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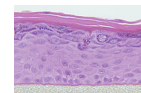NIH Funded HIV Research
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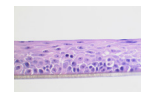## TISSUE MODELS AVAILABLE FROM SLOVAKIA AND USA

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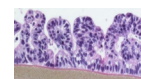
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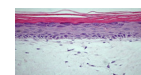
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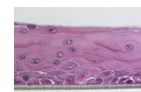## TISSUE MODELS AVAILABLE FROM USA

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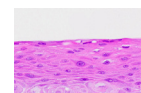
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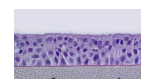
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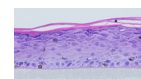
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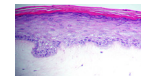
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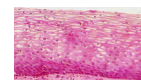
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Welcome address EUSAAT 2019

Dear friends and colleagues,

on behalf of EUSAAT, the European Society for Alternatives to Animal Testing, we welcome you to the 22nd European 3Rs Congress and the 19th EUSAAT Congress in Linz/Austria on October 10-13, 2019.

Since 1991 the "Linz-Congress" has emerged in Europe as the major scientific event in the field of the 3Rs and in 2019 the EUSAAT 2019 3Rs Congress Linz will be the largest international 3Rs congress. We are particularly pleased that our international partners from Japan, JSAAE, the Japanese Society for Alternatives to Animal Experiment, and from China, CCARE, the Chinese Center for Alternatives Research and Evaluation, will report on their activities in our sessions on "3R Centers in Europe & international".

EUSAAT 2019 is hosting oral and poster presentations to facilitate discussions and the exchange of ideas to actively promote the most advanced non-animal methods in the life sciences. The Scientific Committee has identified the subjects that are currently in the focus of 3Rs research in Europe as well as internationally. In 2019 the EUSAAT Congress features sessions on Refinement 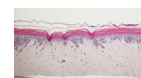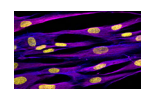and Reduction (e.g. animal welfare of experimental animals, academia and education, ethical and legal issues, drug development and toxicity studies) as well as on Replacement (e.g. disease models based on human cells and tissues, non-animal tools for basic biomedical research, stem cells, bioprinting, 'omics technologies, human 3D tissue culture methods and human multi-organ-chips (MOCs)). Since at EUSAAT 2018 the sessions on establishing 3Rs networks in Europe were most popular, we will have several sessions on this topic as well as a Round Table Discussion.

For the first time, we are holding the YOU EUSAAT 2019 events aimed at young and early career scientists (up to the age of 35) working or planning to work in the 3Rs field. We want to encourage the dialogue among young scientists and with experienced mentors from the 3Rs area to facilitate opportunities for establishing professional networks. In particular, YOU EUSAAT 2019 will provide a free and open atmosphere for exchanging ideas and career experiences and networking with each other!

We are proud that four distinguished colleagues have accepted our invitation to serve as keynote speakers. In addition, we are honored that both the Bjorn Ekwall Memorial Fund (BEMF) will hold the 2019 BEMF Award Ceremony and Lecture at EUSAAT 2019 and also that the editor of ALTEX will present the award for the best article published in the ALTEX journal in 2018 at EUSAAT 2019.

This year we will again host two Round Table Discussions on topics that are of importance for implementing the 3Rs at the international level. The first will cover the "International Impact of Closing Research Animal Facilities in Europe" and the second will be on "Establishing an International 3Rs Centers Network".

EUSAAT is very happy to continue the "Young Scientists Travel Award (YSTA)" program in 2019 and we want to thank the SET Foundation for its generous funding. The YSTA program will provide 20 young scientists with the opportunity to share their ideas on the implementation of the 3Rs in education and novel test systems to reduce the suffering as well as the number of animals in research.

The EUSAAT Board is pleased that the number of sponsors of the EUSAAT congresses has continuously increased, since due to their financial support we can keep the scientific standard high and the congress fee low. This is important to attract young scientists from Europe and abroad. Thus, on behalf of the participants, the EUSAAT Board and the Scientific Committee are grateful to our sponsors.

Moreover, we are also pleased that the abstracts of the EUSAAT 2019 are again published in ALTEX Proceedings and we thank Sonja von Aulock, the editor and CEO of ALTEX Edition, for her generous support.

Finally, we want to thank our colleagues on the EUSAAT Board and the Scientific Committee for their continuous support in planning the EUSAAT 2019 congress.

Horst Spielmann	Winfried Neuhaus	Dagmar Jirova	Dominik Rünzler
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A society for alternatives to animal experiments in India: An update

Mohammad Abdulkader Akbarsha[1,2]

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Although the alternatives movement in India has been initiated and popularized since the late 1990's by a few free-lancers/animal lovers, NGOs such as PeTA, PfA, HSI, etc., and the "Committee for the Purpose of Prevention of Control and Supervision of Experiments on Animals" (CPCSEA; Government of India) [1], it became a serious topic among the academic, scientific and regulatory communities since the establishment of Mahatma Gandhi-Doerenkamp Centre (MGDC) for Alternatives to Animal Experiments in Life Science Education and the Gandhi-Gruber-Doerenkamp Chair for Alternatives by the Doerenkamp-Zbinden Foundation (DZF) at Bharathidasan University, Tiruchirappalli, Tamil Nadu, India, in 2009 [2]. The Centre and the Chair have been doing enormous work by way of research, training workshops, seminars, conferences, etc., especially through national and international collaborations. The pinnacles of glory have been i) decision of University Grants Commission (UGC), the regulatory custodian of higher education in India, to ban dissection of animals as an aspect of Biology education, ii) the Medical and Pharmacy Councils (MCI and PCI) of India prescribing computer-based learning of animal experiments, and iii) the decision of DCGI / CDSCO to ban animal testing of cosmetic finished products and ingredients, to mention a few. The tenure of DZF support to MGDC was over in 2016, following which the taking over of MGDC by the UGC was worked out, with a grant of rupees 500 million for 5 years, and the Centre is now named "National Centre for Alternatives to Animal Experiments" (NCAAE). Thus the alternatives movement in India is going strong to reach newer heights. Further, in order to strengthen this movement so as for propagation, popularization, prescription/adoption, and implementation of alternative methods among different stake-holders, a Society for Alternatives to Animal Experiments-India has been founded with a view to be operating throughout the country, with role from the different stake-holders, in 2018, with the HQ at NCAAE, Bharathidasan University,

Tiruchirappalli. The idea and efforts were reported to EUSAAT at its conference in 2018. Thereafter, the Society was launched at the First National Conference for Alternatives to Animal Experiments (NCAAE) held at Jamia Hamdard (Deemed University), New Delhi, on Nov 27, 2018. The conference was very well organized with about a dozen reputed speakers from abroad more numbers from India. The Society has been registered, and inaugurated. The next conference of the Society is being organized at the National Facility for Biopharmaceuticals, Mumbai, on 13, 14 Dec 2019. This will be accompanied by pre- and post-conference workshops on reconstructed human epidermis (RHE), GARD assay, integrated multiple organ co-culture (IdMOC), alternative model organisms, etc. The Society supports all activities in India connected with alternatives, including by HSI and PeTA-India. The Society looks forward to an Asian Federation of Societies for Alternatives to be established, with support from the Japanese, Korean, China, and other Societies for alternatives in Asian countries, and to widen its horizon of activities with support from several international organizations such as CAAT, CAAT-EU, IIVS, EUSAAT, HSI, PeTA, etc.

References

[1] Akbarsha, M. A., Zeeshan, M. and Pereira, S. (2011). *ALTEX 28*, 153-155. doi:10.14573/altex.2011.2.153

[2] Akbarsha, M. A. and Pereira, S. (2010). *J Pharmacol Pharmacother 1*, 108-110. doi:10.4103/0976-500X.72353

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Glycoengineering as a tool to control the behavior of bone marrow-derived mesenchymal stromal cells in biofabrication processes

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3D Bioprinting is a promising and innovative technique in the field of tissue engineering allowing the generation of highly precise constructs for different purposes. In addition to therapeutic applications, printed tissues are optimal to reduce and finally replace animal experiments in research due to their well-defined composition and structure. One of different challenges to be overcome is related to mechanical shear stress for the cells during the printing process. While a nozzle with a smaller diameter increases the printing resolution, shear forces increase as well, which might lead to an impaired cell viability post-print. Once the cells are printed, their adherence behavior determines distribution in the hydrogel and interaction with the bioink environment. When printing tissue constructs with different cell types, adhesion control becomes more important to guide specific cells to specific areas in the hydrogel. To address these two aspects, our project aims to understand and apply metabolic glycoengineering to enhance (a) glycocalyx mediated cell stability by altering the glycocalyx composition and density as well as (b) cell adhesion within the hydrogel by chemically altering the bioink in addition.

For metabolic glycoengineering, human mesenchymal stromal cells (hMSC) and hMSC-TERT cells were incubated with different tetraacetylated azido sugars (Ac$_4$GlcNAz, Ac$_4$GalNAz or Ac$_4$ManNAz) for 48 h followed by a click reaction. While azido sugar treated cells (Az-hMSC) were incubated for 1 h with DBCO-Cy3 as click molecule (strain promoted alkyne-azido cycloaddition (SPAAC)), in case of the Cu dependent variant (CuAAC) Az-hMSC were incubated for 5 min with alkyne-Cy3 as click molecule in click buffer containing CuSO$_4$, THPTA and sodium ascorbate [1]. For the glycochip assay, commercial pre-treated glass slides were coated with different molecules via amino NHS-ester chemistry and incubated with cells for 24 h followed by HE staining. For 3D bioprinting, alginate and a synthetic poly(2-methyl-2-oxazoline)/poly(2-n-propyl-2-oxazine) block copolymer bioink were optimized for fused deposition modelling [2].

We established the commonly used click reaction variants CuAAC and SPAAC in hMSC as well as hMSC-TERT cells and could successfully detect the azido sugar expression up to 48 h via fluorescence microscopy. Since Ac4ManNAz showed the best results in terms of cell viability and incorporation efficiency into the glycocalyx, it was chosen for future experiments. To identify suitable molecules as binding partners for adhesion mediating glycoproteins like galectin-1, a glycochip assay was designed as screening tool. First experiments revealed no cell adhesion toward different monosaccharides, organic compounds or a highly specific galectin-1 ligand. The adhesion difference between the control glass slide and RGD peptide coated fields as positive adherence control might point to a suboptimal basic functionalization. Interestingly, incubation with the ligand resulted in the appearance of non-adherent cell spheroids, but not in enhanced galectin-1 mRNA expression.

Since the metabolic glycoengineering is working, suitable molecules can now be identified to be introduced into the glycocalyx and evaluated for cell rigidity-increasing effects before and after 3D bioprinting. Furthermore, the overall glycochip design needs to be optimized for the screening of potential galectin-1 binding partners and the galectin-1 ligand impact on cell-cell interactions will be further elucidated.

References

[1] Gutmann, M., Memmel, E., Braun, A. C. et al. (2016). *Chembiochem 17*, 866-875. doi:10.1002/cbic.201500582

[2] Lorson, T., Jaksch, S., Lübtow, M. M. et al. (2017). *Biomacromolecules 18*, 2161-2171. doi:10.1021/acs.biomac.7b00481

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Towards an automated surveillance of well-being in mice using deep learning

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Appropriate refinement methods can only be applied if we are aware that well-being of an animal is compromised [1]. Therefore, tools to assess pain, suffering, and distress in laboratory animals are highly demanded. In recent years, coding systems to analyze the facial expressions of pain were developed for various animal species, for instance for mice, the most commonly used laboratory animals. The so-called Mouse Grimace Scale (MGS) is accurate and reliable [2]. It became a valuable tool for assessing the well-being of mice. However, the use of the MGS is very time-consuming because humans must be thoroughly trained. Moreover, someone must be present and generate live scores. The presence of a human is disadvantageous for well-being assessment since mice are prey animals and often hide signs of weakness, injury, and pain in the presence of humans [3]. Another option to use the MGS is to acquire images/videos to be scored retrospectively, which does not necessarily require the presence of humans. If MGS scores are obtained retrospectively and indicate impairment of well-being, there will be no chance to intervene and apply refinement measures at the right moment. Furthermore, the well-being of a mouse can only be assessed during periods in which the animals are monitored and humans evaluate their status.

Taking into account the great effort and limitations of manual MGS scoring, it is decisive to find a way to automatically monitor well-being of a mouse [4]. Since facial expression analysis has been shown to be useful in mice, we focused on facial expression as a first step and aimed to develop an automated facial expression recognition software for mice [4]. For this approach, we used a data set of images of C57BL/6JRj mice, which had been generated in previous experiments after anesthesia (with isoflurane or ketamine/xylazine), and surgery (castration, under isoflurane and meloxicam) [4]. Images were generated in an observation cages (22×29×39 cm, three white walls, one clear wall) [4]. Since mice were moving freely in the observation cage, images contain natural variation regarding perspective and background [4]. On the one hand, this makes data analysis more challenging, but on the other hand our data set reflects realistic conditions as it would be obtainable without human intervention [4].

Images of the data set were divided into two categories: 1) impaired well-being, 2) unimpaired well-being in order to train a binary classifier [4]. Three convolutional neural network architectures (two pre-trained state of the art deep CNN: ResNet50 and InceptionV3; one CNN without pre-training) were used and achieved an accuracy of up to 99% for the two categories [4]. The result depended on the treatment of the mice. The decision-making process of the CNN architectures was mainly based on the facial expressions of a mouse [4].

Our semi-automated pipeline provides a first step towards the long-term goal to develop a fully automated surveillance ("smart environment") for mice [4].

References
[1] Russell, W. M. S. and Burch, R.L. (1959). *The Principles of Humane Experimental Technique*. London, UK: Methuen.
[2] Langford, D. J., Bailey, A. L., Chanda, M. L. et al. (2010). *Nat Methods 7*, 447-449. doi:10.1038/nmeth.1455
[3] Stasiak, K., Maul, D., French, E. et al. (2003). *Contemp Top Lab Anim Sci 42*, 13-20.
[4] Andresen, N., Wöllhaf, M., Hohlbaum, K. et al. (2019). Towards a fully automated surveillance of well-being status in laboratory mice using deep learning. *bioRxiv*, 582817. doi:10.1101/582817

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Replacing animal tests to improve safety for humans

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Background

Animal safety testing for new medicines is arguably the most difficult use of non-human animals to challenge, for two reasons: first, it is required by governments (regulatory testing); second, protecting patients is a vital goal, and it seems intuitively obvious that animal tests must protect patients. European Union law (European Parliament, 2010, Directive 2010/63/EU) states that animals must not be used if a non-animal method could achieve the same purpose. So, it is crucial to know how well animal tests predict the safety of medicines, and whether any other methods are equally or more predictive. In addition, the efficiency of different methods in terms of time and costs and the ethical acceptability of using animals, if their use is deemed to be of irreplaceable value, must be considered.

Methods and discussion

We reviewed promising new approach methodologies in the context of preclinical testing for new therapies and the barriers to be overcome in order to speed adoption of these methods. We also considered historical and legacy examples of animal testing in drug discovery and examined the impact poor clinical translation had in contributing to the rise in increased reporting of adverse drug reactions (ADRs) in human populations and drug discovery failures. The effectiveness and predictability of both new methods and legacy animal methods was also discussed, with case studies presented of technologies demonstrating success in predicting safety issues for humans, where the current system of mandatory animal-based safety tests had failed (as well as where it had succeeded). In this way, the predictive performance of the new tests could be compared to that of the animal-based methods.

The regulatory position and advances in more flexible and innovative validation of new approach methodologies were also discussed, including the concept of qualification, as introduced by the US FDA and other regulatory bodies; global harmonisation of regulatory requirements between different countries to remove unnecessary barriers to the efficient delivery of safe, innovative, and effective treatments to patients, and a change in regulatory language to state clearly that the test most predictive of human response should (or even must) be used.

Results

Remarkable knowledge and tools are emerging from projects, such as ToxCast; Tox21; Innovative Medicines Initiative; Safety Evaluation Ultimately Replacing Animal Testing (SEURAT); ORgan on a CHip In Development (ORCHID); Integrated European "Flagship" Program Driving Mechanism-based Toxicity Testing and Risk Assessment for the 21st Century (EU-ToxRisk); and the Precision Medicines Initiative. These initiatives have the potential to revolutionize our ability to advance and protect human health, but only if they are implemented. This presentation highlights some of these initiatives and success stories therein while considering what and where barriers to adoption still remain, stalling the move the full replacement of animal tests in human safety testing.

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Use of ethno veterinary medicine techniques for animal welfare and sustainable disease management

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Excessive use of antibiotics and drugs have resulted in development of resistance among livestock species in addition to increased cost of production. The use of these drugs have resulted in decrease immunity along with having ill effect on environment and animal welfare. Tribal farmers have knowledge of ethno veterinary medicine and its significance has been identified by the them through a process of experience over hundreds of years and have been long been using plants and herbs for effectively controlling various ailments with organic rearing and animal welfare in mind. The study was carried out in hilly areas of Jammu and Kashmir,India. The data was collected by means of well-structured questionnaires. Interview schedule was used to collect the information about the use of ethno veterinary practices and their effectiveness. The paper deals with 23 ailments commonly found in different categories of livestock/animals and their treatment with 41 medicinal plant species that occur in forests as well as close vicinity of the rural settlements. Out of the total population, majority of the people (more than 70%) was found dependent on traditional (herbal) system of treatments while rest of the people preferred modern (allopathic) system of treatments for curing veterinary ailments. Moreover, it was found that first line of defence was the use of local herbs and traditional knowledge. Tribal's last option was to use allopathic medicine or a veterinary practitioner. It was observed that old aged people have more knowledge and experience particularly in remote areas for curing veterinary ailments. The traditional system of treatment is one of the most important prevailing systems in the area where modern veterinary health care facilities are still in developing stage due to hilly terrain and long distance. The most frequently occuring ailments included are diarrhoea, afterbirth retention, poisoning, prolapse of the uterus, constipation, liver problems, bloat, pneumonia, bone fracture, cough, fever, indigestion, anorexia, blood in urine, tympany, rheumatism, arthritis, gastric troubles, mastitis, shoulder swelling, mouth blisters, etc. Plant based ethnoveterinary medicine and practices are making an important contribution in improving the veterinary infrastructure and increasing the livestock productivity. This approach offers environmental conservation, animal welfare and management strategies for achieving sustainability, availability, accessibility and affordability of existing ethnoremedies.

References

Katewa, S. S. and Chaudhary, B. L. (2000). *J Vasundhara 5*, 95-98.
McCorkle, C. M. (1986). *J Ethnobiol 6*, 129-149.

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Recent efforts to elucidate the scientific validity of animal-based drug tests by the pharmaceutical industry, pro-testing lobby groups, and animal welfare organisations

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Even after several decades of human drug development, there remains an absence of published, substantial, comprehensive data to validate the use of animals in preclinical drug testing, and to point to their predictive nature with regard to human safety/toxicity and efficacy. Two recent papers, authored by pharmaceutical industry scientists, added to the few substantive publications that exist [1,2]. We argue that the authors' conclusions of animal tests being fit for their stated purpose (with regard to reliable prediction of human toxicity) are not supported by their data, meaning there is still no published evidence to support the current regulatory paradigm of animal testing in supporting safe entry to clinical trials [3]; and that the data in these recent studies, as well as in our own studies (e.g. [4,5]), in fact support the contention that tests on rodents, dogs and monkeys provide next to no evidential weight to the probability of there being a lack of human toxicity, when there is no apparent toxicity in the animals. It must be concluded that animal drug tests are therefore not fit for their stated purpose. At the very least, it is now incumbent on – and we very much encourage – the pharmaceutical industry and its regulators to commission, conduct and/or facilitate further independent studies involving the use of substantial proprietary data.

References

[1] Monticello, T. M., Jones, T. W., Dambach, D. M. et al. (2017). *Toxicol Appl Pharmacol 334*, 100-109.
[2] Clark, M. and Steger-Hartmann, T. (2018). *Regul Toxicol Pharmacol 96*, 94-105.
[3] Bailey, J. and Balls, M. (2019). *BMC Med Ethics 20*, 16.
[4] Bailey, J., Thew, M. and Balls, M. (2015). *Altern Lab Anim 43*, 393-403.
[5] Bailey, J., Thew, M. and Balls, M. (2014). *Altern Lab Anim 42*, 181-199.

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Does the stress of laboratory life and experimentation on animals adversely affect research data?

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Recurrent acute and/or chronic stress can affect all vertebrate species, and can have serious consequences. It is increasingly and widely appreciated that laboratory animals experience significant and repeated stress, which is unavoidable and is caused by many aspects of laboratory life, such as captivity, transport, noise, handling, restraint and other procedures, as well as the experimental procedures applied to them [1]. Such stress is difficult to mitigate, and lack of significant desensitisation/habituation can result in considerable psychological and physiological welfare problems, which are mediated by the activation of various neuroendocrine networks that have numerous and pervasive effects. Psychological damage can be reflected in stereotypical behaviours, including repetitive pacing and circling, and even self-harm [2]. Physical consequences include adverse effects on immune function, inflammatory responses, metabolism, and disease susceptibility and progression [3]. Further, some of these effects are epigenetic, and are therefore potentially transgenerational: the biology of animals whose parents/grandparents were wild-caught and/or have experienced chronic stress in laboratories could be altered, as compared to free- living individuals [4]. It is argued that these effects must have consequences for the reliability of experimental data and their extrapolation to humans, and this may not be recognised sufficiently among those who use animals in experiments [5]. This issue should be taken much more seriously by legislators, regulators, funders, practitioners and advocates of animal experiments.

References

[1] Balcombe, J. P., Barnard, N. D. and Sandusky, C. (2004). *Contemp Top Lab Anim Sci 43*, 42-51.

[2] National Research Council (US) Committee on Recognition and Alleviation of Distress in Laboratory Animals. (2008). Recognition and Alleviation of Pain and Distress in Laboratory Animals. Washington, DC: National Academies Press (US). https://www.ncbi.nlm.nih.gov/books/NBK4032/

[3] Gurfein, B. T., Stamm, A. W., Bacchetti, P. et al. (2012). *Mol Med 18*, 606-617.

[4] Zambrano, E., Martinez-Samayoa, P. M., Bautista, C. J. et al. (2005). *J Physiol 566*, 225-236.

[5] Bailey, J. (2018). *Altern Lab Anim 46*, 291-305.

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CRISPR-mediated gene editing: Scientific and ethical issues

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It is widely claimed that the relatively new method of gene editing, CRISPR, is more specific compared to other methods, producing more animals with the desired modification, less wastage (animals without the desired modification), and less suffering due to non-specific modifications that cause pain and suffering. However, there is increasing concern that the technique remains beset by persistent poor efficiency and specificity, with adverse animal-welfare and scientific consequences. Recent reports have highlighted slightly improved, though still very poor, efficiencies which, when one looks for precision, amount to a few percent at most [1]. Off-target effects are persistent, even with engineered CRISPR components, and can have multiple pathogenic consequences, including cancer. Many off-target effects are missed due to analytical methods that aren't sufficiently comprehensive, and some believe they may never be completely removed, however high on-target specificity may become [2,3]. On-target effects may be are often more significant than intended, causing large deletions and genomic rearrangements. CRISPR is also more likely to be successful in cells in which the p53 gene is deficient, and so which have further cancerous potential – with catastrophic consequences for GM animals and for clinical applications [4]. These concerns are in addition to other, long-standing problems. Poor efficiency and specificity are a serious welfare issue, with every stage of the creation and breeding of GM animals potentially involving pain and suffering to some degree, and which may not be fully appreciated and taken into account in harm-benefit analyses. There exists significant evidence of failed translation of data from GM animals to human benefit, and of their poor human relevance for many diseases. Of salient concern currently is the increased creation of, and experimentation on, GM monkeys, partly in response to greater appreciation of the inadequacies of GM mice. There is no evidence to support claims of their greater human relevance. They will be subject to the same inefficiencies and lack of specificity as GM mice, and there are myriad, confounding differences in gene complement and expression between humans and monkeys. There is therefore a strong argument for GM technology to be used only in an *in vitro* context. Cell and tissue cultures will become more informative and human-relevant with further advances in 3D-culture, organoids, body-on-a-chip approaches, stem cells, and so on. They are already being used to investigate gene function, link genetic mutations/polymorphisms with phenotype, attempt gene therapy, etc. For GM techniques such as CRISPR to be improved to a degree that will enable them to be applied to human patients, this will take place – if it ever does – via these human-relevant *in vitro* methods: there is no scientific basis to assume that successful and "safe" gene therapy in mice or monkeys will translate to the same in patients. In summary: CRISPR is nowhere near ready for *in vivo* use, either scientifically or ethically. Its clinical promise could, and should, be investigated via well designed *in vitro* research, which should include efforts to determine if problems with specificity can be overcome [5].

References

[1] Albadri, S., Del Bene, F. and Revenu, C. (2017). *Methods 121*, 77-85.
[2] Bassett, A. R. (2017). *Mamm Genome 28*, 348-364.
[3] Liang, P., Xu, Y., Zhang, X. et al. (2015). *Protein Cell 6*, 363-372.
[4] Haapaniemi, E., Botla, S., Persson, J. et al. (2018). *Nat Med 24*, 927-930.
[5] Bailey, J. (2019). Genetic modification of animals: scientific and ethical concerns. In K. Herrmann and K. Jayne (eds.), *Animal Experimentation: Working Towards a Paradigm Change* (443-479). Boston, USA: Brill.

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Modernising research and regulatory policies to advance human health

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The use of animals has been the dominant paradigm in biomedical research and toxicology. However, as detailed analyses continue to document limitations in translating results from animal studies to humans for numerous disease areas and toxicological studies, animal tests can no longer be considered the "gold standard". For example, the US National Institutes of Health reports a 95% failure rate of clinical trials for new pharmaceuticals following preclinical success in animals [1] because the drugs are either not safe or not effective [2]. Moreover, the current research paradigm is cost prohibitive. Moving a new drug to market may cost up to US$2 billion (approximately €1.7 billion or £1.5 billion) and take as long as 15 years. Furthermore, a 2015 analysis concluded that the prevalence of irreproducible preclinical research was between 50 and 89%, which, at the most conservative US estimate, results in approximately US$28 billion (approximately €25 billion or £22 billion) per year spent on misleading research [3]. These failure rates cannot be supported economically or ethically, and efforts to transform the research environment are urgently needed.

However, remarkable advances in research technology are already revolutionising biomedical research and regulatory testing, and more progress is expected in the coming years. The transition away from research relying on animals to model human disease or predict human responses to drugs or other substances and towards human biology-based methods is changing policy and practice around the globe. In 2016, the Dutch government announced a plan to reduce animal testing and focus its efforts on non-animal technologies for biomedical and regulatory research [4]. Governments that mandate a move away from animal experimentation and towards more advanced scientific methods have the opportunity to expand job growth rapidly in science and technology and reduce healthcare costs for their citizens. New technologies will streamline drug development and toxicity testing, making the pro-

cesses safer, cheaper, and more effective. Developing these technologies also allows for the creation of interdisciplinary research teams that will be fundamental in creating the human disease and toxicology models of tomorrow.

PETA has published a report and strategy offering a robust blueprint for translating limitations in animal use within the research paradigm, along with the increasing availability of human-relevant biotechnology, into actions aimed at eliminating inefficiencies. We highlight a number of priorities regarding areas of both regulatory and non-regulatory research where opportunities lie for the immediate and forthcoming replacement of animal use.

References

[1] National Center for Advancing Translational Sciences (NCATS). About NCATS. https://ncats.nih.gov/about (updated 03.07.2018; accessed 03.07.2018).

[2] US Department of Health and Human Services, Food and Drug Administration (2004). https://www.who.int/intellectualproperty/documents/en/FDAproposals.pdf (accessed 10.07.2019).

[3] Freedman, L. P., Cockburn, I. M. and Simcoe, T. S. (2015). *PLoS Biol 13*, e1002165. doi:10.1371/journal.pbio.1002165

[4] NCad (2016). https://www.ncadierproevenbeleid.nl/documenten/rapport/2016/12/15/ncad-opinion-transition-to-non-animal-research (accessed 10.07.2019).

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Stakeholder collaboration to advance human-relevant nonclinical methods for drug development in the United States

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Recent initiatives in North America, such as the United States Food and Drug Administration's (FDA) Predictive Toxicology Roadmap, represent a shift in the way drugs will be developed and regulated. Regulators now state the need to integrate modern tools that better predict human outcomes than the traditionally used animal tests. In support of such initiatives and with the goal of increasing the human relevance of nonclinical drug testing, a growing group of professionals from federal agencies, the private sector and patient, health and research organizations collaborate under the Nonclinical Innovation and Patient Safety Initiative (NIPSI).

Through a Drug Discovery Today publication, NIPSI outlined factors that impede integration of new approaches and provided recommendations for addressing these factors. Ongoing projects focus on changing policy, supporting human-based science, and offering industry and regulator training. One policy project involves changing FDA and International Council on the Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) regulations from requiring "animal" data to "nonclinical," which encompasses animal *in vivo* and human and animal-based *in vitro* and *in silico* approaches. Another project aims to establish an evaluation pathway for regulatory acceptance of human biology-based nonclinical approaches at FDA.

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California Cruelty-Free Cosmetics Act establishes first North American law banning the sale of animal tested cosmetics and ingredients

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The United States Food and Drug Administration does not require or review safety information prior to the marketing and sale of cosmetics in North America. Instead, individual companies are responsible for ensuring their cosmetic products are safe for human use. In 2018, the state of California joined the European Union and other regions in banning the sale of cosmetic products or ingredients that have been tested on animals, with limited exceptions. Starting January 1, 2020, no cosmetic may be sold within the state if it, or its ingredients, were tested on animals after January 1, 2020. Enumerated exemptions allow for testing conducted in response to a requirement by a state, federal or foreign regulatory body under certain conditions. However, the law also requires companies selling cosmetics or ingredients tested on animals under one of the exemptions must also use nonanimal methods to substantiate the safety of the product. Because the law applies to cosmetic companies as well as any third-party suppliers or contractors, many ingredient manufacturers will be required to begin using *in vitro* or *in silico* methods, even if they are also conducting an *in vivo* study for the same endpoint to support the ingredient in another sector. This presentation will outline the law and describe approaches available for use to meet California's legislative requirements.

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3R-alternatives to skin testing of bovine PPD tuberculins

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Tuberculin purified protein derivatives (PPDs) are heat-treated, protein- enriched products of lysed mycobacteria. Tuberculins reveal a delayed hypersensitivity in individuals earlier sensitised to mycobacteria and are thereby used to detect tuberculosis in humans and animals. For batch potency testing, the relevant European Pharmacopoeia monographs [1-3] advise intradermal injection of tuberculins into previously sensitised guinea pigs and to compare the size of the resulting skin lesions to a reference tuberculin of known potency. Since the procedure is quite compromising and results are highly variable, we aim to establish an ELISA assays on the basis of *in vitro* T cell proliferation. This approach no longer requires skin testing but allows for *in vitro* stimulation to assess the immunological functionality of tuberculins. In addition, ELISAs yield objective quantitative readouts which are well accepted and thus holds potential for a reduction and refinement alternative. Results obtained so far show promising reproducible, specific, and dose dependent responses.

In addition, we established a mass spectrometric method to record semi-quantitative protein profiles for the *in-vitro* characterization and batch to batch consistency testing of tuberculins. Analytical liquid chromatography (LC)-electrospray ionization (ESI)-MSE method was applied for recording protein profiles. Altogether, we tested up to 4 batches of bovine PPD tuberculins from three manufacturers and the International and European Standard for PPD of Mycobacterium bovis tuberculin. We identified more than 147 proteins in PPDs of which app. 19 proteins were considered for quantification. Ten proteins were consistently found in all batches of all products. Interestingly, we observed significantly different relative amounts of proteins in products with the same or similar qualitative protein patterns. These findings include powerful immunogens, latency markers or virulence markers.

LC-MSE provides a comprehensive insight in the quality determining components of these complex immunologicals. It is a promising technique following the "consistency approach", which implies the assessment of batch potency and safety on the basis of *in vitro* data [4]. In addition, the MSE data provide the basis for the development of a targeted MS method (multiple reaction monitoring) for the absolute quantification of selected key immunogens.

References

[1] Monograph 07/2014:10000, corrected 9.2. General notices. In *European Pharmacopoeia*. 9th edition. Council of Europe, Strasbourg, 2017.
[2] Monograph 01/2008:0536. Tuberculin purified protein derivative, bovine. In *European Pharmacopoeia*. 9th edition. Council of Europe, Strasbourg, 2017.
[3] Monograph 01/2018:50214. Substitution of in vivo methods by in vitro methods for the quality control of vaccines. In *European Pharmacopoeia*. 9th edition. Council of Europe, Strasbourg, 2017.
[4] De Mattia, F., Hendriksen, C., Buchheit, K. H. et al. (2015). *Pharmeur Bio Sci Notes 2015*, 30-56.

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Italian Centro3Rs commitment:
1 year after the opening of the activities

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The Italian Centro3R was spearheaded by the Universities of Pisa and Genova back in March 2018, and today boasts some 300 members from Turin and of Milan Polytechnics, the Milan-Bicocca University and researchers from other universities, such as Pavia, Bologna and Padova who will shortly join this network.

The Centro3R represents a platform for the promotion of Russel and Burch's Principles of Reduction, Refinement and Replacement of animal use in scientific and teaching environments according to the European Union directive 2010/63/UE for the protection of animals used for scientific means [1]. Each of its 3R principles provides a responsible and conscious approach for biomedical research by firstly reducing the number of animals used in experiments, secondly, limiting any suffering by the animals, and thirdly to replace traditional animal-testing methods with alternative ones with equal or improved validity and translational value.

The Center is organized into Operating Units (OUs) (i.e. the Universities) and each OU works within its university to implement and organize degree courses on the 3Rs and foster the concept of humane research. The members possess a wide range of competence in disciplines such as pharmacology, engineering, law, biology, medicine and philosophy and these are shared on the website (www.centro3r.en) and in regular meetings, newsletters and active social media channels.

The 2nd Annual meeting "3Rs in Italian Universities", hosted by the Genoa OU, was generously supported by the Genoa University Rector on 20th-21st June of this year. There was an overwhelming response to the event: 3 weeks before the registration deadline, the event sold out with a record number of 110 participants. Besides the national interest in the 3Rs theme, the high level of response was also thanks to a packed programme including representatives from European and National institutions, as well as from Italian Ministers of Health, Education, University and Re-

search, and numerous scientists from the Centro3R UOs, and Research institutions and companies engaged in the field of innovative technologies and alternative methods. Two keynote speakers, Susanna Louhimies and Chantra Eskes, focused their speeches on Directive 2010/63/EU and on the 3Rs through research, education and communication implementation, respectively. The topics included: involvement of national/international institutions in 3R implementation; ethical and legal issues; *in silico* models; application of reduction and refinement in animal testing; disease models using human cells/tissues/organs; application of replacement in research and the commitment of Centro3R in University training. Moreover, to encourage the attendance of young scientists, two prizes were awarded for the best six abstracts and posters, and 30 young researchers presented their work. A roundtable on the role on Center 3R contributed to consolidating the scientific network on 3R teaching and research. The multi-disciplinarity nature of the speeches was highly appreciated since it paved the way for synergic collaborations among the researchers. In conclusion, the conference attracted the interest of many researchers and the media who contributed to the dissemination of Centro3R's mission in promoting it as a point of reference for research and teaching resources in Italian academia and an invaluable platform for discussions.

Reference

[1] Ahluwalia, A., Bassi, A. M. and Milazzo, P. (2018). *ALTEX 35*, 260-261. doi:10.14573/altex.1803201

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How can the final goal of completely replacing animal procedures successfully be achieved?

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Article 23 of EU-Directive 86/609/EEC [1] required that member states promote the development and validation of alternative technologies and stated that the European Commission "shall report before the end of 1987 on the possibility of modifying tests and guidelines" (European Parliament, 1986, Article 23). This Directive was replaced by Directive 2010/63/EU [2] on the protection of animals used for scientific purposes which requires that Member States develop and validate alternative approaches much more precisely. It furthermore specifies that the ultimate objective is the "full replacement of procedures on live animals for scientific and educational purposes, as soon as it is scientifically possible to do so" (Recital 10) [2]. The logical consequence thereof should be a concrete strategy, a masterplan, including all stakeholders, on how to achieve the final phasing out of animal procedures by focusing on new, better, human relevant research and testing methods.

In 2015 we started collating individual measures in order to tackle the problems we encounter in our day-to-day work as an animal rights organization, defining five categories (or pillars) that should be included in a masterplan. We found many of the ideas aligning with the Netherlands National Committee for the protection of animals used for scientific purposes (NCad) plan, Transition to Non-Animal Research [3], which was first presented in 2016. The Netherlands were the first EU member state to present a road map for phasing out animal procedures and stimulating innovation without laboratory animals. No EU Member State, so far, has publicly spoken out in favor of the Dutch initiative (with exception of the region Brussels in Belgium). On the contrary, its timeline has been criticized as being unrealistic, risking the safety of medical treatment, and hampering basic research.

Under the leadership of politics, it is incumbent upon the stakeholders – science, industry, authorities and animal rights/welfare – to develop a master plan and monitoring for a paradigm change as well as to engage actively in its implementation. We therefore propose the use of the NCad-plan as a basis for a joint EU-project, to create a concrete phase-out-strategy – a masterplan – supported by all member states. A serious strategy to end animal experiments requires suitable resources that purposefully pursue that

end by reducing animal experiments and increasing market-ready animal-free methods. Such a master plan's foundation, which our organization has previously described, should rest on five pillars. These pillars entail measures like a strong increase in funding for new, alternative, human relevant research and testing methods, the expansion of animal-free methods in teaching and scientific research, the consolidation and expansion of bans with an aim to reducing animal experiments, a success monitoring of the increase in animal-free methods and the reduction of animal experiments. Furthermore, complementary measures like the drastic shortening of time needed for assessing and approving animal-free research methods, the introduction of the class action suit for animal welfare in the EU and its Member States and adequate resources for law enforcement authorities, should be considered.

References

[1] The Council of the European Communities (1986). Council Directive of 24 November 1986 on the Approximation of Laws, Regulations and Administrative Provisions of the Member States Regarding the Protection of Animals Used for Experimental and Other Scientific Purposes (86/609/EEC). *Official Journal of the European Communities L 358/1*, 5.

[2] European Parliament and the Council (2010). Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes. *Official Journal of the European Communities L 276*, p. 33-79.

[3] Netherlands National Committee for the protection of animals used for scientific purposes (2016). Transition to Non-Animal Research. Publication number: 201609EN. https://english.ncadierproevenbeleid.nl/

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Bioprinting of 3D organ models for virus and cancer research

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9th Bioprinting technologies hold a great potential to generate 3D organ models to replace animal experiments. In addition to contributing to the 3R principles, the possibility to use human cells makes these models valuable research tools to study human diseases [1]. We use the pneumatic extrusion technology to generate humanized lung and liver models. To this end, A549 cells (an alveolar epithelial cell line) or HepaRG cells (a hepatocellular carcinoma cell line) are embedded in a hydrogel composed of alginate and gelatin. In addition, Matrigel and human ECM, respectively, are added as an extracellular matrix component. The optimized bioinks permit bioprinting of tissue models that are characterized by high cell viability and desirable physiological parameters.

As a proof of concept, the lung model was infected with influenza A virus. We found the virus to efficiently replicate and induce an immune reaction of the infected organ model [2]. The liver model was infected with human adenovirus 5, which causes severe hepatic infections in immunocompromised patients. In addition, we demonstrated that the bioprinted liver model is suitable to study transduction efficiency of adeno-associated virus (AAV) vectors [3].

More recent efforts aim at improving the models. The second-generation lung model will consist of a bioprinted 3D layer of human lung fibroblast on top of which a single layer of human alveolar cells will be cultivated. To improve intracellular interactions, the rigid encapsulation of the cells by alginate which is required during the printing procedure will be loosened by sodium citrate treatment.

Finally, we use bioprinted tissue models for cancer research. Tissue damage induced by UV and aflatoxin treatment is being investigated in 3D liver models. Furthermore, cancer models are generated by printing tumor cells in a healthy environment.

References

[1] Weinhart, M., Hocke, A. C., Hippenstiel, S. et al. (2019). *Pharmacol Res 139*, 446-451.
[2] Berg, J., Hiller, T., Kissner, M. S. et al. (2018). *Sci Rep 8*, 13877.
[3] Hiller, T., Berg, J., Elomaa, L. et al. (2018). *Int J Mol Sci 19*, 3129.

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Replacement and reduction examples from Novo Nordisk

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The pharmaceutical sector performs extensive research and development activities to find new, better and safer solutions for patients with chronic and/or debilitating diseases. A proportion of these activities makes use of live and sentient animals. This cause a justified ethical concern which the pharmaceutical sector and Novo Nordisk takes very seriously.

Although the pharmaceutical sector has initiated and achieved much progress within Refinement, the talk will focus on Replacement and Reduction and include a presentation of Novo Nordisk's approach to Replacement and what we are doing to deal with the inherent obstacles. It will also demonstrate how we share our 3R initiatives with colleagues in the pharmaceutical sector (EFPIA) as well as academia, other external collaboration partners and with NGOs and authorities.

The representation will give an example of a full Replacement achievement, including what this has required of legal and regulatory obligations, and examples of partial replacement where a Reduction in the number of used animals have been achieved.

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Enabling technologies for skin permeation investigation

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The assessment of molecular diffusion through cutaneous tissues is an important step in the evaluation of any transdermal delivery system able to administrate either antiseptic compounds or biomolecules for cosmetics [1,2]. Traditionally, *in vitro* models for skin permeation exploit bulky and expensive diffusion Franz cells [3,4], featuring poor integration with traditional optical or photonic detection systems. These issues clearly limit the possibility of a cost-effective, real-time, and high-throughput study of skin permeation. Furthermore, Franz cells involve time-consuming set-up procedures and operate with large reservoirs causing a significant consumption of reagents. Therefore, new strategies based on fabrication and detection technologies coupling should be explored, in order to overcome the above-mentioned limitations.

Here we present a compact, competitive, and easy to use 3D-printed diffusion cell (3D-PDC) as an original *in vitro* tool for skin permeation study [5]. As proof-of-concept, a polylactide diffusion cell is fabricated and characterized. It is composed of a donor chamber, which allows direct access to the epidermis for vehicle applications (e.g. liquids, creams, patches), and a small receptor compartment (500 μL) integrating two channels for diffusers and metabolites collection and a glass window for visual inspection and microscopy investigation. An O-ring inserted under the explanted skin prevents liquid leakage and the double-compartment device can be easily mounted with either stainless steel screws or magnets. The reduced dimensions of the 3D-PDC (1 cm x 3.5 cm x 3.5 cm) allow for its incubation in a sterile environment, to maintain the experimental conditions as closest as possible to the physiological ones.

Good viability of mice skins up to 24 h incubation in the cell was demonstrated via colorimetric assays (MTT), indicating that tissue integrity is preserved during moderately long incubation times. Due to the pocket-size of our design, the 3D-PDC is compatible with a standard fluorescence inverted microscope, for real-time permeation assays. Diffusion studies through healthy mice skin by means of a model molecule (FITC-dextran, 4 kDa) were performed acquiring automatically fluorescence images every 5 minutes over 24 hours, at a selected depth of the receptor compartment. Our results led to a diffusivity of $\sim2\times10\text{-}10$ m^2/s, in agreement with the expected value. Permeation studies have been extended to treated and injured tissue (microneedles, UV-light damage), demonstrating the versatility of our tool in assessing molecule permeation under different conditions. We are now in the process of validating our method by means of commercially available synthetic membranes (Strat-M® membranes, Merck) and standard compounds (e.g. caffeine, vanillin), with the multiple aim of (1) eliminating the need of animal skin supply, (2) further broadening the 3D-PDC adaptability to artificial/*in vitro*-reconstructed skin membranes, and (3) widening the coupling with other detection techniques (e.g. photo-spectroscopy). Given the promising preliminary data, we foresee potential applications of our assessment pocket-size tool in emerging fields, such as nano-toxicology, nanotherapeutics and regenerative medicine.

References

[1] Bartosova, L. and Bajgar, J. (2012). *Curr Med Chem 19*, 4671-4677. doi:10.2174/092986712803306358

[2] Romgens, A. M., Bader, D. L., Bouwstra, J. A. et al. (2015). *J Mech Behav Biomed Mat 50*, 215-222. doi:10.1016/j.jmbbm.2015.06.019

[3] Franz, T. J. (1975). *J Inv Derm 64*, 190-195.

[4] Ng, S. F., Rouse, J. J., Sanderson, F. D. et al. (2010). *AAPS PharmSciTech 11*, 1432-1441. doi:10.1208/s12249-010-9522-9

[5] Suarato, G., Spanò, R., Bertorelli, R. et al. (2018). *Proceedings 2*, 945. doi:10.3390/proceedings2130945

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What can (Q)SAR modelling tell us about fish toxicity?

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Quantitative Structure-Activity Relationship models (QSARs) are one of the identified alternatives to experimental tests accepted for use in many regulatory contexts. They are statistical models based on semi-empirical approaches used to predict chemical properties based on their molecular structure. These tools are supported by the 3R principles promoting the Refinement, the Reduction and the Replacement of experimentation performed on animals when appropriate. In recent years, several QSARs have been developed to predict ecotoxicological and physico-chemical properties which can predict physico-chemical and environmental properties of compounds as well as ecotoxicity. Despite their clear advantages of speed and cost, QSARs were not heavily employed as alternatives for REACH registration dossiers between 2009 and 2016 compared to other alternative approaches in order to determine fish toxicity [1]. However, modelling can be used to predict the toxicity of very hydrophobic or miscible substances where analytical monitoring is difficult to perform. Specifically, they can be used to predict the chronic toxicity of compounds which are difficult to maintain stable during long-term exposure [2]. Therefore, QSARs may also be useful to predict a result where experimental test would likely fail. As recommended by the newly updated OECD Guideline 203 [3], QSARs may be used to replace the preliminary range-finding test, thus avoiding additional use of fish. But some QSARs are moving to a new dimension where the Mechanisms of toxic Action (MechoAs) [4] can be taken into account based on the molecular structure of the test chemical. The MechoA determines the first key events in the Adverse Outcome Pathway of the toxicity of a compound providing critical information on the understanding of the specific toxic action of the substance, for example the neurotoxicity or the endocrine disruption. Indeed, MechoA determination can complement *in vivo* experiments such as Fish Embryo Toxicity tests by detecting a neurotoxic potential not classically measured in such tests. Consequently, QSARs which integrate the MechoA scheme are an interesting source of information in the framework of Acute Fish Toxicity test replacement. Indeed, existing data on different organisms combined with QSAR results can be used in a Weight-Of-Evidence approach to assess the toxicity to fish. This methodology is presented within the OECD Integrated Approaches to Testing and Assessment for Acute Fish Toxicity.

References

[1] ECHA (2017). The use of alternatives to testing on animals for the REACH Regulation: Third report under Article 117(3) of the REACH Regulation. ISBN 978-92-9495-760-3.

[2] Thomas, P.C., Bicherel, P. and Bauer, J. F. (2019). *Integr Environ Assess Manag 15*, 40-50. doi:10.1002/ieam.4108

[3] OECD (2019). Test No. 203: Fish, Acute Toxicity Test. OECD Guidelines for the Testing of Chemicals, Section 2. doi:10.1787/9789264069961-en

[4] Bauer, F. J, Thomas, P. C., Fouchard, S. Y. et al. (2018). *Comput Toxicol 7*, 36-45. doi:10.1016/j.comtox.2018.06.004

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A recipe for the development of High-Accuracy QSAR models based on toxic mechanisms of action

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For historical reasons, the ultimate objective of QSAR models was often to prioritise chemicals for further testing (screening purposes) rather than to predict an exact experimental value. Therefore, many QSAR models tend to lack the precision required to replace experimental studies. The OECD proposed five validity criteria to ensure the reliability of a model, and consequently, the accuracy of its prediction. However, these conditions remain general, and they are not always completely satisfied in practice. For this reason, it seems necessary to distinguish between QSAR models created to provide approximations of experimental values and those specially designed to substitute for specific experimental endpoints, the High-Accuracy QSAR models (HA-QSARs). In order to develop this kind of model, the five validation criteria of the OECD need to be elucidated and specified. Furthermore, the mechanistic explanation suggested by the OECD as being only facultative, actually appears to be critical, especially in the field of toxicology. In this poster recommendations are given to base the models on toxic Mechanism of Action (MechoA) instead of chemical structure. The aquatic toxicity predicted by commonly used QSARs and HA-QSARs is compared to experimental values which have been validated and appear to be reliable. The analysis shows that the HA-QSAR gets closer results to these experimental reference values than the screening QSARs. This increases confidence in HA-QSARs to such an extent that they should be considered as serious candidates to replace experimental test when the applicability domain is known.

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Charité 3[R] – the 3R Center of Charité Universitätsmedizin Berlin

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Charité 3[R] aims to bundle and coordinate the 3R research activities at Charité – Universitätsmedizin Berlin for a better translation in biomedicine. The centre is committed to finding the best therapies by using animal-free methods whenever possible, establishing meaningful human disease models, and increasing animal welfare. Charité 3[R] supports 3R research with project funding dedicated to the interdisciplinary development of novel research approaches, refinement methods and 3R measures within the scope of existing research projects. The center further offers 3R education, e.g. for PhD students by developing curriculum modules especially dedicated to increase awareness to the thematic of the 3Rs within the young research community and present novel alternative methods. Charité 3[R] aims at developing a communication channel among important stakeholders to better communicate the challenges, needs and opportunities of 3R research. We are implementing the above-mentioned goals in close collaboration with local, national and international partners from public research institutions, as well as pharmaceutical and biomedical companies.

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Similarity of a career in the field of 3Rs and a trans-alpine crossing

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Careers in the area of Pharmacology and Toxicology with the focus on the 3Rs are always starting and developing differently but all of them have the same destination: reliable risk assessment for humans and the environment by good scientific practice with the focus on no/less animal testing.

Barbara Birk, currently Labteam-Leader responsible for the development of Alternatives to Animal testing at BASF SE, Experimental Toxicology and Ecology will compare her professional development with a Trans-Alpine Crossing.

Barbara studied veterinarian medicine, gained her expertise in a start-up company and in big industry, has been working as research but also regulatory scientist by using *in vivo*, *in vitro*, and *in silico* techniques focusing always in 3Rs.

A professional career and a Trans-Alpine Crossing require the same skills and face similar challenges.

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Adaptation of skin sensitization *in vitro* methods (OECD 442C, D, E) for nanomaterials

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During the last decade, numerous Federal Ministry of Education and Research (BMBF)- and EU-funded project have generated methods and know-how on the characterization, testing and grouping of nanomaterials (NM). From 2020, additional data requirements apply to nanomaterials under the REACh regulation and will require toxicological data of nanoforms of materials. Also for NM, non-animal methods are a priority for data generation under REACh. Recently, a battery of three *in vitro* methods to test for skin sensitization potential was validated and respective OECD test guidelines were published (DPRA [1], LuSens [2], h-CLAT [3], the OECD 442C, D and E, respectively). In BMBF-funded project Aerosafe (031L0128C), the applicability of these *in vitro* methods to test the skin sensitization potential to nanomaterials was evaluated.

Different NM (i.a. CeO2, BaSO4, amorphous SiO2) were tested in three different assays to predict skin sensitization based on the common adverse outcome pathway (AOP) concept 1) protein interaction (DPRA), 2) activation of keratinocytes (LuSens) and 3) activation of dendritic cells (h-CLAT). Homogeneous and reproducible suspension of the NM in culture medium/water (0.05 wt%) were prepared by using cuphorn sonication according to a defined protocol (prewetted with 0.5% ethanol, sonication for 16 min at 400 W). Suspensions were then added to the test systems.

h-CLAT and LuSens assays include cytotoxicity testing. The applicability of the standard MTT cytotoxicity test was investigated. If any interference between NM and read out was expected, WST-8 cytotoxicity assay was performed in parallel. The h-CLAT assay assesses the matured cell population by flow cytometry. High NM concentrations may impair the flow cytometer. Therefore, "density gradient centrifugation" as an additional step in the test item/test system incubation phase (by Ficoll-Paque PLUS) was included in the protocol to separate the NM from the cells before applying to the flow cytometer. Assessment of results in DPRA assay, a cell-free assay, is for NM challenging: NM are applied normally not in solution but in suspension. An assessment is only applicable, when the maximal concentration shows positive results. Due to this fact, in many cases DPRA deliver "inconclusive results" for NM.

In summary, the well-established assays on skin sensitization (DPRA, LuSens, h-CLAT) are applicable also for nanomaterials considering adaptation of the protocols: 1) appropriate test item preparation for all assays, 2) checking and avoiding interference with the cytotoxicity tests with LuSens and hCLAT and 3) density gradient centrifugation to avoid impairment of the flow cytometer with the h-CLAT assay. Since the diversity of nanomaterials is high, further tests are needed to learn more about the applicability of the OECD442ff test battery for nanomaterials [4].

References

[1] Gerberick, G. F. et al. (2004). *Toxicol Sci 81*, 332-343. doi:10.1093/toxsci/kfh213
[2] Ramirez, T. et al. (2014). *Toxicol In Vitro 28*, 1482-1497. doi:10.1016/j.tiv.2014.08.002
[3] Nukada, Y. et al. (2011). *Contact Dermatitis 65*, 343-353. doi:10.1111/j.1600-0536.2011.01952.x
[4] Potter, T. M. et al. (2018). *Methods Mol Biol 1682*, 197-210. doi:10.1007/978-1-4939-7352-1_17

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Using the OECD QSAR toolbox as an *in silico* modelling method for computational aquatic toxicology of endocrine disruptors

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Computational analyses of toxicological processes have enabled high-throughput screening of chemical substances and prediction of their endpoints in biological systems. In particular, algorithms used in molecular taxonomy and property classification have resulted in quantitative structure-activity relationship (QSAR) models [1], which automatically correlate the chemical structures and biological activities of a given chemical database and implement *in silico* screening of a wide range of chemical substances according to their medicinal or toxicological actions [2]. Computational tools calculate predictive toxicity profiles as a function of feature vectors of molecular descriptors (e.g., physicochemical parameters) of chemicals of interest to create categorical or numerical endpoints [3]. In recent years, some of the most highlighted types of toxicants are endocrine disruptors (EDs) (i.e., chemicals that can interfere with any type of hormone-related metabolism) [4]. As EDs may significantly affect animal development and reproduction, rapidly predicting the adverse effects of EDs using *in silico* techniques is required. Demonstrating how to use available *in silico* approaches, such as the OECD QSAR Toolbox, will be an effective way to promote alternative animal testing of urgent chemical issues in aquatic toxicology. In this presentation, we demonstrate a means of computationally assessing the potential risks of EDs in aquatic environments. Utilizing the OECD QSAR Toolbox, we show *in silico* screened results for analysing acute toxicity of EDs in fish. The numerical correlations between the concentration for 50% of lethality (LC50) in 96 h and the octanol-water partition coefficient (Kow) are calculated, and the output performances are presented in which the 96-h LC50 values determined in experiments are compared with those generated by computations. The dependences of Kow and 96-h LC50 on the estrogen receptor binding affinity are also analysed.

References

[1] Najarian, K., Najarian, S. and Gharibzadeh, S. (2017). *Systems Biology and Bioinformatics: A Computational Approach*. 1st edition. CRC Press. ISBN: 9781138118034

[2] Roy, K., Kar, S. and Das, R. N. (2015). *Understanding the Basics of QSAR for Applications in Pharmaceutical Sciences and Risk Assessment*. 1st edition. Cambridge: Academic Press. ISBN: 9780128015056

[3] Raies, A. B. and Bajic, V. B. (2016). *Wiley Interdiscip Rev Comput Mol Sci 6*, 147-172. doi:10.1002/wcms.1240 4

[4] Hayes, T. B. (2005). *Integr Comp Biol 45*, 321-329. doi:10.1093/icb/45.2.321

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Fish acute toxicity: Which data do we need and where do we get these from?

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Originally designed as an alternative for the acute fish toxicity test according to, e.g., OECD TG 203, the fish embryo test (FET) with the zebrafish (*Danio rerio*) has been optimized, standardized and validated during an OECD validation study and adopted as OECD TG 236 as a test to assess toxicity of embryonic forms of fish. Given its correlation with the acute fish toxicity test (AFT) and the fact that non-feeding developmental stages of fish are not categorized as protected stages according to the European Directive 2010/63/EU on the protection of animals used for scientific purposes, the FET was deemed ready for use not only for range-finding, but also as a true alternative for the acute fish toxicity test.

In a recent study conducted by ECHA, however, the potential of the FET test to predict acute fish toxicity, which is required by the Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) regulation (EC 1907/2006), the Biocides Regulation (EU 528/2012) and the Classification, Labelling and Packaging (CLP) Regulation (EC 1272/2008) was challenged (Sobanska et al. 2017): The purpose of this study was to re-analyze the toxicity of substances to fish embryos and to adult fish and to investigate whether certain factors (e.g., physicochemical properties, modes of action, or chemical structures) could be used to define the applicability boundaries of the FET test. The application of a rigid filter procedure to exclude all studies that would not fulfil state-of-the-art requirements, the original data set with 2065 FET studies on a total of 1415 substances was reduced to a final dataset of 156 studies (7.6%) on 123 substances (8.7%). On the basis of this final data set, the authors concluded that, given some limitations (e.g., neurotoxic mode of action) and/or remaining un-

certainties (e.g., deviation of some narcotic substances), the FET test alone is currently not sufficient to meet the essential information on AFT as required by the REACH regulation.

A similar filter set was applied to a data set consisting of 2936 acute fish toxicity (AFT) studies covering a total of 1842 substances. Elimination of (1) studies with LC50 entries not precisely defined, (2) duplications, (3) studies with LC50 values above half the level of water solubility, (4) studies into substances with log POW > 4 with test concentrations not verified by chemical analysis, and (5) inorganic substances resulted in a data set of 1108 studies on 682 substances. Finally, when only zebrafish (*Danio rerio*) studies were considered (as done in the ECHA FET study), the potential for elimination of data from the database was similar for both FET and AFT data. However, the rationale for only using zebrafish data is debatable, because data from other fish species is used for regulatory purposes and also correlates well with FET data (Belanger et al., 2013). Since this new exercise indicates that the robustness of FET and AFT data may be similar, the suitability of the FET for REACH and other regulatory purposes should be re-considered at the science-policy interface, including OECD working groups.

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Establishing *Xenopus laevis* oocytes as a novel model system for blood brain barrier analyses

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Outline

Alternative brain-barrier models to test potential drug uptake mechanisms are currently discussed. As the tight junction between endothelial cells play a central role in the formation of the blood-brain barrier, *Xenopus laevis* oocytes were established for heterologous expression and analysis of tight junction proteins [1]. Claudin-5 is considered the predominant sealing protein of the tight junction in the brain [2]. Therefore, the current study aimed to develop human claudin-5 expressing *Xenopus laevis* oocytes as a model for testing medical substances, as exemplarily demonstrated for sodium caprate. This medium chain fatty acid is an established absorption enhancer for drug uptake in the intestine [3] as is leads to an opening of the paracellular barrier [4].

Methods

Oocytes were collected from adult female African claw frogs in a surgical laparascopy and injected with 1 ng cRNA encoding for human claudin-5, or RNase-free water as controls, respectively. After 3 days, oocytes were devitellinized and clustered in pairs of claudin-5-expressing and control oocytes as follows: cldn5-cldn5 (n = 5-8) and ctrl-ctrl (n = 6-7), respectively. After 24 h, sodium caprate in final concentrations of 50, 100 and 500 µmol or culture medium was added to the oocyte pairs and width of contact area was measured 30, 60 and 120 min after addition via bright field microscopy. The contact area was calculated by using the circle equation ($A = \pi \times$ (measured width/2)2).

Results

Oocyte contact areas showed an initial decrease of contact width after addition of sodium caprate or culture medium in both claudin-5 and control oocytes, respectively. After 60 and 120 min the observed decrease was reversed in all tested combinations with or without the addition of different sodium caprate concentrations. After 30 min claudin-5 expressing oocytes showed a significantly larger contact area compared to water- injected oocytes in caprate concentrations of 50 µmol and 500 µmol ($p < 0.05$, student's t test).

Conclusion

In our study, we employed the heterologous *Xenopus laevis* oocyte expression system to analyze the effect of sodium caprate on claudin-5. An effect of sodium caprate on claudin-5 expressing oocytes was not observed in our current study, which might indicate that additional factors are required. However, with our experimental setup we were able to verify a strengthening effect of claudin-5 expression on the contact area of oocyte pairs.

References

[1] Vitzthum, C., Stein, L., Brunner, N. et al. (2019). *FASEB J 33*, 5312-5319. doi:10.1096/fj.201801451RR

[2] Amasheh, S., Schmidt, T., Mahn, M. et al. (2005). *Cell Tissue Res 321*, 89-96. doi:10.1007/s00441-005-1101-0

[3] Lindmark, T., Nikkilä, T. and Artursson, P. (1995). *J Pharmacol Exp Ther 275*, 958-964. PMID:7473188

[4] Krug, S. M., Amasheh, M., Dittmann, I. et al. (2013). *Biomaterials 34*, 275-282. doi:10.1016/j.biomaterials.2012.09.051

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Initiative for better access and digitalisation to EU agencies safety data

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ECHA (European Chemicals Agency), EFSA (European Food Safety Authority) and EMA (European Medicines Agency) are related to the Committee on Environment, Public Health and Food Safety (ENVI) at the European Parliament (EP). All three agencies play an important role in collecting safety data for all manufactured goods that are introduced to the EU market. In all sectors represented by the agencies, similar types of data (toxicological endpoints) are collected, but they differ in format, transparency and confidentiality level assigned to them. Such differences are not due to legal requirements, but they are mostly linked to EU Agency internal policies. The lack of harmonization has dire consequences for the implementation costs of EU regulations, for the performance of the different industry sectors and for the excessive/redundant use of animals for safety testing. Moreover, the efficacy of the agencies themselves is reduced. This oral presentation intends to sum up some of the current agencies' initiatives (e.g. OpenFoodTox [1]) or collaborative activities (e.g. AMBIT [2]). Last but not least the author will describe the adoption of a pilot project funded by the EP on "Feasibility on a common open platform on chemical safety data [3]" currently led by the European Commission. The goals of the pilot project are to facilitate seamless sharing of data between authorities and provide public access to researchers, regulators, industry and the citizen at large. This will promote: a) transparency and trust in EU decision making, b) research and data analytics, c) innovation d) less animal testing & more predictive toxicology, and e) better regulatory decision making and informed consumer choices.

References

[1] https://data.europa.eu/euodp/data/dataset/openfoodtox-efsa-s-chemical-hazards-database
[2] http://cefic-lri.org/toolbox/ambit/
[3] http://www.europarl.europa.eu/doceo/document/BUDG-DT-634494_EN.pdf?redirect (budget line 07 02 77 50)

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Make scientists better: Altertox Academy mission

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Altertox Academy connects international experts to provide hands-on-training in human-relevant alternative methods and technologies for toxicologists of all levels of experience, from entry level technician (BS) to laboratory or department manager (MS/PhD). The participants will become familiar with new technologies and their critical steps.

Like "a picture is worth a thousand words", a hands-on-training is a perfect way to quickly approach a method, understand it with all tiny details that the trainer will share with the participants. The training allows not only to understand methods but also the data analysis that could be a critical step when submitting results to regulators.

Since several years, Altertox Academy has organized more than 30 hands-on-trainings with a format allowing a detailed and practical description of the methods (the training plan includes 20% lectures and 80% hands-on-training or case studies).

With a maximum of 15-20 participants, divided in small groups for practical parts of the training, it allows networking and connect experts to people that will daily use their method. After one of our training a participant said: "The group size was also very good to stimulate discussion and work in focused groups on the case studies. There was also sufficient time spent on the sessions to allow discussion and interaction."

Focused on alternatives to animal testing, the topics covered by our trainings are: *in silico* methods (Endocrine disrupting compounds, *in silico* models for cosmetics) *in vitro* methods (lung inhalation, skin sensitization, hepatotoxicity, proarrhytmia cardiac assay and more).

Promoting education and training brings improvement in scientists' day-to-day work and can also have a positive impact on the general scientific community. Participating in our trainings will improve your skills for a specific method, make you know theirs limitations, and give you the capacity to challenge the tests and interpret data.

Altertox Academy follows the mission to make scientists better, also by organizing once or twice a year training for young researchers ("Skills 4 Science"). At this occasion, they have the opportunity to meet their peers and face the challenges of oral presentations and posters as well as grant writing skills, lab skills, oral communication and paper writing skills.

Reference
Busquet, F. and Vinken, M. (2019). *Toxicol In Vitro 59*, 51-54. doi:10.1016/j.tiv.2019.04.006

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Modelling the human blood-brain-barrier microvasculature and nanocarrier transport on a microfluidic chip

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The blood-brain-barrier (BBB) is a highly selective barrier that help to maintain brain homeostasis; however, it also represents a near-impenetrable hurdle against the delivery of therapeutics to the central nervous system. Since only small compounds can cross the BBB, this hinders most neuropharmaceuticals from eliciting a desired dose and effect, reducing treatments available for neurodegenerative diseases and cancer [1]. Polymer nanoparticles (NPs) have emerged as a potential solution for delivering therapeutics across the BBB to brain targets. One of the problems that has slowed down the development and approval after clinical trials of new drug candidates for brain diseases is the lack of preclinical models that accurately reproduce the human BBB. Indeed, current models such as *in vitro* transwells or mouse models fail to reproduce the anatomical complexity of the human BBB [2]. For these reasons, an innovative and reliable *in vitro* BBB model that adequately reflects the human *in vivo* morphologies and characteristics is required. Moreover, as transport across the BBB represents the first evidence of NP delivery capabilities, the development of an *in vitro* testing platform and method for quantifying NP transport behavior provides an invaluable tool to assess therapeutic efficacy. To address these limitations, a microfluidic *in vitro* microvascular model of the human BBB was developed, containing human induced pluripotent stem cell-derived endothelial cells, brain pericytes, and astrocytes supported in 3D fibrin gel matrix as self-organized microvasculature. The microvascular BBB model was developed via vasculogenesis to accurately replicate the *in vivo* neurovascular organization. The microvasculature of the BBB model was perfusable within 5-7 days, showing permeability coefficient comparable to previous *in vitro* models and similar to *in vivo* measurements in rat brain [3]. Gene expression of tight and adherent junctions (ZO-1, occludin, and claudin-5), extra-cellular matrix proteins (Laminin and Collagen IV), and membrane transporters (PG-P, LAT1, LRP1) was higher in tri-culture condition consistently with quantitative immunocytochemistry analysis indicating maturation and differentiation into more BBB-like structure. Microvessel-pericytes/astrocytes con-

tact-interactions were validated using laser confocal microscopy. When pericytes and astrocytes were included to form microvasculature, the vascular parameters such as vascular diameter, branches length and vascular network area coverage became lower compared to mono-culture of endothelial cells. This revealed that morphological changes were induced by not only the secretion of pro-angiogenic and vasculogenic growth factors but also contact signaling between cells. After characterization, this established 3D *in vitro* model of the human BBB was preliminarily exploited to evaluate nanocarrier permeability such as nanoparticles. Indeed, ongoing experiments are showing that the 3D BBB model might be capable to elucidating differences in 3D transport between Polymeric NPs compared to Transwell assays. This robust and translational BBB microvascular model could be potentially applied to patient-specific and neurodegenerative diseases modelling [4], offering a novel platform to study both drug candidates transport as well as neurovascular functions within a physiologically-relevant BBB microvasculature. This is the first version of an innovative BBB model that potentially might change how therapeutic compounds are designed and transported across the human BBB, reducing and refining the use of animal models.

References

[1] Pardridge, W. M. (2016). *Expert Opin Drug Deliv 5247*, 1-13.
[2] Crawford, L. et al. (2016). *J Control Release 240*, 251-266.
[3] Campisi, M. et al. (2018). *Biomaterials 180*, 117-129.
[4] Osaki, T. et al. (2018). *Sci Rep, 8*, 5168.

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In vitro models of human cardiac fibrotic tissue based on bioartificial scaffolds

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Cardiomyocytes represent 70-85% of cell population in the adult heart and are the main responsible of the cardiac contractile activity. Myocardial infarction causes the loss of billions of cardiomyocytes and the progressive formation of fibrotic scar. Fibrotic tissue is mechanically stiffer than healthy cardiac tissue, and is mainly populated by cardiac fibroblasts, unable to undergo contraction [2]. *In vitro* models of infarcted tissue represent a key tool to evaluate new therapies for cardiac regeneration. In this work, a model of fibrotic heart tissue was designed and fabricated by culturing human cardiac fibroblasts (HCFs) on bi-dimensional (2D) and three-dimensional (3D) bioartificial scaffolds.

Polycaprolactone (PCL, Mw = 43,000 Da) 2D scaffolds were prepared by electrospinning from chloroform/formic acid (70/30 v/v) solutions to obtain fibrous membranes with both aligned and random morphology. PCL 3D scaffolds with different geometries were produced by melt-extrusion additive manufacturing technique, using a hybrid bio 3D printer (Rokit Invivo). Subsequently, scaffolds were functionalised with gelatin through a mussel-inspired approach based on two steps: (i) 3,4-Dihydroxy-D,L-phenylalanine (DOPA, 2 mg/mL in Tris/HCl 10 mM, pH 8.5) polymerisation on the PCL surface; (ii) incubation in gelatin solution for 16 h [3].

HCFs isolated from human ventricle were cultured at a density of 7×10^4 and 30×10^4 cells/cm^2 on 2D and 3D scaffolds, respectively. Their survival, adhesion, proliferation and morphology were investigated through biochemical assays and fluorescence microscopy analyses.

SEM showed that 2D PCL-scaffolds consisted of homogeneous nanofiber membranes, while 3D PCL-scaffolds presented a reproducible interconnected porous structure. QCM-D analysis was performed to follow the functionalisation steps: polyDOPA deposition followed by gelatin grafting. ATR-FTIR and colorimetric assay confirmed successful surface modification for both 2D and 3D scaffolds. Bulk properties did not change after the surface modification, as suggested by unchanged mechanical (tensile stress-strain test) and thermal (DSC analysis) properties. HCFs cultured on gelatin grafted scaffolds showed better attachment and proliferation compared to non-functionalized scaffolds, as well as the deposition of cardiac extracellular matrix (ECM). Moreover, HCF morphology and F-actin expression were investigated as a function of morphology and surface composition.

In conclusion, biomimetic scaffolds able to support the proliferation of HCFs were developed and proposed as promising models of human cardiac fibrotic tissue. The effect of scaffold properties (composition, structure and surface mechanical properties) on the expression of fibroblast markers (α-SMA, Vimentin, DDR2), ECM composition and on direct cardiac reprogramming to cardiomyocytes will be evaluated. Moreover, the obtained models will be validated through the comparison with human cardiac tissue and used to test *in vitro* new cardiac regenerative strategies, e.g. direct cardiac reprogramming.

References

[1] Ponikowski, P., Anker, S. D., AlHabib, K. F. et al. (2014). *ESC Heart Fail 1*, 4-25. doi:10.1002/ehf2.12005

[2] Porter, K. E. and Turner, N. A. (2009). *Pharmacol Ther 123*, 255-278. doi:10.1016/j.pharmthera.2009.05.002

[3] Nardo, T. Chiono, V., Ciardelli, G. et al. (2016). *Macromol Biosci 16*, 288-298. doi:10.1002/mabi.201500241

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CALT-BIO's efforts to promote alternative methods in China

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In China, the driving force for alternative methods be widely accepted is inseparable from the changes of regulatory. The bottom-up strategy from industries application to national research and validation is the positive pathway to influence the changes in government regulations. considering the China's situation, CALT-BIO is now as a leader of non-animal testing institute in china, which was established in 2010, has made countless efforts in this nearly 10 years. CALT-BIO has insisted on organizing an annual conference of alternative methods in China. The ninth conference will be held in November at Shanghai this year. More than 300 participants and 50 companies will attend the conference, which is by far the largest alternative method seminar in China. At the same time, in order to promote the practical application of non-animal testing in the industry, CALT-BIO actively introduced the non-animal testing method of OECD and ECVAM, carried out several technical training courses in the country to promote non-animal testing methods. In addition to influencing the industry to promote the 3R concept, we also realized that education is the key to the future development of 3R. Therefore, CALT-BIO also established alternative method courses with Shanghai Jiaotong University and Sichuan University to educate college students about 3R concepts and non-animal test method theory. Education is inseparable from textbooks, so CALT-BIO has also participated in the publication of several books on the application of alternative methods, including the "Guides of Alternative Methods Standards for Cosmetics Assessment", which is currently used by most cosmetic engineers in China. CALT-BIO, the gateway between China and the world for non-animal testing, brings the latest developments and technologies for non-animal testing around the world to China. It will see up the organ-on-chip platform cooperation with TISSUSE and Shanghai Jiaotong University in 2019 and will conduct test and research service for industries in China. In general, CALT-BIO has been insisting for nearly 10 years to promote the science and technology development of the 3R concept in the Chinese industries, it also insists on the bottom up approaches is an effective way for regulation changes.

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3D CNS model of iPSCs derived neuron and glia for high-throughput neurotoxicity screening in Mimetas' OrganoPlate®

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Prediction of neurotoxicity remains challenging due to the lack of relevant models of the human brain. Current neurotoxicity assessments rely heavily on expensive and time-consuming *ex vivo* and *in vivo* animal testing. They are not always predictive for human outcome and not amenable for high-throughput toxicity screening. Here, we describe the development of a human *in vitro* 3D CNS model in the OrganoPlate® for neurotoxicity screening. Mimetas' OrganoPlate® are microfluidic cell culture plates that enable culturing and screening of a range of miniaturized 3D organ and tissue models.

Human iPSCs derived neuron and glia are embedded in ECM and seeded into the OrganoPlate® allowing formation of complex 3D neuronal network in 96 individual chips. Medium in the adjacent compartment provided the cultures of nutrients and growth factors by diffusion. This model supports the assessment of various neurotoxic endpoints in 3D and is applicable for functional readouts addressing physiological and morphological criteria that leads to improved neurotoxicity screening of compounds and safety assessment in early stages of drug development.

Complex network formation of neurons and astrocytes is seen within 24 hours and immunofluorescent staining confirms the presence of mature neurons, including GABAergic and glutamatergic sub-population and supporting astrocytes. Application of neurotoxicant, methylmercury, demonstrates concentration-dependent reduction in the integrity of the neurites and cytotoxic effect is further identified using mitochondrial toxicity assay and cell viability assay.

Spontaneous firing of the co-culture is seen after 4 days of culture using calcium imaging assay and changed upon stimulation of inhibitory and excitatory compounds like GABA and TTX. To further demonstrate the applicability of the CNS model for assessing seizure liability, we performed compound library screening of potential convulsant compounds. The burst pattern of each single detected neuron in the culture was extracted, including the number of bursts, burst intensity and burst duration. Exposure to the compounds show increased bursting pattern and induced synchronicity in the co-cultures, while anti-seizure compounds was able to inhibit the increased activity. This CNS-on-a-chip model provides promising usability of iPSCs derived neuronal co-culture models for screening purposes. This paves the way towards the development of a predictive and relevant brain model which could be used for high-throughput neurotoxicity studies, including seizure liability testing and can contribute to diminishing the use of animal models.

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Animalstudyregistry.org – a federal database for preregistration of animal research

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The reproducibility of results gained from animal experiments and their extrapolation to humans is currently in focus of the scientific discourse. Reporting bias, HARKing (Hypothesizing after Results are Known) and p-hacking have been identified as major factors contributing to the reproducibility crisis. As greater transparency of animal experiments is requested both by the scientific community and the public, the German Centre for the Protection of Laboratory Animals (Bf3R) has launched a preregistration platform for animal studies, animalstudyregistry.org. The registry is designed for exploratory and confirmatory studies within the scope of fundamental and preclinical research. The registration form serves as a checklist for the researchers, helping them to plan the experiments thoroughly and making sure they won't miss any crucial points concerning study design, methods or statistical planning. Every study registered in the Animal Study Registry receives a DOI, which marks it as intellectual property of the researcher. As an additional measure to protect the contents from the theft of ideas, it's also possible to restrict the visibility of a registered study for a period of up to 5 years. During this embargo period, only a short summary is accessible in the data base.

The Bf3R is part of the Federal Institute for Risk Assessment (BfR), a scientifically independent federal institution, which guarantees continuity.

Registering a study in the Animal Study Registry enables editors, third party donors and the general public to see researcher's commitment to transparency and to quality of scientific data.

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Skin-on-a-chip-based skin irritation evaluation method as an alternative to animal testing

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Recently, the trend in animal testing needed for drug screening and etc. is transferring into applying alternative test methods, prohibiting animal testing throughout the world. When test substances were evaluated *in vivo*, Draize scores based on edema and erythema were used to classify irritants. With an expanding demands of non-animal tests method, many *in vitro* alternative methods have been developed and recommended to be used for classifying irritant and non-irritant substances. However, current *in vitro* models for evaluating skin irritation only measure cell viability. Thus, development of biochip was needed for mimicking *in vivo* skin irritation. After researching on both domestic and international trends in biochip, skin-on-a-chip-based irritation evaluation method was developed. The microfluidic device was fabricated using PDMS and porous membranes were placed between each layer to separate the chambers as demonstrated in previous study [1]. Skin on a chip consists of three layers for keratinocyte, fibroblast and endothelial cell culture, representing epidermal, dermal, and endothelial layers of human skin structure. Test substances were applied to the top chamber and the chip was evaluated for skin irritation hazard identification. Irritation evaluation method includes observing the cell viability and the tight junction. Cell viability was measured adapting the OECD TG439 protocol for chemical evaluation. Furthermore, cell-to-cell junctions, or tight junctions, in endothelial cells were observed and measured to assess physiological responses, such as edema, to chemicals. Total of 20 chemicals were evaluated using skin on a chip which was then compared with LLNA *in vivo* data. Comparison between *in vivo* data and skin on a chip method for evaluating chemicals resulted in 90% accuracy, 100% sensitivity, and 78% specificity for irritation evaluation. Therefore, developed skin on a chip could serve as a momentum for a cutting-edge integration of *in vitro* toxicity evaluation methods and the result of this research suggests that biochip could better represent *in vivo* or human physiology for evaluation of skin toxicity.

Reference

Wufuer, M., Lee, G., Hur, W. et al. (2016). *Sci Rep 6*, 37471. doi: 10.1038/srep37471

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Using big data for developing QSAR models to predict interaction of chemicals with neuronal proteins

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The *in vitro* high-throughput screening (HTS) of cellular and protein elements allows one to measure the biological activity of large libraries of chemicals in a cost efficient and timely manner. HTS has generated an enormous volume of data that has already been collected and stored in a variety of public databases, such as the PubChem and ChEMBL. Although HTS programs provide a significant amount of information about the biological activities for thousands of chemicals, a great deal of information for millions of chemicals still remains missing. Computational methods in combination with HTS data, offer a great opportunity to address this information gap and to provide hypotheses for future mechanistic experiments. Quantitative Structure-Activity Relationships (QSARs) are analytically powerful methods for estimating physicochemical and biological properties of a chemical from its molecular structure. QSAR modeling is a data-driven approach as it relies on a dataset of biological responses for a training set of chemicals and uses machine learning techniques to correlate a set of molecular descriptors of these chemicals to their activity.

Neurotoxicity is initiated by the interaction of a chemical ligand with one or multiple neurological proteins. The recently introduced framework of the adverse outcome pathway links this molecular interaction (Molecular Initiating Event) with a series of key events on different biological levels that result in an adverse outcome effect. We mined several public data sources (ChEMBL, PubChem, BindingDB and ExCAPE-DB) and extracted active and inactive chemicals for 8 major neuronal targets: dopamine receptor D2 (DRD2), serotonin receptor 1a (HTR1A), opioid receptors (OPRD1 and OPRK1), muscarinic acetylcholine receptor (CHRM1), metabotropic glutamate receptor (GRM4), dopamine transporter (SLC6A3) and voltage-gated calcium channel (CAC-

NA1B). The selected protein targets have from 600 to > 4000 active compounds, and from several thousand up to a million inactive compounds. Two sets of data were generated: one set with both active and inactive compounds was used for classification, while the second dataset containing affinity values (Ki) for active compounds was strictly used to develop regression models to prediction Ki values for unknown chemicals of interest. In the present study we used the Online Chemical Modeling Environment (http://ochem.eu) to develop highly accurate QSAR models for selected neurological proteins. The developed models were validated against a set of chemicals that were not used in model development. The classification results showed 90-99% accuracy for the training and validation sets, while the predictions of Ki demonstrated accuracy with R^2 in the range of 0.6-0.77 depending on the neurological target. Our results demonstrate that *in vitro* HTS data and the QSAR modeling approach, can be used to identify and predict interaction of chemicals with neurological targets.

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Biodegradable polyphosphazene scaffolds demonstrate biocompatibility in MucilAir-fibroblast-cocultures

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A new biodegradable bio-inorganic scaffold consisting of polyphosphazenes was tested for biocompatibility in MucilAir-fibroblast-cocultures. Polyphosphazene is a polymer consisting of a phosphate-nitrogen backbone. It exhibits two chlorine functions per monomer that allow for substitution with amino acids and derivatives, drugs, targeting moieties, solubilizing groups and adjustment of material properties such as biodegradability, pH sensitivity, mechanical characteristics.

Scaffolds with a diameter of 6 mm and a thickness of appr. 500 µm were prepared according to Rothemund et al. They were produced with and without covalently bound glutathione to enhance hydrophilicity.

To test the biocompatibility of the material MucilAir-fibroblast-cocultures were incubated in inserts in medium conditioned by eluting one part of scaffold material in 100 parts of culture medium for three days.

The conditioned medium was applied either (i) basally or (ii) apically to the culture system. To test as well (iii) solid exposure of the material, sterile scaffolds were placed apically to the surface of the tissues. Cultures without conditioned medium and cultures without solid exposure served as controls.

Biocompatibility endpoints were tested (i) transepithelial electrical resistance (TEER) to demonstrate physiological barrier function, (ii) cilia beating frequency (CBF) to reveal physiological functionality of the tissues, (iii) LDH release to show integrity of cellular membranes and (iv) Interleukin-8 (IL-8) release to test induction of inflammation.

No differences between controls and the two scaffolds conditions were observed regarding the route of application and all biocompatibility endpoints with exception of application of the solid material and IL-8 release. Since the application of the solid material did neither affect TEER, CBF and LDH release the increase is considered being due to mechanical deterioration of the tissues.

The results demonstrate that polyphosphazene scaffolds can be regarded as biocompatible since they do not change physiological functions like barriers and cilia beating, and do not affect cellular membrane integrity and secretion of inflammatory mediators. This result does not only recommend the material as basis for scaffolds for tissue engineering, its biodegradability recommends the material also for applications in medical devices.

Reference

Rothemund, S., Aigner, T. B., Iturmendi, A. et al. (2015). *Macromol Biosci 15*, 351-363. doi:10.1002/mabi.201400390

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Developing a new simulator of the rat for laboratory animal training courses using 3D printing

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Introduction
In accordance to the Directive 63/2010/EU courses involving rats and mice are classified as animal experiments [1]. As the directive additionally demands the implementation of the 3Rs, simulators of the rat and mouse were developed, intended to serve as an initial training device for various techniques, prior working on live animals. Nevertheless, only little is known about the simulators' general suitability and anatomically correctness. For this reason, the research project "SimulRATor", consisting of a team of anatomists, laboratory animal scientists, epidemiologists and an engineer with a medical background, currently evaluates all six commercially available rat and mouse simulators. In this multi-perspective approach, amongst other things, their anatomical correctness in comparison with live rats, as well as their robustness and functionality will be determined. In parallel to the evaluations, the basic framework and structure of the simulator is virtually designed and test prints are made. This evaluation will help to define technical and structural specifications needed for the development of a new, anatomically and haptically realistic and cost-effective 3D-printed simulator of the rat, which will enable the training of all relevant practical techniques.

Materials and methods
For the creation of the required 3D model, already existing computed tomography scans of a 12-month-old Wistar rat were used. To create a virtual and printable 3D model, various software such as Slicer, Blender and CATIA V5 were used. The anatomy, posture and functional elements have been modified and bone defects caused by the segmentation algorithm have been corrected manually. 80% of the simulator parts will then be produced using the 3D-printing technology. To achieve the different haptical characteristics of the rat, Multi-Jet-Modelling (MJM, Stratasys J750)

was the choice out of a high variety of techniques. This enables the combination of different types of soft and hard materials within the building process.

Results
The initial evaluations confirm specific technical and structural specifications needed for the development of the new, anatomically and haptically realistic simulator of the rat. The simulator, which was designed in accordance with the results of the initial evaluations, is currently under construction. To determine its haptic, some test prints have already been performed and both hard and soft materials were processed. The material spectrum currently available in the field of additive manufacturing, is not suitable for the production of a soft, flexible and durable structure, since a degree of softness cannot be undercut. Therefore, especially for the construction of the skin, a conventional casting technique by mold-modelling using silicone rubber is used. Besides the skin, all remaining structures will nevertheless be produced by means of MJM printing, in order to achieve the most realistic possible haptic.

Reference
[1] Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes (2010).

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Next generation risk assessment of coumarin in personal care products

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Next Generation Risk Assessment (NGRA) is defined as an exposure-led, hypothesis-driven risk assessment approach that integrates one or more new approach methodologies (NAMs) to ensure the safety of materials without the use of animal testing data. The International Cooperation on Cosmetics Regulation (ICCR) principles [1] were applied to assess the safety of hypothetical consumer products (face cream and shampoo) containing 0.5% coumarin. For the purpose of evaluating the use of NAMs, all existing animal and human data on coumarin were excluded. Exposure calculations using specific consumer habits, *in vitro* plasma protein binding and clearance data were used to build a physiologically based kinetic model for dermally applied coumarin. For the systemic toxicity assessment, a battery of *in vitro* NAMs were used to identify points of departure (PoDs) for a variety of biological effects such as genotoxicity (ToxTracker®), receptor-mediated and immunomodulatory effects (Eurofins Safety44™ screen and BioSeek® Profiling, respectively), and non-specific pathways/general bioactivity [ToxCast data, *in vitro* cell stress panel and high-throughput transcriptomics (HTTr)]. A novel statistical Bayesian approach was applied to the cell stress panel, HTTr and Toxcast dose-response data. The PoDs from the *in vitro* assays identified as demonstrating a dose response were plotted against the calculated consumer exposure (Cmax with associated uncertainty) in order to calculate a margin of safety (MoS). From these results, we concluded that coumarin is not genotoxic, does not bind to any of the 44 receptors, and does not show any immunomodulatory effects. The predicted Cmax values for face cream and shampoo were lower than the all PoDs. However, the lower predicted Cmax for shampoo results in a MoS that can be more confidently used to assure safety. Further refinements to the risk assessment are discussed. This case study demonstrates the value of integrating exposure science with computational modelling and *in vitro* bioactivity data that form the basis of non-animal safety assessments.

Reference

[1] Dent, M. et al. (2018). *Comput Toxicol 7*, 20-26.

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Biotransformation of antifungal azoles in rainbow trout cell lines

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Environmental risk assessment of chemicals requires the characterization of the exposure and of bioaccumulation. In aquatic ecosystems, exposure is mainly based on predicted or measured environmental concentration that is not representative of the actual dose at the target tissue where they can trigger an adverse effect. In this context, there are growing investigations to understand the toxicokinetic of environmental chemicals into aquatic organisms. In particular, *in vitro* cell lines appear as a good alternative to investigate bioaccumulation and elimination of organic contaminants, allowing extrapolation to the full organism level by implementing mechanistic models [1]. Nevertheless, only few studies have focused on the biotransformation capacity of these cell lines [2]. Therefore, in the present study, we investigated the biotransformation of three antifungal azoles (i.e. prochloraz, tebuconazole and propiconazole) in two rainbow trout cell lines (liver RTL-W1, gill RTgill-W1). These azoles are detected in aquatic ecosystems, in particular in aquatic organisms [3] and are described as endocrine disruptors and also able to alter fish growth [1,4,5]. Thus, there is a need to better understand what is the fate of these chemicals and their biotransformation products (bioTPs) in organisms and how this links to effects. After preliminary experiments dedicated to the determination of non-toxic concentration [6], a 24 h exposure was performed in 24 well plates in order to identify transformation products of the three investigated chemicals in the two cell lines. To this end, we implemented a high-resolution mass spectrometry workflow allowing to check for predicted and already known bioTPs related to these chemicals. As previously reported in invertebrates [7], we identified more transformation product candidates for prochloraz than for tebuconazole or propiconazole. These englobe mainly oxidation products although the structural identification of these candidates is still ongoing. These data will be further used together with a PBTK model to extrapolate the toxicokinetic parameters at the whole organism level in fish.

References
[1] Stadnicka-Michalak, J., Schirmer, K. and Ashauer, R. (2015). *Sci Adv 1*, e1500302.
[2] Stadnicka-Michalak, J. et al. (2018). *Environ Sci Technol 52*, 3091-3100.
[3] Munz, N. A. et al. (2018). *Environ Sci Technol 52*, 10347-10358.
[4] Matthiessen, P. and Weltje, L. (2015). *Crit Rev Toxicol 45*, 453-467.
[5] Chen, Z. F. and Ying, G. G. (2015). *Environ Int 84*, 142-153.
[6] Stadnicka-Michalak, J. et al. (2018). *ALTEX 35*, 37-50.
[7] Rosch, A., Anliker, S. and Hollender, J. (2016). *Environ Sci Technol 50*, 7175-7188.

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Simulating an arthritic joint *in vitro* by combining multiple tissue components

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Our ultimate goal is to study the influence and efficacy of potential drug candidates in an experimental setting of arthritis. Therefore, we aim to develop a valid human *in vitro* 3D joint model mimicking features of joint inflammation by applying inflammatory conditions namely immune cells and pro-inflammatory cytokines. The *in vitro* 3D joint model consists of different components including an osteogenic and chondrogenic part, the joint space with synovial fluid and the synovial membrane. As an alternative experimental setup for animal models, our *in vitro* 3D joint model will enable us to study the influence and efficacy of potential drug candidates.

Based on human bone marrow derived mesenchymal stromal cells (hMSCs), we developed the different 3D tissue components of the joint that were characterized in detail (e.g. cell vitality, morphology, structural integrity) using histological, biochemical and molecular biological methods, μCT and scanning electron microscopy. To mimic the 3D osteogenic component, we populated β-tricalcium phosphate (TCP) – mimicking the mineral bony part –, while the 3D cartilage component was generated by cellular self-assembly without scaffold (fzmb GmbH). Osteogenic differentiation was verified demonstrating an increase in mineralized bone volume and the induction of bone-related gene expression (*RUNX2, SPP1* and *COL1A1*) as compared to the corresponding control using μCT and quantitative PCR. Chondrogenic phenotype was verified by HE and Alcian Blue staining as well as by the reduced expression of *COL1A1* and an abundant

expression of *COL2A1*. Interestingly, co-cultivation of the osteogenic and the chondrogenic part for up to 3 weeks demonstrated colonization, connectivity and initial calcification implying a functional transitional bridging area. As synovial membrane, we successfully and reproducibly created a viable confluent hMSC-layer for up to 3 weeks of cultivation on top of the cavity filled with hyaluronic acid. To simulate the inflamed joint, we applied sorted CD15^{+} cells on the membrane and injected cytokines such as TNF-α, MIF and IL-6 into the synovial fluid. In response, the simulated synovial membrane showed a significant upregulation of *IL8*, *IL6* and *MMP13* expression on mRNA level. Furthermore, during co-cultivation the MSCs enhanced the survival of CD15^{+} cells.

The results of the single components analysis confirmed viability, integrity and morphology pointing towards an ultimately successful development of the anticipated *in vitro* 3D model. By combining multiple components in a standard 96 well format, we aim to provide a mid-throughput system for preclinical drug testing as well as a valid *in vitro* human-based 3D disease model to study the pathogenesis of arthritis.

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From a 3Rs intent to a company's strategy: Perspective from the industry

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GSK identifies, develops and manufactures innovative medicines, vaccines and consumer healthcare products. Animal studies, conducted with high standards of humane care and treatment, represent a small but vital part of our procedures to develop and release vaccines. GSK's ethical concerns have inspired a company-wide effort to reduce animal use by developing non-animal technologies (NAT) to test the safety and efficacy of new and existing vaccines. In line with this 3R commitment, GSK also actively engages in collaborative studies to promote 3R and to develop and validate NAT that can provide alternatives to the use of animals.

To ensure a meaningful reduction, systematic review on the overall animal use in Vaccines is performed annually. 3R driven by a strong focus on replacement guides our global vaccines strategy. Following our current internal 3R program, we expect to reduce the animal use in Quality Control for Vaccines by 75 percent by 2025.

As part of this strategy GSK shares practices and activities with regulatory authorities as soon as a NAT is considered, to engage and align all parties in a consolidated and joined effort to reduce animal use while ensuring the safety of the patient.

As an example, GSK recently replaced the standard rabbit pyrogen test for Menveo vaccine by the monocyte activation test (MAT) performed in human blood assays. This effort led to a reduction of animal use, but also to a faster release of vaccines for our patients. GSK also developed a couple of years ago an internal position paper describing the company's position regarding the suppression of the General Safety Test (GST), demonstrating the willingness of the company to be pro-active in terms of 3Rs.

While continuing to work toward an era of non-animal-based research and development, GSK remains committed to acting ethically and practicing good animal welfare when animal use is still required.

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Development of an *in vitro* trabecular human bone model integrated in a perfusion system to simulate glucocorticoid-induced osteoporosis

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Bone homeostasis is characterized by a balanced, dynamic and continuous remodeling process which consists of bone formation by osteoblasts differentiating into matrix mineralizing osteocytes and of bone resorption by myeloid multinuclear giant osteoclasts. Bone homeostasis is debalanced during osteopetrosis and osteoporosis to either more bone formation or more bone resorption. The latter case can be a result of aging-related hormonal changes but also due to long-term treatment with anti-inflammatory glucocorticoids, which are most commonly used to treat patients with e.g. autoimmune diseases. The ultimate goal of our project is to simulate the process of bone homeostasis *in vitro*. Therefore, we aim to (i) develop an *in vitro* trabecular human bone model, (ii) integrate the bone model in a perfusion system to provide biomechanical load and (iii) mimic glucocorticoid-induced osteoporosis by the application of prednisolone.

Here, we present our preliminary results on the differentiation of osteoblasts and osteoclast in a 3D trabecular environment and its integration into a perfusion system.

Firstly, incubation conditions for osteogenic differentiation of primary human bone-marrow-derived mesenchymal stromal cells (hMSCs) were optimized. To this end, hMSCs were treated with osteogenic medium and mineralization of hMSCs was evaluated by Alizarin-red staining. Under these qualified incubation conditions, hMSCs were seeded on 3D β-tricalcium phosphate (βTCP) mimicking the trabecular bone matrix under perfused conditions and analyzed for viability by Live/Dead staining and osteogenic gene expression (RUNX2, OSX). Secondly, incubation conditions for osteoclastogenic differentiation of primary human peripheral CD14+ monocytes were established. To this end, monocytes, MACS™-purified from buffy coats, were differentiated into osteoclasts using following protocol: incubation for 3 days with 25 ng/ml M-CSF followed by an 18-day incubation with M-CSF and 50 ng/ml RANKL. Osteoclast differentiation was determined by immunofluorescence staining. Under these qualified incubation conditions, osteoclasts were differentiated on βTCP and analyzed according to viability by Live/Dead staining and to morphology by scanning electron microscopy. Differentiation conditions for both osteogenesis and osteoclastogenesis could be optimized and verified by either positive alizarin red in terms of osteoblasts/osteocytes or by the identification of osteoclasts as multinucleated giant cells positive for DAPI and ß-actin as well as being positive for TRAP and Cathepsin K expression. 3D-incubation of hMSCs on βTCP within a perfused system demonstrated an unaltered viability and an induction of osteogenic gene expression as compared to osteogenic differentiated hMSC in monolayer. We were able to populate the βTCP scaffold with monocytes which were differentiated into osteoclasts (morphological changes, e.g. formation of filopodia) without any effect on cellular viability.

The first results of our approach are promising and in the next steps, we will focus on the co-cultivation with optimization of incubation conditions. By combining several cell types with a suitable scaffold and biomechanical load (perfusion), we ultimately aim to provide a valid and physiological and pathophysiological relevant trabecular bone model to simulate bone homeostasis and GC-induce osteoporosis.

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MTS application to Italian veterinary autogenous vaccines as alternative *in vitro* method to assess cytotoxicity: Preliminary data

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Experimental Zooprophylactic Institutes are public companies operating within the National Health Service that guarantee the technical support and scientific collaboration in hygiene and public veterinary health. As it stated in Italian Ministerial Decree [1], Experimental Zooprophylactic Institute Umbria and Marche has the authorization for the production of veterinary autogenous vaccines and auto-vaccines, avian and bovine veterinary diagnostic reagents and keeps vaccines stocks for emergencies. Vaccination is a control method to prevent bacterial disease spreading and is a valid tool to overcome the antibiotic-resistant conditions. In the vaccine production process, the Quality Control (QC) testing is necessary to ensure the product effectiveness and safety. The abnormal toxicity test (ATT) *in vivo* was developed, in the early 1900s, as an auxiliary assay to ensure safe and consistent antiserum production, then was applied as a quality control release test for vaccines but the test involve large numbers of animals with significant pain and distress. Nowadays the ATT test has no longer been mandatory in the monographs of the European Pharmacopeia [2] and in other regulatory requirements because of the increased ability of manufactures to control and analyze the production process of reagents with advanced technologies. In line with the 3Rs philosophy (replacement, reduction, and refinement) of animal testing in scientific purposes [3], new validated *in vitro* methods are requested, able to gradually substitute *in vivo* assays. Cell-based colorimetric assays are often used for screening collections of compounds to determine if the molecules tested have effects on the cell proliferation or show direct cytotoxic effects that lead to cell death. According to these new safety issues, we evaluated the cytotoxicity of six different veterinary autogenous vaccines produced at Pharmaceutical Unit, by a colorimetric end-point dilution assay (MTS test). Among different tetrazolium compounds we choose [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium-inner-salt;MTS] with electron coupling reagent (phenazine-ethosulfate;PES), which is effective, simple and rapid. Mouse fibroblast L929 cells were incubated with vaccines and adjuvants in two-fold dilution for 24 h, then the MTS substrate was added and the absorbance was read at 492 nm after 2 hours. Cell viability was evaluated comparing with an untreated and a positive control, in order to assess the percentage of metabolic active cells. A progressively positive metabolic effect was recorded as the vaccine dilutions increased, ranging from 1:64 for the vaccine against ovine lameness to 1:256 for the vaccine against ovine colibacillosis infection. To our knowledge, this is the first report of MTS test dilution endpoint assay applied to veterinary autogenous vaccines, according to 3Rs principle. These preliminary results represent an interesting point to continue the study of QC in autogenous vaccine with alternative *in vitro* methods that combined with the traditional *in vivo* assays, could determine a substantial refinement and reduction in the use of animals. Further tests are necessary to obtain solid results, in line with the consistency approach, based on vaccine's characterization during development. The final prospective is the standardization of protocols and the application of validated alternative test.

References

[1] DECRETO 17 marzo 1994, n. 287. Regolamento recante norme sulla produzione, l'impiego ed il controllo dei medicinali veterinari immunologici inattivati, aventi caratteristiche di vaccini stabulogeni ed autovaccini.
[2] EDQM: Updated Work Programme of the European Pharmacopoeia (June 2016).
[3] Directive 2010/63/EU of the European Parliament and the Council on the protection of animals used in scientific purposes. *OJ L 276,* 33–79.

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Thematic review of animals used for scientific purposes: A mechanism for advancing replacement

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Article 58 of Directive 2010/63/EU instructs that "periodic thematic reviews of the replacement, reduction and refinement of the use of animals in procedures" be conducted. Such systematic thematic reviews, which consider progress in technological developments and new scientific and animal welfare knowledge, provide a potential mechanism for achieving "the final goal of full replacement", as cited in Recital 10 of the Directive, in a realistic and practicable manner. Currently, no concrete steps have been taken by the European Commission to implement structured thematic reviews. This process could, however, be advanced by individual Member States, or even individual organisations with an interest in beginning the process of phasing out animal experiments wherever this is possible. Animal Defenders International propose a 3-step process for thematic review to systematically examine specific uses of animals in research, with a view to implement a phase out strategy that will replace or eliminate their use. The audience of the European Society for Alternatives to Animal Testing annual conference are in prime position to discuss how thematic review should be carried out and to identify specific uses of animals in research that should be a priority. The uses of animals in specific areas of education and training, namely trauma training, will be presented as an example for how thematic review can be implemented in order to progress replacement.

References

Dodkin, C. and Jayne, K. (2017). Laboratory and Farm Animal Law: Opportunities for Ending Animal Use. *Relations Beyond Anthropocentrism 5*, 177. doi:10.7358/rela-2017-002-dodk

Herrmann, K. and Jayne, K. (eds.) (2019). *Animal Experimentation: Working Towards a Paradigm Change*. Vol. 22. Leiden: Brill, Human Animal Studies. doi:10.1163/9789004391192

Pawlowski, J., Feinstein, D., Crandall, M. L. and Gala, S. (2019). Modernizing biomedical training: replacing live animal laboratories with human simulation. In K. Herrmann and K. Jayne (eds.), *Animal Experimentation: Working Towards a Paradigm Change*. Vol 22. Leiden: Brill, Human Animal Studies. doi:10.1163/9789004391192

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Reliable and representative *in silico* predictions of freshwater ecotoxicological hazardous concentrations

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The majority of the chemicals in the REACH registry have experimental ecotoxicological data included for between one to three taxonomically distinct freshwater species [1], causing large uncertainties when quantifying hazardous concentrations for comparative risk assessment and life cycle assessment [2]. Addressing the data gap with experimental data is resource intensive and when considering vertebrates in particular is also contrary to progress towards eliminating animal testing. One alternative to avoid testing is the use of *in silico* approaches to predict ecotoxicity values from chemical properties or species characteristics. Here, we developed a method to quantify hazardous concentrations of chemicals ($HC50_{EC50}$) on freshwater ecosystems without the need for *in vivo* testing. The $HC50_{EC50}$ is defined as the hazardous concentrations for which 50% of the species are exposed above their acute EC50, where the EC50 is the concentration causing an acute effect in 50% of the population. To this end we combined two *in silico* approaches: quantitative structure activity relationships (QSARs) to derive acute ecotoxicity values for *Daphnia magna*, *Pseudokirchneriella subcapitata*, and *Pimephales promelas* [3,4] and interspecies correlation estimation (ICE) models [5] for 51 chemicals with at least ten reported acute EC50s. We quantified two types of uncertainty: the statistical uncertainty and the bias. The bias is calculated as the ratio of the $HC50_{EC50}$ based on measured ecotoxicity data and the *in silico* $HC50_{EC50}$. This statistical uncertainty was quantified as the ratio of the 95th and 5th percentile of the $HC50_{EC50}$ derived with a Monte Carlo simulation. For the *in silico* $HC50_{EC50}$, the statistical uncertainty reflects uncertainty in the QSAR and ICE models as well as uncertainty due to the limited species number. Our results show that the bias of the $HC50_{EC50}$ estimated from combining QSARs and ICE ranges from 0.2 to 6.2 (90%-range) with a median bias of 1.2. If only the three QSARs are applied, the bias ranges from 0.1 to 4.8 with a median estimate of 0.7. The median statistical uncertainty of the $HC50_{EC50}$ based on QSARs and ICE is $6.1 \cdot 10^2$ ($5.2 \cdot 10^2 - 1.1 \cdot 10^3$; 90%-range], while the uncertainty in the $HC50_{EC50}$ derived from three QSAR EC50s is larger, i.e. $4.6 \cdot 10^3$ with a range of $1.7 \cdot 10^3$ to $7.4 \cdot 10^6$. As a comparison, we also evaluated the bias and statistical uncertainty when applying three experimental toxicity values (preferably from algae, daphnia, and fish) only. In this case the bias is 1.2 [0.3-5.4] and the statistical uncertainty $9.2 \cdot 10^1$ [$3.2 - 4.6 \cdot 10^5$]. Based on our analysis, we conclude that ICE and QSAR typically provide representative results of the $HC50_{EC50}$ and that adding the ICE estimates increases the reliability of the $HC50_{EC50}$. We also found that three experimental values typically result in lower statistical uncertainty of the $HC50_{EC50}$ compared to our approach, but with notable exceptions for 27% of the chemicals included. We plan to assess the usefulness of this approach to estimate other HC values (e.g. HC5) and more widely support the application of hazardous concentration approach in impact assessment.

References

[1] Saouter, E., Wolff, D., Biganzoli, F. and Versteeg, D. J. (2019). *Integr Environ Assess Manag 15*, 796-807.

[2] Van Zelm, R., Huijbregts, M., Harbers, J. V. et al. (2007). *Integr Environ Assess Manag 3*, 203-210.

[3] Sangion, A. and Gramatica, P. (2016). *Environ Int 95*, 131-143.

[4] IRFMN Welcome to the VEGA HUB. https://www.vegahub.eu/

[5] Raimondo, S., Lilavois, C. R. and Barron, M. G. Web-based Interspecies Correlation Estimation (Web-ICE) for Acute Toxicity: User Manual. Version 3.3. https://www3.epa.gov/webice/iceSSDSpecies.html?filename=as (01.2019).

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Sensitive and automated assessment of DNA strand break by AUREA gTOXXs in HepaRG™

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Notably, numerous drugs, chemicals or food contaminants develop toxicity only upon metabolic activation in specific organs. In the liver primarily through the action of cytochrome P450 enzymes, thereby mainly causing hepatotoxicity, the most common reason for a drug to be withdrawn from the market. In the case of aflatoxin B1 (AFB1) from Aspergillus fungi, toxicity unfolds upon AFB1 epoxidation by P450 1A2 and 3A4. The AFB1 exo-8,9-epoxide is the aflatoxin metabolite that reacts with DNA to form the adducts that presumably account for the biological effects of the toxin. The HepaRG™ cell line is considered to be the most stable model for mapping the metabolic response of human-derived hepatocytes to chemicals like aflatoxin B1.

AUREA gTOXXs solution based on the automated FADU (Fluorimetric Detection of Alkaline DNA Unwinding) assay provides a sensitive and reliable *in vitro* test system for detection of DNA strand breaks. It is based on progressive DNA unwinding under specific conditions of alkaline pH, time and temperature and fluorescence labeling of double-stranded DNA [1].

As previously shown by the comet assay, dose-dependent DNA damage is observed after treatment of differentiated HepaRG cells with AFB1 [2]. Here, the applicability of HepaRG™ for the automated determination of AFB1 genotoxicity by AUREA gTOXXs is demonstrated. HepaRG™ cells were treated for 3 or 20 h with the genotoxic agents, etoposide, methylmethansulfonate (MMS),

or aflatoxin B1 (1-15 µM). Both, etoposide and MMS, which unfold genotoxicity without the need for bioactivation, and AFB1 are shown to produce clear dose-dependent effects on the DNA strand break percentage in HepaRG™ in the absence of cell damage. In Jurkat cell line, however, merely etoposide and MMS produced significantly increased percentage of DNA strand breaks contrary to AFB1. Thus, AFB1 genotoxicity is clearly uncovered by gTOXXs testing on HepaRG™ at a sensitivity comparable to the one of the comet assay.

Interestingly, the DNA damage caused by MMS treatment is, as yet, diminished with time of AFB1 exposure, indicative of a DNA repair mechanism in the HepaRG™ cells. It will be interesting to test additional substances with affinity to CYPs and to evaluate the repair mechanism of HepaRG™ cells within the gTOXXs.

References
[1] Moreno-Villanueva, M. et al. (2011). *ALTEX 28*, 11.
[2] Josse, R. et al. (2008). *Drug Metab Dispos 36*, 1111.

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Cosmetic perfumes and their potential of endocrine disruption, sensitization and genotoxicity

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Given the wide scope of mixture toxicity testing, there is an urgent need for closer links and involvement of many scientific disciplines, i.e. analytical chemistry, biomedicine and toxicology. Hazard identification of mixtures composed of variable concentrations of substances is recommended as necessary. Cosmetic products are mixtures consisting of many ingredients, therefore the assessment of their hazardous properties and health safety is more complex than in case of individual chemicals. Animal testing is prohibited in the European Union for cosmetic ingredients and final products. Alternative *in vitro* methods and clinical studies on human volunteers are the only safety assessment approaches available for cosmetics and their ingredients. It is necessary to introduce *in vitro* methods suitable for detecting multiple toxicological endpoints for testing of cosmetic products. Very recent data suggest that substances such as fragrances may exhibit multiple biological effects, e.g. cytotoxicity, skin sensitization, (photo) genotoxicity and endocrine disruption. In our pilot study we selected 10 samples of deodorants, EDT and EDP and intended to integrate unique results from a number of novel alternative toxicological methods suitable for detection of the following toxicological endpoints:

- cytotoxicity with the use of 3T3 Balb/c fibroblasts,
- skin sensitization potential using the *in chemico* method DPRA,
- skin sensitization potential using the LuSens method based on human keratinocytes,
- genotoxic potential by the Comet Assay with the use of 3T3 Balb/c fibroblasts,
- endocrine potential by means of the YES/YAS assay based on two genetically modified *Saccharomyces cerevisiae* strains with stably incorporated human estrogen receptor α and human androgen receptor into the genome.

For support of skin sensitization detected by the DPRA and LuSens methods, the content of allergens and prohibited substances was determined by gas chromatography. The highest cytotoxicity was reported for samples 1, 2, 3, 5, while the other samples were not toxic up to concentration 500 µg/ml. Skin sensitization potential using the LuSens method was detected starting at concentration 12.5 µg/ml in case of sample 6 and at concentration 100 µg/ml in case of samples 7, 8, 9. The DPRA method detected skin sensitization potential in case of samples 5, 6, 7, 8. The Comet assay did not detect any significant genotoxic potential at the selected concentration of 250 µg/ml. However, a concentration dependent fragmentation of DNA, which was evaluated to be statistically significant, was detected in case of samples 6, 8, 9, 10. Endocrine disruption potential was identified in case of samples 1, 2, 5, 6, 7, 8, 9, 10. Consequently, a GC/MS analysis of 24 allergens was performed and the most loaded samples were identified. This battery of tests with parallel biological and chemical analysis may be a promising approach for future testing of this type of cosmetic products.

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Microphysiological flux balance platform unravels the dynamics of drug induced steatosis

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Drug development is currently hampered by the inability of animal experiments to accurately predict human response. While emerging organ on chip technology offers to reduce risk using microfluidic models of human tissues, the technology still mostly relies on end-point assays and biomarker measurements to assess tissue damage resulting in limited mechanistic information and difficulties to detect adverse effects occurring below the threshold of cellular damage. Here we present a sensor-integrated liver on chip array in which oxygen is monitored using two-frequency phase modulation of tissue-embedded microprobes, while glucose, lactate and temperature are measured in real time using microfluidic electrochemical sensors. Our microphysiological platform permits the calculation of dynamic changes in metabolic fluxes around central carbon metabolism, producing a unique metabolic fingerprint of the liver's response to stimuli. Using our platform, we studied the dynamics of human liver response to the epilepsy drug Valproate (Depakine™) and the antiretroviral medication Stavudine (Zerit™). Using E6/E7LOW hepatocytes, we show TC50 of 2.5 and 0.8 mM, respectively, coupled with a significant induction of steatosis in 2D and 3D cultures. Time to onset analysis showed slow progressive damage starting only 15–20 hours post-exposure. However, flux analysis showed a rapid disruption of metabolic homeostasis occurring below the threshold of cellular damage. While Valproate exposure led to a sustained 15% increase in lipogenesis followed by mitochondrial stress, Stavudine exposure showed only a transient increase in lipogenesis suggesting disruption of β-oxidation. Our data demonstrates the importance of tracking metabolic stress as a predictor of clinical outcome

Reference

Ehrlich, A., Tsytkin-Kirschenzweig, S. et al. (2018). *Lab Chip 18*, 2510-2522.

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In vitro approaches to identify hepatotoxic chemicals

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What is known about idiosyncratic reactions comes essentially from studies dealing with drugs. However, it is likely that basic mechanisms are also relevant in the case of chemical-induced liver injury. There is growing evidence, that adaptive immune responses and inflammatory stress both play a role in the development of IDILI and tumor necrosis factor alpha (TNF)-signaling seems to play a crucial role. Interactions between hepatocytes and immune cells as well as inflammatory episodes are thus important in the alteration of the individual susceptibility to idiosyncratic liver reactions. In order to mimic this cell crosstalk in predictive *in vitro* models, hepatocytes have to be exposed to the test chemicals in the presence of immune cells or at least pro-inflammatory stimuli, e.g. TNF.

To test the hypothesis that chemical-induced liver injury is mediated by TNF signalling, HepG2 cells were exposed to the solvents Dimethylacteamide (DMA) or Dimethylformamide (DMF) at EC20 in the presence or absence of TNF and then the cytotoxic effect on the cells was analyzed using the WST-assay. DMA and DMF are frequently used in industry and also known to be associated with occupational liver diseases. Co-exposure to 10 ng/mL TNF for 24 h resulted in a significant decrease in viability compared to solvent treatment alone, the viability levels being 21.6% ±3% for 3% (v/v) DMA and 37.2% ±7%for 3.2% (v/v) DMF, respectively. These results point to a synergy between these solvents and TNF in HepG2 cells.

To examine whether this effect could also be observed in a complex liver-derived *in vitro* model which correlated more closely to the *in vivo* situation, we started experiments on human precision-cut liver slices (hPCLS). Liver slices (Ø 8 mm, 200-300 µm thick) from healthy tissue of patients, undergoing partial hepatectomy, were prepared and cultured as described previously by Granitzny et al. [1] with minor modifications. After a pre-incubation period, slices were treated for 24 h with increasing concentration of DMF (0-15 vol/vol%) and cytotoxicity was evaluated using WST-assay. Preliminary results indicate a dose-dependent toxicity and suggest an EC50 of approximately 160 mM.

In conclusion, our results are in line with the hypothesis that organic solvents exert their liver toxicity in conjunction with inflammatory stress. As a consequence, *in vitro* test systems including immune cells or utilizing inflammatory stimuli are needed to detect chemicals with the potential to induce idiosyncratic liver reactions.

Reference

[1] Granitzny, A., Knebel, J., Schaudien, D. et al. (2017). *Toxicol In Vitro 42*, 200-213. doi:10.1016/j.tiv.2017.05.001

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3Rs education in academia: Experiences from the Swiss 3R Competence Centre

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The Swiss 3R Competence Centre (3RCC) is a non-profit association founded in March 2018 to promote the Replacement, Reduction and Refinement of animal experimentation (3Rs principle) in Switzerland and facilitates its implementation in life sciences. The 3RCC is formed as a joint initiative of academia, the industry, regulators, the government and an animal welfare association. Located in Bern, it promotes the 3Rs principle by financing high-quality research projects, developing dedicated educational programmes and promoting communication and dissemination of the 3Rs principle (www.swiss3rcc.org).

During its first year, a mapping of existing educational programmes on 3Rs was conducted at the under-graduate, graduate, professional and continuing education levels. Furthermore, a survey counting with the participation of 176 research groups working or interested on the 3Rs in Switzerland, enquired about current gaps and opportunities for implementing the 3Rs in Switzerland. The outcomes of the educational programmes mapping and of the survey led to the recommendation for the implementation of a 3Rs educational program at bachelor level, including a list of recommended topics to be addressed.

The 3RCC is also collaborating with the EC for the development of an e-Learning module to promote the implementation of alternatives to animal testing, addressing in particular the development of reliable and relevant *in vitro* methods and approaches for scientific purposes and regulatory use. The 3RCC is furthermore collecting information about the use of alternative methods for educational purposes in Switzerland. Finally, the centre has established a biostatistics task force to help fostering good experimental design and biostatistics practices and support.

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Development of a fibrosis on-chip tool for drug efficacy testing

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Introduction

Fibrotic diseases account for an increasing burden of disease-associated morbidity and mortality worldwide. The intricate underlying biomechanism involving several cell types and metabolic molecules, is subject to many research projects but remains not fully elucidated. Morphologically, a stiffening of the parenchymal tissue through the accumulation of extracellular matrix (ECM) and the loss of organ specific functions occur during the progression of fibrosis. The increased deposition of ECM is mediated through mesenchymal cells, such as myofibroblasts in the lung and activated stellate cells in the liver. Anti-fibrotic drug responses are patient specific, and for new compounds to be tested there is a unfortunate lack of predictive *in vitro* models [1,2,3].

Objective

The aim of this research is to create an advanced *in-vitro* tool for preclinical efficacy testing of anti-fibrotic drugs. Ultimately, this simple and robust method will become a decision-making tool for clinicians.

Methods

Applying a given pressure onto a thin, elastic and soft membrane results in a measurable deflection, which heights varies depending on the stiffness of the material. To gain such a flexible membrane with a low stiffness, which is necessary to increase the sensitivity for the natural ECM production from cells cultured upon it, a mixture of two different kinds of polydimethylsiloxane (PDMS) was used, namely PDMS 184 and 527, as described by Palchesko et al. [4]. The determination of mechanical stiffness of these membranes, using the bulge test method, was conducted using an upright Axioplan 2 in reflective mode. The same microscope was then used to measure the deflection height of membranes with cells cultured on top. The cell culture conditions mimicking a diseased state were supplied with human TGF-β for 5 to 6 days in concentrations of 1 ng/ml and 5 ng/ml. Sirius red 80 staining was used to image the collagen deposition and to measure the collagen absorption with a TECAN reader.

Results

Using curve fitting on the pressure/deflection curve according to Small and Nix [5], the elastic modulus of the used membranes could be determined to be around 60 kPa. Cell cultures of human lung fibroblasts showed then a significant decrease in deflection height, when supplemented with TGF-β, suggesting an increase in ECM deposition and therefore representing a diseased, fibrotic state compared to the untreated samples. This could be confirmed through absorption measurements of the collagen from the cell cultures. Similar results were obtained using primary hepatic stellate cells.

Conclusion

Taking a soft polydimethylsiloxane membrane as culture substrate with subsequent deflection measurements using a generic microscope, a facile approach with minimal equipment requirements could be established to test different cells for their change in ECM production under varying conditions. This should allow for a patient-specific determination of the degree of fibrosis on a cellular level, while at the same time enable optimized drug testing and therefore not only refine, but also reduce and ultimately even replace animal testing.

References

[1] Friedman, S. L. (2010). *Nat Rev Gastroenterol Hepatol 7*, 425-436. doi:10.1038/nrgastro.2010.97
[2] Asmani, M., Velumani, S., Li, Y. et al. (2018). *Nat Commun 9*, 2066. doi:10.1038/s41467-018-04336-z
[3] Thannickal, J. V., Zhou, Y., Gaggar, A. and Duncan, S. R. (2014). *J Clin Invest 124*, 4673-4677. doi:10.1172/JCI74368
[4] Palchesko, R. N., Zhang, L., Sun, Y. and Feinberg, A. W. (2012). *PLoS One 7*, 1-13. doi:10.1371/journal.pone.0051499
[5] Small, M. K. and Nix, W. D. (1992). *Journal of the Materials Research Society 7*, 1553-1563. doi:10.1557/JMR.1992.1553

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Non-technical summaries provided by the Austrian Federal Ministry of Education, Science and Research

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The EU directive 2010/63/EC requires that its member states publish non-technical summaries (NTS) of animal experiments authorized by the EU. In 2013 a working document on NTS was published offering both a template and an illustrative example of a completed NTS. In 2017 Taylor et al. published a review about the quality of NTS in Europe with a special emphasize on Germany and the UK [1]. They also provided some suggestions of how to improve NTS in Europe. Thus, I wanted to have a closer look at NTS provided in Austria. More precisely, I had a closer look at NTS from 2014 to 2018 by focusing on later updates. In Austria these NTS are published every 3 months as one PDF-file and publication is still about 6 months delayed, as already mentioned by Taylor et al. (2017). Concerning content and quality I investigated 50 of the last publicly available NTS (by 12.06.2019) published for the third quarter of 2018.

I'd like to highlight some problems concerning the provision of the NTS by the Austrian Federal Ministry of Education, Science and Research. The PDF-files are not useable for analysis. Updates are presented at the end of each PDF-file. Bold letters illustrate the respective parts of the new information within the NTS, though not all new parts. On top of the updated NTS you get the information when they have been published before, sometimes these references are wrong and it is nearly impossible to find the proper NTS. In more than 90% the change affected the number of animals which increased in general. Just to give one example: In 2016 384 NTS were published. Until the third quarter of 2018, in 51 of these NTS an update was published highlighting an increase in the number of animals and further 35 times these updates were updated again. In general, the new number of animals and not the increase in the number of animals was published. The increase ranges from 1 animal to more than 8 times the number of animals applied for in the first place (e.g. 2018, Q3, page 184 [2]). In only a few cases a reason for the increase in the number of animals was given.

Concerning the content of NTS: NTS still lack a project title as well as a clearly defined project duration. The section of harms is often presented in general terms like "Husbandry conditions are optimized for the well-being of the animals" or at least once lack any logic at all, translated and summarized as follows "Due to our aging society and the increase of fractures or osteoporosis, the expected degree of severity is medium" ([2]; 2018, Qu.3, page11).

To get an overview of animal experiments done in Austria a standardized NTS database is necessary as well as links between different versions and an exact presentation of the increase in animal numbers as well as a reason for this increase. If NTS are not provided properly they are for no use to anyone.

References

[1] Taylor, K. Rego, L. and Weber, T. (2018). *ALTEX 35*, 193-210. doi:10.14573/altex.1708111

[2] https://bmbwf.gv.at/forschung/national/forschungsrecht/tierversuche/nichttechnische-projektzusammenfassungen-veroeffentlichung-gemaess-tierversuchsgesetz-2012/ (Download 12.06.2019).

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The biological activity of medium-strength square-wave electric impulses on the proliferation patterns of different animal cells

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Medium-strength electric forces can influence the immune responses of animal or human by inducing [1] or augmenting different immune response elements [2]. It is known [3], that exogenous electric shock is able to induce the production of antiviral substance which is "interferon-like" and not interleukins or tumor necrosis factor (TNF). It was also found [4] that medium-strength short electric pulses affect the human blood and enhanced the number of cells expressing: CD-11a, CD-19, MHC I, MHC II and ICAM-1. The presented experiments were aimed to investigate the influence the medium-strength square-wave electric impulses on the proliferation pattern of different animal cells. The following cell lines were used: (I) Cells: (a) Lymphoblastoids: FB1, K562 (b) Adherent cells: WiREF, VERO, MDBK, PLA, MRC, WISH and HeLa. (II.) Cell multiplication: The suspension cell lines were multiplied in the RPMI-1640 + 10% FCS. The monolayer cell cultures were resuspended in 60 ml EMEM complemented with surplus Ca++ and 2% FCS. (III.) Impulse generator device was developed to provide 1-300 V square-wave impulses of 1 µs - 10 ms duration with a repetition option of 1-9 and also a continuous work option. [5] (IV.) Electroshocks: The aliquots of 3 x 20 ml of cells suspensions were put in two electro induction chambers with built-in platinum wire electrodes, for treatment. Samples were subjected to 1 and 3 medium-strength square-wave pulses of 10 ms with a field force of 100 V/cm. (V.) Analysis of obtained data: (1) Hela: after 72 h the control has GI: 13,698, after 1 shock GI 10,163 and after 3 shocks 5,372; (2) MRC-5: after 72 h control has GI: 5,582, after 1 shock GI is 4,186 and after 3 shocks: 2,605; (3) Vero: after 72 h control has GI: 5,396, after 1 shock GI is:5,396 and after 3 shocks GI is 5,698; (4) MDBK: after 72 h control has GI: 8,396, after 1 shock the GI is 5,396 and after 3 shocks the GI is 5,698; (5) PLA: after 72 h control has the GI 15,884, after 1 shock the GI is 12,535 and after 3 shocks the GI is 8,186; (6) Chicken fibroblasts (CHF): after 72 h

the Control has the GI: 16,768, after 1 shock the GI is 15,814 and after 3 shocks the GI is 7,093. (7) FB1: after 72 h the control has GI: 6,559, after 1 shock the GI is 13,489 and after 3 shocks the GI is 12,256; (8) K562: after 72 h the Control has the GI: 9,070; after the shock, the GI is 12,372 and after 3 shocks the GI is 13,552. (VI.) From all the data obtained it can be concluded: (1) One or three electroshocks in monolayer cells causes the inhibition of GI, depending from the nature of cells, like primary cells (CHF) are more susceptible than VERO, MDBK and PLA. (2) Electroshocks in lymphoblastoids cause the increase of GI.

References

[1] Petrini, M., Polidori, R.,Ambrogi, F. et al. (1990). *J Bioelectric 9*, 159-166. doi:10.3109/15368379009119803

[2] Holan, V., Machala, O., Dao, T. and Minowa, J. (1994). *Immunobiology 190*, 368-371. doi:10.1016/S0171-2985(11)80608-X

[3] Pretnar, G., Filipič, B., Golob, A. et al. (1991). *Bioelectrochem Bioenerg 25*, 183-193. doi:10.1016/0302-598(91)87002-X

[4] Filipič, B., Kovacs, K., Somogyvari, F. et al. (2000). *Bioelectrochemistry 52*, 29-36. doi:10.1016/S0302-4598(00)00079-9

[5] Filipič, B., Koren, S., Kovacs, K. et al. Naprava in postopek za elektrostimulacijo celic (Device and method for electrostimulation of cells) (in Slovenian). Slovenian Patent No. SI 20111 A, 600-214/98-MT-6 z dne: 03.07. 2000. (P-9800214. 1998).

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Repeating past mistakes: The banality and futility of nowadays cigarette smoke-related animal experimentation

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The health hazards of cigarette smoke (CS) have been a subject of countless studies dating back to the beginning of the 20th century. Since the 1950s experiments have been performed with different animals like mice, rats, dogs and monkeys, forced to inhale, ingest or otherwise receive large amounts of CS or its derivatives. Because of a low reproducibility of the results between different species and a poor transferability to humans, these experiments have been widely criticized by the scientific community and partly used as an argument by the cigarette industry in an attempt to deny the link between CS and lung cancer [1]. Eventually, despite these experiments, the collective results from multiple largescale epidemiological studies have conclusively proven that CS is the leading cause of lung cancer and contributes to many other severe respiratory diseases.

Although animal experiments have long been deemed unreliable in assessing the effects of CS in humans, such experiments are still being performed in multiple laboratories in central Europe. Amongst them are extremely invasive procedures associated with immense animal suffering, including acute trauma, hemorrhagic shock and massive inflammation [2,3,4]. Furthermore, with small modifications these studies are merely repeating the effects already described in humans and are frequently failing to reproduce the results of clinical data.

The banality of the reported outcomes, i.e. that CS contributes to various lung-related health problems, the repetition of studies already performed in patients and the direct contradiction of clinical results reveals the extreme inadequacy of using animals for the analysis of CS-related diseases in humans. Luckily, many human-oriented, innovative and personalizable methods like precision cut lung slices, 3D lung epithelium models and lung-on-a-chip systems are readily available and approved for regulatory purposes. Here, we show animal experiments and human-based research on CS effects conducted in Germany, Austria and Switzerland. Taken together, we regard the fact that severely harmful animal experiments are still being performed as scientifically and ethically unjustifiable and demand their immediate replacement with more suitable *in vitro* techniques.

References
[1] Witschi, H. (2007). *Int J Toxicol 26*, 339-344. doi:10.1080/10915810701490380
[2] Jia, J., Conlon T. M., Sarker R. S. et al. (2018). *EMBO Mol Med 10*. doi:10.15252/emmm.201708349
[3] Hartmann, C., Gröger, M., Noirhomme, J. P. et al. (2019). *Shock Augusta Ga 51*, 68-77. doi:10.1097/SHK.0000000000001115
[4] Bucher, H., Mang, S., Keck, M. et al. (2017). *Pulm Pharmacol Ther 44*, 96-105. doi:10.1016/j.pupt.2017.03.008

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Establishment of precision cut bovine udder slices for pharmacological studies

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Precision cut tissue slices are frequently used as *in-vitro* models for pharmacological studies. The aim of this study was the establishment of precision cut bovine udder slices (PCBUS) to investigate possibilities of pharmacological interventions in pathophysiological processes of infection and inflammation. PCBUS can be useful for the characterization of complex pathophysiological processes as *in vitro* culture which is enabling to study cellular interactions. Furthermore, the usage may contribute to the reduction of animal experiments for the development of new therapeutics.

Directly after slaughtering, udders of healthy dairy cows (German Holstein Friesian) were transported to the laboratory. A gland tissue piece of approximately 10 x 10 x 5 cm was harvested with a sterile scalpel blade. PCBUS of 250 μm thickness were prepared using a tissue slicer and were placed into sterile phosphate buffered saline (pH = 7.4; PBS). The slices were repeatedly washed with PBS until a clear solution without milk contamination was achieved. Numerous 6 mm biopsy punches were taken from these PCBUS and transferred into petri-dishes. Another series of washing steps followed in the presence of antibiotics. Afterwards the slices were put into 24-well plates and incubated with 1 ml of RPMI-1640 medium supplemented with FBS, antibiotics and an antimycotic in a humidified atmosphere containing 5% CO_2 at 37°C. The medium was changed every 48 h. Viability was confirmed by MTT test. After an incubation period of 48 h, slices were transferred into medium without any antibiotics or an-

timycotics. In a first experiment, PCBUS were inoculated with 2.5 x 10^7 CFU of *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). Supernatants were removed after 1, 2, 4, 6 and 24 h. Concentrations of IL-1ß, TNF-α and PGE2 were measured with ELISA kits according to the manufacture's instruction. Sample collection for histological examination was taken at the same time points (fixed for 24 h in 4% formalin and embedded in paraffin wax). Tissue sections of 2-3 μm hematoxylin and eosin (HE) as well as Gram stained were used for histological examination.

PCBUS were viable for at least 7 days. Challenging PCBUS with the above-mentioned bacteria led to an increase of the inflammation markers in the samples. An increase of IL-1ß and PGE2 was mainly observed during the first 6 h while an increase of TNF-α concentration occurred between 4 h and 24 h. In the histological Gram-stained sections, bacterial adherence to the PCBUS was confirmed.

This study presents a model for the assessment of an inflammatory process in bovine udders that can be useful for investigations in pharmacological studies.

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From initiation to implementation: The RTgill-W1 cell line assay to predict fish acute toxicity of water samples and chemicals (ISO21115)

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Predicting fish acute toxicity of water samples and chemicals *in vitro* is an attractive alternative method to the conventional approach using juvenile and adult fish. Assuming that the gills are a major target of fish acute toxicity, the rainbow trout (*Oncorhynchus mykiss*) cell line assay with RTgill-W1 cells has been designed for this purpose. It quantifies cell viability using fluorescent measurements for metabolic activity, cell- and lysosomal- membrane integrity on the same set of cells. Application of a specifically designed exposure medium, L-15/ex, and procedures to verify exposure concentrations in the case of chemical testing have moreover been important developmental steps [1]. Results from over 70 organic chemicals attest to the high predictive capacity of this test; as well, numerous municipal and industrial water samples have been explored. Repeatability (intra-laboratory variability) and reproducibility (inter-laboratory variability) of the RTgill-W1 cell line assay have been tested in a round-robin study focusing on six test chemicals involving six laboratories from the industrial and academic sector. Coefficients of variation for intra and inter-laboratory variability for the average of the three fluorescent cell viability measurements were well comparable to other fish-derived, small scale bioassays [2]. Thus, the round robin study attested the robustness of the RTgill-W1 cell line assay and its accurate performance when carried out by operators in different laboratory settings. Thanks to these efforts, the International Standardization Organization (ISO) has recently approved the assay as the first international standard based on a fish cell line [3] – rendering it a forerunner in this regard. The test has as well been submitted to OECD, where it was included into the work programme by the group of national co-ordinators in April 2019, and where it constitutes a part of the development of an Integrated Approach to Testing and Assessment for fish acute toxicity.

In this presentation, we wish to highlight the most important steps and hurdles encountered for the successful implementation of this assay in this decade long effort. As well, we will reflect on its limits and suggestions for future developments.

References

[1] Tanneberger, K., Knoebel, M., Busser, F. J. M. et al. (2013). *Environ Sci Technol 47*, 1110-1119. doi:10.1021/es303505z

[2] Fischer, M., Belanger, S. E., Berckmans, P. et al. (2019). *Toxicol Sci 169*, 353-364. doi:10.1093/toxsci/kfz057

[3] ISO 21115:2019. Water quality – Determination of acute toxicity of water samples and chemicals to a fish gill cell line (RTgill-W1). International Standardization Organization.

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Gene expression upon immune challenge in a fish intestinal *in vitro* model

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The fish intestine comprises an important environment-organism interface that is vital to fish growth, health and pathogen defence. Yet, knowledge about the physiology and defence mechanisms toward environmental stressors, such as bacterial or viral cues, is limited and depends largely on *in vivo* experiments with fish. To overcome this bottleneck, we develop *in vitro* models of the fish intestine, specifically of rainbow trout (*Oncorhynchus mykiss*), with the epithelial intestinal cell line, RTgutGC, as the corner stone. This cell line can be employed 2D as adherent monolayer or 3D in a two-compartment system, both in static as well as flow-through exposures [1-3].

In quest to understand the immune competence of this *in vitro* intestinal barrier model we study how the RTgutGC cell barrier reacts to two immune stimuli, the bacterial lipopolysaccharide (LPS) from *Escherichia coli* and the viral Poly(I:C), by regulating the mRNA abundance of selected genes. Immune stimuli activate the Myd88- and Ticam-dependent signalling cascades, resulting in downstream activation of pro-inflammatory cytokines and interferon, comparable to the regulatory patterns known from *in vivo* [4].

On this basis, we developed the RTgutGC immune-challenger assay. In this assay, RTgutGC cells are exposed to the agent in question (e.g. chemicals of environmental concern, fish nutrients, probiotic feed additives) either in the presence of LPS / Poly(I:C), or after challenge with the latter, and immune-relevant genes are monitored for their mRNA expression levels. In this way, we are not only able to provide mechanistic knowledge on how the intestinal cells respond to the respective agents but as well provide a screening option whereby the RTgutGC immune-challenger assay serves as filter to better decide on the necessity of *in vitro* trials, thus contributing to a reduction of animal testing in toxicology and aquaculture.

References

[1] Minghetti, M., Drieschner, C., Bramaz, N. et al. (2017). *Cell Biol Toxicol 33*, 539-555.

[2] Drieschner, C., Minghetti, M., Wu, S., Renaud, P. and Schirmer, K. (2017). *ACS Appl Mater Interfaces 9*, 9496-9505.

[3] Drieschner, C., Vo, N. T. K., Schug, H. et al. (2019). *Cytotechnology 29*, 835-848. doi:10.1007/s10616-019-00327-0

[4] Schug, H., Yue, Y., Krese, R. et al. (2019). *Fish Shellfish Immunol 88*, 308-317.

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Assay-ready use of KeratinoSens®/LuSens cells in skin sensitization

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The reproducibility of cell-based assays strongly depends on the cell quality, which in turn is influenced by multiple factors such as the choice of the culture media and sera, the source and passage number of the cell line, or even slight differences in cell handling by different operators. Thereby, all these parameters need to be optimally standardized. For this, the use of pre-made and pre-qualified assay-ready cells, which can be applied in a cellular assay basically like a reagent without prior cultivation or passaging, can minimize the variability related to cell culture.

To evaluate the skin sensitizing potential of chemicals, reporter skin cell lines are used to measure the activation of the ARE/Nrf2 pathway, which is one of the key events of this complex cascade. Within the context of the keratinocyte activation, the Kera-tinoSens® and LuSens cell lines have been developed by Givaudan and BASF, respectively, and validated by the ECCVAM. Here we demonstrate that the use of these cell lines in an assay-ready format to test the proficiency substances according to the OECD guideline 442D leads to equivalent results as compared to continuously cultured cells.

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On the uncertainty of toxicological methods: Quantifying the borderline range of prediction models and implications for decision-making

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For hazard classifications, continuous data from animal- or non-animal testing methods are often dichotomised into binary positive/negative outcomes by classification thresholds (CT). Experimental data are, however, subject to biological and technical variability. This results in uncertainty of the positive/negative outcome if the experimental result is close to the CT. The borderline range (BR) addresses this uncertainty and represents a range around the CT in which the likelihood to obtain a positive or negative result is equal and ambiguous outcomes are expected. Several analyses of the intra-assay variability of the animal test, local lymph node assay (LLNA) have been published [1-5]. Previously, a BR of the LLNA has been defined [1], in the following, BRs were determined for three non-animal methods assessing skin sensitization based on pooled standard deviations [6].

Our recent research presented here explores different ways to quantify the BR, using pooled standard deviations, pooled mean average deviation and non-parametric methods (bootstrap analysis) from existing data of eye irritation and skin sensitization non-animal methods as well as the animal method, the LLNA.

The results demonstrate (i) the precision of the methods is determining the size of their BRs, (ii) there is no "perfect" method to derive a BR, alas (iii) a consensus on BR is needed to account for the limited precision of testing methods.

References

[1] Kolle et al. (2013). doi:10.1016/j.yrtph.2012.12.006
[2] Hoffmann (2015). doi:10.14573/altex.1505051
[3] Dumont et al. (2016). doi:10.1016/j.tiv.2016.04.008
[4] Dimitrov et al. (2016). doi:10.1002/jat.3318
[5] Luechtefeld et al. (2016). doi:10.14573/altex.1510055
[6] Leontaridou et al. (2017). doi:10.14573/altex.1606271

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Human-based T cell-skin models for graft-versus-host disease

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Allogeneic hematopoietic stem-cell transplantation (allo-HSCT) might be the last option for patients with high-risk hematologic malignancies or severe stem cell disorders. While allogeneic T cells within the graft elicit the beneficial graft-versus-leukemia (GvL) effect and thereby prevent tumor relapses, they carry the inherent risk of inducing graft-versus-host disease (GvHD) due to major and minor mismatches in the histocompatibility complexes between donor and recipient. Besides gut and liver, the skin is a major target of GvHD. Therefore, the study aimed to set up models of acute GvHD by introducing allo-activated T cells in established skin models. By reflecting the human molecular, cellular and ECM architecture, the models should reproduce central physiological processes during allo-HSCT: (I) presentation of specific human leukocyte antigen (HLA) complex, (II) re-activation of allogenic T lymphocytes by the HLA of the recipient (artificial skin) and (III) T cell-mediated tissue disruption.

In first preliminary studies using reconstructed human epidermis RHEs and epidermal-dermal models, human PBMCs or human T cells were pre-stimulated under various conditions and then applied to the skin models. We found allo-immune destruction of engineered skin if T cells were primed poly-clonally or on allogeneic DCs. In line, allo-primed T cells on such skin models produced IL-6, TNF and especially IFN-γ. The presence of Cyclosporin A (CsA), a calcineurin inhibitor, used in the clinic to prevent or treat GvHD, inhibited tissue destruction. Moreover, putative inhibitors of NFAT activation, mediated by calcineurin, elicited some protective effect. In sum, we could demonstrate that *in vitro* skin models can serve a novel tool to investigate aspects of GvHD and can be implemented in pre-clinical drug discovery.

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The relevance of shear stress for *in-vitro* models of the human blood-brain barrier: A comparative study

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In recent years research on the development of flow-based *in-vitro* blood-brain barrier models has increased progressively. Compared to static Transwell models, micro- as well as millifluidic *in-vitro* models offer the possibility to study the blood-brain barrier (BBB) under shear stress presumably leading to a more *in-vivo* like phenotype. Though all three models are suitable for BBB modeling, they show differences in applicability. Detailed molecular analysis and barrier functional studies with a microfluidic and a millifluidic hollow-fiber model were accomplished to assess the influence of shear stress on human brain capillary endothelial cells. The immortalized human brain capillary endothelial cell line hCMEC/D3 or human induced pluripotent stem cell derived brain capillary endothelial cells (hiPS-BCEC) were cultivated with the three models mentioned above. Depending on the model, cell viability as well as barrier integrity and functionality were assessed using established methods such as glucose and lactate measurements with the BioProfile®100Plus, TEER measurements and permeability studies at different flow rates with the paracellular marker FITC-Dextran 4000 (FD4). Changes at the mRNA level of tight junction proteins including CLDN1-25, ZO1-3, occludin, tricellulin, JAM 1-3 as well as a series of ABC- and SLC-transporters were analyzed with a "BBB-chip", an adapted high-throughput qPCR Fluidigm® chip. Immunofluorescence images were performed to support functional data. The possible cultivation time of hCMEC/D3 as well as of hiPS-BCECs was increased progressively by exposure to shear stress allowing for long-term studies with the millifluidic model over for several weeks. On the contrary, cultivation in the microfluidic chip and the Transwell model was possible for some days. Adjustments in the medium composition showed a serum-dependency not only for cellular growth rates, but also for BBB properties of the hCMEC/D3. Paracellular permeability rates of FD4 were dependent on the applied flow rate. Differences in the gene expression between the models indicate a complex regulation of BBB properties induced by shear stress.

Anyhow, the choice for the most suitable model is dependent on the study question. The Transwell model is highly useful for acute disease modeling. In case of modeling shear stress for short-term studies, the microfluidic system seems to be the model of choice, whereas the millifluidic model can be applied for long-term studies over several weeks or months.

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Human Artificial Lymph Node Model (HuALN) for biopharmaceutical testing and disease modelling *in vitro*

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As modern biopharmaceuticals show a very high degree of species-specificity, conventional animal models are inadequate for drug development and to assess efficacy and safety. The Mode of Action (MoA) is complex, so simplified cell culture models using human cells often fail. Customized animal models, e.g. immune deficient, transgenic, and humanized animals as well as xenograft models are big in business but they show limitations in reproducibility and relevance to a certain extent. In particular for the human immune system, drawbacks in the pharmaceutical arena during the last years have raised significant doubts about their value and predictability for assessing immune modulation and immunogenicity. New "humanoid" models are required for new promising pharmaceutical treatments, e.g. by immune modulators, checkpoint inhibitors, and cell- and gene therapeutics.

The dramatically increased animal consumption triggered by customized animal model technologies pushes the ethical concerns on animal testing for pharmaceutical R&D, efficacy and toxicity testing, and risk assessment.

The Human Artificial Lymph Node Model (HuALN) is a micro physiological system (MPS) mimicking immunity in a continuously perfused 3D culture system and suitable for long-term treatment (e.g. 28 d) and repeated dosing. The MPS serves as a human micro-organoid lymph node model for induction or modulation of cellular and humoral immune responses. The implementation of stromal cells improves organoid formation. The HuALN model is designed for testing immunomodulation (e.g. MoA of checkpoint modulators), to assess unwanted immunogenicity reactions (e.g.

ADA formation, sensitization) or efficacy of vaccines, adjuvants and formulation. T cell responses and shifts in the TH1/TH2 pathway are continuously monitored by cytokine secretion profiles. The induction of primary humoral responses is demonstrated by B cell activation, plasma cell formation and antibody secretion profiles for IgM and IgG. Cells can be harvested from 3D matrix at the end of the MPS culture time and used for flowcytometric analysis and functional tests, e.g. ELISPOT assays.

By integration of tumour cells or tumour spheroids the HuALN platform is extended towards disease models.

The HuALN model will be introduced, selected results of biopharmaceutical testing will be presented and the opportunities using the HuALN for modelling tumour treatment will be discussed.

References

Langley, G. R., Busquet, F. and Giese, C. et al. (2017). *Drug Discov Today 22*, 327-*339*.

Marx, U., Andersson, T. B. and Giese, C. et al. (2016). *ALTEX 33*, 272-321.

Giese, C. and Marx, U. (2014). *Adv Drug Deliv Rev 69-70*, 103-122.

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Regulation of endocrine disruptors: REACH may change, but will the science evolve?

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Changes to the data requirements for substances under REACH are expected as the European Commission works to harmonise the identification and control of endocrine disruptors across a number of regulatory frameworks in an effort to allay doubts on whether current EU chemicals legislation adequately protects human and ecological health. Now that requirements are well established for plant protection products and biocides, the focus moves to other legislation which has fewer provisions for endocrine disruptors, including REACH.

The Commission's 2018 REACH review contends that the standard data requirements specified by REACH, even with a general requirement to collect all relevant available information, have limited capacity for providing sufficient insight into endocrine disrupting properties. Even though some information about endocrine disruption can be gained from the standard tests currently required – such as reproductive toxicity, repeated-dose studies, and long-term toxicity testing on fish – the REACH Annexes may be updated in order to expand the data requirements so that, much like for PBT and vPvB properties, a targeted evaluation of endocrine disruption can be performed.

In an ever-changing landscape, any updates to REACH could lock in animal-based assays at the expense of more human-relevant (and more humane) next generation non-animal methods. We explore the *in vivo* mammalian endocrine disruption tests that may be included in updated REACH Annexes, consider the ramifications for the animals that will be used in those experiments and ask the question: how well can these *in vivo* methods protect human health? We then examine what animal-free methods have to offer and look at how cosmetics testing legislation is driving technological advances. Finally, we make recommendations for a long-term REACH strategy for endocrine disruptors that is proportionate, stimulates and keeps pace with the evolving science of non-animal approaches, and realises the better protection of human health whilst eliminating the suffering of animals used in endocrine disruptor assays.

References

European Commission report on the operations of REACH, 2018: The second REACH review (The REACH REFIT evaluation), downloadable here: https://ec.europa.eu/growth/sectors/chemicals/reach/review_en

European Commission document, 2019: Evaluation and Fitness Check Roadmap – Ares(2019)3752914, downloadable here: https://ec.europa.eu/info/law/better-regulation/initiatives/ares-2019-2470647_en

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Advancing chemical safety assessments through integration of population-based *in vitro* models and computational methods

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Chemical risk assessments are undergoing a transition from traditional animal-based toxicity testing to incorporating New Approach Methods (NAMs), including substitution of mammalian testing with organotypic *in vitro* models and computational approaches supporting data integration and visualization. Among the key challenges in connecting technological advancement with practical decision making are evaluations of inter-individual variability and derivation of chemical-specific toxicodynamic adjustment factors. Integration of population-based organotypic culture models therefore represents a specific opportunity bridging lacking information requirements and application of *in vitro* alternatives in chemical risk assessments. Human induced pluripotent stem cell (iPSC)-derived cardiomyocytes are a genetically-defined, donor-specific model with demonstrated potential for functional and mechanistic cardiotoxicity profiling in combinatorial *in vitro*/*in silico* screening assays. This led to the hypothesis that a population-based *in vitro* cardiotoxicity model is amenable for quantitative assessment of inter-individual variability in cardiophysiologic responses to chemical exposures and for data-integrative extrapolation of critical bioeffects levels to physiologically-relevant concentrations. In this study, cardiomyocytes derived from more than 30 "healthy" individuals were exposed to select pharmaceuticals with known human cardiotoxic modes of action. iPSC-cardiomyocyteswereexposedtotestchemicalsforeither90min or 24 hrs in concentration-response covering nano- and micromolar concentration ranges reflective of physiological relevance based on available human Cmax values. Effects on cardiomyo-

cyte beating were monitored using kinetic calcium-flux measurements, high-content live cell imaging, and targeted transcriptomic analysis. Quality assurance assessment included evaluation of reproducibility of phenotypic responses across batches and time. iPSC-cardiomyocytes exhibited reproducible donor-specific differences in baseline function and drug-induced effects. Therefore, we demonstrate the feasibility of using a panel of population-based organotypic cells from healthy donors to rapidly screen drugs and other classes of chemicals for inter-individual variability in cardiotoxic responses. The presented approach demonstrates utility in quantifying population-level responses to xenobiotic exposures using iPSC-derived cell types.

References

Grimm, F. A., House, J. S., Chiu, W. et al. (2018). *ALTEX 35*, 441-452. doi:10.14573/altex.1805301

Blanchette, A. D., Grimm, F. A., Dalaijamts, C. et al. (2018). *Clin Pharmacol Ther 105*, 1175-1186. doi:10.1002/cpt.1259

Sirenko, O., Grimm, F. A., Ryan, K. R. et al. (2017). *Toxicol Appl Pharmacol 322*, 60-74. doi:10.1016/j.taap.2017.02.020

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Development of a test system for the investigation of the pulmonary mucus barrier

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Airway mucus is a heterogeneous hydrogel which protects our lungs from the environment. All foreign inhaled particles from dust to inhalation drugs will face this first line of defence. Mucus will influence drug efficacy by various effects [1]. In order to reduce animal experiments and to avoid wasting money, mucus should be considered as a significant barrier during an early stage of drug development. The aim of this project is to develop a test system to measure the penetration of new drugs through the human pulmonary mucus barrier. We aim for a robust method with high repeat accuracy. Therefore, different setups were evaluated.

First experiments were performed with a mucus-loaded electrophoresis capillary. In this method the molecules will separate caused of their charge and interaction with the human pulmonary mucus. The second setup was a column loaded with mucus which was applied as in chromatography. In this setup, we evaluated the effect of mucus layer thickness with focus on the diffusion in a transwell™ system. Four different mucus layer thicknesses were investigated: 0.0 cm, 0.1 cm, 0.2 cm and 0.44 cm. We found a reduced transport rate with increasing mucus layer thickness. However, standard deviations were unacceptable high. In consequence, we are currently working on a new setup based on an Ussing Chamber loaded with gelatine for the first experiments and human airway mucus in future studies.

Reference

[1] Murgia, X., Loretz, B., Hartwig, O. et al. (2018). *Adv Drug Deliv Rev 124*, 82-97. doi:10.1016/j.addr.2017.10.009

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A comparison of enzymatic and non-enzymatic strategies to isolate extracellular matrix (ECM) proteins from human placenta and liposuction fat

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Introduction

Human extracellular matrix (ECM) represents the ideal environment for mammalian cells and is therefore used to establish 3D cell culture methods, as 3D printing material or as biomaterial. Various strategies to extract and to decellularize ECM proteins based on enzymatic (e.g. pepsin) or non-enzymatic detergents (e.g. Tris-buffers or activated fetal calf serum; FCS) were published so far, and each is associated with its own bottlenecks. The aim of this study was to compare the biochemical characteristics of ECM proteins extracted by different published strategies.

Material and Methods

ECM proteins from human placenta were isolated by incubation with aqueous solutions of Fetal calf serum; FCS (activated), pepsin digestion, or a by extraction with Tris-NaCl buffer. ECM proteins from liposuction fat were extracted using FCS solutions (heat-inactivated control group) or by pepsin digestion. The ECM proteins were biochemically characterized by using a DNA (HOECHST 33342) assay, sodium dodecyl sulfate/polyacryl-amide gel electrophoresis (SDS-PAGE) analysis and an angiogenesis array.

Results

The mean DNA content was significantly reduced in placenta-extracted ECM using Tris-NaCl (33%), activated FCS (69%) and pepsin (85%) and in fat-extracted ECM using pepsin (90%), but not when using heat-inactivated FCS. A Tris-NaCl treatment showed various protein bands ranging from 20 kDa up to around 500 kDa on SDS-PAGE whereas pepsin treatments led to a reduction of bands. ECM isolates from Tris-NaCl buffer extractions showed the highest levels higher levels of bioactive cytokines, chemokines and growth factors determined using an angiogenesis array compared to pepsin-digestion derived isolates. Pepin-digested ECM from placenta and liposuction fat formed a solid 3D clot upon heating up to 37°C.

Discussion

The extraction strategy to isolate and to decellularize ECM proteins from tissues strongly determines the final characteristics of the ECM isolates (1) in terms of conservation of ECM proteins and (2) safety for potential clinical applications (DNA remnants, toxic chemicals). For instance, pepsin or active FCS efficiently reduced the DNA remnants in tissues. However, the treatment with pepsin leads to partial or complete digestion of most ECM proteins and growth factors. Tris-NaCl conserved most ECM proteins and angiogenesis-related enzymes, but the DNA level was far away from being decellularized (below 50 ng/mg ECM dry weight). Hence, more research is needed to optimize current isolation strategies.

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Parallelized precision medicine applications with a microprocessor-controlled, 3D-printed mini-bioreactor

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The reduction and replacement of animal experiments requires novel strategies for 3D cell culture that better reflect the complex 3D interaction of different cell types in living tissue. Compared to static 3D methods such as hanging drops, v-bottom plates or magnetic levitation *in vitro* cultivation using agitation-mediating devices significantly improves many physiological parameters such as oxygen supply and nutrient uptake. Usually, orbital shakers or stirrer-tank bioreactor are used for the formation of compact spheroids and organoids, however these commercially available devices do not support parallelization for e.g. drug screening. As these devices occupy significant incubator space, only minimal condition testing is possible. To overcome these limitations, we developed a microprocessor-controlled, fully 3D-printed mini-bioreactor system that allows stirred agitation of human cell spheroids and tissue aggregates in 12 or 24 well plates in a highly parallelized manner – i.e. simultaneous cultivation of up to 384 (16x 24 well plates) wells per bioreactor. The bioreactor housing and stirrer-plates were designed in open source CAD software and 3D-printed in conventional, low-cost FDM and DLP printers. Each bioreactor is controlled by an Atmega 328 microprocessor allowing exact control over rotation speed / direction and includes programmable routines for specific cultivation procedures. The system is successfully used for growing brain-organoids and tumor cell / fibroblast spheroids. With these mini-bioreactors we now also developed a protocol to culture and expand tumor spheroids isolated from the ascites of ovarian cancer patients, which allows us parallel testing of multiple anti-cancer drugs. These free-floating tumor spheroids might be responsible for the spreading of ovarian cancer throughout the abdomen and the poor prognosis of patients with this malignancy. Such spheroids cannot be cultivated in static cell culture and would have to be expanded in immune-compromised mice. Interestingly, tumor-spheroids frequently exhibit significant resistance to standard drugs used for ovarian cancer therapy (e.g. cisplatin), suggesting that these spheroids either represent a drug-resistant subpopulation of cancer cells shed off from the primary tumor or that these spheroids have an entirely different metabolism making them resistant to therapeutic drugs, which in both cases is detrimental for the patient. Parallelized testing with the mini-bioreactor system now provides an option to identify those chemotherapeutics that eradicate also ascites-derived tumor-spheroids and to study the underlying drug resistance mechanism. We believe that the depletion of freely-floating, metastasis-inducing tumor spheroids in the ascites of ovarian cancer patients is critical to improve the long-term survival of these cancer patients and the use of mini-bioreactors for parallelized drug screening provides an option to identify effective chemotherapeutic drugs.

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The effects of Lisinopril on wound healing response using *in vitro* human skin equivalent (EpiDermFT) after 5 day-repeated topical application

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Lisinopril, angiotensin-converting enzyme (ACE) inhibitor, is used for the treatment of high blood pressure and heart failure. Recent studies have shown that some ACE inhibitors improved re-epithelization during wound healing process and reduced scar formation. In this study, we performed wound healing experiment by using *in vitro* human skin equivalent model (EpiDermFTTM), which consist of human epidermal keratinocytes and dermal fibroblast with collagen matrix. Incised or burn wounds in epidermis were induced in EpiDermFTTM tissues by 3 mm biopsy punch (one circle area/tissue) or cauterizer (2 long square areas/tissue) and then 1% Lisinopril was treated topically each tissue for 5 days (20 µL/tissue/day). To compare the effect of wound healing, DPBS for negative control was concurrently treated topically and tissues with culture media including growth factors (2% human serum) was used for positive control. Each group was collected for hematoxylin and eosin (H&E) tissue slides or whole mount staining at two time point (Day 2 or 5) of recovery and evaluated for re-epithelization ratio (%, keratinocyte migration length/wound length), morphology, and expression of epidermal differentiation marker (cytokeratin 14). In the assessment of re-epithelization, biopsy punch-induced wounds were fully recovered (100%) on Day 5 in 1% Lisinopril-treated tissues, which was relatively higher ($p < 0.01$) than in DPBS group (79.2%). In the burn wounds, re-epithelization ratio was also higher ($p < 0.01$) on Day 2 in 1% Lisinopril-treated tissues (69.1%) than in DPBS group (45.1%). However, in burn wounds at Day 5, the re-epithelization ratio showed no significant differences between all tissues since most of wound areas were fully recovered by keratinocytes. In microscopic examination, epidermal differentiation was relatively more enhanced in 1% Lisinopril-treated tissues with cornification in stratum corneum of wound areas. In immunostaining, the expression of cytokeratin-14 was identified in tissues of all groups but depended on the degree of re-epithelization reflecting overall morphologic results. These data indicate that 1% Lisinopril have benefits in wound healing, and may have clinical utility in the future if the effects of ACE inhibitor on wound tissue were supported by further mechanistic studies.

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Novel approaches for evaluation of xenobiotic-mediated liver enzyme induction

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Xenobiotics may induce hepatic metabolizing enzymes (XMEs) normally resulting in increased ability for elimination and modulating the toxicological effect of substances. The induction of XMEs is preceded by the activation of nuclear receptors which control the transcription of the respective XME-encoding genes [1]. XME induction is commonly determined by measuring the XME activity [2]. In order to detect earlier events of XME induction, two methods measuring either mRNA or protein in rat liver were investigated and compared.

Rats were treated with standard liver enzyme-inducing substances, either with Aroclor 1254 or with Phenobarbital and b-Napthoflavon. In liver tissues, XME gene expression was measured by NanoString nCounter [3] and XME protein expression by peptide group-specific immunoaffinity enrichment combined with LC-SRM-MS [4]. The results were compared to XME activities obtained with model substrates.

All three methods were able to detect induction of target XMEs. With Aroclor 1254, CYP1A2 activity (EROD/BROD) was increased 30-fold/13-fold, mRNA 13-fold and protein 50-fold; CYP2B2 activity (PROD/BROD) 11-fold/13-fold, mRNA 223-fold and protein 789-fold. With Phenobarbital and b-Napthoflavon, CYP1A2 activity (EROD/BROD) was increased 5-fold/11-fold, mRNA 3-fold and protein 4-fold; CYP2B2 activity (PROD/BROD) 12-fold/11-fold, mRNA 133-fold and protein 258-fold. The x-fold changes of XME induction on mRNA, protein and activity level were not identical but clearly showed comparable relative magnitudes and correspond to known induction patterns [2].

Allowing for a lower amount of liver samples and being less time-consuming as well as more cost-effective, mRNA determination by NanoString nCounter and protein quantification by peptide group-specific immunoaffinity enrichment coupled to LC-SRM-MS might be convenient alternatives to the established activity measurements for evaluation of xenobiotic-mediated liver enzyme induction.

References
[1] Oesch, F. (1988). *Mutat Res 202*, 335-342. doi:10.1016/0027-5107(88)90196-0
[2] Oesch, F., Fabian, E. and Landsiedel, R. (2018). *Arch Toxicol 92*, 2411-2456. doi:10.1007/s00204-018-2232-x
[3] Geiss, G. K., Bumgarner, R. E., Birditt, B. et al. (2008). *Nat Biotechnol 26*, 317-325. doi:10.1038/nbt1385
[4] Weiß, F., Schnabel, A., Planatscher, H. et al. (2014). *Sci Rep 5*, 8759. doi:10.1038/srep08759
[5] Schmalix, W. A. et al. (1995). *Eur J Pharmacol 293*, 123-131. doi:10.1016/0926-6917(95)00008-9

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Vaccine batch to vaccine batch comparison by consistency testing (VAC2VAC)

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VAC2VAC brings together a unique consortium of pharmaceutical companies, academia, translational research organisations, Official Medicines Control Laboratories and regulatory bodies with the overall objective to demonstrate proof of concept of the consistency approach for batch release testing of established vaccines. This means that non-animal assays – instead of animal tests – can be used to ensure that each vaccine batch produced is consistent with a batch already proven to be safe and efficacious.

The three main steps to reach these objectives are:

1) Development of new or optimisation of existing non-animal methods for consistency testing: the core activity of the project, focuses on development and optimisation of physicochemical methods, immunochemical methods, cell-based assays, and multi-parametric assays & bioinformatics.
Thus far in VAC2VAC project Mass spectrometry (LC-MS) assays for Leptospira, DTaP intermediates and final vaccines and veterinary tetanus toxoid were successfully set up.

Progress has been made towards the objective of developing immunoassays that can substitute existing animal potency tests, as part of a consistency approach for veterinary rabies, thickborne encephalitis, DTaP and clostridium Chauvoei vaccines.

To replace the Rabbit Pyrogen Test for TBEV vaccine, the monocyte-activation test (MAT) was optimized, validated and transferred to the respective industry partner.

Significant progress was made towards development of a human B-cell assay for consistency testing of DTaP antigens.

Characterization of Clostridium tetani seed strains was performed using DNA, RNA and protein analysis.

For the development of platform technology to study interaction of vaccines/adjuvants with APC, suitable cellular platforms were identified.

2) Pre-validation of selected methods
For selected methods developed in VAC2VAC, small-scale multi-centre studies will be set up to assess transferability and inter-laboratory reproducibility of the methods.

3) Regulatory acceptance of the consistency approach
To maximise the chances of global acceptance and regulatory acceptance of the consistency approach, the development of methods in VAC2VAC involves close cooperation between public partners and industry partners in consultation with the regulatory bodies.

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Refinement on the way towards replacement: Are we doing what we can?

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This year marks sixty years since the inception of Russell and Burch's 3Rs framework of replacement, reduction and refinement [1]. It took several decades, however, until the importance of their principles was acknowledged, and for the 3Rs to be incorporated into legislation. Today, the 3Rs are a center piece of animal research regulation in many countries around the world. Thus, it seems reasonable to expect changes within the biomedical research industry, particularly the replacement of animals with non-animal approaches. However, the number of animals used has continually increased since the year 2000 [2], especially in the field of basic and applied research [3]. In opposition to the 3Rs hierarchy postulated by Russell and Burch who put replacement first and refinement last [1], many scientists who use animals apparently consider refinement to be more pressing and more achievable than reduction and replacement [4]. Due to this, the paper first explores how comprehensively and adequately refinement methods are being applied in practice, commencing with current housing and husbandry standards, and a discussion about the benefits of a "culture of care", followed by assessing important experimental refinements. To further assess the quality of animal-based research, I examine necessary refinements in planning, conduct, and reporting practices of animal studies. Then the other two Rs are reviewed, first discussing tools to appraise animal studies that could lead to a significant reduction of animal experiments. I subsequently reflect on what the scientific community has been doing to move towards replacement of animal use in research, testing, and education. The results of this review illustrate that much more can and must be done to truly apply the 3Rs framework, in which animal replacement is a clear focus. Thus, recommendations for steps are given that would accelerate the use of animal-free, human-relevant approaches.

References

[1] Russell, W. M. S. and Burch, R. L. (1959). Potters Bar, Hertfordshire, England: Universities Federation for Animal Welfare. http://altweb.jhsph.edu/pubs/books/humane_exp/het-toc

[2] Taylor, K. and Rego, L. (2016). *ALTEX Proc 33*, 465-468.

[3] Taylor, K. and Rego Alvarez, L. (2019). *ALTEX 36*, 314-319. doi:10.14573/altex.1812211

[4] Franco, N. H., Sandøe, P. and Olsson, I. A. S. (2018). *PLoS One 13*, e0200895. doi:10.1371/journal.pone.0200895

[5] Herrmann, K. (2019). *Human-Animal Studies*. Vol. 22, 3-64. Leiden: Brill. doi:10.1163/9789004391192

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Full implementation of refinement methods in practice through education and training

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Retrospective reviews of the implementation of refinement methods in practice revealed that the knowledge we have gained from refinement research is not fully applied in practice and thus, laboratory animals may endure unnecessary suffering [1-4]. Experimental refinements, including anesthesia and peri- and postoperative analgesia protocols, less-inhumane endpoints and killing methods were reviewed in over 500 basic and applied animal research proposals. Furthermore, a literature review was conducted to assemble the latest best practice-approaches in regards to housing and care. In all areas of refinement, flaws in the application of available approaches which could help to reduce unnecessary pain, distress and suffering of animals used in science were detected. One potential reason that was identified in the study of experimental refinement use was that researchers might not be sufficiently aware of existing refinement methods. In addition, a recent international survey, conducted by the European Commission's Joint Research Centre found that very few courses could be identified at university level that are specifically teaching the 3Rs [5]. To avoid needless suffering of laboratory animals, refinement methods need to be sufficiently implemented and fully applied in practice. Thus, an 8-week module-based course for university students and early career scientists is currently being established at Johns Hopkins University to teach about best practice-approaches and to foster a culture of care for laboratory animals. Several modules cover classical housing and experimental refinement, others comprise refinements of planning, incl. identifying the most appropriate research model, proper data analysis and comprehensive reporting. Consequences of poor refinement method use for the animals' welfare and for the validity of collected data are also discussed. The lectures are complemented by interviews with experts in laboratory science to explore reasons for the currently low refinement implementation rates and to find possible approaches to improve the situation.

References

[1] Herrmann, K. (2019). *Human-Animal Studies.* Vol. 22, 3-64. Leiden: Brill. doi:10.1163/9789004391192_002
[2] Herrmann, K. and Flecknell, P. A. (2018). *Altern Lab Anim 46*, 317-333.
[3] Herrmann, K. and Flecknell, P. A. (2018). *Altern Lab Anim 46*, 273-289.
[4] Herrmann, K. and Flecknell, P. A. (2018). *Altern Lab Anim 36*, 65-80. doi:10.14573/altex.1804011
[5] Holley, T., Bowe, G., Campia, I. et al. (2016). *EUR – Scientific and Technical Research Reports*.

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Education towards non-animal approaches in basic and applied biomedical research

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Directive 2010/63/EU, with its goal of replacing all procedures on live animals, is fundamental not only for furthering the implementation of the 3Rs but for helping to change the current research paradigm. Unfortunately, the Directive does not provide a plan of action on how to achieve this important goal. It is not unrealistic that this crucial shift can be accomplished, given the scientific and ethical shortcomings of animal modelling, which forms the basis of the prevailing paradigm [1]. However, these limitations are not yet widely taught to future scientists, and there is evidence that they are not appreciated or simply ignored, especially by experimenters who currently base their work on animal use [2,3]. In the EU, over 65% of laboratory animals are used in basic and applied research [4] which account for 68% of all animal experiments [5]. An EU requirement to use animals in these fields is to complete a FELASA-accredited course, which is generally 40-hour long and includes only a 1-hour lecture on non-animal methods, mostly only applicable for regulatory testing. Clearly, there is further scope for additional education in non-animal, human-relevant approaches. This paper will describe such a comprehensive course. Its eight modules cover themain shortcomings of animal use in science, how to fully apply the 3Rs principles, how to properly conduct literature searches, and how to plan, conduct, analyse and report research studies. The course further teaches how to critically appraise the validity of animal and non-animal models and methods in order to choose the best means for particular research interests.

References

[1] Herrmann, K. and Jayne, K. (eds.) (2019). *Human-Animal Studies*. Vol. 22. Leiden: Brill. doi:10.1163/9789004391192

[2] Franco, N. H., Sandøe, P. and Olsson, I. A. S. (2018). *PLoS One 13*, e0200895. doi:10.1371/journal.pone.0200895

[3] Fitzpatrick, B. G., Koustova, E. and Wang, Y. (2018). *Lab Anim 47*, 175-177. doi:10.1038/s41684-018-0088-6

[4] Daneshian, M., Busquet, F., Hartung, T. and Leist, M. (2015). *ALTEX 32*, 261-274. doi:10.14573/altex.1509081

[5] Taylor, K. and Alvarez, L. (2019). *ALTEX 36*, 314-319. doi:10.14573/altex.1812211

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Microfluidic *in vitro* lung model to replace murine infection and ARDS models

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Acute infections of the lung and the Acute Respiratory Distress Syndrome (ARDS) cause several million deaths worldwide every year. These diseases are associated with a strong influx of inflammatory cells (e.g. neutrophils) into the lung. Sepsis and pneumonia models in rodents are widely used for lung disease studies. The disadvantage of these *in vivo* models is their limited comparability with the situation in humans and the use of animals. Therefore, there is an urgent need to replace these animal experiments with innovative *in vitro* models.

The aim of this study was to develop a microfluidic *in vitro* lung model to replace murine infection and ARDS models. A microfluidic chip system was fabricated with the following main components: (1) a transparent, thin pore membrane, (2) two microfluidic channels, (3) two electrodes for impedance measurement. The pore membranes of silicon nitride with a thickness of 1000 µm and pore diameters of 3 to 5 µm were micro fabricated on a silicon wafer by chemical vapor deposition, lithography and wet chemical and reactive ion etching. The pore membrane was integrated into the fluidic module. Gold electrodes were included in the chip system for impedance measurements. Human bronchial epithelial cells (primary cells and 16HBE) were cultured in the microfluidic chip system under continuous flow (flow rate 100 µl/h) and used to test the migration of neutrophils from the serosal to the

mucosal side of the lung barrier. Neutrophils were isolated from whole blood of healthy donors by density gradient centrifugation. To pre-validate the microfluidic *in vitro* lung model, the migration of neutrophils across the epithelial barrier was investigated. Migration of neutrophils was promoted by the addition of *Pseudomonas aeruginosa* or the chemokine N-Formyl-Met-Leu-Phe. Neutrophil numbers were analyzed in the microfluidic channels and the morphology of the neutrophils was evaluated via staining and microscopy.

In our proof-of-concept study, we present a microfluidic *in vitro* lung model that allows a detailed biological analysis of the migration of neutrophils across pulmonary barriers *ex vivo*. It can be used in basic research as well as in the pharmaceutical industry and offers advantages over *in vivo* studies: (I) the number of animal experiments can drastically be reduced, in line with the 3R concept. (II) The application of human cells under fluidic conditions provides a much better prediction for the situation in humans.

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Combination of *in vitro* reporter gene assays, zFET and FETAX reveal cyanobacteria as producers of teratogenic retinoid-like compounds

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Formation of cyanobacterial water blooms and related production of diverse bioactive and toxic compounds have been often linked with adverse effects on exposed organisms and potential risk to human health. Newly detected group of compounds produced by cyanobacteria are retinoid-like compounds, but there is little information on their production, levels in the environment and namely potential adverse effects and risks associated with their occurrence. Our studies employed bioanalytical approaches using transgenic reporter cell lines for the characterization of the retinoid-like potencies of extracts and exudates from laboratory cultured cyanobacteria from different orders and samples of environmental water blooms and their surrounding surface water collected from field studies. The retinoid-like potencies were characterized by *in vitro* reporter gene assays focused on the interaction of the samples and isolated compounds with retinoid acid receptor (RAR). The relevance of the detected bioactivities of selected samples and compounds for whole organism was evaluated in zFET assay using embryos of zebrafish (*Danio rerio*) and FETAX test with frog embryos (*Xenopus laevis*), which are alternative methods for the assessment of developmental toxicity and embryotoxicity. Exposure studies documented correspondence of retinoid-like activity in cyanobacterial samples with developmental effects in embryos. Malformations typical for retinoid signaling disruption were detected after exposure to the cyanobacterial samples and those were compared to effects of standard retinoid compounds. The effects showed high morphological resemblance and the same dose-response pattern, suggesting presence of compounds affecting retinoid signaling in early vertebrate development. Observed effect phenotypes in both fish and frog embryos and effective concentra-tions of cyanobacterial samples corresponded to all-trans retinoic acid (ATRA) equivalents, which supports the hypothesis that the teratogenic effects of cyanobacterial samples are probably associated with retinoid-like activity. Sensitive analytical methods (LC-MS-MS) were used to characterize compounds contributing to the detected retinoid-like activities. The results document production of compounds with this bioactivity into surface waters by various cyanobacterial species and environmental water blooms. In some cases, the level of retinoid-like activity reached values that can cause adverse developmental effects in exposed organisms. We have identified a set of compounds contributing to the detected retinoid-like activity in both laboratory and field samples. These include ATRA, 9/13cis retinoic acid (RA), as well as several novel cyanobacterial metabolites, such as 5,6epoxy-RA or 4keto-ATRA with high retinoid-like potency. Our studies document that the production of retinoids by cyanobacteria into the aquatic environment is a common phenomenon, since retinoid-like activity and presence of individual retinoids have been shown to be associated with cyanobacterial water blooms dominated by many different species.

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Orally inhaled drug products – the strengths and weaknesses of simplified *in vitro* methods

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Complex co-culture systems consider cell-cell communication and give valuable insights in these interactions [1]. However, they generate more costs in acquisition and human resources. Simple *in vitro* models such as Calu-3, A549 or differentiated THP macrophage-like cells, dTHPs, are easy in handling, generate lower costs but do not mimic the physiological situation as close as more complex systems. In both cases, the relevance for human data and therefore the drug development itself is often not proven. This might be a reason why some companies still bet on data generated with animal experiments. In order to address this challenge and to reduce costs for pre-clinical developments, we focused on the following questions:

(1) Can dTHP macrophages replace primary human alveolar macrophages?

(2) Is there a correlation of the IC50 (tested on Calu-3 and A549) to FDA approved excipient concentrations?

(3) Do we need a co-culture for studying an acute inflammation *in vitro*?

Reference

[1] Hittinger, M., Schneider-Daum, N. and Lehr, C. (2017). *Eur J Pharm Biopharm 118*, 73-78. doi:10.1016/j.ejpb.2017.02.019

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Animal experiments or humane alternatives: Awareness raising campaign in Belarus

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In modern society, the successful solution of ethical problems connected with animal use in experiments is corresponded with institutionalization of bioethical principles. In Belarus, this process is on the initial level. There is still no law that can protect laboratory animals. The government does not register the quantity of animals used in experiments. The establishment and operation of ethical committees are not mandatory requirements of the Belarusian legislation. There is no ban on cosmetic products testing on animals and no special centers for development of alternatives.

Researchers, teachers of schools and universities, students and pupils know little about modern principles of animal protection legislation and alternatives to animal experiments that are already used in many countries in toxicology, medicines testing, and in teaching of the life sciences.

In order to enlighten Belarusian citizens in the field of animal experiments and humane alternatives Center for the Ethical Attitude Towards Nature (ECOetika) has initiated the project "Humane Alternatives to Animal Experiments". It was executed in 2018-2019 by our Center and with the support of International Sakharov Environmental Institute of Belarusian State University (ISEI BSU), International Association Against Painful Experiments on Animals (IAAPEA), International Network for Humane Education (InterNICHE), Lush – The Charity Pot Fund and the Doctors Against Animal Experiments Germany (DAAE).

The activities of the project were the following:
– development and distribution of E-Newsletter through the Internet;
– conducting of training module for high school students profiled in the life sciences;
– organization of Online Quiz on Bioethics;
– holding of republican contest of creative works;
– survey of public opinion among high school students;
– information campaign in the media and on the sites of educational establishments;
– informing via social networks;
– translation of articles;
– collaboration with universities;
– distribution of printed materials.

While carrying out the project we published 6 issues of E-Newsletter. Training module for high school students from 54 schools, gymnasia, lyceums, supplementary education institutions was conducted. We managed to cover not only Minsk, but also 12 provincial districts of Belarus. 109 high school students took part in the Online Quiz and more than 80 creative works were presented on the contest. The survey of public opinion among high school students showed that 33.5% of respondents considered animal experiments to be meaningless and their results irrelevant when transferred to humans. 62.5% of respondents spoke against conducting animal experiments in educational process. Totally 460 people took part in the survey. During the project execution, we translated more than 30 articles from English into Russian and published them on social networks. In the context of cooperation with universities, we attracted distinguished scientists and teachers as experts. We also made the report during the round table "Actual problems of biomedical ethics" in Minsk and read a lecture on biological faculty of Belarusian State University. The leaflets and brochures on the topic of animal experiments and humane alternatives, that we published, were distributed among schoolchildren, students, teachers and public. The project had great media coverage.

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Current status of animal-free research into Parkinson's disease

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Parkinson's disease, earlier also known as shaking palsy, is considered the second most common neurodegenerative disease after Alzheimer's disease. 4.1 million people worldwide are reported to suffer from Parkinson's disease, with numbers rising.

In Parkinson's disease, the death of dopamine (L-dopa)-producing neurons in the substantia nigra (in the midbrain) causes typical motor symptoms such as tremor at rest, slowness of movement, muscle rigidity or rigid facial expressions. In addition to the death of the nerve cells, so-called Lewy bodies accumulate in the nerve cells of the substantia nigra. Damage to complex I in the electron transport chain of the mitochondria seems to be central to the development of the disease, but in about 90% of cases, the cause is not known or not sufficiently known, with only 10% being due to genetic reasons. Earlier encephalitis, environmental toxins, pesticides or medicines may also play a role. Currently available treatment methods cannot halt the disease and there is no cure yet. For instance, disease symptoms are treated by orally administered L-dopa, but new findings have shown that gut bacteria inactivate L-dopa [1]. Bacterial metabolites could be used as biomarkers, inhibitors against material decomposition could be developed. There is a strong need for new treatment methods.

Animal species or breeding lines can only develop symptoms of a particular human disease. In these animals, symptoms are induced genetically or chemically. However, results from these experiments with animal models are not readily transferable to humans.

There are increasing alternative methods (*in vitro* and *in silico*) with which the causes can be investigated and drugs can be tested, e.g. the "midbrain-on-a-chip" model and many others. In Germany, the German Research Foundation supports the development of *in vitro* disease models using patients' own cells (DACaION project). Scientists are also working on solutions for the especially complex systemic approach. Developers of pharmaceutical drugs are very interested in human-specific procedures.

The authors want to give an introduction about the current development of animal-free methods.

Reference

[1] Rekdal, V. M. et al. (2019). Discovery and inhibition of an interspecies gut bacterial pathway for Levodopa metabolism. *Science 364/6445*. doi:10.1126/science.aau6323

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Current status of the implementation of new methods in cardiotoxicity safety testing

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Cardiotoxicity, the harmful effect of drugs on the heart, is the reason for about a third of all new drugs withdrawn from the market and for the end of many active ingredients in the late clinical development phase. The costs for drug development now amount to approximately 2.6 billion dollars over a production period of between 10 and 15 years.

Cardiotoxicity tests for drug research and development are defined in Directive S7B of the International Conference on Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). The tests provide for a combination of animal testing and animal-free procedures. Among other things, the regulation describes the hERG test, a test procedure on a calcium channel of the heart, from which it was assumed in the 90s that solely its inhibition seems to be the molecular mechanism of the cardiac arrhythmia caused by drugs. Meanwhile, however, it is known that The exclusive measurement of hERG can lead to incorrect results. A Prolongation of the action potential can be induced by both, a reduced inactivation of the inwardly directed $Na+$- or $Ca2+$ currents, increased activation of the $Ca2+$ current or inhibition of one or more of the outward $K+$-currents can result. Furthermore, heart tissue and whole hearts of rabbits are used, although scientists are increasingly critical of Langendorff's heart preparations because they cannot integrate pathological and pharmacological conditions into an autonomously functioning nervous system.

In recent years, scientists have carried out state-of-the-art cardiotoxicity tests with human heart cells from stem cells on chip technologies. The methods are often used already in validation studies with the industry, however they are not mandatory.

With the CiPA Initiative [1], scientists of the United States together with other international researchers have developed a new testing strategy and integrated these new methods. However, their inclusion in the test regulations is being delayed. Meanwhile, the industry is still using the outdated tests [2]. The rapid implementation of new test guidelines under incorporation of the new human-specific methods into the guidelines is demanded.

We want to find out what the reason is for this slow implementation process.

References

[1] Initiative Comprehensive In Vitro Proarrhythmia Assay (CiPA) der Food and Drug Administration. http://cipaproject.org/about-cipa/
[2] Authier, S., Pugsley, M. K., Koerner, J. E. et al. (2017). *J Pharmacol Toxicol Methods 86*, 34-43.

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Social enrichment by pair-housing of male C57BL/6JRj mice

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Standard housing in laboratory animal facilities does not reflect social structures of free-living mice [1] and, therefore, comes along with animal welfare issues. A common code of housing practice is to keep mice in single-sex groups. However, in group-housed adult males, aggressive territorial and resource-related behavior is often observed, which in turn can lead to stress due to repeated social defeat and subordination. This may even result in injuries or death [2]. When an unacceptable level of aggression is reached, male mice are separated and housed individually, though the lack of social contact can increase anxiety- and depression-like behaviors as well as stress levels [3].

As a strategy to refine laboratory housing of male mice another housing system called "pair housing" was introduced. To provide social enrichment for individually housed mice, two male mice, separated by a transparent, perforated wall, were kept in a cage together. Pair housing allows olfactory, visual and acoustic contact. Short-term effects of the transition to this housing system were reported for male Hsd:NMRI mice: pair housing increased heart rate, body temperature, and motor activity [4]. However, long-term effects on behavioral, physical and biochemical parameters have not been examined yet. Therefore, we investigated whether long-term pair housing (for eight weeks) fosters well-being of male C57BL/6JRj mice, the most commonly used mouse strain in animal research, when compared to single and group housing (pair: n = 16, single: n = 16, group: n = 16). Besides burrowing and nesting, we assessed anxiety-related behavior in the free exploratory paradigm, the ease of handling by using a rating scale and social behavior in a social interaction test. Moreover, body weight and stress hormone (metabolites) concentrations in feces and fur were measured.

Whereas the housings systems did not affect burrowing performance, pair-housed mice built more complex nests than single-housed mice. The nest position suggested that pair-housed mice preferred the close proximity to their cage mates. Although the free exploratory paradigm did not indicate any effects of the housing systems on trait anxiety, pair-housed mice showed a higher latency to voluntarily interact with the experimenter's hand than single-housed mice. In contrast, single-housed mice were more difficult to catch. The social interaction test did not reveal any differences between the housing systems, except from the distance moved. Pair-housed mice moved less in the test arena when compared to single- and group-housed mice. Stress-hormone (metabolites) concentrations were not significantly influenced, but a higher body weight gain was found in group-housed mice.

Overall, our study did not reveal any long-term beneficial effects of pair-housing on stress levels of male mice, though nest complexity may indicate fostered well-being. However, the ventilation in the IVCs can also influence nest complexity. Taking into account that male mice prefer dwelling near other males to staying alone [5], pair housing rather than single housing can meet this need. The decrease in exploratory behavior may suggest higher anxiety-related behavior in pair-housed mice. However, to correctly interpret these findings, state anxiety-related behavior and home cage activity should be further investigated.

References

[1] van Zegeren, K. (1979). Variation in aggressiveness and the regulation of numbers in house mouse populations. *Neth J Zool 30*, 635-770. doi:10.1163/002829679X00241

[2] Weber, E. M., Dallaire, J. A., Gaskill, B. N. et al. (2017). *Lab Anim 46*, 157-161. doi:10.1038/laban.1219

[3] Berry, A., Bellisario, V., Capoccia, S. et al. (2012). *Psychoneuroendocrinology 37*, 762-772. doi:10.1016/j.psyneuen.2011.09.007

[4] Rettich, A., Käsermann, H. P., Pelczar, P. et al. (2006). *J Appl Anim Welf Sci 9*, 277-288.

[5] Van Loo, P. L., de Groot, A. C., Van Zutphen, B. F. et al. (2001). *J Appl Anim Welf Sci 4*, 91-103. doi:10.1207/S15327604JAWS0402_1

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P. aeruginosa infected co-culture of human cystic fibrosis bronchial epithelial cells as a preclinical test system for anti-infectives

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In the lungs of infected cystic fibrosis (CF) patients, *P. aeruginosa* (PA) is one of the persistent bacteria. Chronic biofilm formation is still a challenge in CF treatment, as classical anti-infectives fail in the course of CF patients´ life. We established a new co-culture model of CFBE41o- cells homozygous for ΔF508 mutation infected with a pre-formed PA biofilm. The aim is to create a predictive *in vitro* system for human CF lung infection in order to test novel anti-infectives pre-clinically and thereby minimizing the number of animal trials.

 Human bronchial epithelial cells were cultured in the Transwell® system at the air-liquid interface (ALI), allowing for aerosol deposition. Cells were infected with a 72 h pre-grown mature PA biofilm for 1h prior to treatment. After restoring ALI conditions, Tobramycin or vehicle was nebulized on the co-culture of biofilm and cells via Aerogen® Aeroneb® Lab Nebulizer.

Tobramycin (10 µg dose) preserved barrier functions and cell viability after 4 and 24 h treatment. CFU of *P. aeruginosa* biofilm was reduced by 3 and 4 logs, respectively. Infected cells treated with vehicle showed approx. 60% cytotoxicity after 4 h and an increased release of IL-8; after 24 h the cells did not survive the infection. To check the relation between bacterial killing and host cell survival, Tobramycin was nebulized at concentrations of 1 µg and 100 µg on infected cells. At 1 µg dose, no reduction of CFU was observed whereas the host cells did not survive after 24 hours. The nebulization of 100 µg efficiently killed almost all bacteria (10^1 CFU of surviving bacteria), whereas the host cells were still alive and showed preserved barrier function after 24 h.

To assess if this infected co-culture could be suitable for successive antibiotic administration, 10 µg Tobramycin was nebulized on infected cells previously treated for 24 h with the same dose of antibiotic. Bacterial survival was checked after 24 hours as well as epithelial barrier integrity. Even with Tobramycin re-administration, the bacteria were not completely eliminated on the cells. Nevertheless, the host cells were alive, and showed a TEER of approximately 200 Ω x cm^2.

In conclusion, the present model is a valuable system to test efficacy of antibiotics and other anti-infectives against *P. aeruginosa* as well as the host response to the treatment. Furthermore, this model allows to analyse host-response in a chronic state of infection.

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Refinement in Laboratory Animal Science education – an inquiry concerning the use of rat and mouse simulators among LAS instructors

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Qualified education of persons involved in animal experiments is, in itself, a means of applied refinement in Laboratory Animal Science (LAS). This is implemented in practice by LAS courses in which basic theoretical knowledge is taught and essential manual skills are trained prior to working with live animals. Since, to date, none of the available alternative methods can fully provide an adequate acquisition of practical skills under realistic conditions, most courses predominantly include training involving live animals, usually rats and mice. According to the Directive 2010/63/EU, this training is categorized as an animal experiment and the implementation of the 3Rs is requested [1]. A further refinement strategy is the use of simulators prior to training with live animals, by which the stressful impact of training procedures on the animals can be reduced to an indispensable minimum. Currently, five rat simulators and one mouse simulator for first practice in handling and procedural techniques and one rat simulator for microsurgical techniques are commercially available on the international market. Since only few data concerning the suitability and efficiency of these simulators exist, a team of scientists from the Department of Veterinary Medicine, *Freie Universität Berlin*, set out to systemically evaluate all currently available rat and mouse simulators in a multi-perspective approach.

In order to investigate the implementation and suitability of rat and mouse simulators in LAS courses, a questionnaire for trainers and advisers was developed concerning the use of and satisfaction with these simulators. Furthermore, requirements for the improvement of future simulators were determined. The survey, in which 37 course trainers and advisers from Germany and neighboring countries took part in, was accessible online from 2018-05-31 until 2019-06-30 and is currently being statistically analyzed. The outcome of which will be presented at the EUSAAT congress in October 2019.

Reference

[1] Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes (2010).

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Development of 3D skin model and 3D skin infection model, as advanced testing tools for the bio-evaluation of novel antimicrobial biomaterials for treating infected wounds

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EU 7th amendment (Dir. 2003/15/EC) of the "Cosmetics Directive" (76/768/EEC) has required the replacement of animal trials with reliable *in vitro* tests for assessing cutaneous resorption since 2009. *In vitro* bio-evaluation would be more accurate using biomimetic 3D testing systems respect to 2D cell cultures. In this work, *in vitro* 3D human skin equivalent (HSE) and wound infection model (c-HSE) were developed and used for testing commercially available Ag-dressings, then compared to 2D cultures.

Initially murine *in vitro* dermal construct (MDC) and human *in vitro* dermal construct (HDC) were generated, and different viability assays were optimized for 3D system [1]. Then, in-house reconstructed human epidermis (RHE) and reconstructed murine epidermis (RME) [2] were developed with the aim to understand the relevance of outcomes obtained from human- and animal-based systems reducing the animal trials. The human skin equivalent (HSE) having both dermal and epidermal compartments was obtained by optimizing 3D cell culture conditions based on serum/animal component-free and fully-defined media to obtain epidermal differentiation mimicking as closely as possible native human skin (NHS). Skin infection model (c-HSE) was created by full-thickness incision and colonization with *S. aureus*.

To validate the 3D systems, Ag+ and several commercially available Ag-dressings were bio-evaluated using HSE and c-HSE. On the other hand, cell monolayer cultures (based on primary cells and cell lines) were used as 2D cytocompatibility evaluation systems. These materials were also evaluated for their antibacterial activity against a range of clinically relevant pathogens using different growth culture conditions including simulated wound fluid (SWF). Moreover, additional efforts were addressed to the bio-evaluation of novel drug-free biomaterials intended for infected wound healing applications.

Histology showed characteristic well-differentiated epidermal layers. Immunohistochemistry for proteins' tissue-distribution analysis showed Ki-67 & K14 positive basal keratinocytes; K10 positive supra-basal keratinocytes; loricin, filaggrin, & involucrin positive sub-corneal & corneal keratinocytes; and laminin 5 positive basement membrane at dermal-epidermal junction. TEM re-vealed the ultrastructure of basement membrane demonstrating lamina lucida, lamina densa, regular hemidesmosomes, and anchoring fibres. The epidermal compartment showed abundant of intracellular keratin filaments, desmosomal connections, and tight junctions between keratinocytes. SEM revealed the interwoven network architecture of ECM with embedded dermal fibroblasts. The contact angle of 82.5°±8.9° demonstrated the barrier function of HSE with respect to 90.0°±5.1° for NHS. This model represented a fundamental construct with highly resembling features to that of NHS. Histology of c-HSE demonstrated bacterial aggregations and early biofilm formation while TEM revealed interesting features including *S. aureus* induced ECM degradation, fibroblasts/keratinocytes cell lysis, bacterial internalization by keratinocytes, and dissociating epidermal layers. Grape-like bacterial clusters were found encased in extracellular polymeric substance at wound site. The c-HDC results suggested that co-existence of *S. aureus* & *P. aeruginosa* have a significant impact on bacterial colonization and pathogenicity in wounds. The bio-evaluation outcomes were different in 2D monolayer vs. 3D HSE based cell culture systems, and conventionally used microbial methods vs. advanced c-HSE system.

With an increasing need for reliable *in vitro* testing systems, we were successfully able to establish closely mimicking and verify our advanced 3D models, to serve as a risk assessment platform for cytocompatibility and antibacterial properties.

References

[1] Idrees, A. et al. (2018). *Int J Artif Organs 41*, 779-788. doi:10.1177/0391398818775519
[2] Segrelles, C. et al. (2011). *BMC Dermatol 11*, 9. doi:10.1186/1471-5945-11-9

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Development of a subacute 28-day respiratory toxicity assay using the EpiAirway *in vitro* human airway model

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Knowledge of subacute (28-day) respiratory toxicity potential is an important component of establishing safe use of chemicals and consumer products. The current work describes efforts to develop an alternative, non-animal method for determining subacute respiratory toxicity using the EpiAirway™ *in vitro* human airway model. Initial acute toxicity experiments were conducted by exposing EpiAirway tissues to four concentrations of test chemicals via apical application using either aqueous or corn oil vehicles for three hours. After exposure, the test chemicals were rinsed off and the tissues were incubated for an additional 21 hours. An IC75 concentration (concentration required to reduce the endpoint value to 75% of vehicle exposed controls) was determined from the dose-response data using barrier function (determined by measuring transepithelial electrical resistance (TEER)) and tissue viability (MTT assay) as endpoints. Based on the determined acute IC75 value, EpiAirway tissues were exposed to additional serial dilutions of the test chemicals, using the IC75 as the baseline dose. Tissues were apically exposed for three hours, followed by rinsing, every Monday, Wednesday and Friday, with TEER measured prior to each dose application. Experiments were continued for at least 30 days to determine no-observed-adverse-effect level (NOAEL) doses. Rank ordering of NOAEL levels obtained for 8 chemicals was as follows: formaldehyde $\ll$ butyl amine $<$ oxalic acid $\ll$ vinyl acetate $<$ morpholine $<$ methyl methacrylate $\ll$ dimethylacetamide $<$ ethanol. Expansion of the data set to include additional chemicals of different classes, chemical structures and physical properties is ongoing. These results indicate that *in vitro* airway tissue models using TEER as a convenient non-destructive endpoint are a promising alternative to animal tests for assessment of subacute 28-day respiratory toxicity and NOAELs. With further *in vivo* correlation and validation, this test may be a useful non-animal alternative for determining safe human subacute exposure levels for inhaled chemicals.

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Transcriptomic, metabolic and toxicological comparison of the human liver derived HepaRG with the human kidney derived RPTEC/TERT1 cells

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The human proximal tubule RPTEC/TERT1 and the human hepatic line HepaRG are considered the most differentiated and stable cell lines currently available for their respective tissues. They are fairly unique for cell lines, in that they become contact inhibited which initiates a differentiation program (analogues to Caco-2 cells). Here, we compared and contrasted both cell lines with respect to gene expression, morphology, metabolic activity and response to toxins. Both cell types represented an oxidative phenotype, with a high mitochondrial reserve capacity. Each represented well their tissue of origin, with specific characteristics and features. Due to dynamic dome formation, which is a consequence of apical to basolateral transport of water and solutes, differentiated RPTEC/TERT1 exhibited oscillations in monolayer impedance, as measured by the xCELLigence device. HepaRG as expected exhibited significantly higher expression of genes encoding plasma proteins, including albumin (ALB), haptoglobin (HP), transthyretin (TTR), alpha fetoprotein (AFP), apolipoproteins (APOA1, APOC1, APOE), fibrinogen (FGG, FGB) and complement proteins (C3, C1R and CFH), genes involved in xenobiotic metabolism, including cytochrome P450s (CYP3A4, CYP3A5, CYP2E1), N-acetyltransferase 2 (NAT2) and carboxylesterase 1 (CES1). Both cell types exhibited unique alterations in the transcriptome when exposed to a small panel ($N = 6$) of compounds for 24 hours. The implications of these results are discussed.

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A study on skin irritation test of industrial chemicals using RhE model for prevent occupational skin diseases

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Exposure to chemical substances can cause health hazards to workers, such as occupational skin diseases and systemic toxicity. NIOSH (National Institute for Occupational Safety & Health) estimated that more than 13 million workers are potentially exposed to chemicals that can be absorbed through the skin.

However, there is a lack of database on MSDS skin irritant/corrosiveness data for domestic workers. Therefore, we have selected 3 chemical substances (EDTA tetrasodium, Indene, n-Butyl Lactate) based on volume of the domestic chemical distribution and the latest issues, performed a skin irritation test on reconstructed human epidermis (RhE) model. Consequently, it is anticipated that the result of the study will be possible to apply on MSDS.

It is fact that skin irritation test has been performed on skin of rabbits to evaluate hazard of skin from since. To fulfil of animal welfare and 3Rs, this study was performed on human epidermis model (SkinEthic™ RHE) accordance with OECD guideline TG 439 and the manufacturer (EPISKIN)'s Protocol. NSCliving(Non-specific colour) control and MTT analysis were performed to evaluate the hazards of EDTA tetrasodium(Cas No.13235-36-4), Indene(Cas No.95-13-6), n-Butyl Lactate(Cas No.138-22-7). Skin irritation hazard assessment results showed that the standard deviation (SD) of Indene and n-Butyl Lactate satisfied the allowable range (SD ≤ 18) specified in the guidelines. However, EDTA tetrasodium was found to require additional testing beyond the acceptable range of SD = 41,341. The skin irritation hazard classification of Indene and n-Butyl Lactate was revealed as a skin irritant (GHS "Category 2")

Through this research project, it is anticipated that it will be possible to preemptively cope with the prevent of occupational skin diseases by evaluating skin irritation hazard for prevention of workers health disorder for industrial chemical substance.

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A new skin-on-chip for anti-UV evaluation

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As we all know, UV radiation can induce oxidation and inflammation to skin which may cause skin senescence, irritation, showing hyperpigmentation, erythema, wrinkle, or even cause cancer.

Our team has designed and conducted test for UV-induce skin senescence in cell and recombined skin tissue level. However, there was still limitation existed. We needed a test system more flexible and closer to human, which could extend the treatment and sample dynamically.

The skin-on-chip which combined two different tissues could be the best option for our needs. 3D epidermis model (keratinocyte) was used as barrier and metabolic component which stimulates directly UV. And fibroblast cell from human skin was server as target cell. After UVA radiation to recombined epidermis for 5, 8, 10, 12 J/cm^2, we found that the target cells were different from viability, morphology. Radiation at 5 and 8 J/cm^2 caused no obvious damage to the keratinocyte or fibroblast. As we know, UVA can reach the dermis through the epidermis. Next, we tried to extend our radiation at 5 and 8 J/cm^2 for 7 days (once a day). By sampling the medium for cytokines, such as IL-1alpha, IL-6 and TNF-alpha, we found that they all increase differently; suggesting inflammation was induced during the radiation. The beta-galactosidase was stained as the market of cell senescence. More senescent cells were found than single radiation. Then 2 test substance resveratrol and procyanidine were added to the medium before UVA radiation (Pretreat with test substance for an hour before every day's radiation). For 7 days radiation, the cytokines from medium were sampling 24 h after radiation every day. The cytokines were decreased compared to the radiation group (medium only,

without test substance). Less cytokines (at Day 8) was detected than any other days.

We summarize that our skin-on-chip test system combining epidermis model and fibroblast cell was able to mimic the UVA radiation to skin causing inflammation for 7 days or more. However, only cytokines were evaluated. Now, our team is developing a test system using skin-on-chip for subchronic/chronic skin aging induced by UV radiation, not only UVA, but also UVB or both. Besides that, we are trying to consider the mRNA level of cytokines for inflammation and ROS or SOD for oxidation.

References

Wufuer, M. et al. (2016). *Sci Rep 6*, 37471

Schimek, K. et al. (2018). *Bioengineering 5*, 43. doi:10.3390/bioengineering5020043

Arik, Y. B., van der Helm, M. W., Odijk, M. et al. (2018). *Biomicrofluidics 12*, 042218. doi:10.1063/1.5023041

Zhang, Q., Sito, L., Mao, M. et al. (2018). *Microphysiological Systems 2*, 4. doi:10.21037/mps.2018.08.01

Sriram, G., Massimo, A., Dancik, Y. et al. (2017). *Mater Today 21*, 326-340. doi:10.1016/j.mattod.2017.11.002

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Integrated evaluation for skin sensitization and phototoxicity of plant extracts

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Skin sensitization and phototoxicity are two of the important risks of topical applied products, such cosmetic. Plant extracts are more and more popular as ingredients of cosmetic and they are quite different due to seasons, environment, parts of plants and extract methods. Our group has set up a project for plant extracts evaluation trying to establish a test procedure for skin sensitization and phototoxicity.

Total components of the extracts were first analyzed using LC-MS, GC-MS, NMR.

For skin sensitization, the optimized DPRA using cysteine-containing heptapeptides (Ac-RFAACAA), lysine-containing heptapeptides (Ac-RFAAKAA) and heptapeptides Cor1-C420 (Ac-NKKCDLF) was used. After 24 h, 48 h and 72 h reaction, the concentration of the peptides was analyzed. Then calculate the percent depletion. All the sample with positive or negative result was then tested using KerantinoSens and/or H-CLAT. H-CLAT was performed only if KerantinoSens gave a negative result. 8 samples were evaluated by the Episkin and THP-1 co-culture system which was established for lubricants before due to the solubility.

For phototoxicity, we used 3T3-NRU-PT test (OECD TG 432) or EpiSkin-based-PT test (only for insoluble sample). In EpiSkin-based-PT test, Then UVA radiation of 6 J/cm^2 was performed after test article was applied on the top for 18-24 h. Control well was kept in dark at room temperature. Then incubated for 18-24 h. MTT test was used to determine the tissue viabilities of UV+ or UV-.

Our results show that 4 of 30 were identified as potential skin sensitizer (3 of which were DPRA KerantinoSens and H-CLAT were all positive, 1 of which was tested by co-culture system showing positive) and 7 of 30 were identified as potential photo toxicants (under testing).

1 of the 8 samples tested by co-culture system also induced the upregulation of gene of EpiSkin, such as HSPA6 and IL8. We later compared the components of the 4 positive sensitizers. These 4 samples were come from different parts of 2 kinds of plants. And 3 skin sensitization relative components (structure) were found in these 4 skin sensitizers based on former research. Interestingly, we found that not all the sample with special structure related to skin sensitization, would be classify as potential skin sensitizer.

References

Gibbs, S., Corsini, E., Spiekstra, S. W. et al. (2013). *Toxicol Appl Pharmacol 272*,529-541. doi:10.1016/j.taap.2013.07.003

Saito, K., Nukada, Y., Takenouchi, O. et al. (2013). *Toxicol In Vitro 27*, 2213-2224. doi:10.1016/j.tiv.2013.08.007

Cottrez, F., Boitel, E. Auriault, C. et al. (2015). *Toxicol In Vitro 29*, 787-802. doi:10.1016/j.tiv.2015.02.012

Wong, C. L., Lam, A.-L. et al. (2016). *Front Pharmacology 7*. doi:10.3389/fphar.2016.00053

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3Rs Center at the National Institute of Public Health in the Czech Republic

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Activity of the 3Rs Center has started in the 1990s and followed the worldwide scientific vision of replacing animal experiments with *in vitro* methods. A number of *in vitro* methods was implemented and applied in toxicological studies focused on hazard and risk assessment of consumer products, medical devices and their ingredients. The Czech 3Rs Center was nominated by the Ministry of Agriculture in accordance with the Directive 2010/63/EU as a national single contact point to provide advice on alternative methods, a representative of the CR in PARERE network and a qualified laboratory in EU-NETVAL, the ECVAM´s network of laboratories for the validation of alternative methods.

The 3Rs Center applies for routine toxicological testing the following methods:
- Skin corrosion and irritation: EpiSkin/ EpiDerm /SkinEthic RHE models of epidermis
- Phototoxicity: 3T3 NRU test, 3D skin models
- Eye irritation: 3T3 NRR test, HET-CAM test, 3D cornea models
- Inflammation markers: ELISA – IL1α, IL6, IL8, TNFα
- Skin absorption / penetration: *Ex vivo* porcine skin,3D skin models
- Skin sensitization: DPRA, LuSens
- Genotoxicity / Mutagenicity: Comet assay, Ames test, *In vitro* chromosomal aberration test
- *In vitro* micronucleus test

- Acute toxicity: Cytotoxicity test for LD50 estimation
- Cytotoxicity test: EN ISO 10993-5 for medical devices
- Reprotoxicity: Estrogen and Androgen receptor transactivation tests.

Experts of the 3Rs Center are engaged in cooperation with scientific institutions and universities, provide education and demonstrations of *in vitro* methods, enhance spread of information via public communications, participate in authorization of proposals for animal experiments and in the Central Commission for Animal Welfare, take part in validation trials and in Peer Reviews of new methods for EU-NETVAL, OECD, EURL-ECVAM, and provide scientific and technical support for responsible authorities and legislative bodies.

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Generation of functional macrophages from human induced pluripotent stem cells

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Monocytic lineage cells (monocytes, macrophages and dendritic cells) play important roles in immune responses and are involved in various pathological condition. Primitive macrophages arise from embryonic yolk sac-derived early or late erythro-myeloid progenitors (EMPs) during primitive hematopoiesis. These primitive macrophages seed at fetal tissues following initiation of blood circulation and subsequently give rise to tissue-resident macrophages in all other tissues such as brain (microglia), liver (Kupffer cells), bone (bone marrow macrophage) and skin (Langerhans cells). Development of tissue-resident macrophage cells from human induced pluripotent stem cells (hiPSCs) is of particular interest because it provides an unlimited cell source in various research fields. In the present study, we demonstrated efficient generation of functional macrophages from hiPSCs. Hemangioblast-like hematopoietic cells were induced by sequential addition of BMP4 and the combination of bFGF, VEGF and SCF. Then, we generated CD45+ hematopoietic cells with defined cytokines. To induced CD14+ monocytes, we used Flt3 ligand, GM-CSF and M-CSF. These cells were cultured with FBS and M-CSF for differentiation into macrophages. hiPSC-derived macrophages (mPs) were identified by the cell surface marker expression at each step. In response to LPS and Th2 cyto-

kine, hiPSC-mP were polarized into distinct inflammatory (M1) or anti-inflammatory (M2) subtypes. These subtypes showed specific gene and cell surface protein expression, respectively. A phagocytic function of hiPSC-mP were confirmed by phagocytosis assay Kit. To determine whether hiPSC-mP give rise to liver-resident macrophages (Kupffer cells), we co-cultured hiPSC-mP with hiPSC-derived hepatocyte-like cells (hiPSC-HLCs) and then induced inflammatory response. High-magnification imaging showed a direct physical interaction between hiPSC-mP and hiPSC-HLC. hiPSC-mP secreted TNF-α, which is Kupffer cell-specific inflammatory cytokine by LPS stimulation. Also, expression of CD163 in hiPSC-mP suggests hepatic inflammatory response. In conclusion, hiPSC-mP have the potential to be developed into tissue-resident macrophages that can be used for pathophysiological and toxicological studies.

Submission declaration:
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Neuroprotection via an iNOS-inhibitor in a porcine retina organ culture model

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Aims

The aim of this study was to test a therapy for oxidative stress induced retinal degeneration using our *ex vivo* organ model. Nitrite oxide (NO) plays an important role in the pathogenesis of various retinal diseases, especially when hypoxic processes are involved. This degeneration can be simulated by incubating porcine retinal explants with cobalt-chloride (CoCl2).

Methods

Degeneration through 300 µM CoCl2 (for 48 h) and treatment with the iNOS-inhibitor 1400W (for 72 h) were applied simultaneously to explants of porcine retinae from day one on. Three groups were compared: control, CoCl2, and CoCl2+iNOS-inhibitor. At days 4 and 8, retinal ganglion cells (RGCs), bipolar, and amacrine cells were analyzed via immunohistology and qRT-PCR. Furthermore, the influence on the glia cells and different hypoxic/stress markers (HIF-1α, VEGF, HSP70, iNOS) were evaluated.

Results

First, the expression of the transcription factor HIF-1α and its downstream target genes could be used to prove that treatment with 1400W was effective. Further treatment with CoCl2 re-sulted in a significant loss of RGCs already after 4 days, which was counteracted by the iNOS-inhibitor. After 8 days, the CoCl2 group displayed a significant loss in amacrine cells and downregulation of PVALB mRNA expression. A drastic reduction in bipolar cells was observed after 8 days, which was prevented by 1400W. A significant decrease in microglia numbers was found in the CoCl2 group after 4 and 8 days, which could not be prevented by the inhibitor.

Conclusion

CoCl2 induces strong degeneration in porcine retinae by mimicking hypoxia, damaging RGCs, amacrine, bipolar, and microglia cells. Treatment with the iNOS-inhibitor counteracted these effects to some extent and clearly prevented the loss of retinal ganglion and bipolar cells. Hence, this inhibitor seems to be a very promising treatment for retinal diseases.

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Innovations in veterinary education and training and the feasibility of full replacement

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This presentation will explore some new tools and approaches within veterinary education and training. A number of innovative methods for teaching and learning anatomy and surgery will be described, supported by film that illustrates the methods and allows the developers and end users to share their experience. With reference to these and other humane alternatives, as well as the myths that are common within education and training, the feasibility of full replacement will be addressed.

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Using ex *vivo* human skin for the assessment of drug transport across the skin barrier with label-free Raman microscopy

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Assessment of drug delivery to the skin has a long history of testing, ranging from using live animal models, like hairless mice and pigs, to excised animal tissue as well as *in vitro* reconstructed cell-based models. Here, the use of excised human skin tissue has emerged as a gold standard for physiologically relevant testing conditions for the application of dermal drug products. However, the thorough assessment of penetration and permeation behaviour of both API and excipients across the skin barrier remains a challenging task. The analysis is either limited to quantitative determination of skin permeation via *in vitro* permeation testing (IVPT) or destructive techniques that evaluate the API and excipient content in single skin layers via tape stripping or similar techniques.

The dense and complex structure of the human skin is both a challenge for the formulation of topical drug products and the analysis of penetration abilities of the active pharmaceutical ingredient (API) as well as additives. Active substances often face the problem of crossing the biological barrier of the skin in sufficient quantities to achieve a therapeutic effect and have to rely on the penetration enhancing abilities of excipients. Over the last decade, carriers have gained attention within the research field of pharmaceutical technology as a way to increase solubility and enhance the penetration of APIs into the skin tissue.

In this study, carrier substances with penetration enhancing effects on active pharmaceutical ingredients and their interaction with the human tissue were evaluated. Rather than following solely the permeation of the drug in IVPT studies using Franz diffusion cells, the penetration of the substance into the outermost skin layer was assessed using chemically-selective confocal Raman microscopy to observe the penetration of the carrier in a non-invasive manner. Combination of a non-destructive technique for monitoring superficial interactions and IVPT to quantify drug transport across the barrier yielded correlating results, highlighting the complementary nature of the two methods to gain a better understanding of formulation-tissue interactions. Depending on the selection of the carrier system, an enhanced penetration of the carrier compound into the *stratum corneum* could be observed, promoting permeation of the API through the tissue compared to other carriers or reference substances.

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The use of rainbow trout liver hepatocytes in three-dimensional cell cultures for the study of graphene-related material internalization

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Graphene-related materials (GRM) are undoubtedly one of the most promising nanomaterials (NMs). Their unique physico-chemical properties have been shown to have huge potential application in various areas of technology and biomedicine. As the production and use of GRM rapidly increase, so does the likelihood of their release into the aquatic environment. Concerns over their safety have been raised, however, the available toxicity data set is limited or conflicting. Thus, there is an urgent need to develop approaches for the toxicity testing and screening of these NMs. Isolated rainbow trout (*Oncorhynchus mykiss*) hepatocytes have repeatedly demonstrated their suitability for the screening of the cytotoxicity of different environmental contaminants, and have recently been employed successfully to assess the ecotoxicity of a limited number of NMs. Nevertheless, primary fish hepatocytes in three-dimensional cell cultures represent a more powerful tool to investigate the *in vivo* effects of NMs. Despite this, very few studies on fish liver spheroids exist. The present study aims to investigate the suitability of this 3D model system for screening toxicity and to reveal any interactions of GRM with hepatic spheroids of rainbow trout.

Primary hepatocytes were isolated using an *in-situ* collagenase perfusion method and spheroid formation was achieved by the gyratory platform technique. 8-day old hepatic spheroids were ex-posed to relevant concentrations of different forms of GRM, two graphene oxides of sheet-like structure and one tubular-shaped carbon nanofiber for 72 h. Transmission electron microscopy was used to investigate the ability of these NMs to enter the spheroids.

In general, no obvious alterations in cell structures were induced by any of the NMs tested. Micrographs showed extensive presence of metabolism-related organelles mitochondria and rough endoplasmic reticulum, as well as tight junctions between adjacent cells. NM uptake, if any, was limited only to a few cells at the periphery of the spheroid. Ongoing research will further investigate the cytotoxic effects of these GRM in spheroidal hepatocytes and evaluate the relevance of 3D cell culture systems based on primary hepatocytes in nanoecotoxicology.

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Brief introduction of user-driven microphysiological system development in Japan

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MPS (Microphysiological System) is *in-vitro* cell-culture environment, which is precisely maintained in a micro space manufactured using MEMS (Micro Electro Mechanical Systems) technology, to derive *in-vivo* like functions with human cells. MPS is widely expected as an innovative technology for drug discovery process [1], leading to the reduction and replacement of animal testing as well. The national projects on these subjects governed by NCATS (National Center for Advancing Translational Sciences, NIH, USA) achieve significant results [2] and the same goes in EU.

In July 2017, the Japan Agency for Medical Research and Development (AMED) initiated a research project to develop a chip-based MPS. The authors have been involved in the project and Kanamori is in charge. Because we pointed out that substantial information exchange among developers and users in pharmaceutical, cosmetic and chemical companies is a must to make the project successful, we established an R&D center in Tsukuba, Japan, where any members involved in the project can anytime experiment with MPS chips, which have been developed in the project.

We think the five element technologies are needed to commercialize MPS chip [3]; 1) Source of organ-specific cells, 2) Induc-tion of organ-specific functions in cells, 3) Perfusion in MPS chip, 4) Implementation and integration of screening, detection, and analysis technologies on MPS chip, 5) Manufacturing of MPS chip.

In this presentation, we will give a brief introduction for the project as well as a part of results achieved in the R&D center.

References

[1] Ewart, L., Fabre, K., Chakilman, A. et al. (2017). *Exp Biol Med 242*, 1579-1585. doi:10.1177/1535370217715441

[2] Low, A. and Tagle, D. A. (2017). *Clin Transl Sci 10*, 237-239. doi:10.1111/cts.12444

[3] Kanamori, T., Sugiura, S. and Sakai, Y. (2018). *Drug Metab Pharmacokinet 33*, 40-42. doi:10.1016/j.dmpk.2017.11.006

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Validation of the *in vitro* photo-toxicity test using 3D reconstructed human epidermis model – building on 20 years of experience

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Assessment of the phototoxicity hazard and phototoxic potency (i.e. phototoxic risk) of compounds and mixtures is a crucial step in the safety assessment of cosmetic, pesticide and pharmaceutical products absorbing UV and visible light. The validated and regulatory accepted *in vitro* assay, the 3T3 NRU PT (OECD TG 432), provides a high level of sensitivity, however, it has been reported that it also generates a high rate of false positive results due to the lack of barrier properties naturally appearing in the human skin or other targeted tissues.

In vitro reconstituted human skin models are increasingly being investigated for their usability in hazard identification and safety testing, because of their organotypic structure with a functional stratum corneum that allows for assessment of bioavailability of topically applied compounds and mixtures. An *in vitro* photo-toxicity test using the human reconstructed epidermis model EpiDerm™ (EpiDerm™ H3D-PT) has been developed and pre-validated almost 20 years ago [1] and can be used either as a stand-alone method for the phototoxicity testing of topically applied materials, or in combination with the 3T3 NRU PT, to minimise the potentially false positive results from this assay [2].

In the current study we internally validated the method with six reference substances, of which four were known phototoxins (chlorpromazine hydrochloride, two types of bergamot oil and anthracene) and two compounds were UV-absorbing, but without phototoxic potential (cinnamaldehyde, p-aminobenzoic acid). In the next step, the method has been transferred into other two laboratories and the test was assessed with a set of blind-coded substances. The reproducibility of the predictions between the laboratories was 100% confirming the robustness of the protocol and the prediction model. Submission of the data to the OECD for the implementation of the EpiDerm™ H3D-PT into the regulatory framework is ongoing.

References

[1] Liebsch, M., Traue, D., Barrabas, C. et al. (1999). Prevalidation of the EpiDerm phototoxicity test. In D. Clark, S. Lisansky and R. Macmillan (eds.), *Alternatives to Animal Testing II: Proceedings of the second international scientific conference organised by the European Cosmetic Industry, Brussels, Belgium* (160-166). Newbury, UK: CPL Press.

[2] Kandarova, H. and Liebsch, M. (2017). The EpiDerm Skin Irritation Test (EpiDerm SIT). In Ch. Eskes, E. van Vliet and H. Maibach (eds.), *Alternatives for Dermal Toxicity Testing* (41-57). ISBN 978-3-319-50351-6

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Slovak National Platform for Three Rs (SNP 3Rs) in Science, Education, Research and Development

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The principles of 3Rs (Replacement, Reduction and Refinement) were developed over 50 years ago by Russell and Burch providing a framework for performing more humane animal research. The 3Rs principles have been since then embedded in national and international legislation and regulations on the use of animals in scientific procedures, as well as in the policies of organisations that conduct animal research.

The idea for establishment of 3Rs center in Slovakia was under the discussion among the Slovak scientists, regulators and industry already since 2015. Strategy concept of a national 3Rs centre was presented at the TOXCON 2016 Conference, organized by the Slovak Toxicology Society SETOX in High Tatras, and further discussed between several members of the National Committee for Alternative Methods (NOVS) at the EUROTOX 2017 Congress in Bratislava.

As a follow up, the Ministry of Agriculture and Rural Development of the Slovak Republic organized in February 2018 an informal meeting of NOVS members, invited experts in toxicology and pharmacology, representatives of Slovak Academy of Science and academia, industry representatives and governmental organizations involved in the implementation of the EU legislation on animal welfare and protection in order to discuss the collaboration between the parties and to express an interest and formal support to the national 3Rs centre. The attendees of that meeting fully endorsed an establishment of the Slovak National Platform for Three Rs (SNP 3Rs) that should stimulate development and implementation of the alternative methods in Slovakia.

The platform has officially been launched at the annual meeting of the Slovak Toxicology Society SETOX on June 21st 2018 at the Toxicology Conference TOXCON 2018. The SNP 3Rs operates with support of SETOX and its mission is to provide information, resources, and practical guidelines on the 3Rs principles of Russell and Burch in science, education, research and development. One of the recent activities of the platform included organising informative session on the implementation of 3Rs in Slovakia during TOXCON 2019 Congress. This Session was organized with support of Slovak National Focal Point for Scientific and Technical matters with European Food Safety Authority (EFSA) and Slovak National Contact Point for the PARERE Network. For details see Web-page: www.snp3rs.com

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Lessons learnt from validation studies of *in vitro* methods for topical toxicity testing

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The development of alternative methods to animal experimentation has progressed rapidly over the last 30 years. *In vitro* methods have an important role in the hazard identification and assessment of toxicology profile of compounds. This would not be possible without well-structured and highly organised validation projects, that proved reliability of the *in vitro* systems. In general, validation process ensures that alternative methods developed by academic or industrial scientists will be scientifically valid and thus, eventually accepted by regulatory authorities for classification and labelling, product approval or safety testing purposes [1].

Validation criteria for new toxicological test methods in use today were developed as collaborative efforts of lead scientists from both the *in vivo* and *in vitro* communities, regulators and other experts beginning in the early 1980's. The process was carried out under the auspices of three organizations: the Organisation for Economic Cooperation and Development (OECD), the European Centre for the Validation of Alternative Methods (ECVAM), and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). These international organizations have worked together with external experts and national organizations such as FRAME, ZEBET at the BfR and CAAT on harmonizing the validation criteria so that there are no major differences between them amongst different countries and continents [2].

Based on these principles, several prospective international validation studies for topical toxicity testing were performed since 2000, followed by smaller "catch-up" validations. Almost all of these projects led to the new OECD test guidelines e.g. OECD TG 431, 432, 435, 437, 438, 439, 491, 492. The process has not always ben straightforward and the methods developers as well as validation teams and regulators had to discuss number of findings related to e.g. the reliability and reproducibility of the *in vivo* as well as *in vitro* studies, predictive capacities of the new tests and defining of performance standards for the new methods. Even studies, that were not immediately successful contributed significantly to the knowledge on the applicability domains and failures helped to the test method developers to come up later with improved and better-defined *in vitro* systems and novel prediction models.

The presentation will show on practical examples from several validation projects how important is to standardise methods before entering the validation and will also discuss the lesson learnt in the validation process.

References

[1] Kandárová, H. and Letasiová, S. (2011). *Interdiscip Toxicol 4*, 107-113.
[2] Worth, A. P. and Balls, H. (2004). *Altern Lab Anim 32, Suppl 1*, 623-629.

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Transcriptional regulatory mechanism of CYP450 genes in human pluripotent stem cell-derived hepatocytes

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Human pluripotent stem cell-derived hepatocytes (hPSC-Heps) have been considered as an alternative cell source to human primary hepatocytes (hPHs) for drug discovery and hepatotoxicity testing. Although hPSC-Heps exhibit morphological and functional liver-specific characteristics, they cannot fully reflect the drug metabolic function of hPHs or adult liver. Due to the low expression of drug metabolizing enzymes such as Cytochrome P450s (CYPs), hPSC-Heps have a limitation to use as a hepatotoxicity testing model. Several studies have reported low CYP expression and activity in hPSC-Heps, however the regulatory mechanism correlated with CYP gene expression is yet to be elucidated. Here, we showed the expression of nine major CYP genes in each of two types of human embryonic stem cell-derived hepatocytes (hESC-Heps), human induced pluripotent stem cell-derived hepatocyte (hiPSC-Heps), and hPHs. Transcript levels of nine CYP genes in hESC-Heps and hiPSC-Heps were significantly lower than those of hPHs. We hypothesized that low expression of CYP genes in hPSC-Heps would be closely related with epigenetic modifications including DNA methylation and histone modification. In order to explain the mechanism of limited expression of CYP genes in hPSC-Heps, we analyzed the methylation status of CpG sites in the regulatory region to which transcription factors bind. In addition, we performed the chromatin immunoprecipitation (ChIP) analysis to identify the enrichment of active histone mark H3K-27ac in the regulatory region of CYP genes. We confirmed that DNA methylation and histone modification in regulatory regions of CYP genes differed between hPHs and hPSC-Heps. Therefore, our finding suggests that epigenetic modifications are key transcriptional regulatory mechanism for the expression of nine CYP genes in hPSC-Heps. Furthermore, these findings will provide an important clue to establishing an alternative *in vitro* cell model for hepatotoxicity testing.

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The need for non-animal recombinant antitoxins

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The demand for therapeutic antitoxins is global, as are concerns about their availability, production, and efficacy [1]. Most of the currently available antitoxins are harvested from the blood of immunised animals. They are commercially prepared by repeatedly exposing large mammals, such as horses, to repeated doses of a toxin, causing the animals' immune systems to produce large quantities of antibodies against the toxin. For the animals, this process can cause local and systemic adverse effects, including injection-site oedema, thrombosis, phlebitis, abscesses, fistulas, and fibrosis. The resulting drugs derived from animal serum have well-documented safety issues – including hypersensitivity reactions and serum sickness – and maintaining reliable stockpiles for quick transportation to patients in need of treatment is challenging [2,3,4]. Fortunately, recombinant human antitoxins can be developed and produced in cell culture, without the use of animals [5]. Several recombinant antibody-based therapeutics have been approved for clinical use, and more are in development. This presentation will discuss the limitations of animal-derived antibody products and will summarise the use of the phage display approach to generating recombinant human antitoxins without immunising and bleeding animals. The PETA International Science Consortium Ltd. is funding the development of recombinant human monoclonal antibodies that neutralise the diphtheria toxin, with the ultimate goal of replacing the use of equine-derived diphtheria antitoxin.

References

[1] UNICEF Supply Division (2017). https://www.unicef.org/supply/files/Diphtheria_Antitoxin_Market_Update.pdf

[2] Both, L., White, J., Mandal, S. et al. (2014). *Euro Surveill 19*, pii 20830. doi:10.2807/1560-7917.ES2014.19.24.20830

[3] Bradbury, A. and Plückthun, A. (2015). *Nature 518*, 27-29. doi:10.1038/518027a

[4] Ryan, N. M., Downes, M. A. and Isbister, G. K. (2015). *Toxicon 108*, 181-183. doi:10.1016/j.toxicon.2015.10.012

[5] Unkauf, T., Meithe, S., Fuhner, V et.al. (2016). *Adv Exp Med Biol 917*, 55-76. doi:10.1007/978-3-319-32805-8_4

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InSilc: An *in silico* platform for advancing BVS design and development and replacement of animal testing

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Coronary Artery Disease (CAD) is the leading cause of death in Europe and worldwide with more than 17 million deaths [1]. Atherosclerosis, the major disease process of CAD, is a chronic inflammation driven by the build-up of atherosclerotic plaques inside the coronary arteries. Bioresorbable Vascular Scaffolds (BVS) revolutionised the field of interventional cardiology by providing targeted drug delivery, mechanical support and complete resorption overcoming the barriers of bare-metal and drug-eluting stents. In fact, animal model systems are the translational link between the bench-top experiment and the clinical trial. Using animal models to test new BVS is a crucial component for allowing the release of the BVS to the market, since it allows researchers to evaluate the BVS performance in living systems. However, despite their many physiological and morphological similarities, BVS perform differently in humans than they do in animal models. In addition, there is a growing public pressure against animal experimentation in most developed countries that is leading to the development of alternative methods for pre-clinical assessment, where *in silico* clinical trial (ISCT) can play a key role.

InSilc is an ISCT platform that accounts on the biological and biomedical knowledge and available advanced modelling approaches for simulating the short and medium/long term BVS performance. This is achieved by the integration of multidisciplinary and multiscale models that simulate the BVS mechanical behaviour, the deployment and degradation, the fluid dynamics and the myocardial perfusion. The development of the: (i) Mechanical Module, that reproduces the standard mechanical tests that are currently performed by the Stent Industry, (ii) 3D Reconstruction and plaque characterisation Module, that enables the reconstruction of the arterial tree and the scaffold, (iii) Deployment Module, that simulates the post deployment BVS configuration, the stresses/ strains within the BVS and the arterial wall, (iv) Fluid Dynamics Module that provides the patient specific shear stress, the flow patterns and the process of in stent restenosis, (v) Myocardial perfusion Module, that captures ischemia and revascularization in the myocardium, (vi) Degradation Module, that predicts the degradation and long-term mechanical performance of the BVS, provides the final users (Stent Industry experts, Contract Research Organizations –CROs-, Animal study experts, Interventional Cardiologists, Researchers) with fruitful information on the BVS behaviour.

InSilc has the potential to contribute to a faster and cheaper BVS development process, to overcome difficulties inherent in the design of clinical trials (such as underrepresented high-risk subgroups within the recruited cohorts of patients), and to minimise animal experimentation in BVS testing, as recognised with the 3Rs Prize for the Replacement, Refinement and Reduction of animals in research.

References
[1] WHO, Cardiovascular diseases (CVDs). http://www.who.int/mediacentre/factsheets/fs317/en/
[2] InSilc: In silico trials for drug-eluting BVS design, development and evaluation, Cordis, European Commission. https://cordis.europa.eu/project/rcn/212941/factsheet/en

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A comparison of hyperuricemia model mice loaded with oxonic acid and the silkworm "o06" strain as an alternative for evaluating voluntary oral administration

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Introduction

We proposed the silkworm "o06" strain as a hyperuricemia model. When silkworms were given either a single subcutaneous or single intestinal dose of medication for hyperuricemia (Feburic®, Allopurinol, etc.), the medication effectively reduced the uric acid (UA) levels [1]. Here, we examined the optimal conditions for evaluating long-term voluntary oral dosage.

Methods

Silkworms were given fresh mulberry leaves previously soaked in the sample to imitate non-invasive oral administration. The silkworm groups given mulberry leaves soaked in CPPG solution [2], Feburic® suspension, and Milli-Q water formed the evaluation, treatment, and control groups, respectively. Hemolymph was collected regularly and the UA quantified by high-performance liquid chromatography. The change in UA over time was compared and the optimal conditions for evaluation were examined.

Results

A significant difference in the UA level 20 hours after starting was observed between the treatment and control groups. Evaluation of the UA levels was possible. We plan to make comparisons with the sample dose (g/kg) in mice and evaluate whether the silkworm is a useful model of hyperuricemia.

References

[1] Tanaka, R. et al. The use of translucent larval skin mutants of the silkworm "o06" as animal models of hyperuricemia, XXV ICE2016 proceedings.
[2] Tanaka, R. et al. The Xanthine Oxidase Inhibitory Activity and Hypouricemic Effects of new Phenylpropanoid obtained from aerial parts of Matricaria chamomilla L., J.JSMUFF, submitted.

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Application amounts affect skin penetration of caffeine and testosterone in rat and pig skins

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Skin penetration is an important factor to risk assessment of toxic compounds. Recently, due to limitation of animal study, *in vitro* alternative skin permeation approaches such as Franz-type diffusion cells are being utilized. However, there are various opinions on the amounts of application. So, we conducted *in vitro* skin penetration of caffeine and testosterone by application amounts to provide the appropriate information. A various amounts of caffeine or testosterone, 5, 10, 25 and 50 mg (or µl)/cm^2 were used as solid (cream formulation containing 1% caffeine or 0.1% testosterone) and liquid (1% caffeine in distilled water or 0.1% testosterone in 50% ethanol (v/v)) forms in rat and Micropig skins (Micropig® Franz cell membrane). After 24 h application, residue on the skin surface and stratum corneum was recovered by ethanol swab and tape, respectively. To calculate the skin absorption, absorbed caffeine or testosterone in skin and receptor fluid were analyzed and quantified by LC-MS or UPLC, respectively. The amounts of skin penetrations were increased as application amount increased in solid and liquid formulations. Specially, the amounts of penetration in 50 µl/cm^2 was different from those in 5 or 10 µl/cm^2 by Tukey's multiple comparison test in caffeine and testosterone. The amounts of penetration per unit area (cm^2) decreased with increasing applied amounts, but did not show significant difference in rat skin, while 5 or 10 µl/cm^2 of Micropig skin was significantly differed with those of 25 or 50 µl/cm^2 in micropig skin. The caffeine absorption rates (%) of solid and liquid formulations ranged from 9.7 to 56.4% and from 16.4 to 90.7%, respectively. In case of testosterone, 50 mg/cm^2 was significantly differed with the others in rat and Micropig skin. The absorption rates (%) of solid and liquid formulations ranged from 13.2 to 41.6% and from 24.2 to 72.4%, respectively. Generally, absorption rates of liquid form were higher than those of solid formulation, and the smaller the applied amount, the greater the absorption rate. In this study, there were differences in the absorptions of caffeine and testosterone by different amounts applied and we recommended that a small amount, 5 or 10 mg (or µl)/cm^2, were preferably used for the *in vitro* skin absorption test.

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Generation of hepatic organoids from human induced pluripotent stem cells

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Organoids are *in vitro* 3D tissues that display architectures and functionalities similar to target organs and that self-organize from organ-specific stem cells and/or induced pluripotent stem cells (iPSCs). Organoids have a wide range of applications in developmental research, toxicity screening, and regenerative medicine. Human adult liver-derived organoids have been established by growth factor-defined conditions, including Wnt agonist R-spondin1, Noggin, EGF, FGF, and HGF inside drops of polymerized Matrigel (Matrigel drops). However, the hepatic organoids derived from human iPSCs have not been made except from the liver buds, which are combined with hPSC-derived hepatic endoderm, endothelium, and septum mesemchyme. In this study, hepatic organoids were generated from human iPSC-derived hepatic progenitor cells by growth-factor defined conditions not only Matrigel drops and but also Matrigel suspension, floating in a medium containing ~10% Matrigel. In addition, we found that expansion of hepatic organoids is possible without R-spondin1, which is a key factor in the generation and expansion of intestinal organoids.

hPSC-derived hepatic organoids were expanded without chromosomal abnormality and could be self-organized into 3D cystic structure comprised of a single-layer epithelium. The hepatic organoids expressed hepatic progenitor markers such as SOX9, CK19, and EpCAM and could readily be converted into functional hepatocytes in differentiation condition. Differentiated hepatic organoids expressed mature hepatocyte markers and have hepatic functions such as glycogen storage, albumin secretion, CYP450 activity, and high drug sensitivity compared to hiPSC-derived hepatocytes cultured under 2D condition. Therefore, we suggest that hPSC-derived hepatic organoids can be applied to liver disease modeling and hepatotoxicity screening model.

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3R funding of research activities 2014-2019 in Denmark by the Danish 3R-Center

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The Danish 3R-Center promote the 3Rs by initiating research projects focusing on one or more of the 3Rs. Researchers affiliated to organizations, institutions or businesses in Denmark can apply. Applications will be evaluated from clear 3R perspectives, scientific quality and feasibility. The applicant has to declare which of the 3Rs are central to the project.

Every year €200,000 is reserved for research funding of 3-4 projects.

In the period from the start of the 3R grant in 2014 to 2019 a total of 117 applications were submitted and 19 were supported financially. All supported projects which are successful, result in scientific publication in a peer reviewed journal indexed in Pub Med.

I searched in Pub Med for all applied projects on principal investigator and title and more than 10 projects from the first two years resulted in publication even without financing from the 3R center. This could be seen as increased alertness to the 3Rs in the community resulting in financing from other sources than the 3R center.

The applicants declared which of the 3Rs central to the projects. In total 55 out of 117 concerned replacement/reduction and the rest refinement/reduction. Projects with focus on refinement/reduction is increasingly being applied for during the 6-year period. Applications for support of replacement studies have to justify how many animals to be saved which is impossible for applications setting up new approaches not related to animal studies as such, but paving the way for new concepts as Adverse Outcome Pathways and *in silico* methods increasingly part of the modern biomedicine.

In my opinion, more reference to publications arising from the 3R grant in Denmark and the concept of Replacement may increase the discussions related to modern research including more attention to a variety of research approaches prior to use of animals.

https://en.3rcenter.dk/research/

Reference

Knudsen, L. E., Smith, A., Törnqvist, E. (2019). *Basic Clin Pharmacol Toxicol 124*, 560-567.

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Placenta-on-a-chip model for assessing the transport and toxicity of xenobiotics *in vitro*

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Objectives

The study of the transport and toxicity of xenobiotics in women is limited for ethical reasons. *Ex vivo* placenta models have high variability and low success rates. Animal models *in vivo* differ from a human in anatomy, genotype, and proteome. The placenta-on-a-chip model is a compromise. The cells BeWo b30 in combination with endothelial cells are often used as models of the placenta *in vitro* [1-4]. Cultivation of cells with the circulation of the medium allows the microenvironment to be brought closer to the real organism [5]. We studied the transport of the components of the FAC chemotherapy regimen for breast cancer in this model.

Methods

BeWo b30 cell line was grown in the DMEM with L-glutamine, 4.5 g glucose/l and Earle's salts containing 10% FBS, 1x MEM NEAA, 100 U/ml penicillin and 100 μg/ml streptomycin in inserts cut from 96-well Transwell plate and placed in a microfluidic chip. Cells were seeded with a density of 10,000 cells per insert. After 7 days, 5-fluorouracil (25 μg/ml), doxorubicin (50 μg/ml), cyclophosphamide (150 μg/ml), or all three drugs were added to the cells for 1 hour. Control cells were cultured in the presence of 0.05% DMSO. The impedance spectrum was measured before and 1 and 24 hours after the addition of the drug. The concentration of the drug was determined by HPLC-MS/MS. Cell viability was assessed using the CellTiter-Blue Assay.

Results

After 1 h incubation with drugs, TEER decreased in experiment and control groups from an average of 90 to 25 $\Omega \cdot cm^2$, and after 24 h TEER was 67.3 ±17.9 $\Omega \cdot cm^2$ for control, 67.8 ±16.4 $\Omega \cdot cm^2$ for cyclophosphamide, 90.0 ±20.1 $\Omega \cdot cm^2$ for 5-fluorouracil, and decreased to the background for doxorubicin and drug mixture. Cell viability did not differ significantly between the control, 5-fluorouracil, and cyclophosphamide, but decreased to 40 ±9% of the control when exposed to doxorubicin and drug mixture. The placenta-on-a-chip model transported the drugs from the apical to the basolateral side.

Conclusion

The developed placenta-on-a-chip model is suitable for assessing the transport and toxicity of xenobiotics *in vitro*.

References

[1] Knyazev, E., Zakharova, G., Astakhova, L. et al. (2019). *Bull Exp Biol Med 166*, 321-325. doi:10.1007/s10517-019-04342-1

[2] Knyazev, E., Petrov, V., Gazizov, I. et al. (2019). *Bull Exp Biol Med 166*, 369-372. doi:10.1007/s10517-019-04352-z

[3] Knyazev, E., Maltseva, D., Zakharyants, A. et al. (2018). *Bull Exp Biol Med 164*, 749-752. doi:10.1007/s10517-018-4072-3

[4] Knyazev, E., Maltseva, D., Zakharyants, A. et al. (2018). *Bull Exp Biol Med 164*, 757-761. doi:10.1007/s10517-018-4074-1

[5] Sakharov, D., Maltseva, D., Knyazev, E. et al. (2019). *BMC Syst Biol 13, Suppl 1*, 19. doi:10.1186/s12918-019-0686-y

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Asian Consortium for Three Rs

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In 2016, the Japanese Society for Alternatives to Animal Experiments (JSAAE) launched the first Asian Congress on Alternatives to Animal Experiments to promote throughout Asia the Three Rs – Replacement, Reduction, and Refinement – as guiding principles for a more ethical use of animals in scientific testing. Following the First Congress in Karatsu, Japan, during November 2016 and the Second Congress in Guangzhou, China, during October 2018, a Third Congress is now being planned for Korea during 2021. And to promote closer collaboration between our Asian colleagues, we are also now exploring the possibility of organizing for 2020 an Asian Consortium for Three Rs, which will provide funding and human resources in support of future Congresses in Asian countries.

JSSAE has concluded a memorandum of agreement with the European Society of Alternatives to Animal Testing (EUSAAT), the American Society of Cellular and Computational Toxicology (ASCCT), and the European Society of Toxicology in Vitro (ESTIV). Also, China's Toxicity Testing Alternative and Translational Toxicology (TATT) has concluded a similar memorandum with ESTIV.

Although our Asian colleagues are deeply involved in research and promotion of the Three Rs, countries in the EU and the USA are still the leading advocates for these principles, and we look forward to strong support for the Asian Consortium from our colleagues in EUSAAT and ASCCT. This Consortium has also a one of mission to coordinate Three Rs on pharmaceutical regulation.

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OECD DRP on *in vitro* assays in reproductive toxicology

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The Hand1 Luc-Embryonic Stem Cell Test (EST) was developed by Sumitomo Chemical, Co. Ltd between 2006 and 2010 as part of the New Energy and industrial technology Development Organization (NEDO) project. The goal of this development was to improve the original EST as a screening for developmental/reproductive toxicity, which was not submitted for the guideline application. The developers continued to improve the protocol between 2010 and 2013, based on results previously obtained with the support of the Ministry of Economy, Trade and Industry (METI). Since that time, the current Hand1-Luc EST was established [1,2], in which the D3 cells were replaced by KOB1-ES cells transfected with the promoter of the Hand1 gene followed by the luciferase gene, and suppressing the use of 3T3 cells to simplify the protocol. From February 2013 to February 2016 when the validation study was completed, the Validation Management Team (VMT) held meetings between each phase or important protocol improvement to obtain the understanding and approval of all members supported by Japanese Center for the Validation of Alternative Methods (JaCVAM). The validation study achieved confirmation of transferability in three different laboratories and of between- and within- laboratory reproducibility (higher than 75%). Japan submitted the Standard Project Submission Form (SPSF) with the validation report and peer review report to Organisation for Economic Co-operation and Development (OECD) in 2017.

The Working Group of National Co-ordinators of the TGs programme (WNT) agreed the project on the Review and feasibility of EST: *In vitro* assay detecting disruption to differentiation of rodent embryonic stem cells into cardiomyocytes using the Hand1 gene. However, they suggested to develop the Detailed Review Paper (DRP) of available methods and evaluation of utility and application prior to developing the Test guideline on Hand1 Luc-EST. So, JaCVAM has coordinated the development of DRP of EST with the international experts in this field and this report was submitted to OECD before summer in 2019.

References

[1] Le Coz, F., Suzuki, N., Nagahori, H. et al. (2015). *J Toxicol Sci 40*, 251-261.
[2] Nagahori, H., Suzuki, N., Le Coz, F. et al. (2016). *Toxicol Lett 259*, 44-51.

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The many cards up "computational sleeves": Reshaping *in silico* tools used in drug discovery to design safer and functional chemicals

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Computational methods have been increasingly used in hazard and alternatives assessments, alleviating economic and ethical burdens of animal testing. Nonetheless, their potential to inform safer chemical design in bridging the fields of toxicology and green chemistry has not been realized. Over the past 50 years, computers have revolutionized drug discovery by being both fast, virtually screening vast chemical libraries to find drug candidates against biological targets of therapeutic interest, and accurate, providing state-of-the-art tools to optimize said candidates for greater activity. Nowadays, all pharmaceutical companies employ computational modeling, and many drugs on the market are an outcome of computer-guided drug design. In developing pharmaceuticals, we seek to impart specific biological activity to a molecule but also to minimize any side effects caused by unintended activity. The latter is true for all commercial chemicals as our society has grown increasingly aware of the adverse effects chemicals can have on human and environmental health. Regrettably, to test every new chemical on animals to ensure its safety is unfeasible. Current *in vitro* and *in silico* methods offer a promising alternative; however, their role is limited to screening existing chemicals rather than providing a holistic platform for safer chemical design. Inspired by the successes of computer-aided drug discovery, our group has focused on transforming said techniques to aid in rational design of safer chemicals. Mimicking Lipinski's rules for druglikeness, we have developed broad, property-based guidelines that inform design of chemicals with minimal ecotoxicity. More recently, we have explored the utility of statistical free energy perturbations used in lead optimization of drug candidates to redesign existing toxicants for increased safety. Herein, we demonstrate our approach on organophosphorus flame retardants, and show that high-volume chemicals of concern, such as triphenyl phosphate, can be rationally modified to significantly decrease their activity against specific targets while preserving intended functionality. Our approach is i) fast, relying on validated computational methods, ii) suitable for other chemical classes, for which mechanism(s) of action are known, and iii) cost-effective, imposing minimal structural changes to existing commercial chemicals and thus applicable to incumbent product development processes.

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Advanced *in-vitro* management of three-dimensional cell cultures and explanted tissue

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The use of 3D cell cultures (*ex vivo* tissue and *in vitro* models) gain increasing importance in medicine and pharmaceutical research considering the 3R rules. However, currently, commercially available systems are operated under suboptimal conditions as no tailor-made minimally invasive monitoring systems with integrated sensors are available to monitor, optimize, and standardize culture conditions.

While 2D cultures are too artificial, not reflecting the cellular physiology in 3D, most 3D cell cultures lack adequate methods for proper examination while mostly being destructive endpoint analysis or highly dilutes samples from supernatants.

Integration of Open Flow Microperfusion (OFM) [1] in combination with biosensors allows continuous monitoring of metabolism, secretome, as well as functional maturation of time resolved 3D cell- and tissue-based models (4D applications) in dynamic cultivation systems. Samples can be gathered nondestructively for on- and offline analysis enabling continuous monitoring of the same samples allowing long-term studies as well as studies on pharmacokinetics, pharmacodynamics, toxicity, or cell based therapies resulting in more relevant conclusions than separate 2D endpoint analyses.

Due to the vast amount of culture media usage for 3D perfusion systems the metabolome and secretome of the cultured cells is highly diluted. To still evaluate the metabolome of the cells within a tissue/matrix in a non-destructive, minimally invasive method within a small sample volume, *in vitro* models are placed in the newly developed dynamic cultivation and monitoring system.

Cells were cultured within a hydrogel or collagen matrix inside the monitoring system. Samples were taken from within the 3D cell culture via a perfused catheter embedded within the cell laden matrix. A physiologic perfusate is guided through a minimally invasive catheter trough the tissue. Within the tissue an equilibration between the perfusate and the surrounding interstitial fluid occurs. The perfusate with substances from within the tissue can be collected and analyzed in a small, undiluted sample volume. Perfusion culture media supernatant was analyzed as reference.

While the concentration of glucose and lactate was steady within the supernatant and comparable to the fresh medium, the analysis of the perfusate revealed a reduced glucose concentration within the matrix and a local increase in lactate. Both presumably due to the cellular metabolism, however, not detectable from the medium supernatant.

By using the monitoring system in a preliminary study, it was already feasible to monitor glucose and lactate metabolism of the cells within a 3D perfusion culture. Implementation of additional sensors will achieve online monitoring of various parameters (e.g. glucose, lactate, pH, and oxygen concentration).

Due to repetitive time-resolved non-destructive sampling of the same model, sample size and variation will be reduced, bridging the gap between *in vitro* and *in vivo* studies to obtain more reliable and conclusive data from 3D cultures reducing the amount of animal experiments (3R).

Reference

[1] Höfferer, C., Tutkur, D., Fledelius, C. et al. (2015). *Diabetes Obes Metab 17*, 121-127. doi:10.1111/dom.12394

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Hepatic non-parenchymal cell isolation methods in rodents for development of hepatic co-cultures

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Liver cells and for the most part hepatocytes are established as *in vitro* models for toxicology studies in 2D and 3D cultures. Nevertheless, cell cultures are only useful when they are able to mimic the *in vivo* situation as good as possible. Next to the parenchymal cells, non-parenchymal cells (NPCs) fulfil important functions inside hepatic lobules. Therefore, the next steps to expand the specificity of cell culture models are cultivation methods of total cell populations of the liver. However, the isolation and cultivation of these non-parenchymal cells like Liver Sinusoidal Endothelial Cells (LSEC), Kupffer cells (KC) and stellate cells (SC) remains an issue. Percoll and Iodixanol are density gradient media used for density centrifugation of cells, organelles and viruses. Percoll gradients for cell separation of non-parenchymal cells have been established, but the yield of NPC per gram liver tissue can be low. Iodixanol gradients as a tool for NPC isolation is a newly implemented method to isolate rat non-parenchymal cells [1]. At PRIMACYT we were able to apply the cell isolation by the Iodixanol gradient media for rat and mouse NPC. Further experiments were done establishing the Iodixanol method also for Cynomolgus and Beagle NPC. Here, NPC from Wistar rats (n = 13) and CD-1 mice (n = 13) were isolated, cultivated and tested for their purity and viability. Purity of the cells was tested by Phagocytosis assays and Immunofluorescence detection. The comparison of Percoll- and Iodixanol gradient in rat liver showed a slightly higher yield of whole NPC population when cells were isolated by an Iodixanol gradient (+20%) compared to Percoll. In mice, this effect was more obvious by providing about 54% higher NPC yield when the Iodixanol gradient isolation method was applied. In other species, Cynomolgus or Beagle, NPC isolation showed similar tendencies. Fernández-Iglesias and co-workers described in their protocol two interphases that separated the stellate cells from the rest of the NPC (LSEC, KC) population as an additional advantage of the Iodixanol gradient. However, we could not confirm this observation for any of the species, neither by phagocytosis assays or immunofluorescence microscopy. Further research will be done to analyse NPC in co-cultures with hepatocytes and the potential of cryopreserved co-cultures. The approach to utilise tissue-like co-cultures should ultimately reduce the number of laboratory animals used for toxicity studies and drug development.

Reference

[1] Fernández-Iglesias, A. and Ortega-Ribera, M. (2019). *J Cell Mol Med 23*, 877-886. doi:10.1111/jcmm.13988

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At crossroads between regulation, science and industrial application: The EPAA, a public-private initiative for the 3Rs

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The EU has been pioneering progress in refining, reducing and replacing animal testing ever since animal welfare has been enshrined in the Treaties [1], both within Europe and by using its strong international role as a regulator of markets.

As a public private initiative, EPAA is a voluntary collaboration between the European Commission and companies and European trade associations from seven industry sectors. The partners are committed to pooling knowledge and resources to support and accelerate the development, validation and regulatory acceptance of alternative approaches to animal testing. The overall aim is the replacement, reduction and refinement (3Rs) of animal use in regulatory testing.

The presentation will describe the set up and functioning of the EPAA and its activities, and it will provide examples of recent and ongoing projects supported by the EPAA. The three examples relate to the areas of Biologicals, Skin sensitization, and Carcinogenicity testing in agrochemicals. The presentation will also point to the new EU institutional set up, highlight major upcoming events in 2019 and 2020 and look at the next EPAA Action Programme.

The "Harmonisation of 3Rs in Biologicals" project includes cooperation from the European Medicines Agency, EURL-ECVAM, EDQM, several industry sectors and the Council of Europe; it is led by K. Schutte and F. Delannois and has already delivered concrete results. The next steps and ideas generated from this project are being discussed, including a new EPAA-supported project on the safety assessment of monoclonal antibodies.

The "Carcinogenicity of agrochemicals" project is coordinated by RIVM (National Institute, the Netherlands) and, like all EPAA projects, includes experts from the industry and regulatory bodies.

The project "Optimal strategies for skin sensitization" aims to compare new *in vitro* models, to share knowledge across sectors about their applicability and to promote new approach methodologies for safety assessment.

Reference

[1] Article 13 of the TFEU; previously: Protocol on Protection of Welfare of Animals (Annex to Treaty of Rome).

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Combining *in vitro* and *in silico* modelling to simulate cartilage degradation during osteoarthritis

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Our project aimed at computing an *in silico* model based on the results of our recently developed *in vitro* osteoarthritis (OA) model to enhance validity and translatability towards a more sophisticated simulation of OA. In detail, the used 3D *in vitro* model was based on 3D chondrogenic constructs generated solely from human bone marrow derived mesenchymal stromal cells (hMSCs). Besides studying the model under standard conditions over 3 weeks (analyzed weekly), it was treated with interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNFα) to mimic an OA-like environment (analyzed after 3 weeks). In order to provide suitable dataset for the *in silico* model, image analysis pipelines had to be optimized for determining cell and matrix concentrations. As a subsequent mathematical strategy, we described the biological processes by differential equations considering, e.g., the change in cell numbers and collagen II (Col II) concentrations in different areas of the constructs to include spatial resolution over time.

Our chondrogenic *in vitro* model revealed the expression of the cartilage specific markers collagen 2 (Col II) and aggrecan (ACAN) and the deposition of glycosaminoglycans (GAGs) in in the extracellular matrix. The cell concentration was slightly decreased over 3 weeks of untreated cultivation. After stimulation with pro-inflammatory cytokines, the constructs showed an increased expression of inflammatory markers (IL-1, -6 and -8) and matrix degrading enzymes (matrix metalloproteinase (MMP)-1, -3 and -10) compared to untreated controls. During histological and histomorphometric analysis, we observed a decreased compactness of extracellular matrix, a loss of Col II, a significantly reduced cell concentration, and altered cell morphology. Based on the developed set of partial differential equations, we were able to simulate the distribution processes within the constructs including the diffusion from the outside to the interior of especially IL-1β and the corresponding Col II degradation and cell number reduction. Optimal parameter values were determined by calibrating the *in silico* model data to the *in vitro* observations. Our next step was to use the model to simulate also the effects after 3 weeks of stimulation assuming an inhomogeneous spatial and temporal distribution. The mathematical simulations were performed with KARDOS and Matlab. The reproduction of the experimental results by a mathematical *in silico* model is considered to facilitate the refinement of our *in vitro* model in the future and to plan and determine experiments and outcome parameters more precisely. By combining methods used in biological research and those used in mathematical systems biology, we aim at developing a valid, efficient and attractive alternative approach to test possible treatments for OA, examine underlying mechanism of OA and cartilage repair to further support translation in OA research.

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A holistic approach for the toxicological assessment of phytogenic substances in human and animal nutrition

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Natural compounds have been a good source for new bioactive drugs for a long time and provide unique structural diversity. Nowadays, phytogenic substances are widely-used in human nutrition and animal production. However, there is little or no information about possible negative effects of phytochemical addition to feed and food on animal and human health. Within this regard, the cytotoxic potential of phytogenic substances might be currently underestimated, as a natural compound is not necessarily safer than other products. It is therefore of critical importance to assess the toxicological potential of such substances. Nowadays, the European food and feed market is strictly regulated and monitored by different authorities, most important the European Food Safety Authority (EFSA) and the European Union Reference Laboratories (EURL). New food and feed additives are evaluated by scientific panels, which deliver opinions on the safety of food supplements, mainly based on available literature data. Furthermore, novel food and feed ingredients have to undergo a science-based safety assessment demonstrating that the additive has no harmful effects, on human and animal health and on the environment, before being placed on the European market. Therefore, there is a great demand for toxicity testing, not only in basic science, but also for companies. Importantly, alternative methods to animal testing are promoted and in general desirable, to minimize the use of experimental animals. Within this regard, alternative approaches are gaining momentum.

Here we present a holistic approach for the toxicological assessment of phytogenic substances in human and animal nutrition using comprehensive *in-vitro*, *in-ovo* and *in-vivo* evaluation methodologies. To reduce the number of experimental animals, fast and adaptable toxicity testing in living organisms is carried out using different cell culture models, the nematode *Caenorhabditis elegans*, the hens egg test on the chorioallantois membrane (HET-CAM) and the chick embryo screening test (CHEST). In a first step, various toxicological endpoint measurements were set up and validated with respect to reproducibility and applicability by using known toxic modulators. By combining the data from *in-vitro* cell cultures as well as from whole animal approaches (HET-CAM, CHEST and *C. elegans*) with intact and metabolically active digestive, reproductive, endocrine, sensory and neuromuscular systems, we can provide a toxicity screening approach which will be as predictive as rat our mouse LD50 ranking screens. Furthermore, the assays allow for detailed sophisticated single cell/organism analysis but can also be easily adapted for high-content and high-throughput screening purposes.

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Clicker training for small rodents – cognitive enrichment and beyond

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The development of new refinement strategies for laboratory rodents is a challenging task that contributes to the fulfillment of the 3Rs of laboratory animal science. Laboratory animals can experience several stressful situations while they are being kept and bred or used for experiments. Even though there is a positive impact of gentle handling protocols on the well-being of laboratory rodents, the interaction between the laboratory rodents and the responsible persons is often still rather limited. Therefore, a trusting relationship cannot develop. This can elicit increased anxiety and stress in reaction to handling and experimental procedures, which negatively affects the well-being of the regarded animal. Further, stress induces changes in behavior and in other body parameters, including the autonomic nervous system, the neuroendocrine system and the immune system. Therefore, stress is often referred to as a possible source of unexpected deviations across animal experiments and should be clearly avoided in high-quality animal research. Further, anxiety behaviors make it harder to work with the animals. Especially inexperienced people can struggle with such behaviors, resulting in increased animal numbers for training of procedures and possibly also for experimental purposes. Trained animals therefore can help to reducing stress for both, experimenter and animal. Further anxiety behavior can also reduce the participation in behavioral tests like the catwalk.

Our aim is to continuously improve the well-being of our laboratory animals. Therefore, we established clicker training as a cognitive enrichment for small rodents, which did show to have beneficial effects on the animals. Clicker training is a form of positive reinforcement training using a conditioned secondary reinforcer, the "click" sound of a clicker, which serves as a time bridge between the strengthened behavior and an upcoming reward. We could show, that clicker training works very effectively in mice and rats, not only by direct training but also by social observation. As training procedures can easily be adapted, we also aim to implicate training to prepare animals for experimental procedures, as the voluntary cooperation of the animals in the procedure can improve the well-being. First results show that clicker training can improve performance of mice in the CatWalk behavioral test. Further mice trained for educational courses show reduced anxiety behaviors during the course and were perceived as easier to handle. Also, they more friendly by the course participants.

Clicker training can help to reduce anxiety in laboratory rodents and to build a relationship of trust, therefore improving experimental procedures and the well-being of the animals.

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ALT4EI: Assessment of eye irritating potential of 59 chemicals using EpiOcular™ time-to-toxicity (EpiOcular ET-50) neat and dilution protocols

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Assessment of the acute eye irritation potential is part of the international regulatory requirements for testing of chemicals. The objective of the ALT4EI (ALTernatives for Eye Irritation) project was to confirm the testing strategy developed in the CON4EI (CONsortium for *in vitro* Eye Irritation testing strategy) project. These projects focused on the development of tiered testing strategies for eye irritation assessment for all drivers of classification and evaluation of whether the test methods can discriminate chemicals not requiring classification for serious eye damage/eye irritancy (No Category) from chemicals requiring classification and labelling for Category 1 (Cat 1) and Category 2 (Cat 2).

A new set of 59 chemicals (41 liquids: (un)diluted, and 18 solids) was tested using the reconstructed human cornea-like epithelium (RhCE), EpiOcular, in two EpiOcular time-to-toxicity Tests (Neat and Dilution ET-50 protocols). The set of chemicals contained 32 chemicals not requiring classification (No Cat) and 27 chemicals requiring classification (16 Cat 2 and 11 Cat 1).

The chemicals were tested blinded in two independent runs by MatTek In Vitro Life Science Laboratories. In this study, a testing strategy to achieve optimal prediction for all three classes that was developed in CON4EI project (which combines the most predictive time-points of both protocols and which tests liquids and solids separately) was used.

Using the CON4EI testing strategy, we were able to identify correctly 63.6% of the Cat 1 chemicals, 56.6% of the Cat 2, and 76.6% of No Cat chemicals. Reproducibility between both runs was 88.7%. The combination of the EpiOcular ET-50 neat and dilution protocols seem to be promising in an integrated testing strategy (ITS) for eye irritation assessment.

Submission declaration:

Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.

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Development, pre-validation and validation of the EpiDerm *in vitro* skin irritation protocol for the medical devices extracts

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Evaluation of dermal irritation is an essential component of the safety evaluation of medical devices. Reconstructed human epidermis (RhE) models have replaced rabbit skin irritation testing for neat chemicals (OECD TG 439). However, medical device (MD) extracts are dilute solutions with low irritation potential, therefore the validated RhE-methods needed to be modified to reflect needs of ISO 10993.

A protocol employing RhE EpiDerm was optimized in 2013 using known irritants and spiked polymers [1]. In 2014, a second laboratory assessed the transferability of the assay. After the successful transfer and standardization of the protocol, 17 laboratories worldwide were trained in the use of the protocol in the preparation for the validation. All laboratories produced data with almost 100% agreement of predictions for the selected references [2].

Moreover, several medical devices benchmark materials (5 irritants and 2 vehicles) were evaluated in the controlled human patch testing (4 h and 18 h) and in EpiDerm *in vitro* skin irritation protocol, results were then compared to existing rabbit skin irritation test data [3].

In 2016, an international round robin validation study was conducted to confirm the ability of the RhE models to correctly predict the intra-cutaneous irritation of extracts from MDs. Four irritant polymers and three non-irritant controls were tested. Blinded polymer samples were extracted with sesame oil and saline per ISO 10993-12. Positive and negative solvent controls were included [4].

EpiDerm tissues were able to correctly identify virtually all of the irritant polymer samples either in the saline or in the sesame oil or in both solvent extracts. Our results indicate that RhE tissue models can detect the presence of skin irritants at low concentrations in dilute medical device polymer extracts [4]. The use of the reconstructed tissue models, as replacements for the rabbit intra-cutaneus test is currently being implemented into the ISO 10993 standards used to evaluate medical device biocompatibility.

References
[1] Casas, J. W., Lewerenz, G. M., Rankin, E. A. et al. (2013). *Toxicol In Vitro 27*, 2175-2183. doi:10.1016/j.tiv.2013.08.006
[2] Kandarova, H., Willoughby, J. A., De Jong, W. H. et al. (2018). *Toxicol In Vitro 50*, 407-417. doi:10.1016/j.tiv.2018.02.007
[3] Kandarova, H., Bendova, H., Letasiova, S. et al. (2018). *Toxicol In Vitro 50*, 433-438. doi:10.1016/j.tiv.2018.02.009
[4] De Jong, W. H. et al. (2018). *Toxicol In Vitro 50*, 439-449. doi:10.1016/j.tiv.2018.01.001

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Evaluation of *in vitro* embryotoxicity tests for Chinese herbal medicines

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Chinese herbal medicines (CHMs) have been widely used during pregnancy, but feto-embryo safety tests are lacking. Here we evaluated *in vitro* embryotoxicity tests (IVTs) as alternative methods in assessing developmental toxicity of CHMs. Ten CHMs were selected and classified as strongly, weakly and non-embryotoxic. Three well validated IVTs and prediction models (PMs), including embryonic stem cell test (EST), micromass (MM) and whole embryo culture (WEC), were compared. All strongly embryotoxic CHMs were predicted by MM and WEC PM2. While all weakly embryotoxic CHMs were predicted by MM and WEC PM1. All non-embryotoxic CHMs were classified by EST, MM, but over-classified as weakly embryotoxic by WEC PM1. Overall predictivity, precision and accuracy of WEC determined by PM2 were better than EST and MM tests. Compared with vali-dated chemicals, performance of IVTs for CHMs was comparable. So IVTs are adequate to identify and exclude embryotoxic potential of CHMs in this training set.

Reference

Li, L. et al. (2019). *Reprod Toxicol 89*, 45-53.

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The blood-saliva barrier: An optimized *in vitro* model of the oral epithelium and its relevance for biomarker research

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Saliva has gained popularity as a diagnostic fluid in the past decades as it offers the advantage of non-invasive collection and easier handling or storage in comparison to blood. The proteomics, transcriptomics and microbiological composition of saliva has been screened for biomarkers for systemic diseases such as cancer, HIV or cardiovascular disease. Due to the rapid development in regard to the discovery of salivary biomarkers the term "salivaomics" was first coined in 2008 [1]. However, until today their appearance in saliva and how they are transported from blood to saliva crossing the blood-saliva barrier is still not clear.

The blood-saliva barrier is defined as the sum of epithelial cell layers, infiltrated by other cell types, from the oral cavity and salivary glands. Based on the human epithelial cell line TR146 from a buccal carcinoma, first described in 1980s [2], a Transwell model was established at AIT. As the majority of the molecules are assumed to cross the barrier using the passive transport route, the paracellular integrity of tight junctions was intensively investigated by TEER (transepithelial electrical resistance) measurements and permeability of paracellular marker. For optimization of the model different media supplements and cultivation set-ups (airlift, submerged) were tested. The set-ups were characterized by the "Barrier-Chip" using a high-throughput qPCR platform (Fluidigm), Hematoxylin-Eosin and immunofluorescent staining. Highest increase of TEER over time was achieved by cultivation in a media containing human keratinocyte growth factors under airlift condition.

With the optimized model transport studies with tryptophan, a biomarker for Alzheimer´s disease, were performed, whereby results indicate an active transport to the saliva compartment. CRP (C-reactive protein), a biomarker for cardiovascular disease and inflammation has shown to correlate to health status of diabetic patients as well in saliva [3]. In our model we investigated the transport of CRP from blood to the saliva compartment and *vice versa*, whereby first results indicate an enhanced transport to the saliva side. To investigate the effect of the microenvironment on buccal epithelial cells, the model was co-cultivated with primary human oral fibroblasts. However, first results show no improvement in barrier functionality compared to the model in monoculture.

Future studies include co-cultivation of TR146 with other cell types from the microenvironment such as endothelial cells, characterization of *in vitro* models representing the salivary glands and investigating the transport of biomarkers across both parts of the blood-saliva barrier.

References

[1] Wong, D. T. W. (2012). *J Am Dent Assoc 43*, 19S-24S. doi:10.14219/jada.archive.2012.0339
[2] Rupniak, H. T., Rowlatt, C., Lane, E. B. et al. (1985). *J Natl Cancer Inst 75*, 621-635.
[3] Dezayee, Z. M. and Al-Nimer, M. S. (2016). *Indian J Dent Res 27*, 388-391. doi:10.4103/0970-9290.191887

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Normalizing the unthinkable: The report of the Oxford Centre for Animal Ethics

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This paper will discussion the conclusions of a report by the Oxford Centre for Animal Ethics on the ethics of using animals in research. The report is the most comprehensive and detailed analysis of the moral dimension of the subject. It is estimated that 115.3 million animals are used in experiments worldwide per annum. In terms of harm, pain, suffering, and death, this constitutes one of the major moral issues of our time. The use of animals in invasive, regulated research represents the institutionalization of a pre-ethical view of animals. The normalization of animal experiments over the last 150 years has been based on flawed moral arguments that can no longer be justified given a changing philosophical paradigm and a deeper contemporary understanding of animal sentience. The only logical conclusion is that animals must be afforded special moral consideration that precludes them from use in experiments.

Reference

Linzey, A. and Linzey, C. (eds). (2018). *The Ethical Case Against Animal Experiments*. Urbana, IL, USA: University of Illinois Press.

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Assessment of uncertainties – ECVAM experience from recent *in-vitro* validation studies

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Performance evaluation of (*in-vitro*) alternative methods is a key element in the EURL ECVAM validation process as it shall provide sufficient information for the informative decision on the regulatory acceptance of the method. Two factors are important here, the experimental design of the study and the evaluation of the data generated. For prospective validation studies, the challenge is to find good balance between constraints for experimental design of the validation study and enough information generated to make the assessment feasible. For retrospective studies the situation might be more restricted. EURL ECVAM proposed recently new ways for the performance evaluation of single methods and defined approaches taking into account associated uncertainties. Two main performance measures are under the focus, reliability and relevance, where the comparison of traditional single valued statistics versus confidence intervals or distributions is discussed. Illustrative data examples from validation studies on skin sensitization methods are provided too.

References

Liska, R. (2018). Evaluation of skin sensitisation DA uncertainty and reproducibility. EURL ECVAM Internal Report.

Liska, R. (2015). Re-analysis of the within and between laboratory reproducibility of the human Cell Line Activation test (h-CLAT). EURL ECVAM Internal Report.

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In vivo measurement of 7-methoxycoumarin-O-demethylase activity in zebrafish (*Danio rerio*) embryos

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The lack or insufficiency of biotransformation has for long been a hurdle in the development of alternative models for toxicity testing. Since quantitative and qualitative differences in biotransformation activities between distinct developmental stages or species might lead to an over- or underestimation of toxic potentials, a thorough characterization of the biotransformation capacities of a test organism is imperative for a correct interpretation and extrapolation of toxicological data. Early life-stages of zebrafish (*Danio rerio*) have been extensively used as an alternative model in (eco)toxicological and pharmacological testing strategies. However, while spatial and temporal expression patterns of xenobiotic metabolizing enzymes (e.g. cytochrome P450-dependent monooxygenases, CYPs) have been investigated extensively at the mRNA level, there is only little and contradictory information about protein functions and biotransformation activities. Therefore, this study was designed to systematically investigate functional CYP-dependent biotransformation activities throughout early zebrafish development. To determine the constitutive spatio-temporal activity patterns and the inducibility of CYP-dependent activities, zebrafish embryos (5.5-120 hours post-fertilization, hpf) were assessed by confocal laser scanning microscopy for *in vivo* 7-methoxycoumarin-O-demethylase (MCOD) activity, a CYP2-dependent process in humans and other mammals. Results demonstrate that zebrafish embryos display constitutive and inducible biotransformation activity from as early as 5.5 hpf onward. Dynamic spatio-temporal patterns were detected for MCOD activities localized in several tissues and organs (e.g. cardiovascular system, urinary tract, digestive system). Exposure to β-naphthoflavone (aryl hydrocarbon receptor agonist) for 3 h resulted in a significant increase in MCOD activity, while exposure to other prototypical inducers (rifampicin and phenobarbital) had no effect on MCOD activity. The results indicate the presence of biotransformation activities in zebrafish from very early stages of embryological development.

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Biotransformation and bioactivation capacities in early life-stages of zebrafish (*Danio rerio*) – a state-of-the-art review

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Major criticism to the use of the fish embryo test (FET) with the zebrafish (*Danio rerio*) as an alternative method for acute toxicity testing in juvenile and adult fish has been based on the assumption of a limited biotransformation capacity in early life-stages of zebrafish. Indeed, a limited biotransformation or bioactivation capacity could lead to significant underestimations of toxic or teratogenic potencies of xenobiotics, which would be bioactivated in later developmental stages. Thus, in order to further develop the acceptance of the FET, there is a need for an improved characterization of biotransformation activities in zebrafish in general and zebrafish early life-stages in specific.

In an attempt to get an overview and to identify gaps in knowledge, a literature review was conducted, and more than one hundred peer-reviewed publications and academic theses were identified by searching the following electronic databases: Google Scholar, Science Direct, PubMed, and Web of Science. This review includes studies on constitutive spatial and temporal expression patterns of CYP genes, catalytic activities towards prototypical fluorogenic and luminogenic CYP probe substrates, bioactivation capacities and metabolite spectra of xenobiotic substances in zebrafish. In the literature, embryonic, juvenile and adult life-stages have been studied to varying extent, and especially early embryonic (< 48 hpf) and juvenile zebrafish have largely been neglected so far. Whenever studied in more detail, biotransformation in zebrafish embryos has been documented on both mRNA and functional levels, and only for rare exceptions (e.g. CYP3C4 [1] and allyl alcohol [2]), a lack of mRNA expression or bioactivation could be demonstrated.

References

Shaya, L., Dejong, C. and Wilson, J. Y. (2014). *Comp Biochem Physiol 166C*, 115-125.
Kluever, N., Ortmann, J., Paschke, H. et al. (2014). *PLoS One 9*, e90619.

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Moving transparency to the next level – non-technical project summaries under Directive 2010/63/EU

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Transparency is essential to develop a trust in the systems of ethical and socially acceptable care and use of animals in science as the basis for the continued research using animals in the EU until such time that their use can be replaced by non-animal alternatives. Comprehensive and accurate information is a prerequisite for decision making, be it for policy development, research funding or simply to understand the status quo. Accordingly, improved transparency was set as one of the key objectives for Directive 2010/63/EU. The tools to facilitate improved transparency include the revised requirements for statistical reporting, and the publication of non-technical project summaries to provide objective information on projects using live animals.

The review of the Directive, completed in 2017, showed that majority of Member States and users considered that the publication of non-technical project summaries was already positively contributing to transparency, although the full impact had yet to be realised. However, not all stakeholder groups, especially those representing animal welfare, shared this view.

The main issues raised concern the accessibility, both in speed and ease of access, the quality of content and absence of any facility to search them at EU level. One of the recommendations of the review called for the Commission, Member States and stakeholders to explore the possibilities of a central EU repository of non-technical project summaries.

A recent Regulation (EU) 2019/1010 on environmental reporting will make this a reality and move transparency to the next level.

The talk will present the actions the Commission has taken in this regard and the progress towards improving both the accessibility and quality of non-technical project summaries.

References

Directive 2010/63/EU. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32010L006

Regulation (EU) 2019/1010. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32019R1010

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Advancing the Three Rs education and training under a European Parliament Pilot Project

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The European Parliament provided additional funding to the European Commission through a Pilot Project to promote the uptake of existing alternatives, to facilitate development and validation of new alternatives, to foster the exchange of information, knowledge and best practices and to provide tools for education and training to facilitate the application of the Three Rs.

Today's users and future scientists are the targets of this project. The project consists of three parts enabling:

- the delivery of six interactive eLearning training modules covering learning outcomes on aspects of Directive 2010/63/EU on the protection of animals used for scientific purposes and non-animal alternatives, including incorporation of key elements from a recently adopted OECD guidance on Good *In Vitro* Method Practices, GIVIMP. The modules will be publicly available for any individuals or course providers to be used as stand-alone training tools or as part of a curriculum.
- to develop, through a project coordinated by EURL ECVAM in the Commission's Joint Research Centre, new Three Rs resources for high schools, universities, and early career scientists. The envisaged deliverables include learning resources and specifications for building guidance on how to include the Three Rs in a curriculum.

- through direct funding to the European Education and Training Platform for Laboratory Animal Science (ETPLAS) to develop guidance on assessment criteria; actual assessment criteria, and tools for competence assessment, under Directive 2010/63/EU Article 23 based on the EC Education and Training Framework. ETPLAS platform will also host the six interactive, open access, eModules.

The session provides an overview of the three actions under the EP Pilot project.

References

EU Education and Training Framework. http://ec.europa.eu/environment/chemicals/lab_animals/pdf/guidance/education_training/en.pdf

Directive 2010/63/EU. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32010L0063

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Project evaluation – importance of a coherent and consistent approach

Susanna Louhimies[1] and David Anderson[2]

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Directive 2010/63/EU requires that all projects are subjected to a project evaluation and sets out in detail the elements to be considered, and the type of expertise which should be considered when conducting such an evaluation.

The evaluation requires that a harm – benefit analysis forms part of the process, to assess whether the harm to the animals is justified by the expected outcomes, taking account of ethical considerations.

Project Evaluation is a cornerstone of the Directive in ensuring the Three Rs are implemented and animal welfare compromises minimised consistent with the scientific objectives.

Coherent approach to project evaluation and consistency in the outcomes are important in ensuring delivery of a level playing field for the scientific community, as well as confidence in the regulatory framework.

Several factors influence consistency, starting from the definition of the term "project". In addition, the level of detail in which projects are scrutinised plays an important role. An equilibrium needs to be established that minimises administrative burden whilst obtaining assurance of a robust scientific design, implementation of the Three Rs and animal welfare obligations in line with the Directive.

The presentation will explore these issues and other difficulties raised by the scientific community in the Directive review and how these may be addressed.

References

Directive 2010/63/EU. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32010L0063

Project Evaluation and Retrospective Assessment Document. http://ec.europa.eu/environment/chemicals/lab_animals/pdf/guidance/project_evaluation/en.pdf

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Implementing Directive 2010/63/EU – current status and next steps

Susanna Louhimies

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Directive 2010/63/EU governs the use of animals for scientific purposes in the EU. It took effect in January 2013 and its review was published in 2017. All key stakeholders contributed to the process, even if with rather limited experience from its functioning due to the early timing of the review. Nonetheless, the results included several recommendations covering the three objectives of the Directive; to improve level playing field for the operators to enhance competitiveness and innovation of the EU research; to ensure high standards of animal welfare and implementation of the Three Rs; and to improve transparency to the general public on the use of live animals for scientific purposes.

2019 represents an important year for the Directive. The first EU report on the implementation of the Directive will be published in November this year. Similarly, in line with Article 57 of the Directive, the first report, under this Directive, on the statistics on the use of animals in the EU is also due in November.

Several recommendations from the Directive review are being followed up by a variety of stakeholders to improve its implementation, efficiency and transparency. However, more efforts are needed, especially to address inconsistencies in project evaluation and authorisation. One of the recommendations taken up by the Commission reached an important milestone: on 5 June 2019, the Council and the European Parliament agreed to an establishment of searchable, open access, central EU databases on animal use information. With this decision, EU is setting a new world standard for transparency.

Reference

Directive 2010/63/EU. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32010L0063

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Retinal protection against oxidative stress via mild hypothermia

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Purpose

Oxidative stress plays an important role in several retinal diseases, such as glaucoma and ischemia. We previously introduced an *ex vivo* oxidative stress-degeneration model with porcine retinal explants [1]. Hydrogen peroxide (H_2O_2) was used to induce stress to the inner retinal layers. Based on this degeneration model, we were interested if processes underlying oxidative stress, can be prevented by a mild hypothermia at 30°C in *ex vivo* cultivated porcine retinal explants.

Methods

In order to analyze possible neuroprotective effects of a mild hypothermia at 30°C, porcine retinal explants were cultivated for five and eight days. Oxidative stress was induced on day one for 3 h via 300 μM H_2O_2. For the treatment, retinae were cultivated at 30°C for 3 h simultaneously to the H_2O_2-induced stress. The effects of hypothermia-treatment were analyzed by comparing three groups: a control group and a H_2O_2 group, which were cultivated at 37°C during the whole period of time, and a hypothermia treated H_2O_2+30°C group. The number of retinal ganglion cells (RGCs), cholinergic amacrine cells, and rod bipolar cells was investigated via immunohistology. Additionally, the apoptosis rate of RGCs was evaluated immunohistologically, but also the apoptotic state of the whole retina was investigated via western blot (Bax/Bcl-2). Likewise, the microglial as well as the microglial response was examined.

Results

Hypothermia-treatment protected RGCs from oxidative stress due to a strongly decreased apoptosis rate (H_2O_2: p < 0.001; H_2O_2 + 30°C: p = 0.51). In consequence, the RGC loss induced by H_2O_2 (5 days: p < 0.01; 8 days: p < 0.001) was totally counteracted after mild hypothermia (8 days: p < 0.001). Furthermore, amacrine cells were rescued via hypothermia after eight days (H_2O_2:

p < 0.001; H_2O_2 + 30°C: p = 0.17). Interestingly, rod bipolar cells were only protected at five days (p = 0.61), but not at day eight (p < 0.01). After eight days, microglial reaction to oxidative stress (p < 0.001) was inhibited via hypothermia (p = 0.60). Also, the amount of activated microglia due to H_2O_2 (p < 0.001) was significantly reduced after hypothermia-treatment (p < 0.05). In contrast, no macroglial reaction was seen in any point in time (for all: p > 0.3).

Conclusion

The simulation of oxidative stress due to H_2O_2 induced strong degenerative processes in porcine retinas. The H_2O_2-induced degeneration models of cultivated porcine retinal explants therefore qualifies as a suitable model to investigate new therapeutic approaches for retinal diseases, such as glaucoma or ischemia. Simultaneously, this model will help to reduce the number of animal experiments in ophthalmic research. In the present project, a strong protection of retinas against H_2O_2-induced oxidative stress through hypothermia was observed. Especially, retinal ganglion cells, which are strongly affected in glaucoma disease, were protected by hypothermia through a decreased apoptosis rate. These effects strongly indicate, that hypothermia might be a promising additional treatment for retinal diseases.

Reference

[1] Hurst, J., Kuehn, S., Jashari, A. et al. (2017). *Altern Lab Anim 45*, 11-25. doi:10.1177/026119291704500105

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Synovial cell characterization for developing a new 3D model for *in vitro* osteoarthritis studies

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Osteoarthritis (OA) is the most common joint disease and the major cause of pain and disability in the aging population. Synovial inflammation is now accepted as an important feature for the symptoms and disease progression.

Synovium is mainly composed of synovial fibroblast (SF) and macrophage (SM) and a low percentage of other cell types. In literature, studies *in vitro* mainly focused their attention on SF not considering the important inflammatory role of SM and there is a lack of *in vitro* systems that well represent the pathological synovial tissue. Our recent studies evidenced an important role of SM in maintaining the production of pro-inflammatory cytokines.

The aim of the study was to characterize OA synovium, to analyze the role of SF and SM and to develop a new biotechnological *in vitro* human 3D cell co-culture OA system that mimics the *in vivo* synovial structure.

Synoviocytes were isolated from synovia of OA patients undergoing total joint replacement. Synoviocytes both at passage 1 and p5 were characterized for the following markers: CD3, CD11b, CD14, CD16, CD55,CD68, CD80, CD86, CD90, CD163, CD206, Collagen type 1 and VCAM-1by flow cytometry, immunocytochemistry or qRT-PCR. Secreted inflammatory factors (IL1ß, IL6, CXCL8/IL8, CXCL1/GROα, CCL2/MCP-1, CCL3/ MIP1α, CCL5/RANTES, TNF) were analyzed by multiplex bead-based sandwich immunoassay on synoviocytes p1 and p5. Macrophages type 1 and 2 were also isolated.

For 3D co-culture experiments, SF or macrophages or mixed population were seeded onto Collagen membrane at different cells density and the construct was inserted into a perfusion bioreactor (Cellec). At different time point (2 and 7 days) we evaluated cell viability by MTT test, a panel of markers (described above) by FACS analysis or immunohistochemistry.

Synoviocytes at passage 1 were positive to CD3, CD14, CD16, CD68, CD80, CD86, CD206 and 90% to CD90 while synoviocytes at p.5 (only SF) were negative to all the markers analyzed

except for CD55, CD90, VCAM-1 and Collagen type 1. Moreover, synoviocytes at p.1 produced a significantly higher amount of IL6, IL8/CXCL8, CCL2/MCP-1, CCL3/MIP1α and CCL5/ RANTES than at p.5, except CCL3/MIP1α that was not detected. We confirmed on serial sections of synovial tissue that the same percentage of synovial cells detected on p.1 isolated synoviocytes.

Preliminary results on dynamic 3D co-culture construct found a homogenous distribution of cells respect to static condition at day 2 and day 7 by MTT assay and histological cells evaluation.

FACS analysis of digested construct evidenced the presence of positive cells for typical M1 (CD80,86), M2 (CD163,206) and fibroblast (CD55, CD90) markers.

We characterized and compared synoviocytes at two cell passages (p.1 and p.5) to define phenotypical and functional role of synoviocytes as a mix of SF and SM (p.1) and as SF alone (p.5).

Basing on these data we reproduced in a 3D system a synovium-like tissue maintaining in co-culture both synovial fibroblast and macrophages that are usually that are lost during passages in culture.

Our data demonstrate that the 3D system better mimic the *in vivo* synovial structure and would be an important tools for validating the effect of new cells therapies or drugs in OA treatment.

Reference

Manferdini, C., Paolella, F., Gabusi, E. et al. (2016). *Arthritis Res Ther 18*, 83.

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A 3D micromodel to study chemotaxis of cancer spheroids exploiting microfluidic channels

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Our ability to study cell migration biological mechanisms, its function and its relation to pathology is either based on simplified two dimensional (2D) cell cultures or on animal models where the ability to interfere with endogenous biological mechanisms is often reduced. The employment of microfluidic platforms for cell culture and analysis leads to a better mimic of a 3D environment since it allows for a more accurate cell monitoring, fluids handling and instauration of chemical concentration gradients [1-3]. *In vitro* 3D cultures provide a third approach that bridges the gap between traditional cell culture and animal models [4].

A 3D micro model for the study of the relations between cancer stimulation and cells chemotaxis is presented. The platform allows tests in a 3D environment, requirement that approximates the *in vivo* conditions, of both single cells and spheroids. Moreover, it allows the spatial and temporal control of the concentration of the chemo-attractants. The chip is produced using different and well known techniques [5], but differently from the commercial ones is made in PDMS (Poly-dimethilsiloxane), a biocompatible and gas permeable material. The use of this material, together with 150 µm microscope glass slices, ensures the possibility of integrating cell culturing and live microscopy. The proposed fabrication process is standardized and allows the production of repeatable chips with high aspect ratio micro-pillars (6:1). These structures ensure that chemotaxis is ruled only by the diffusion phenomenon of the growth factors molecules that is generated between lateral channels and the central one. A Finite Element Model corroborates the results of this study to understand the diffusive gradient formation and obtain a predictive behavior. The chips and in particular the micro-pillars were designed focusing on the peculiarity of Matrigel™, but also ensuring the use of collagen as well. Cells analyses were conducted in time-lapse microscopy at 37°C overnight and a diffusive gradient of the fluorescent protein was successfully observed.

References

[1] Gao, Y., Sun, J., Lin, W. H. et al. (2012). *Microfluid Nanofluidics 12*, 887-895.

[2] Saadi, W., Wang, S. J., Lin, F. et al. (2006). *Biomed Microdevices 8*, 109-118.

[3] Li, J. and Lin, F. (2011). *Trends Cell Biol 8*, 489-497.

[4] Han, S., Yang, K., Shin, Y. et al. (2012). *Lab Chip 12*, 2305-2308.

[5] Marasso, S. L., Giuri, E., Canavese, G. et al. (2011). *Biomed Microdevices 13*, 19-27.

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In vitro neurotoxicity testing: Functional neuron-specific endpoints

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In accordance with the requirement for reduction of animal usage in toxicology testing and with the 3Rs principle, alternative models are actively investigated which would serve to replace the standard neurotoxicity assessments. Nevertheless, detection and characterization of the neurotoxic effects of drugs or chemicals is a demanding task, due to the complexity and the specific features of the nervous system and of the nervous system functioning [1,2]. High-content tools are needed, that by considering the specific features of the nervous system functioning might identify neurotoxic vs cytotoxic effects. In fact, bi-directional communications between neurons and glial cells through release of transmitters and sensors for the transmitters – the receptors – are relevant functional features of the nervous system, required for the neuron and astrocyte-neuron network collective behavior and functioning. To support screening for potential neurotoxicity, tools are required providing neuron-specific endpoints by which toxicity end-points should predict *in vivo* toxicity.

We here propose a multi-disciplinary electrophysiological, neurochemical and immunocytochemical approach. Primary rodent neuron-astrocyte co-cultures were set on engineered culture chamber equipped with 60 electrodes (Micro-Electrode Arrays MEAs [3,4]). Recording of electrical activity of neuron network (long-term multisite recording of collective network behavior) was combined with immunocytochemistry (pre/postsynaptic markers, neurochemical markers) and measurement of transmitter release (HPLC detection) to assess network maturation.

Both glutamatergic and GABAergic neurons, target for relevant neurotoxicity mechanisms and related to Adverse Outcome Pathways relevant to neurotoxicity, matured during network development. In the mature networks synaptic connectivity was mainly dependent on activity of the glutamatergic pathways; in fact, the cerebrocortical cultures coupled to MEAs behaved as sensitive sensors of the glutamatergic transmission functioning. Changes in the network collective behavior (electrophysiological activity; firing and bursting activity) were evoked by pharmacological manipulation of the glutamatergic transmission (activation or blockade of glutamate NMDA or AMPA receptors, or blockade of the plasma membrane glutamate transporters); these changes were related to the drug effects on the release of glutamate. The network was also capable of sensing the subtle transmission variations related to synapse plasticity. Indeed, pharmacological manipulation of the NMDA-dependent NO-cGMP pathway (NO synthase

or guanylyl cyclase inhibitors, NO donors or the cGMP analogue 8Br-cGMP) evoked variations of both the network electrophysiological activity and the glutamate release.

Modulation of the network activity by neuroactive substances such as glutamate or GABA receptor agonists/antagonists, and by known neurotoxic agents (e.g., domoic acid, chlorpyrifos oxon), together with ineffectiveness of molecules not exhibiting acute neurotoxic effects, indicate that neuron networks coupled to MEAs can represent an integrated approach for neurotoxicity testing based on functional neuron-specific endpoints.

We conclude that MEA systems can be developed as effective alternative tool for substance neurotoxicity evaluation [5]. In fact, neuronal networks coupled to MEAs behave as highly sensitive biosensor for manipulation of glutamatergic transmission and can provide neuron-specific endpoints (collective behavior of neuron network – intercellular communication through transmitters and transmitter sensors) for neurotoxicological and neuropharmacological purposes. Notably, they also can provide a mechanistic information on glutamatergic/GABAergic receptor function/dysfunction therefore allowing investigation on Adverse Outcome Pathways relevant to neurotoxicity [2].

References
[1] Bal-Price, A., Hogberg, H. T., Buzanska, L. et al. (2010). *Neurotoxicol Teratol 32*, 36-41. doi:10.1016/j.ntt.2008.12.003
[2] Bal-Price, A. and Meek, M. E. B. (2017). *Pharmacol Ther 179*, 84-95. doi:10.1016/j.pharmthera.2017.05.006
[3] Martinoia, S., Bonzano, L., Chiappalone, M. et al. (2005). *Biosens Bioelectron 20*, 2071-2078. doi:10.1016/j.bios.2004.09.012
[4] Cervetto, C., Marcoli, M., Massobrio, P. et al. (2017). *Toxicol Lett 280*, S321-S322. doi:10.1016/j.toxlet.2017.08.070
[5] Vassallo, A., Chiappalone, M., De Camargos Lopes, R. et al. (2017). *Neurotoxicology 60*, 280-292. doi:10.1016/j.neuro.2016.03.019

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Full-thickness human skin and airway tissue models produced using electrospun scaffolds

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Production of *in vitro* full-thickness epithelial tissue models typically involves the use of collagen gels containing fibroblasts as the stromal component. Collagen gel constructs suffer from stability issues and a tendency to contract which can be difficult to control. The current work describes production of full-thickness skin and airway epithelial tissue models produced using electrospun support scaffolds for the stomal components. Non-degradable electrospun scaffolds consisting of randomly oriented fibers with nanometer to submicron diameters (Bio-Spun™-PET) similar to those of native collagen fibers were seeded with human dermal or pulmonary fibroblasts and cultured under submerged conditions. Fibroblasts proliferated within the scaffolds and synthesized additional native collagen and extracellular matrix materials that self-assembled *in situ* to produce robust and stable stromal matrices. Epidermal keratinocytes or bronchial epithelial cells were seeded onto the stromal components and cultured at the air-liquid interface to produce organotypic 3D stratified dermal and airway tissue models. H&E stained paraffin sections revealed robust stomal components populated with fibroblasts. Copious amounts of *in situ* produced extracellular stromal matrix materi-al was evident. The full-thickness skin models displayed a well-developed stratified epidermis consisting of basal, spinous, granular and stratum corneum components. Transmission electron microscopy showed large numbers of striated collagen fibers and a well-developed basement membrane with lamina densa, anchoring fibrils and hemidesmosomes. The full-thickness airway models displayed pseudostratified mucociliary epithelium with abundant mucus secretion evident on the apical surfaces. In summary, new-generation full-thickness skin and airway models were produced without the need for collagen gels using Bio-Spun™-PET scaffolds. These models offer promise as animal alternatives for testing of cosmetics, chemicals and pharmaceuticals. Additional work to fully characterize the functional properties of these novel models is ongoing.

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Effects of fruit proteases on intestinal integrity and permeability in 3D EpiIntestinal reconstructed human tissue model

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Intestinal absorption enhancement via paracellular route is a promising target for drug delivery. Fruit enzymes have been shown to enhance intestinal paracellular uptake of low weight molecules as studied *in vivo*, *post-vivo* and Caco-2 cell lines [1,2]. The present study aimed to validate effects of cysteine proteases such as bromelain from pineapple (*Ananas comosus*) or papain from papaya fruit (*Carica papaya*) on intestinal integrity and permeability using 3D reconstructed human small intestine epithelium (EpiIntestinal, SMI-100).

Purified enzymes in powder form, both from BioChemica, were dissolved in HBSS buffer, pH 6.5, and applied on apical side in concentration 10 mg/ml and volume 0.01 ml per tissue insert for 30 min treatment period followed by Lucifer yellow (LY) permeability test for another 30 min. Transepithelial electrical resistance (TEER) measurement was performed to assess the intestinal integrity before the enzyme application (0 min), after termination of treatment (at 30 min) and after 30 min washout period (at 60 min). The measurements of bromelain (BRM, n = 9) or papain treated inserts (PAP, n = 6) were compared to the untreated control (Ctrl, n = 9).

Results, presented as mean ±SEM, showed significant increase for % LY passage from apical to basolateral side for BRM (2.7 ±1.0, $p < 0.05$) and PAP groups (1.3 ±0.3, $p < 0.01$) compared to Ctrl (0.4 ±0.07). However, the TEER result showed significant decrease of area resistance, $\Omega*cm^2$, at 60 min for BRM group only as compared to Ctrl (67.8 ±17.05 *vs*. 133 ±11, respectively, $p < 0.05$). It is worth mentioning that tissues viability was unaffected neither by PAP nor BRM during exposure period as evaluated by MTT assay.

In conclusion, although both enzymes were administered at the same dose, the papain has shown slighter effects on intestinal integrity than bromelain. Present study has confirmed that 3D EpiIntestinal human tissue model is useful to investigate effects of food bioactive compounds such as fruit enzymes on intestinal integrity and permeability and can be further implemented in nutritional research to improve the modeling of the human intestinal functions comparatively to existing animal models.

References

[1] Guggi, D. and Bernkop-Schnürch, A. (2005). *Int J Pharm 288*, 141-150. doi.org/10.1016/j.ijpharm.2004.09.023

[2] Grabovac, V., Schmitz, T., Föger, F. et al. (2007). *Pharm Res 24*, 1001-1006. doi.org/10.1007/s11095-006-9226-8

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Modeling of drug toxicity and permeability using the reconstructed 3D model of small intestine

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Reliable and reproducible *in vitro* model that mimics function of normal human small intestine is a necessity in prediction of safety and bioavailability of orally administered compounds. Here we describe a 3D *in vitro* human small intestinal (SMI) model and its use in predicting the drug absorption and toxicity. Characterization of the microtissues included evaluation of structural features, barrier properties, and expression of drug transporters and drug metabolizing enzymes. We have shown that the reconstructed tissues have highly reproducible morphology mimicking that of normal tissue with physiological TEER values averaging at approximately 150 $\Omega*cm^2$. The real-time PCR analyses confirmed presence of transcripts encoding all tested drug transporters and metabolizing enzymes known to be present *in vivo*. To evaluate the suitability of the microtissues for prediction of drug absorption, the apparent permeability coefficient (Papp) values for a panel of benchmark drugs with known human absorption values were measured. Drug-drug interactions were examined using drugs known to be substrates or inhibitors of efflux transporters. Drug permeation analysis with selected drugs showed that the intestinal microtissues could discriminate between low and high permeability drugs with higher accu-racy than the CaCo2-based model. The activity of efflux transporters was shown by exposing the tissues to substrates of ABC pumps, the resulting drug efflux ratios were > 2.0. Further experiments were aimed at assessing the usability of SMI model in predicting an incidence of clinical diarrhea by selected drugs. Diarrhea is associated with compromised barrier function, which can be measured using transepithelial electrical resistance (TEER). An effect of drugs with known incidence of diarrhea on TEER was measured and after adjusting to clinical exposure, the threshold was identified. In the subsequent blinded experiment an excellent performance was demonstrated with 80% positive predictivity and 83% negative predictivity. In conclusion, the SMI microtissues appear to be a promising tool for predicting safety and bioavailability of orally administered drugs.

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The impact of closure of specialised mouse research facilities

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The recent closure of two prominent mouse genetics facilities in the UK – the Wellcome Sanger Institute and the Medical Research Council Mouse Genetics Unit – in response to the "rise in the use of alternative technologies" [1] signal a welcome evolution in the biomedical research paradigm, and a brighter future for more human-predictive and ethical tools such as iPSC, organs-on-chips and computational systems biology modelling. It is well established that most animal models are poor predictors of the human response, with over 90% of new candidate drugs never making it to patients. A large contributor to this failure is that pharmaceutical compounds that appear "safe" and "effective" in animal trials fail to deliver the same result when given to people; 55% do not effectively treat the condition for which they are intended, and almost 30% show signs of toxicity that were not seen in animal tests [2].

The traditional response to improve translation has been to pursue the creation of "improved" animal models – particularly genetically modified mice – altering murine genes to enhance disease susceptibility, or inserting human genes in attempts to create more symptomatically realistic models of human disease. Even after decades of repeated failure of such transgenic models, the hope remains, for those still committed to an animal model paradigm, that another tweak of the genome will somehow overcome the lack of translation from rodent to human. Beyond the failure of such disease models to accurately recapitulate a human disease, the breeding and use of genetically modified animals is also a hugely wasteful process. In the UK alone in 2017, almost 50% of all procedures involving animals were for the creation of genetically modified mice [3], which equates to almost 2 million animals never used for any research – simply (over)bred and culled.

Although the news of fewer animal research and breeding facilities can rightly be seen as a positive development, it is possible that such closures could also have negative consequences in the near-term. Government animal use statistics provide a clear indicator of the wasteful nature of the creation of genetically modified animals and maintenance of established strains, but the units to that are to close in the UK were trailblazers in terms of efficient colony management – minimising the numbers of animals bred or used, and providing important advice and guidance on best welfare practices. This presentation will discuss the implications, positive and potentially negative, that the closure of these specialised centres could represent over the short- and long-term, and outline what is needed to streamline genetically modified breeding processes in order to reduce the overbreeding and wasteful culling of animals, while the biomedical research paradigm continues to shift away from animal use altogether.

References

[1] https://chemicalwatch.com/77872/sanger-institute-announces-closure-of-animal-research-facility
[2] https://ntp.niehs.nih.gov/iccvam/meetings/iccvam-forum-2019/06-lee-ncats_508.pdf
[3] https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/724611/annual-statistics-scientific-procedures-living-animals-2017.pdf

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Development of intestinal organoids differentiated from porcine induced pluripotent stem cells

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The pig is a favorable model for the human intestine in biomedical research. Further, there are zoonotic diseases, like the infection with Yersinia enterocolitica leading to a clinical outcome in humans, but not in the pig. Underlying mechanisms are still unknown in detail. The generation of induced pluripotent stem cells (iPSCs) from farm animals gives new opportunities for the development of models, based on target cells differentiated from species-specific iPSCs. Other working groups were already able to produce intestinal organoids directly from porcine intestinal stem cells. Our study aims on the generation of intestinal organoids from porcine induced pluripotent stem cells.

Intestinal/colonic organoids were generated from porcine iPSCs based on a protocol for human iPSCs which is already established in our lab for human iPSCs [1]. These data were compared to the differentiation of human colonic organoids.

It was possible to keep the porcine organoids in culture for 66 days. In three independent experiments, it was demonstrated that stage specific genes were expressed during critical differentiation steps (OCT4/SOX2 for pluripotency; SOX1/FOXA2 for definitive endoderm; CDX2 for hindgut). The expression of colon specific genes was matched between expression in supposed to be colonic organoids and tissue samples from the porcine intestine (MUC2, MUC5B, INSL5, CHGA, LGR5). These findings suggest that porcine colonic organoids have been produced.

The formation of colonic organoids from porcine iPSCs needs further optimization: The majority of intestinal organoids looked more like enterocysts than budding organoids in comparison to the formation of human colonic organoids with a comparable protocol. This event indicates that Wnt and Notch signaling, important to break the symmetry during budding of intestinal organoids, seems to play an important role in the intestinal stem cell signaling of pigs in comparison to the formation of human intestinal organoids.

Further steps are currently being taken in optimization of the differentiation method towards the establishment of budding colonic organoids from porcine iPSCs and the related consideration of Wnt and Notch signaling during intestinal development in the pig.

Reference
[1] Munera, J. O., Sundaram, N., Rankin, S. A. et al. (2017). *Cell Stem Cell 21*, 51-64.e6. doi:10.1016/j.stem.2017.05.020

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Study the effect of cyclosporin A on functionality of endothelial cells differentiated from iPS cells as *in vitro* toxicology model system

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Cyclosporin A (CSA) is known as an immunosuppressive agent in pharmacologic studies, especially in solid organ transplantation. Beside the positive effect of CSA, there is emerging evidence showing its effect on inducing long-term vascular dysfunction and angiogenesis impairment in patients. In order to get deeper insights into these effects and finding out the exact mechanisms, a good *in vitro* model of vascular system is required. Available models of endothelial cells as the main part of vascular system are associated with some limitations such as low expansion rate, inability of specific cells isolation from patients treated with toxic chemicals, and considering 3R principles, have encouraged researchers toward stem cells technology and using induced pluripotent stem cells (iPSCs) as unlimited source of differentiation towards endothelial lineage. Therefore, we designed an efficient protocol for differentiation of iPS cells to endothelial cells due to *in vivo* vascular system development by using small molecules and growth factors from two iPS cell lines, (SbAd03-01, SbAd02-01, available in StemBANCC). Flowcytometry, ICC, tube formation and sprouting assay proved endothelial properties of differentiated cells. In the next stage, we started our toxicity experiments, showed that CSA (Sima-Aldrich, 30024-100MG) has EC50 of 5 µM on differentiated endothelial cells after 72 hours treatment checking viability by resazurin assay. Also angiogenesis impairment of endothelial cells by CSA in EC50, was shown using two functional test of Matrigel assay and sprouting assay which showed endothelial cells treated with CSA in this concentration, lacked tube formation and sprouts spreading, respectively.

So by now, we could design an *in vitro* model of endothelial cells showing exact mechanism of CSA affect which could be the first step of understanding signaling pathways involved in angiogenesis impairment after cyclosporin A treatment in patients requiring organ transplants.

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Animal free development, testing, parameterisation and calibration of human PBPK models for two plasticisers

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Plasticisers from different classes of chemicals are used in the manufacture of plastics. They are used to promote plasticity, that is, the ability to be shaped and moulded, in order to increase flexibility and reduce brittleness. Since plasticisers are physically, rather than chemically bound to the polymer, they can leach into the surrounding media thus entering the environment. Humans are exposed to plasticisers, principally through diet and the application of personal care products.

In this work, physiologically based pharmacokinetic (PBPK) models were developed for two different classes of plasticizers: the phthalate DPHP (Bis(2-propylheptyl) benzene-1,2-dicarboxylate); and the cyclohexane dicarboxylate Hexamoll® DINCH (diisononyl-cyclohexane-1, 2-dicarboxylate), in order to interpret the biokinetics in humans following single oral doses of these respective substances.

Baseline PBPK models were initially parameterised upon *in vitro* and *in silico* derived parameters which described the absorption, distribution, metabolism and elimination (ADME) of ingested parent chemical. Uncertainty and sensitivity analysis techniques were applied in order to study the qualitative behaviour of the baseline models, to identify the key parameter sensitivities, and to assess biological plausibility. A process of further model development and refinement was undertaken based upon a qualitative comparison of the range of predictions from the PBPK models with human biomonitoring data. Key refinements

of the baseline model included the inclusion of a lymphatic compartment which allowed a proportion of administered substance to by-pass first pass metabolism in gut and liver, and the inclusion of enterohepatic recirculation, which slowed the rate of elimination from the body. Calibration of sensitive model parameters in the final PBPK models was achieved using human bio-monitoring (HBM) data, specifically through tuning sensitive model parameters such that model predictions were consistent with datasets of metabolite (of DPHP and DINCH, respectively) concentrations measured in timed urine voids from participants in human volunteer studies following single-dose oral exposure.

The focus of this work will be on the process of initially specifying models using *in vitro* and *in silico* derived parameters and on the subsequent process of refinement, informed through the application of appropriate techniques for uncertainty analysis and global sensitivity analysis, prior to calibration using HBM data. The essential requirement of post-modelling verification is discussed.

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The thyroid hormone disruptors validation project and application of OECD's GIVIMP and Guiding principles on good practices on protected elements in Test Guidelines

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The European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) has embarked on a large scale validation study of *in vitro* methods to detect chemicals with thyroid hormone disruption activity. A set of 17 mechanistically informative alternative methods were identified taking into account the information reported in an OECD scoping document, an OECD Detailed Review paper and feedback received at various expert group meetings [1,2]. The methods cover the possible sites of action of environmental contaminants in the thyroid hormone pathway. With the collaboration of 14 facilities from the European Union Network of Laboratories for the Validation of Alternative methods (EU-NETVAL), and 13 test method developers, the 17 *in vitro* methods will be assessed in parallel for reproducibility and relevance. The validation aims at identifying a battery of *in vitro* methods that can indicate thyroid disruption activity.

Effort is being invested to assure that this validation study is carried out in compliance with two international guidance documents both recently issued by the OECD: 1) Good *in vitro* Method Practices (GIVIMP) [3] and 2) Guiding principles on good practices on protected elements in OECD Test Guidelines (TGs) [4]. GIVIMP provides practical guidance on the development and implementation of *in vitro* methods for regulatory use in human safety assessment, aiming at a reduction of uncertainties in the predictions derived from cell and tissue-based *in vitro* methods. Its drafting was coordinated by EURL ECVAM, and, it tackles ten important aspects related to *in vitro* work, each one addressing key elements of good *in vitro* method practices. The principles outlined in GIVIMP will be applied in the Thyroid hormone disruptors validation study to assure e.g. the characterisation and long-term availability of the test systems, the selection of relevant reference and control items, the preparation of complete SOPs,

etc. The Guiding principles on good practices on protected elements for TGs [4] provide information on intellectual property (IP), which can be linked to an *in vitro* method, and guidance on documenting such IP for an OECD TG. In compliance with this guidance, an inventory of all possible IP elements within the 17 *in vitro* methods is being made, including investigating if all conditions can be met for the methods to be eventually proposed to the OECD as TGs.

References

[1] OECD (2017). New Scoping Document on in vitro and ex vivo Assays for the Identification of Modulators of Thyroid Hormone Signalling. *Series on Testing and Assessment No. 207.*

[2] OECD (2012). Detailed review paper on the state of the science on novel in vitro and in vivo screening and testing methods and endpoints for evaluating endocrine disruptors. *Series on Testing and Assessment No. 178.*

[3] OECD (2018). OECD Guidance Document on Good In Vitro Method Practices (GIVIMP) for the Development and Implementation of In Vitro Methods for Regulatory use in Human Safety Assessment. *Series on Testing and Assessment No. 286.*

[4] OECD (2019). Guiding principles on good practices for the availability/distribution of protected elements in OECD test guidelines. *Series on Testing and Assessment No. 298.*

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Development of an OECD Test Guideline for Androgen Receptor Transactivation Assays (ARTAs)

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Endocrine disrupters (EDs) are chemicals with the potential to interfere with the endocrine system, acting as an endocrine hormone. These chemicals can be naturally occurring in the environment or can be man-made. The OECD has established a conceptual framework (CF) for screening and testing of EDs which lists the OECD Test Guidelines (TGs) available, under development or methods proposed to be used to evaluate chemicals for ED (*in vitro* and *in vivo*) [1]. Screening methods are of importance in the CF level 2, e.g. binding and transactivation assays which involve the receptors for endocrine hormones such as estrogens, androgens, thyroid hormones. The European Commission (JRC) has taken the lead in drafting a TG encompassing several Androgen Receptor Transactivation Assays (ARTAs). Hereto, EURL ECVAM at the JRC coordinated the validation study of the AR-CALUX® method which is an ARTA using osteosarcoma cells U2OS. These cells have been transfected with a human AR and a luciferase reporter gene preceded by androgen responsive elements to which the ligand bound AR can bind and induce the reporter gene. Three laboratories of the EU Network of Validation Laboratories (EU NETVAL), namely RISE, Covance and Charles River Labs and the test method developer BDS participated in the study. A good reproducibility of the method was demonstrated within and between laboratories. The results of this validation study have been peer-reviewed with a successful outcome and the validated AR-CALUX® method will be available in 2019.

Up to date, 3 ARTA's are considered to be included in an OECD TG for ARTAs: the AR STTA method using the EcoScreen™ cell line (TG 458 adopted), the AR STTA method using the 22MTV GR⁻ cell line (peer-review ongoing), and the above described AR-CALUX® method. TG 458 has been updated with the newly validated method AR-CALUX® [2] and is currently being reviewed by OECD WNT with the aim of adoption and publication in 2020.

References

[1] OECD (2018). Revised Guidance Document No 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption. *OECD Series on Testing and Assessment No. 150*. OECD Publishing, Paris. doi:10.1787/9789264304741-en

[2] OECD (2019). DRAFT Test Guideline 458 on Androgen Receptor Transactivation Assays (ARTAs). http://www.oecd.org/env/ehs/testing/DRAFT-TG458-10-07-2019v2.pdf

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The method of spheroid formation for 3D cultures of primary hepatocytes influences hepatocellular functions and hepatotoxicity

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Primary hepatocytes of human and animal origin are the gold standard for all pharmacological-toxicological studies in drug development. They play a major role in eco-toxicological evaluation as well. Three-dimensional (3D) cultures became more popular in recent years since they might mimic the *in vivo* cell morphology, polarity and cell-cell interactions better than traditional two-dimensional (2D) cultures. Here, two types of cell culture plates were used to generate 3D cultures with primary hepatocytes: the GravityPLUS Hanging Drop System with subsequent culture in Gravity TRAP plates in comparison to U-bottom ULA (ultra-low attachment) plates with cell repellent surfaces. Standard 2D cultures were performed as well.

Hepatocellular detoxification functions like urea release and CYP450 activity as well as the response to the hepatotoxin Diclofenac were analysed in these culture systems. The results were normalized to the corresponding volume of culture medium or to protein content.

The secretion of urea was improved and maintained at higher levels in U-bottom ULA plates compared to the Hanging Drop System. Hepatocytes cultured in U-bottom plates maintained an approx. 2 fold higher basal cytochrome P450 enzyme activity compared to hepatocytes in the Hanging Drop System. Also, hepatocytes in U-bottom plates showed a 2-3 fold higher sensitivity and inducibility of cytochrome P450 activities than in the Gravity TRAP plates in Beagle hepatocytes 3D cultures.

Diclofenac, a known and well-described hepatotoxic compound, did show similar effects on hepatocytes with regard to the ATP content in both 3D culture systems. Beside this, the decrease of ATP content due to Diclofenac treatment was higher in 2D culture than in the 3D culture systems.

In summary, our results indicate that major differences seem to exist between different 3D culture systems and in comparison to standard 2D culture methods. These differences may lead to different and conflicting results in the assessment of drug toxicity and drug-drug interaction.

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Development of a blood reference model of New Zealand White Rabbits with the Siemens Advia 2120i

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Animal models such as the New Zealand White Rabbit are widely used for experimental research. When haematological measurements are made, it is necessary to use the most accurate reference values possible in order to minimise the animal numbers. When conducting research with rabbits, studies showed that haematological reference values differ a lot due to variables such as breed, husbandry conditions and gender. Therefore, many studies have to develop their own reference model. The aim of this study was to develop an accurate reference model for New Zealand White Rabbits with the Siemens Advia 2120i. A retrospective study was performed without the usage of further animals. The data was gathered from experiments conducted between 2014 and 2019. 177 complete blood counts, analysed with the Advia 2120i, were evaluated statistically and compared to past literature. In addition, data from 37 blood samples that have been counted manually were used to see differences between the automated CBC and the manual one. Three primary results were found. First, the manual and automatic WBC count match without significant deviations. Second, the reference models, including the one developed in this study, vary a lot compared to each other. Lastly, a new reference model from the evaluated data was established. This consequently leads to a reduction of animal numbers by eliminating the need of further animals.

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An integrated testing strategy for polymer exposure in inhalation risk assessment

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Next Generation Risk Assessment (NGRA) is an exposure-led, hypothesis-driven risk assessment approach that integrates one or more new approach methodologies (NAMs) which can be applied to ensure the safety of consumer products (e.g. cosmetics) without the use of animal testing data [1]. Whilst different *in silico* and *in vitro* tools are already available for testing for lung toxicity there is a need amongst both industry and regulatory risk assessors to create examples that can demonstrate the utility of these tools for decision-making on safety of use regarding pulmonary exposure [2,3]. We have identified polymers present in personal care products for cosmetic use (e.g. hairsprays, antiperspirant) as a case study example to showcase a testing strategy for an NGRA approach in the area of inhalation risk assessment. Impairment of mucociliary clearance, lung fibrosis and lung surfactant inhibition were defined as one of the most critical endpoints under repeated exposure for the consumer.

Adverse Outcome Pathways (AOPs) for both the establishment of lung fibrosis and for impairment of mucociliary clearance by increased mucus production are under development and most of the underlying molecular events are known. The challenge however is still the development of *in vitro* approaches. Therefore, the selection of the appropriate cell model, relevant biomarkers, exposure/dosimetry considerations and the derivation of point of departure from *in vitro* dose response data still needs to be properly addressed. The MucilAir™-HF cell model (Epithelix) is a cell system which shows ciliated as well as mucous producing cells common in the upper pulmonary tract and has been used before for the analysis of inhibition of mucociliary clearance. The Epi-Alveolar™ cell model (MatTek) on the other hand is a cell model representative of the alveolar region including most of the cells of the alveolar region (AT1 and AT2 cells, fibroblasts and macro-phages) which seems promising in being used as a suitable system for measuring toxic endpoints of the lower pulmonary region including surfactant inhibition and a fibrotic response. Testing of the case study materials with additional benchmark substances are ongoing for both cell models.

Linking the *in vitro* point of departures to the relevant *in vivo* exposure through the knowledge about consumer habits and practices (i.e. how a consumer uses an aerosol antiperspirant or a hairspray) was combined with the use of computational exposure tools (i.e. ConsExpo, 2-Box Indoor Air Dispersion models or Multiple Path Particle Dosimetry Modelling (MPPD)) and/or simulated use evaluation testing (i.e. mannequins equipped with an aerosol sampler connected to a particle size spectrometer to measure the inhaled and respirable dose) to estimate a realistic dose which can then be used for the *in vitro* testing strategy. These calculations in combination with the experimental results form a tiered approach NGRA for inhaled materials.

References

[1] Dent, M., Teixera Amaral, R., Amores da Silva, P. et al. (2018). *Comput Toxicol 7*, 20-26. doi:10.1016/j.comtox.2018.06.001

[2] Clippinger, A. J., Allen, D., Jarabek, A. M. et al. (2018). *Toxicol In Vitro 48*, 53-70. doi:10.1016/j.tiv.2017.12.011

[3] Lacroix Ghislaine, K. W., Ritter, D., Gutleb, A. C. et al. (2018). *Appl In Vitro Toxicol 4*, 91-106. doi:10.1089/aivt.2017.0034

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Evaluation of human induced pluripotent stem cell derived podocyte-like cells for toxicity testing

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Glomerular visceral epithelial cells aka podocytes play an essential role in the function and maintenance of the glomerular filtration barrier. Podocytes play a central role in glomerular disease initiation and progression and their health can be adversely affected in specific genetic diseases, in diseases states such diabetes and by injury from xenobiotics. Podocytes are notoriously difficult to culture, due to the fact that terminally differentiated podocytes no longer proliferate. Human induced pluripotent stem cells (iPSC), with their capacity for self-renewal offer a potential to provide a continuous source of patient derived podocytes. The main objective of this work was to investigate the utilisation of hiPSC as a potential source for the production of podocyte-like cells and the evaluation of such cells for toxicological and disease related investigation.

Using a recently published method developed in our laboratories we characterised podocyte-like cells from three different donors. After 10 days of differentiation the iPSC-derived cells no longer proliferated, showed a podocyte-like morphology with the characteristic "foot processes" and distinct expression of podocyte markers synaptopodin, podocin, nephrin and WT-1 as assessed by immunofluorescence staining and Western blot analysis. These terminally differentiated podocyte-like cells were exposed to a panel of compounds, including Cyclosporine A, paraquat and doxorubicin. Cellular viability was determined using ATP and resazurin, mitochondrial toxicity was determined using the Seahorse bioanalyser and Nrf2 activation was assayed using a hemeoxygnease 1 GFP reporter line.

In conclusion, this protocol is robust and reproducible enough to proceed to more in depth toxicological evaluations including transcriptomics. As a follow up to this study we will investigate the possibilities of creating endothelial / podocyte co-cultures w from the same iPSC donor.

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Time for Primates Zero: Replacing primates in research

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According to the latest EU published Statistics on animals' use [1] in Europe the total number of new world monkeys was 700 and old-world monkeys 5312 in 2011, therefore around 5800 primates are still used every year and the numbers, according to EU national statistics, appear increasing. The majority of primates comes from China, but also India and Mauritius, and many are still taken from Nature. Many primates enter EU through accredited EU animal providers and then are bred and sold around Europe. The cost of each primate is variable, according to age and breed. Latest published national statistics [1] in Italy shows that two species of non-human primates are used "Java Macaques" (*Macaca fascicularis*) 545 animals for 582 procedures and Macaques Rhesus (*Macaca mulatta*) 3 animals for 4 procedures, with a reduction for reusing by 6,3% and 25%, respectively. The number of primates increased comparing to the 2 previous years, from 545 in 2017, 418 in 2016, and 224 in 2015, showing for 2017 an increase of Java Macaques to 23,3% comparing to 2016 and a 58,8% increase comparing to 2015. Animal suffering was classified as "Moderate" for 53% of Java Macaques, 90% are imported from Africa and 10% from Asia. Projects using primates are continuously been funded in EU, despite warnings by the IUCN Red list [2] and latest statistics will be analyzed. It is well known that primates are the species of animals closer to humans and their capacity to live in organized society and understanding, using tools and performing complex tasks, misleading humans to experiment on primates to draw conclusions for humans and building so called "animal-models". Analysis of research results using primates has shown that primates do not provide a good model for humans and that much of the research funded and performed does not result in therapies or drugs useful for humankind. However, repeated and similar research keeps been funded, also at EU lev-el, giving credit for usefulness that finds no scientific and practical evidence in reality. An example is a EU-Italian project aiming at reproducing surgically in primates a human neuropsychological and neurophysiological condition, called "blindsight", to supposedly restoring visual awareness in humans. This assumption was made despite the fact that much research worldwide has failed in demonstrating such condition in animals, and that any improvement in humans has never been obtained from such experiments. Something in research has gone terribly wrong. Animals need to return to be considered living beings, and not "living-objects" on whom to perform experiments, and science need to become objective again, despite the desperate need for some scientists for funding and publications. It is time now to respect the Nature of animals and begin a new era of "Primates Zero" research now, totally Replaced, obtaining high standard of scientific and moral ethics, where it will become illegal to use any primate for research, importing, breeding or collaborating with countries with lower standards than EU, and this needs to be done for all primates, as well as for humanity.

References

[1] http://ec.europa.eu/environment/chemicals/lab_animals/member_states_stats_reports_en.htm
[2] IUCN Red list: https://www.iucnredlist.org/

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Time to rethink the 3Rs after 60 years. Time for just 1R: Replacement and non-animal research

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The concept of 3Rs was invented by Russell and Burch in 1959 and described in the book "The Principles of Humane Experimental Techniques" where for the first time the concept of 3Rs as "Replacement, Reduction and Refinement" was made. Then the 3Rs arrived in the reality of the European legislation, first in 1989 and then to our time, with the EU Directive 2010/63/EU and in the national legislations of the Member States.

The aim of the 3R was the final replacement of animals in research, since up to 1959 animals had been used as materials and methods for scientific experiments. After 60 years, now in 2019, we can finally conclude that the concept of 3Rs has not worked to replace animals and so it needs to be changed.

Today we still talk about "animal welfare" in EU and national law, almost forgetting that where there is a need to guarantee minimum standards in animal welfare, the reason is that animals suffer, are still used for experiments, reused again and finally killed. This is the reality of animal welfare in the laboratories and this is the result of the 3Rs 60 years on: an increasingly large number of animals.

In Italy the latest statistics shown more than 611,707 live animals used and killed [1], 4160 animals used more than once, 1787 animals used in training still today. In basic research 216,654 animals have been used and killed, although not obligatory by law and not aimed at finding drugs or cures for human diseases.

In Europe in one year have been used and killed 11,500,000 animals [2] (Seventh Report on the Statistics on the Number of Animals used for Experimental and other Scientific Purposes, 2011) with an increase of "other mammals" new species for new experiments (bats, boars, shrews, lamas, moles, bisons, deers) increased by 38.3% comparing to the previous statistics (2009) and 28.5%

of fish, and still are used 408 dogs in Italy and 17,896 dogs and 3713 cats in EU and even more, 450 primates in Italy and 6095 in EU.

The 3R are an old concept that in 60 years has not worked: the results are clear. The number of animals used has increased and actually more animal experiments have been developed, financed and published. There is still no culture and education for Replacement and no willingness for Non-Animal Research, the number of animals used speak for themselves. It is now time for just 1R, Replacement, to Replace animals in all areas of research and human-based medicine, and to use non-animal methods, including advanced methods applicable to more areas of research, computer modelling, big data and next generation sequencing, design experiments with no animal use, use of human samples, materials from surgical operations and creation of biobanks for research. We need to go beyond the old accepted science, to begin a new era for research, that is specific, reliable, responsible, ethical, sustainable and humane: the Non-Animal Research (NAR) working in the present towards the future of research.

References
[1] http://bit.ly/2kZrv1c
[2] https://bit.ly/2n7ngS1

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Combining fluidic chips and 3D bioprinting – an approach to study tumor-microenvironment and angiogenesis *in vitro*

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Additive 3D bio-manufacturing is a young, rapidly evolving research field that allows defined 3D architecture for artificial tissue equivalents. In contrast to other 3D culture techniques, 3D-bioprinted tissue can be designed to contain channel geometries for optimized perfusion or areas with specific cell types, such as immune cells, tumor cells or organoids. In a previous project we discovered that in neuroblastoma tumors the transcription factor FOXO3 promotes tumor-angiogenesis in chorion allantois membrane (CAM) assays and in xeno-graft transplantation mouse models. An ongoing study identified small compounds that bind to the FOXO3-DBD and inhibit the activity of this transcription factor. To study possible anti-tumor effects of these compounds and in parallel to replace above animal experiments we now developed a fully self-contained, mostly 3D-printed, microprocessor-controlled perfusion system, designed fluidic chips and bioprinted into these chips hydrogels that contain various cell types and channel geometries for optimized perfusion. The perfusion controller has a footprint of 24x24 cm^2, three independent stepper-motor driven peristaltic pumps, temperature control and an automated atmosphere system with air-pump, valves and sensors for humidity / air-pressure, CO2, and O2 to also induce hypoxic conditions. Microfluidic devices are made of glass and PMMA which is cut and engraved with a laser engraving machine. Using our 3D Discovery bioprinter we directly manufacture conduits-con-taining hydrogels in such custom-designed fluidic chips, which allows direct connection of hydrogel channel-geometries to engraved channels and perfusion circuits. In parallel, we also developed a bioink that shows excellent printability for extrusion- and microjet bioprinting. This bioink was optimized to support growth and adhesion of human fibroblasts, human umbilical vein endothelial cells (HUVEC) and adipocyte-derived stem cells and it promotes the formation of microvessel networks. The hydrogel between imprinted conduits can be therefore micro-vascularized with HUVEC-based vessel networks below the resolution of the bioprinter. Perfusion controller, fluidic devices, and perfused 3D bioprinted hydrogel represent a novel system for developing *in vitro* tumor-microenvironment / tumor angiogenesis models to study the impact of tumor cells, immune cells, chemotherapeutics and FOXO3-inhibitory compounds on (tumor) cell growth, tumor-microenvironment and micro-angiogenesis.

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Adverse outcome pathway network-based testing strategy for thyroid disruption

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The applicability of *in vitro* bioassays in human risk assessment of endocrine disruptive compounds (EDCs) can be strongly supported by including of adverse outcome pathway (AOP) concept [1], which aims to link molecular initiating events (MIE) detectable *in vitro* with adverse outcomes relevant to the individual or even population level. The concept of AOP network is used in currently starting H2020 ERGO project focusing on thyroid hormone (TH) system, which is highly conserved across vertebrate classes, making it cross-species relevant. The project aims to design a battery of *in vitro* assays for thyroid-responsive endpoints and evaluate the endpoints suitable for extrapolation of effects from fish and amphibian tests to humans and other mammals.

The *in vitro* battery at the current state focuses on prioritized molecular initiating events (MIEs) such as the iodide uptake by thyroid cells by Na+/I- symporter (NIS), iodination mediated by thyroperoxidase (TPO), TH transport by transthyretin and thyroxin binding globulin (TTR, TBG), TH de/activation by deiodinases (DIO1,2) and TH receptor interactions. The bioassays are being designed with an emphasis on 3R principles minimizing the use of *ex vivo* materials. Prioritization criteria have been established for designing a set of human health-relevant model compounds for cross-validation of the bioassays. The set will serve for characterization of the bioassays, their relevance to the MIEs of interest and for prioritization of bioassays for pre-validation. The pre-validation step will be coordinated with EU-NETVAL validation study for *in vitro* methods for detection of thyroid disruptors that aims to update existing or develop new OECD standardized testing guidelines.

Reference

[1] Villeneuve, D. L., Crump, D., Garcia-Reyero, N. et al. (2014). *Toxicol Sci 142*, 312-320. doi:10.1093/toxsci/kfu199

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Design of experimental animal surgical models: Creation of a mouse uterus 3D-model for learning the *in-utero* electroporation

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In 2017, about 1.4 mio. mice were used for research in Germany, with 2% of all animals used for teaching and education [1]. Based on the principles of the 3R, our projects aim to develop surgical simulation models for procedures used on experimental animals. By creating artificial models to train surgical procedures, the number of animals used in the education for biomedical research will be reduced. With the upcoming of 3D-printing technologies in the last years, we took the possibility of designing a simulation model for the *in-utero*-electroporation (IUE). This technique is commonly used in Neurosciences that allows genetic modification of the developing brain of a mouse embryo inside the uterus by intraventricular injection of a vector and applying an electrical pulse [2]. So far, each trainee uses 25 mice, until this complex procedure can be performed independently. The creation of our Uterus model will be introduced, a crucial part of the IUE simulation to replace animals in learning the IUE.

The process used for creating a silicone model, as used for our work, encompasses biomedical imaging for three-dimensional information of the desired organ, segmentation by software, 3D-Design in sophisticated software, as well as the 3D-printing and post-processing. We will introduce the working steps as used for our Mouse Uterus Silicone Model, which brings along complications due to the hollow space, which is to carry the Silicone Embryo Model.

By applying the procedure we developed, we believe it will be possible to replace animals in the learning process of surgical techniques, the major principle of Replacement after Russell and Burch.

References

[1] Bundesministerium für Ernährung und Landwirtschaft. (2017). Animals used under § 7 (2) of the Animal Protection Act by species. 7(2):12, Tabelle 11. https://www.bmel.de/SharedDocs/Downloads/Tier/Tierschutz/Versuchstierdaten2017.pdf?__blob=publicationFile

[2] Baumgart, J. and Grebe, N. (2015). *J Neurosci Methods 240*, 116-124. doi:10.1016/j.jneumeth.2014.11.004

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In vitro distribution kinetics and neurotoxicity of the cardiac drug amiodarone in the iPSC-derived human 3D model BrainSpheres

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The nervous system is one of the most complex organ systems in terms of both structure and function and is particularly vulnerable to toxic insult. Guidelines on neurotoxicity testing prescribe *in vivo* animal tests to determine neurotoxic effects. Since these tests are expensive, time-consuming and give rise to ethical issues, alternatives are needed, and regulatory authorities are strongly encouraging the development and use of human-based *in vitro* models for toxicity testing. However, nervous system complexity and cell-cell interactions are difficult to mimic *in vitro*. This, together with the lack of information on the *in vitro* distribution kinetics of the chemicals, has limited the use of *in vitro* neurotoxicity data for safety assessment.

Amiodarone, a Class III antiarrhythmic medication, is a non-competitive inhibitor of alpha- and beta-adrenergic receptors, used to treat patients with refractory ventricular tachycardia and paroxysmal atrial fibrillation. Side effects including neurotoxicity, characterized by symptoms such as headache, dizziness, fatigue, tremor, peripheral sensorimotor neuropathy, proximal muscle weakness and ataxia, were reported by patients under therapeutic treatment, during which the drug reached plasma concentration in the range of 1.3-2 µM.

In this study, we aim to evaluate the neurotoxicity and distribution kinetics of amiodarone, using an iPSC-derived human 3D model called BrainSpheres (BS). This model comprises neurons, astrocytes and oligodendrocytes, strongly interacting. The presence of pre- and post-synaptic proteins, as well as the recording of electrophysiological activity, suggest the presence of functional synapses. All these features make this model a very useful tool for neurotoxicity testing.

BS were repeatedly exposed to amiodarone between week 6 and 7 *in vitro*. Samples were collected immediately at the end of exposure and after a washout period of one week. Cytotoxicity was evaluated by MTT assay in BrainSpheres exposed to concentrations ranging from 0.625 to 10 µM of amiodarone. IC50 and IC20 were determined (2.5 µM and 2.4 µM, respectively immediately at the end of the exposure; 1.7 µM and 1.4 µM, respectively after the washout period). Gene expression of cell type-specific markers was assessed. For further *in vitro* to *in vivo* extrapolation of our neurotoxicity data, the *in vitro* distribution kinetics of amiodarone was evaluated. To that end, BS were exposed to 1, 2 and 3 µM of amiodarone, and chemical extracts from medium, cell and well plate plastic were collected after 1, 3, 6, 24 and 48 h of exposure. Furthermore, extracts were also collected after 1 week of repeated exposure to test for potential intracellular accumulation of amiodarone, as well as after the washout period. Amiodarone levels were quantified at each time point in medium, plastic and in cells by HPLC-UV/fluorescence.

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High-throughput phenotypic profiling for bioactivity screening of environmental chemicals

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The United States Environmental Protection Agency (USEPA) is exploring the use of new approach methodologies (NAMs) for rapid bioactivity screening and hazard evaluation. The first tier of the screening strategy specifies the use of high-throughput profiling methods, i.e. high-throughput transcriptomics and high-throughput phenotypic profiling (HTPP). Phenotypic profiling is an imaging-based screening method that combines automated microscopy and image analysis to measure a large variety of morphological features at the single cell level. Here, we adapted an existing assay that evaluates changes in organelle morphology [1] for use in high-throughput bioactivity screening of environmental chemicals. The USEPA is currently exploring two potential applications of HTPP. (I) Estimating the threshold for chemical bioactivity for comparison with human exposure estimates. (II) Using phenotypic profiles to discern putative mechanisms-of-action to guide further bioactivity testing.

In these studies, U-2 OS cells were plated in 384-well plate format and after 24 h treated with 8 concentrations (1/2 log10 spacing, n = 3) of test chemicals, with reference chemicals run on each plate. After 24 h of exposure, cells were labeled with MitoTracker (mitochondria), fixed, permeabilized and labeled with Hoechst-33342 (nuclei), SYTO14 (nucleoli) and fluorescent conjugates of concanavalin A (ER), phalloidin (actin cytoskeleton), and wheat germ agglutinin (Golgi/plasma membrane). Confocal images were acquired using an Opera Phenix HCS system and analyzed using Harmony software, yielding ~1300 features per cell. Cell-level data were normalized to DMSO controls using median absolute deviation and benchmark concentration (BMC) modeling was performed on well-level median values with BMDExpress. The threshold for *in vitro* bioactivity was defined as the median BMC of the most sensitive category of morphological features. *In vitro* potency estimates were converted to administered equivalent doses (AED) using the httk R package and compared to *in vivo* oral toxicity data available in the ToxValDB database and to high-throughput exposure estimates from the Systematic Empirical Evaluation of Models (SEEM3) framework.

A set of 462 unique chemicals of mostly bioactive chemicals was screened, and 441 (95%) were active in the HTPP assay. For 68% of chemicals (286/420), the estimated AED was comparable or lower than the *in vivo* toxicity estimate. While for most chemicals the bioactivity estimate was orders of magnitude above the estimated exposure, for 17/433 (4%) chemicals, the lower bound of the bioactivity estimate (AED 95th) was below the upper bound of the exposure estimate (SEEM 95th). This overlap indicates a potential for humans to be exposed to bioactive concentrations of these environmental chemicals.

Moreover, it was observed, that chemicals with similar chemical structure produced similar phenotypic profiles. Similar profiles were also observed for chemicals with the same putative mechanism-of-action. Overall, these findings indicate that the phenotypic profiling assay could be used for bioactivity screening of environmental chemicals and provision of NAMs data for potential use in chemical safety assessment.

This abstract does not necessarily reflect USEPA policy.

Reference

[1] Bray, M. A., Singh, S, Han, H. et al. (2016). *Nat Protocol 11*, 1757-1775. doi:10.1038/nprot.2016.105

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A totally defined animal product free cell culture medium for a 3D human tumour outside the body

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Cell culturing has been used for the testing of potential anti-cancer compounds for about 50 years. Implicit in the word "cell culturing" is the meaning animal free for many, but cell culturing still bears the burden of history of including animal-derived products. At the dawn of cell culturing, the knowledge of cell nutrition was sparse and serum was added to obtain stable cancer cell lines. The best serum was that from unborn calf fetuses extracted from the womb of the cow and subjected to heart puncture to obtain as much blood as possible. Despite the knowledge we have today about cellular requirement for nutrients, fetal bovine serum (FBS) is still one of the most common additions to cell culture medium recommended by vendors selling cell lines. We have used the knowledge of today and set together a totally defined animal product free medium. This medium supports the routine culturing of several human normal and cancer cell lines. Besides removing the use of an animal ethically questionable product, a totally defined medium will contribute to increase the reproducibility of experiments using cell lines. FBS is non-defined and subjected to large batch variation which has a negative impact on reproducibility.

Thousands of chemicals have been evaluated for anti-cancer activity using cancer cell lines grown in the traditional way of cell culturing i.e. in two dimensions (2D). This is far from how a tumour grows in the body in three dimensions (3D). Another deviation from reality in these testing systems is the fact that a tumour contains different types of normal cells that influence the cancer cells by e.g. protecting them from insult. These factors may contribute to the high failure rate of finding new compounds for cancer treatment i.e. the 2D culturing results in the selection of inappropriate compounds. We are culturing human cancer cells and fibroblasts in a 3D mesh of biocompatible polycaprolactone fibres using the totally defined medium. The histological feature of this human tumour outside of the body is similar to that of a patient's tumour. To increase the complexity, we add different kinds of immune cells. The cultures have also been subjected to treatment with conventional chemotherapeutic drugs and experimental compounds. We believe our human tumour outside the body can result in a true reduction of animal experiments in the testing of compounds for anti-cancer treatment.

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Engineering *in vitro* lung microbiota for antimicrobial treatment

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Cystic fibrosis (CF) mucus exhibits altered chemical and viscoelastic features, limiting its clearance and leading to chronic bacterial infections. Current bacterial culture fails to recreate bacteria communities and microenvironments of lung microbiota. Additionally, it is difficult to induce representative human multi-bacterial infections in animals.

Three-dimensional hydrogels (Bac^{3}Gel) were engineered to recreate lung microbiota by modelling the physicochemical properties of CF mucus, and supporting bacteria growth. Bac^{3}Gel exhibits a polysaccharidic backbone, typical of CF mucus, and mucin. Extensive rheological analyses were carried out to control final viscoelastic properties. Additionally, *in vitro* infections were induced within Bac^{3}Gel by culturing *Pseudomonas aeruginosa* and *Staphylococcus aureus*, the prevalent bacteria colonizing the airway CF mucus. Finally, Bac^{3}Gel, infected for 24 h with *P. aeruginosa*, were treated for 24 h with three different antibiotics, to which *P. aeruginosa* is sensitive, and compared in effectiveness to standard bacteria cultures.

Bac^{3}Gel exhibits similar viscoelastic properties alterations to those reported for CF sputum. Bac^{3}Gel successfully sustains growth of *P. aeruginosa* and *S. aureus* either in monoculture or co-culture with a bacterial concentration of 10^9 CFU/mL after 24 h of infection. Bacteria resulted more susceptible to antibiotic treatment under planktonic conditions than when cultured within Bac^{3}Gel, where these instead displayed increased antibiotic tolerances even at high antibiotic concentrations (10 MIC). The sensibility difference between Bac^{3}Gel and planktonic cultures confirmed the well-reported mismatch between planktonic conditions and clinical outcomes [1,2]. Additionally, *P. aeruginosa* created aggregates, which size and shape resemble those observed in sputum from CF patients [3] fibrosis. These results indicate that Bac^{3}Gel is a promising substrate to recreate lung microbiota for antimicrobial screening. The versatile production process of Bac^{3}Gel allows to generate microgradients of viscoelastic properties, nutrients, and gases, which are typical of lung microbiota. Overall, Bac^{3}Gel holds the potential to recreate relevant microbiota environments, including the intestinal microbiota.

References

[1] Macià, M. D. et al. (2014). doi:10.1111/1469-0691.12651
[2] Quilès, F. et al. (2016). doi:10.1016/j.bbamem.2015.10.015
[3] Bjarnsholt, T. et al. (2013). doi:10.1016/j.tim.2013.06.002

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Accounting of uncertainties for complex endpoints: What do we want to know?

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The uncertainties of the standard animal testing and assessment approach for carcinogenicity were systematically reviewed by using the OECD Guidance document that was originally developed for reporting defined *in vitro* approaches [1]. The OECD format is suitable for this re-purposing and shall ease a future comparative uncertainty assessment of the current animal approaches with new approaches. Similar assessments may support other current OECD projects. Such analysis requires recognition of the protection target's variability and may support objective decision criteria for the acceptance of new approaches: In principle, a new approach may be considered acceptable, if its protection target related performance-metrics is similar or better than for the agreed reference approaches. Moreover, where the relevance of the current approach for the protection target cannot be supported easily by quantitative correlation data, the validation of the new approach needs to focus more on mechanistic considerations. Furthermore the reliability as well as the testing and assessment throughput of the new approach becomes of central importance [2]. Ultimately the recognition of uncertainties of the current *in vivo* approaches may lead to an evolution of regulatory toxicology towards: 1) accepting frankly rough toxicity estimates from current and new approaches, where more precision is not possible at present; 2) recognizing a conceptual similarity of *in vivo* and *in vitro* data streams, in that both need data context for interpretation;

3) using more computational approaches for providing this context; 4) conceptualizing toxicological adversity at the molecular and cellular level, eventually promoting the formation of new GHS *in vitro* mode of action hazard classes. Such approaches shall allow a meaningful comparison of toxicity over many chemicals and consequently a significant reduction of the overall toxic chemical burden for the environment and human health.

References
[1] OECD (2016). *OECD Series on Testing and Assessment No. 255*. http://www.oecd.org/env/ehs/testing/series-testing-assessment-publications-number.htm
[2] Paparella, M., Colacci, A. and Jacobs, M. N. (2017). *ALTEX 34*, 235-252. doi:10.14573/altex.1608281

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Establishment of a murine 3D cell culture model of the endometrium

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In reproductive research, animal studies are not fully replaceable due to the complexity of the reproductive cycle, the embryonic implantation and development. However, *in vitro* models may benefit toward reduction of animals necessary. The combination of a murine endometrial co-culture comprised of stromal and epithelial cells with a trophoblast body generated *in vitro* or an *ex vivo* blastocyst may at least partially mirror early embryo implantation and facilitate the analysis of receptivity markers and early embryonal-endometrial-interaction *in vitro*, thereby contributing to the 3R concept.

Endometrial epithelial and stromal cells were separately isolated from uteri of Hsd:ICR (CD-1) mice 3.5 days after plug detection by multiple digestion and filtration steps using modified protocols [1-3]. Based on previously published articles [1,4], stromal cells were suspended in GrowDex® or Matrigel® of various concentrations and seeded in 24-well inserts. After gel solidification, epithelial cells were seeded onto the gel surface and the 3D cell culture was incubated basolaterally and apically with respective cell-specific medium for up to six days. Following cryo-, paraformaldehyde (PFA), or methanol-carnoy fixation, matrix structure and cellular preservation were examined macroscopically and histologically by hematoxylin-eosin (HE) staining. Cell immunophenotyping was performed via cytokeratin AE1/AE3-, Ki67- and active caspase 3-antibodies (n = 4).

For *in vitro* trophoblast body generation, murine trophoblast stem cells (TS cells) were cultivated on gelatin-coated MEF (mouse embryonal fibroblasts) pre-seeded plates. Cells were passaged and banked over several weeks according to established protocols [5].

Only 10.8 mg/ml Matrigel® provided acceptable handling, highest stability, as well as optimal macroscopic and, in combination with PFA-fixation, histologic preservation of the gel-structure compared to GrowDex® and lower concentrations of Matrigel®. Fixation with methanol-carnoy resulted in a loosened gel-structure whilst cryo-fixation caused difficulties in cutting with a consequently massive loss of structure. Histology revealed some stromal cells forming cellular extensions in both GrowDex® and Matrigel®. Epithelial cells, which were positive for cytokeratin, formed gland-like structures in Matrigel®, but tended to aggregate apically rather than forming a monolayer and lacked high prismatic cellular morphology. Some of the epithelial cells were positive for Ki67, whilst most of the cells were caspase negative. TS cell colonies displayed a typical roundish colony morphology with defined demarcation toward MEF cells and increasing density over time.

This modified protocol provides reproducible isolation and separation of both endometrial stromal and epithelial cells from the same organ in one process for respective mouse strain and time point of cycle. Hence, it contributes to the 3R by reducing the numbers of organs necessary compared to protocols facilitating the isolation of only one cell type at a time. In combination with 10.8 mg/ml Matrigel® and consecutive PFA-fixation, it also yielded best structural results in terms of 3D culture setup and preservation, in which co-cultured epithelial cells show active proliferation for up to six days with a low rate of apoptosis. However, epithelial cell density and morphology requires further optimization. The generation of a trophoblast *in vitro* body from cultivated TS cells and its combination with the co-culture remains to be established.

References

[1] Arnold, J. T., Kaufman, D. G., Seppälä, M. et al. (2001). *Hum Reprod 16*, 836-845. doi:10.1093/humrep/16.5.836

[2] Chen, J. C. and Roan, N. R. (2015). *Bio Protoc 5*, e1623. doi:10.21769/BioProtoc.1623

[3] De Clercq, K., Hennes, A. and Vriens, J. (2017). *J Vis Exp 121*, e55168. doi:10.3791/55168

[4] Wang, H., Pilla, F., Anderson, S. et al. (2012). *Mol Hum Reprod 18*, 33-43. doi:10.1093/molehr/gar064

[5] Sozen, B., Amadei, G., Cox, A. et al. (2018). *Nat Cell Biol 20*, 979-989. doi:10.1038/s41556-018-0147-7

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Using platelet-rich human platelet lysate to substitute fetal calf serum for cultivation of mesenchymal stromal cells

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The worldwide use of fetal calf/bovine serum (FCS) as a supplement for cell culture media remains the "gold standard" when working with e.g. human primary cells or cell lines despite the well-known disadvantages such as its unknown immunogenicity, composition and inter-batch variation and last but not least ethical issues. Thus, it is not surprising that chemically defined media as well as human platelet lysate become more and more interesting in order to replace FCS in cell culture media e.g. DMEM.

Here, we compared the influence of FCS, self-made platelet-rich human platelet lysate (pHPL) and a commercially available HPL on the behavior of mesenchymal stromal cells (MSCs).

To this end, we incubated primary human MSCs (n = 4) under either 10% FCS, 10% pHPL, 2.5% pHPL or 10% HPL in DMEM under standardized cell culture conditions (37°C, 5% CO2) and analyzed cell growth, differentiation potential towards adipogenic and osteogenic lineage as well as the presence or absence of typical surface markers (CD73+, CD90+, CD105+; CD45-CD34-, CD20-, CD14- and HLADR-). MSCs were expanded using 10% FCS containing DMEM until passage 3 and transferred into DMEM supplemented with either 10% FCS, 10% pHPL, 2.5% pHPL or 10% HPL. Additionally, we freshly isolated and freeze-thawed hMSCs and transferred the cells into DMEM supplemented with the respective sera.

Using hMSCs expanded until passage 3 in FCS-containing DMEM, we observed no significant differences in cell growth but after passaging, cells cultivated with 2.5% pHPL showed the highest growth capacity, whereas the cells cultivated with HPL grew very slowly. All hMSCs incubated under the variety of conditions analyzed, differentiated towards osteoblasts or adipocytes without any differences between the groups. hMSCs incubated under the varying conditions were positive for the expression of CD73, CD105 and negative for CD45 CD34, CD20, CD14 and HLADR as analyzed by flow cytometry. Interestingly, only hMSCs maintained in the presence of FCS also expressed the typical surface marker CD90. Finally, using freshly isolated and freeze-thawed hMSCs, growth, expansion and differentiation completely failed in the absence of FCS.

From our observations, we assume that pHPL but not HPL could replace FCS in many cell culture settings. However, certain hMSC-properties have to be carefully monitored when replacing FCS with pHPL.

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An *in vitro* fracture hematoma model as a tool for preclinical drug testing

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Approximately 10% of fractures lead to significant fracture healing disorders. In an ageing society, it is almost certain, that these problems will increase in the near future. Besides the enormous costs, the patients´ well-being is strikingly affected. In order to test new therapeutic strategies and find appropriate drug candidates, in most cases animal studies are conducted. We established an *in vitro* fracture hematoma model (FH model), only consisting of autologous human material. To qualify the established *in vitro* FH model, we compared the *in vitro* results obtained to human *ex vivo* data as well as to *in vivo* data from animal studies and identified profound similarities.

To further proof the validity of the established *in vitro* FH model, we here studied the impact of (i) the glucocorticoid dexamethasone (DEX) which is well known to impair fracture healing and (ii) the iron chelating hypoxia-inducible factor stabilizer deferoxamine (DFO) known to promote fracture healing.

Therefore, we incubated the FH models under hypoxic conditions in osteogenic differentiation medium (in order to resemble the situation found *in vivo* most appropriately) for 48 h with either 10-7 DEX, 250 µmol DFO or left them untreated. We analyzed cell composition, gene expression of osteogenic (RUNX2, SPP1), angiogenic markers (VEGF) and hypoxia markers (HIF, PGK1 and LDHA) as well as secretion of cytokines/chemokines using flow cytometry, quantitative PCR and multiplex suspension assay, respectively.

The treatment with DEX significantly favored the survival of immune cells over mesenchymal stromal cells (MSCs), enhanced the secretion of pro-inflammatory cytokines and suppressed the expression of RUNX2, SPP1 and VEGF. On the other hand, the treatment with DFO lead to an enhanced expression of the osteogenic marker RUNX2 and genes relevant for the adaptation towards hypoxic conditions (HIF, PGK1, and LDHA). Additionally, DFO did not significantly reduce the frequency of MSCs in comparison with the frequencies of immune cell populations. Interestingly, the DFO not only enhanced the secretion of pro-inflammatory but also of anti-inflammatory cytokines/chemokines.

In summary, we were able to show that both fracture-healing disrupting and fracture-healing promoting substances can influence the *in vitro* FH model in a similar way as it was observed in the *in vivo* situation. Therefore, we believe that our model is able to correctly mimic human fracture hematoma and reduce the number of animal experiments in early preclinical studies.

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An *in vitro* 3D fracture gap model as a tool for preclinical testing procedures

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Approximately 10% of fractures lead to significant fracture healing disorders. In an ageing society, it is almost certain, that these problems will increase in the near future. Thus, our ultimate goal is to study the influence and efficacy of potential therapeutics in an experimental setting of fracture healing *in vitro* in order to provide a preclinical test system for fracture healing disorders. Therefore, we developed an *in vitro* 3D fracture gap model (FG model), solely consisting of autologous human cells and composed of an *in vitro* fracture hematoma model (FH model consisting of peripheral blood and MSCs) co-cultivated with scaffold-free bone-like constructs, correspondingly produced from autologous MSCs.

To proof the validity of the established *in vitro* FG model, we here studied the impact of (i) the bone-like construct on the *in vitro* FH model with regard to its osteogenic induction capacity and (ii) the iron chelating hypoxia-inducible factor stabilizer deferoxamine (DFO) known to enhance bone healing.

To this end, we co-cultivated the fracture hematoma and the bone-like construct for 48 h under hypoxic conditions (n = 3), in order to reflect the *in vivo* situation after fracture most adequately. To analyze the impact of the bone-like construct on the *in vitro* FH model with regard to its osteogenic induction capacity, we cultivated the fracture gap models in either osteogenic differentiation medium or medium without osteogenic supplements. To analyze the impact of DFO on the FG model, we further treated the FG models with either 250 µmol DFO or left them untreated. After incubation and subsequent preparation of the fracture hematomas,

we evaluated gene expression of osteogenic (RUNX2, SPP1), angiogenic (VEGF, IL8), inflammatory markers (IL6, IL8) and markers for the adaptation towards hypoxia (LDHA, PGK1) as well as secretion of cytokines/chemokines using quantitative PCR and multiplex suspension assay, respectively.

As a result, we demonstrate that the bone-like constructs induced the upregulation of osteogenic markers (RUNX2, SPP1) within the FH models irrespective of the supplementation of osteogenic factors in the medium. Furthermore, we observed an upregulation of hypoxia-related, angiogenic and osteogenic markers (RUNX2, SPP1) under the influence of DFO and the downregulation of inflammatory markers (IL6, IL8) as compared to the untreated control. The latter was also confirmed on protein level.

In summary, our findings demonstrate that the established *in vitro* FG model provides all osteogenic cues to induce the initial bone healing process, which could be enhanced by fracture-healing promoting substance DFO. Therefore, we believe that our model is able to correctly mimic the human fracture gap situation suitable to study the influence and efficacy of potential therapeutics and reduce the number of animal experiments in early preclinical studies.

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Why are alternative methods (not) chosen? Presentation of a project on the investigation of value judgments for or against alternatives in animal testing in basic research

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Background

Scientists have made great progress in developing and using alternative methods for animal testing. However, alternative methods are not always used frequently, even if they are validated. While stricter regulations as in toxicology testing (e.g. the ban on the use of animals for cosmetics in the EU) can force the use of (some) alternative methods, there is still enough leeway for scientists to also choose the animal test instead of an alternative. This is especially true in basic research, where there are significantly fewer requirements. To promote the use of alternative methods, it is therefore important to better understand why animal testing is chosen when there is an (alleged) alternative.

Aim

The project "Structure of ethically relevant value judgments referring decision-making for or against replacement methods" which started in February 2019 thus examines how principal investigators (Pls) especially in basic research make a decision for or against the use of an alternative method. In detail, this includes working out the crucial normative and empirical premises and presuppositions of a decision. The analysis should then enable a critical examination of the value judgements and their justifications, or should at least help to identify where further research is necessary to check the plausibility of the value judgements involved.

Method

In a first step a theoretical model will be created to define value judgments. A second step will identify and analyse literature that includes statements with value judgments for or against alternative methods. At the end of this step, there should be a qualitatively sufficiently differentiated and theoretically categorized spectrum of possible value judgements and their justifications. To verify and to complete the catalogue of possible value judgements, a third step envisages conducting expert interviews with principal investigators. The fourth step comprises a normative-ethical evaluation of all identified value judgements and their justifications. This involves critically examining the values, the empirical assumptions and the relationship between the two.

Expected results

Besides gaining a better theoretical understanding of how decisions are made and justified for or against alternative methods, the project plans the formulation of key questions to help decision-makers navigate through the decision-making process by supporting the identification of value judgements involved and by taking them into account in an appropriate way. For the conference, the project itself as well as preliminary results, e.g. how value judgments can be analysed, as well as exemplary analyses of concrete value judgments will be presented.

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3D *in vitro* scaffold based cell model of neuroblastoma for evaluating genotoxic and miRNA-targeted therapeutics

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Neuroblastoma is an aggressive paediatric cancer of the sympathetic nervous system [1]. Nearly ten new cases are reported annually in Ireland, 100 in the UK and 800 in the US. Current therapies are not effective in the long term for almost 80% of patients with clinically aggressive high-risk disease [1].

The accurate representation of the tumour architecture and patient diversity are two primary challenges in the identification of new agents for paediatric drug development particularly with the limited number of patients eligible for clinical trials. The main challenge in treating neuroblastoma is to combat tumour metastasis and resistance to multiple chemotherapeutic drugs.

We aimed to bridge the gap between conventional 2D culture and *in vivo* tumours for neuroblastoma by developing a 3D neuroblastoma tissue-engineered platform and exploring its therapeutic relevance to genotoxic and targeted drugs.

Chemotherapeutic sensitive Kelly and resistant KellyCis83 neuroblastoma cell lines [2] were cultured in a 3D *in vitro* model on collagen-based scaffolds containing either glycosaminoglycan (Coll-GAG) or nanohydroxyapatite (Coll-nHA) and compared to 2D cell culture and an orthotopic murine model [3]. The 3D models were characterised by cell proliferation (DNA content) and viability, secretion of Chromogranin A (CgA) and fluorescent microscopy (DAPI). Chemosensitivity to cisplatin treatment was assessed in all tested models.

Neuroblastoma cell lines actively infiltrated and proliferated over the 21-day timeframe and exhibited physiological activity by secreting CgA demonstrating the correlation between cell numbers and concentration of CgA. Both cell lines responded to cisplatin, a genotoxic drug commonly used in neuroblastoma treatment, dis-playing > 100-fold increased resistance to cisplatin treatment when compared to 2D cultures, exhibiting chemosensitivity similar to orthotopic xenograft *in vivo* models. Our findings are supported with previous studies showing a significant increase in cancer cell resistance to cytotoxic agents when grown in 3D when compared to their 2D monolayer counterparts in various cancer types [3]. The efficacy of cellular uptake and gene knockdown by liposomes containing miR-324-5p was similar in both 2D and 3D *in vitro* culturing models highlighting the proof-of-principle for the applicability of this model for validation of miRNA function.

We successfully established and characterised a physiologically relevant, scaffold-based 3D neuroblastoma model, strongly supporting its potential value in the evaluation of chemotherapeutic and miRNA-based drugs and investigation of neuroblastoma biology.

References

[1] Cheung, N.-K. V. and Dyer, M. (2013). *Nat Rev Cancer 13*, 397-411. doi:10.1038/nrc3526
[2] Piskareva, O. et al. (2015). *Cancer Lett 364*, 142-155. doi:10.1016/j.canlet.2015.05.004
[3] Curtin, C. (2018). *Acta Biomater 70*, 84-97. doi:10.1016/j.actbio.2018.02.004

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The need to address human relevance and measure return on investment in biomedical research

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Animal models have been traditionally used in biomedical research to recapitulate human disease features and develop new drugs, as they are generally purported to resemble some of the major hallmarks of human diseases. However, these animals do not develop the disease as it occurs in humans, and their use has not paved the way to the development of drugs effective in human patients. Indeed, despite conspicuous research and economical endeavours, the clinical failure rate in drug development still remains very high, with an overall likelihood of approval from Phase I of about 9.6%. On the other hand, the expanding toolbox of non-animal methods, accounting for e.g., induced pluripotent stem cells derived from patients, next-generation sequencing, omics and integrated computer modelling can be used to study human diseases in human-based settings, identify new potential druggable targets, and evaluate treatment effects.

Research proposals based on the use of both animal and/or non-animal approaches have been extensively funded at European level. Notwithstanding, defining indicators to measure return on investment of research funding strategies is necessary to retrospectively assess public health trends, and readdress funding strategies when needed. Here we discuss these aspects, presenting a list of indicators that could be suitable to measure return on investment in biomedical research.

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An AOP-driven assessment of developmental neurotoxicity induced by chemical mixtures using human iPSC-derived neuronal/glial cultures

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Human induced pluripotent stem cell (hiPSC)-derived neurons and astrocytes represent unique cellular models to study *in vitro* key brain developmental processes. These models are nowadays considered for the assessment of developmental neurotoxicity (DNT) *in vitro*, enabling mechanistic understanding of chemically-induced adverse effects. Moreover, since infants and children are co-exposed to more than one chemical at a time, novel mixture risk assessment strategies for the evaluation of DNT should be implemented. Here we used hiPSC-derived neural progenitors differentiated into mixed cultures of neurons and astrocytes to assess the acute and repeated dose effects of chemicals belonging to different classes (e.g., pesticides, industrial chemicals, heavy metals, polychlorinated biphenyls, endocrine disruptors, and drugs), an approach that better mimics real-live exposure. Selected chemicals, all associated with learning and memory deficits in children, were grouped based on their mode of action (MoA) into "similar" and "dissimilar" MoA compounds. Cells have been treated with both single compounds and different chemical mixtures in order to assess their effects on DNT specific endpoints (i.e., synaptogenesis, neurite outgrowth, and brain derived neurotrophic factor (BDNF) protein levels), identified as common key events in different DNT-related adverse outcome pathways. Data suggest that single chemicals (in particular those working through similar MoA), at non-cytotoxic concentrations, can have neurotoxic effects in mixtures. This methodological approach represents a valuable conceptual framework to evaluate chemical mixtures with potential to cause learning and memory impairment in children, which nowadays represents an ever increasing public health concern.

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Initiatives to promote the Three Rs in education and training

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Although much knowledge exists on the Three Rs (Replacement, Reduction and Refinement of animal procedures) better education, communication and outreach could enhance knowledge sharing and promote their uptake. This is the main finding of a recent study carried out by the Joint Research Centre (JRC) of the European Commission [1].

With this in mind the JRC has launched a project to investigate the feasibility of including the Three Rs in educational curricula under a European Parliament Pilot Project, which aims to promote alternatives to animal testing. The project focuses on three educational levels of learning (high school, university and early career scientists) and will result in specifications for the actual guidance aiming at advising on effective ways of creating, adapting and implementing curricula and practices specific to the Three Rs. A set of free access teaching resources targeting the three levels of educations will also be produced.

In addition to this work, the JRC is engaging in other initiatives to boost education and training in this area. For example, it is now completing an extensive overview of the Three Rs education and training landscape by mapping currently available courses and resources worldwide. The intention is to analyse current trends and identify areas of strength and demand to inform further actions in accelerating the development and uptake of the Three Rs within an education and training context.

Reference

[1] Holley, T., Bowe, G., Campia, I. et al. (2016). Accelerating progress in the Replacement, Reduction and Refinement of animal testing through better knowledge sharing. EUR 28234 EN. Publications Office of the European Union, Luxembourg. http://publications.jrc.ec.europa.eu/repository/handle/JRC103906

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Animal research for Alzheimer disease: Failures of science and ethics

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In recent decades, perhaps the most impactful and foreboding development in chronic diseases has been the increasing prevalence and awareness of various dementias, especially Alzheimer disease (AD). As with other diseases that have no meaningful methods for prevention and treatment, research targeting AD has primarily focused on preclinical approaches, predominantly using animals. Nonetheless, decades of animal research have failed to translate into significant advances in the prevention or treatment of AD. In view of this failure, a different and human-relevant approach is critically needed. Here the epidemiology and current understanding of AD as a scientific and societal challenge are presented, highlighting the uses and results of animal research in basic science and drug development, the risk factors and funding strategies. Additionally, current and in-development, human-relevant approaches are presented. The ethical responsibility to AD patients, their families, and the larger community, demands reliable and useful results, which in turn demand a revised research approach, emphasizing human-relevant methods.

Reference

Herrmann, K. and Jayne, K. (eds.) (2019). *Animal Experimentation: Working Towards a Paradigm Change*. Vol. 22. Leiden: Brill. doi:10.1163/9789004391192

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The Animal Protection Quality Certificate.
A tool to assess Refinement
methods in animal experiments

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The 3R concept was developed in 1959 by Russel and Burch to banish inhumanity from animal testing. After extensive discussion, these principles were finally implemented in the EU Directive 2010/63, which imposed the obligation to implement them into national legislation of the member states. This signifies the demonstrable documentation of 3R procedures used in practice. The 3Rs are including Replacement and Reduction of animal experiments *per se* as well as Refinement of the methods applied in animal experiments and in the husbandry and care of animals in order to reduce animal suffering to a minimum. The last years have shown that communication about direct Replacement methods is more popular and has a longer tradition than discussions about Refinement. This could be due to a better measurability of the success of Replacement methods. However, it is more difficult to prove the effects of Refinement on animal studies. Despite urgent efforts to replace or at least minimize animal experiments, Refinement is the only way to ensure the welfare of animals during an experiment. The improved methods in husbandry and experimentation automatically lead to a Reduction in the number of animals in individual experiments. The Austrian 3R Initiative (The RepRefRed Society, MUI animal free research) is developing the Animal Protection Quality Certificate (APQC) in animal research. This Certificate will assess the animal welfare in the animal research facilities during the housing and during animal experiments. With the APQC we will be able to show the direct impact of Refinement methods to the Reduction of animal numbers.

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Using PLHC-1 topminnow liver cells to characterize and predict the effect of plastic additives in fish

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Marine plastic fragments are widespread environmental contaminants. They contain complex mixtures of chemicals, including residual or unreacted monomers, and different plastic additives that are no chemically bound to the plastic polymer. Once in the aquatic environment, they can leach out and aquatic organisms be exposed either directly or through micro-plastics ingestion. These additives are classified as endocrine disrupters and obesogens in terrestrial vertebrates. However, its toxicity and effects in fish lipid homeostasis are almost unknown.

This work explores the use of PLHC-1 cells as an alternative model to characterize and predict the effect of plastic additives in fish, using dibutyl phthalate (DBP), diethylhexylphthalate (DEHP), bisphenol A (BPA), bisphenol F (BPF), and chlorinated bisphenol A diglycidyl ether (BADGE·2HCl) as models. Cell lipid extracts were analyzed by flow injection coupled to high resolution mass spectrometry (FIA-ESI(+/-)-Orbitrap-Exactive) after 24 h exposure. Cytotoxicity, concentration of the compounds in culture medium and intracellular accumulation were assessed.

These analysis revealed the highest intracellular concentration of BADGE·2HCl, DBP and DEHP, which in turn induced the highest cytotoxicity and the strongest alteration of the cells lipidome. BADGE·2HCl induced a significant depletion of triacylglycerides (TGs), while DEHP and DBP stimulated the accumulation of TGs. These effects were observed at environmentally relevant concentrations. BPF which is currently used as an alternative to BPA, showed the lowest cytotoxicity and intracellular concentration, but induced the generation of reactive oxygen species in PLHC-1 cells and a significant depletion of phosphatidylcholine (PC)-, phosphatidylethanolamine (PE)-plasmalogens, and TGs. TGs act as cell depots of polyunsaturated fatty acids, and after exposure to BPF were hydrolyzed, possibly to provide fatty acids to replace damaged/oxidized lipids.

Overall, this study highlights different modes of action of the selected plastic additives in topminnow liver cells, which lead to different lipid fingerprints in exposed cells. Moreover, PLHC-1 cells shows comparable sensitivity and responses to plastic additives to those previously reported in mammalian models, which support its use as an alternative model for the screening of metabolic and lipidic disrupters in aquatic organisms, particularly considering that the liver is the main organ involved in lipid metabolism and homeostasis.

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Are researchers moving away from animal models as a result of poor clinical translation in the field of stroke? A systematic analysis of opinion papers

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Background

Despite decades of research using animal models to develop pharmaceutical treatments for stroke patients, few therapeutic options exist. The vast majority of interventions successful in preclinical animal studies have turned out to have no efficacy in humans, or to be harmful to humans. The main treatment options include admission to a stroke unit, secondary prevention, and recanalization (thrombolysis or thrombectomy) for a minority of ischaemic stroke patients. In view of this ongoing unmet need and given translational disappointments, we explore whether there is any evidence of a move away from animal models and towards human focused approaches in this field.

Methods

We conducted a systematic search for opinion papers within the literature on animal models of stroke using electronic (MEDLINE, Embase and Web of Science) and manual searches. Data were extracted from retrieved papers in chronological order and analysed qualitatively.

Findings

Eighty eligible papers were identified, published during the period 1979 to 2018. Most authors were from academic departments of neurology, neuroscience or stroke research. Authors were unanimous that translational stroke research was in crisis. While a minority voiced caution about the use of animal models (some as early as the 1990s), most felt that the translational crisis could be explained by factors such as poor internal and external validity, problems with clinical trials, extrapolation from the findings of poorly conducted animal studies, and publication bias. While most authors' explanations did not challenge the use of animal models then, some raised the issue of animal-human species differences and one felt that translational problems were due to a lack of human *in vitro* models. In terms of solutions to the crisis, most authors proposed fine-tuning animal models, although there was disagreement about how effective such modifications would be. A minority proposed using human-focused (non-animal) approaches alongside animal models ("paradigm bridgers") and one proposed abandoning animal models altogether. The sorts of human-focused approaches suggested by authors included *in vitro* models (e.g. of oxygen/ glucose deprivation) using human cells, tissues or organoids, induced pluripotent stem cell technologies, high-throughput assays, and MRI and PET scanning.

Conclusion

Despite recognising that animal models have been unsuccessful in the field of stroke, most researchers exhibited a strong resistance to relinquishing them, preferring instead to modify existing approaches. Nevertheless, evidence suggests that a new paradigm is emerging in the field of stroke, in the form of human relevant technologies. With the increasing development of these technologies it seems likely that the proportion of "paradigm bridgers" will gradually increase. Whether or not animal models will ultimately be supplanted by human focused approaches will depend in part upon the extent to which the latter are effective and fit for purpose. Government support and strategic funding to explore the evidence base of new technologies could expedite a transition to human relevant approaches.

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Of mice, chicken and human induced pluripotent stem cells: Studying midbrain dopaminergic neuron development and survival in the context of Parkinson's Disease

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Dopamine (DA)-synthesizing nerve cells located in the human midbrain play crucial roles in the control and modulation of voluntary movements, rewarding/aversive behaviors and other cognitive functions of the brain. The age-dependent and progressive degeneration of the substantia nigra pars compacta (SNc) subset of these neurons underlies the appearance of the motor symptoms characteristic for Parkinson's Disease (PD), whereas the dysfunction of the ventral tegmental area (VTA) subpopulation is associated with neuropsychiatric disorders such as addiction and schizophrenia. The etiology of these diseases, in particular of the neuropsychiatric disorders, is thought to have a neurodevelopmental component. Therefore, a better insight into the developmental pathways directing the generation of these midbrain dopaminergic (mDA) neurons is needed for an improved understanding of these still incurable neurodegenerative and psychiatric disorders, and for devising new therapeutic approaches to these diseases [1]. Furthermore, the reprogramming of somatic cells into so-called induced pluripotent stem cells (iPSCs) directly derived from the patient's or healthy donor's skin or blood cells has opened up new avenues to study and treat these neuropsychiatric disorders. However, such approaches require a precise knowledge of the inductive and specifying cues of the mDA cell fate during development. Only this knowledge allows the directed differentiation of iPSCs into mDA neurons in general and, as a future outlook, into the specific subsets of these neurons in particular [2]. The differentiated cells can subsequently be used for the analysis of patient-specific disease phenotypes and/or pathways (disease modeling), to correct or attenuate these phenotypes by small-molecule agonist/antagonist treatment (drug testing), or for the stem cell-based replacement therapy (transplantation) of the diseased or dying SNc DA neurons in the case of PD.

I will provide a brief review of our journey trying to understand the embryonic generation of the mDA neurons and their adult survival, starting with mouse development and the use of genetic mouse models, proceeding with chicken development and the use of *in ovo* electroporation techniques [3], and ending with the use of human iPSCs from idiopathic PD patients and healthy controls and (in the latter case) their CRISPR/Cas9-mediated genetic manipulation. Critical issues encountered along this journey arguing against the use of animal models for a better understanding of mDA neuron development in the context of human disease, but also ahead of us arguing in favor of these animal models prior to the clinical implementation of these approaches in the treatment of e.g. PD will be discussed as well.

References

[1] Zhang, J., Götz, S., Vogt Weisenhorn, D. M. et al. (2015). *Neurobiol Dis 82*, 32-45. doi:10.1016/j.nbd.2015.05.015

[2] Fukusumi, Y., Meier, F., Götz, S et al. (2015). *J Neurosci 35*, 13385-13401. doi:10.1523/JNEUROSCI.1722-15.2015

[3] Klafke, R., Alwin Prem Anand, A., Wurst, W. et al. (2016). *Development 143*, 691-702. doi:10.1242/dev.126748

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Replacement of the potency assay for tick borne encephalitis virus vaccines

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The potency of tick borne encephalitis virus (TBEV) vaccines has to be determined in a lethal challenge assay in mice according to Ph.Eur. monograph 1375. The Austrian Official Control Medicines Laboratory (OMCL) is the only OMCL that performs Official Control Authority Batch Release for the TBEV vaccines on the European market. In accordance with Directive 2010/63/EU and the Austrian *Tierversuchsgesetz* 2012, the Austrian OMCL decided to work on alternative tests for the potency assays of TBEV vaccines. ELISA based methods using structure specific monoclonal antibodies were developed. Here we present the first results and our proposed way forward to introduce the presented potency assays for TBEV vaccines into the *European pharmacopoeia*. This project was supported by the Innovative Medicines Initiative 2 (IMI2) project "Vaccine batch to vaccine batch comparison by consistency testing" (VAC2VAC).

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Recreating a human pulmonary alveolar-capillary barrier on a lung-on-chip

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The pulmonary alveolar capillary barrier, with its large surface and extremely small thickness, is one of the most important entry routes for xenobiotics into the blood. Recreating a biologically relevant model of the pulmonary air-blood barrier is of crucial importance for inhalation toxicology and drug development studies. However, due to the complex microenvironment and physiology of the lungs, which is challenging to recreate, and the poor availability of human primary cells, there is a lack of physiologically relevant models.

To answer this need, we have developed a Lung-on-a-Chip (LOC) model that integrates human primary alveolar epithelial cells (hAEpCs) and endothelial cells into a tight co-culture that allows cell-cell crosstalk [1]. The thin porous PDMS membrane confers a soft support to the cells, and the bio-inspired micro-diaphragm transmits the cyclic mechanical movements to the cells, mimicking the lung expansion.

In this study, we aimed at establishing a reliable and stable alveolar-capillary barrier similar to *in vivo* conditions, by integrating freshly isolated human alveolar primary cells with pulmonary endothelial cells on the Lung-on-Chip.

hAEpCs from patients undergoing lung resections were cultured on Transwell inserts in parallel to four commercially available human alveolar epithelial cells from different suppliers. Their differentiation state was investigated by RT-qPCR and their ability of building a tight barrier was assessed by measuring trans-barrier electrical resistance (TER). Subsequently, hAEpCs were co-cultured with four different endothelial cell types, in order to select the best match for a stable co-culture. In addition, the effect of cyclic mechanical stretch and differentiation of hAEpCs over time were assessed on the LOC.

Our results showed that co-culture with pulmonary microvascular endothelial cells allowed to reconstruct a stable and tight barrier over time. Moreover, a synergism was observed when epithelial cells were in co-culture with endothelial cells compared to the monocultures, suggesting that we need both cell types to better reconstruct a tight alveolar-capillary barrier. Only freshly isolated hAEpCs expressed type II alveolar epithelial cells markers and built a tight barrier, either in monoculture or in co-culture with endothelial cells. Cyclic mechanical stretch did not distort the tightness of the alveolar-capillary barrier on-chip.

In summary, we have established a stable air-blood barrier human model which resembles more closely the *in vivo* physiological conditions in terms of alveolar markers, barrier tightness and mechanical forces. This innovative system is a promising tool that can be tailored for many animal-free approaches including toxicology studies, drug development and translational medicine.

Reference

[1] Stucki, J. D., Hobi, N. et al. (2018). *Sci Rep 8*, 14359. doi:10.1038/s41598-018-32523-x

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An *in vitro* lung-on-chip system to model inflammation of the alveolar-capillary barrier

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Pulmonary inflammatory diseases are characterized by the disruption of the alveolar-capillary barrier, leading to infiltration of proinflammatory immune cells, edema, and impaired gas exchange. Although people are suffering from those diseases worldwide, therapeutic possibilities are currently limited. This lack of treatment options underlines the necessity of developing new models that can better emulate the complex human lung physiology and could therefore provide an alternative for pharmaceutical and patient's needs.

The aim of this study was to reproduce inflammation of the alveolar-capillary barrier in an advanced *in vitro* system, the lung-on-chip (LOC), integrating human immune cells (peripheral blood mononuclear cells, PBMC), proinflammatory stimuli (bacterial lipopolysaccharide, LPS) and dynamic features of the alveolar *in-vivo* microenvironment.

Our results show that cell-cell crosstalk between the immune component and the epithelial/endothelial barrier leads to barrier disruption upon proinflammatory stimulation. After cotreatment with PBMCs/LPS, both rise of barrier permeability and reduction of transepithelial electrical resistance were associated with high proinflammatory cytokine release (IL-8), all hallmarks of inflammation. Our findings suggest that cyclic 3D stretch favored endothelial tightness ($p < 0.01$). We additionally reproduced the in-flammatory cascade on a lung-on-chip membrane, from the generation of a confluent endothelial layer and activation through proinflammatory stimuli, to the rolling and attachment of perfused PBMCs. Besides, upon adhesion, PBMCs could transmigrate through the endothelial layer and the LOC ultrathin porous membrane.

In summary, we developed an *in vitro* model of inflammation at the alveolar level that while reproducing physiologically relevant lung-mimicking mechanics, can recapitulate key processes of inflammation: epithelial/endothelial barrier disruption as well as immune cell recruitment and transmigration under flow. Hence, this innovative *in vitro* system constitutes a promising tool that may provide insights into alternative therapeutic approaches and enable animal-free preclinical drug testing.

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Advances in *in silico* research and safety testing: Recognition and reward by the Lush Prize

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Lush Prize, Manchester, United Kingdom

The Lush Prize is very pleased to attend and sponsor the European Society for Alternatives to Animal Testing (EUSAAT) Congress 2019 and the 22nd European Congress on Alternatives to Animal Testing in Linz.

Since its launch in 2012, the Lush Prize has awarded over €2.5 million in funding for innovative scientific research, campaigning and lobbying initiatives, aiming to bring forward the day when safety testing takes place without animals and uses only human relevant technologies. All prizes are "1R" based, working directly towards replacement of animals in toxicology and research. The Prize continues to offer awards in the following five categories: Science, Training, Young Researcher, Public Awareness and Lobbying. The Young Researcher category offers early career scientists the opportunity to overcome potential challenges in gaining funding for their work.

The session at EUSAAT 2019 on "*In Silico Models: toxicology and efficacy of drugs, chemicals and cosmetics, new approaches for biomedical research*" aligns perfectly with this year's Lush Prize interest in rewarding the best in computational toxicology. This year, Lush Prize is reviewing and celebrating some achievements of Lush Prize winners in the field of *in silico* research.

As always, the Prize promotes a "1R" message: the entire replacement of animals, or animal derived materials, in research and safety testing.

Nominate your work for a 2020 Lush Prize:
Nominations open for the 2020 Lush Prize in September 2019. All eligible nominations are reviewed and chosen for award by an independent judging panel. For further information visit www.lushprize.org

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Effect-based study of human platelet lysate in various cell lines

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Background and novelty
In this study, the growth promoting efficiency and functional metabolic influence of a human platelet lysate (hPL), GMP-manufactured from platelet apheresis, on various cell lines was investigated considering the postulate of a full replacement for Fetal Bovine Serum (FBS) in cell culture. Although FBS is still widely used as supplement in cell line cultivation, it is not suitable for future therapeutic purposes. For mesenchymal stem cells, hPL is recognized as growth supplement superior to FBS; however, it needs to be validated with respect to its metabolic activity in various cell types which may find applications in regenerative medicine or cell-based therapy.

Experimental approach
As model cells of primary origin, the HaCaT, human Gingival Fibroblasts (hGF) and human Dental Pulp Mesenchymal Stromal Cells (hDPSCs) were used. In addition, seven permanent cell lines from differing origins were cultured in media supplemented with either 5-10% FBS or 5% hPL. Cell morphologies and proliferation efficacies were analyzed using the WST-1 assay or manual cell counting. Enzymatic and immunofluorescent assays were performed to elucidate cell specific markers.

Results and discussion
Comparing the cell proliferation between media supplemented with hPL versus FBS showed active support of proliferation with minor differences. Cellular morphologies, however, revealed major differences in the case of HepG2: the cells lost adherence and grew as slightly attached spheres when cultured in the presence of hPL. In conclusion, growth curves and cell viabilities pointed to a growth-promoting effect of hPL in various permanent and primary cell lines, comparable to FBS or even better. Several cell lines responded unexpectedly to the hPL treatment whose mechanisms are elucidated by determination of cell specific markers. The present study shows the need of in-depth investigations for each cell type with respect to future applications in cell therapy.

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Implementing 3Rs by the pharmaceutical industry

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The European Federation of Pharmaceutical Industries and Associations (EFPIA) represent the pharmaceutical industry operating in Europe. Through its direct membership of 36 national associations and 40 leading pharmaceutical companies and Partners in Research including CROs, we are involved in a number of initiatives, which affirm the key principles of the 3Rs and support animal welfare. As part of this commitment, the European pharmaceutical industry promotes these initiatives internally and externally.

The pharmaceutical industry collaborates within the research field recognising how the sector operates to meet the requirements to effectively implement the requirements of Directive 2010/63/EU and also where researchers and technicians go beyond regulatory requirements to develop practices leading to improved animal welfare and focused 3Rs efforts in every day practice. We continue to work with regulators to ensure the fastest possible uptake of new approaches that balance increased effectiveness in safety and efficacy assessment and impact on 3Rs. We engage all stakeholders and disciplines in dialogue and collaborations and facilitate exchange of good practice between life science community stakeholders to improve animal welfare and scientific outcome by addressing emerging animal welfare issues and mechanisms that share practices that promote good science, animal welfare and the 3Rs. The presentation will describe exciting advances which offer the prospect of radical new approaches to how we go about enhancing 3Rs where we put considerable resources into new and more predictive research methods.

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A user perspective towards improving the processes of project evaluation

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Project evaluation can be carried out locally and/or centrally by the Competent Authority. In transposing the Directive 2010/63, Member States developed differing structures to meet its requirements. In some, single national authorities deal with all applications in the country. In others, there are regional committees, or committees within user establishments, often integrated with the AWB. There are also difference in composition and governance. Despite these variations which were also identified by the Commission during the review of the Directive in 2017, generally, the user community consider the project evaluation and authorisation processes to be effective and efficient. It's not the differences in evaluation and authorisation frameworks that influences the quality of project evaluation but the expertise of and questions asked by the individuals involved in the reviews. There should therefore be more focus on the outcomes related to the use of the animals and the good implementation of 3Rs.

Some proposed improvements or recommendations include:

Project Evaluation is a complex area however awareness and use of existing EU and other National Guidance is improving. EU guidance on Project Evaluation (and Retrospective Assessment) of projects to facilitate the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes has been developed. It is important for Competent Authorities to disseminate this and support the guidance within all research establishments.

To ensure good decisions are made, it is key, for example, to implement training for evaluators, to cross-fertilize works of Ethics committee (e.g. through networks) to ensure consistent outcomes of project evaluation.

Well-structured templates for licence applications with a standard set of questions could enhance and assist Project Evaluation by ensuring that the benefits of the research and the harms to the animals can be assessed in a more consistent way.

There needs to be a mechanism for ensuring provision of sufficient good quality information, with evidence that the applicant has considered and understood all the relevant issues, to facilitate a well-informed harm-benefit analysis.

It is important to ensure good practice and knowledge is applied by the establishments – they could voluntary pre-review license applications by their AWB prior to submission to CA to ensure local expertise ad good practice is incorporated.

There is a need to improve in the assessing of relevant literature – an expert training resource on effective literature searching for 3Rs (by ECVAM, ETPLAS, 3Rs centres) would be useful as well as potentially a literature searching service that would deliver high quality information and reduce duplication.

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Austria goes 3R – the RepRefRed society

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The awareness of the impact of animal well-being on research result quality, together with the revision of the law for animal experiments in Austria 2012 made animal welfare and animal ethics unavoidable topics for persons involved in animal studies. Thus, in the field of biomedical research the 3Rs principles (replacement, reduction and refinement of animal experiments) became increasingly important within the last years. To critically analyze animal experiments and husbandry, as well as to encourage alternative means, we founded the association, the "RepRefRed society". Together with experts in veterinary medicine, laboratory animal science, animal keeping and alternative methods, the RepRefRed society organizes congresses, meetings and workshops and is involved in several Austrian- and EU-wide projects such as the harmonization of the work of the Austrian animal welfare committees, the harmonization of education and training strategies, the establishment of novel cell culture standards and the EU project COST.

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Interpreting irregular LLNA data for evaluation of non-animal methods for skin sensitization

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For the assessment of non-animal approaches to prediction of toxicity, be they *in vitro*, *in chemico*, *in silico* or any other, historic animal data are necessary. For skin sensitization there is a large body of information, which has increased by the release of regulatory databases, on testing of chemicals in the mouse local lymph node assay (LLNA), in which potency is quantified by the EC3 value. In the course of work on structure-activity and quantitative modelling with LLNA data, it has become apparent that some LLNA results are not straightforward to interpret, and in some cases published EC3 values are questionable. In some cases, extremely low EC3 values, derived by extrapolation, have been reported, and in other cases chemicals expected to be strong sensitizers have been classified as non-sensitizers. These cases usually arise where the dose-response pattern does not show a monotonic increase of the SI value with increasing dose, but is bell-shaped or shows a decrease in SI with increasing dose over the whole dose range tested. Here a mechanistic rationale will be presented to explain these irregular dose-response patterns and it will be demonstrated how they can be interpreted to obtain more realistic estimates of potency. Some examples that will be discussed are:

- 4'-Hydroxychalcone and ethyl (4-bromomethyl)benzoate – unrealistically low extrapolated EC3 values
- 2-Amino-3-hydroxypyridine and 4-nitrophthalonitrile – non-sensitizer classifications questionable

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The scope of physical organic chemistry in toxicology read-across, in particular skin sensitization

David Roberts

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Physical organic chemistry is a long-established scientific discipline and provides a large repertoire of experimental methodologies for determining chemical properties that may be of interest for various reasons, such as product development, synthesis, optimising manufacturing processes and toxicology. It has provided a strong theoretical basis, firmly supported by experimental evidence, to understand, interpret and predict, the properties of chemicals and how they depend on chemical structure.

For any toxicological endpoint, the toxicity of a chemical depends on its structure. However, the nature of that dependence is different for different endpoints. Skin sensitization, although a biologically quite complex process is chemically quite simple. Sensitization potency depends on the chemical's ability, as such or after metabolic or abiotic activation, to covalently modify skin protein.

It follows that for read-across purposes in skin sensitization, similarities in reaction mechanism, in reaction kinetics and in partitioning properties, are more relevant than similarities in chemical structure that can be important in endpoints dependent of the chemical's ability to bind non-covalently to biological receptors.

Examples will be presented illustrating the value of physical organic chemistry in estimating skin sensitization potency by read-across and how inappropriate similarity parameters can be misleading.

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Development of recombinant antibodies to replace animal sera

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The most widely used *in vitro* technology for animal-free antibody generation is antibody phage display. This method allows to select recombinant monoclonal antibodies from universal antibody gene libraries, through a highly customizable selection method named panning. The panning procedure can be designed to reduce unwanted cross-reactivities, to obtain cross species-specific antibodies, target post-translational modification or conformation specific antibodies. With this approach it is possible to generate highly specific recombinant monoclonal scFv antibodies ready to be cloned and expressed in any desired format. These antibodies can be documented by their sequence, providing infinite reproducibility of studies where they had been used, even if the original clone was lost, since it can always be reconstituted from the electronically stored or printed amino acid sequence.

Nowadays, phage display for antibody selection is one of the pillars of therapeutic human antibody generation, and is progressively replacing animal immunization and hybridoma technology in the generation of diagnostic and research antibodies as well.

In this presentation we will illustrate the potential of the *in vitro* technology for antibody selection in comparison to the most known immunization approach. We will provide examples of the applicability of the method to replace animal sera in therapy (diphteria toxin) as well as for improving the quality of diagnostic and research antibodies.

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Collaboration for non-animal cosmetic safety assessment globally by 2023

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There has been significant progress globally over recent years in advancing the science to underpin non-animal cosmetic safety assessment. This has facilitated legislative change within some countries; however, to achieve a global ban on the use of animals in cosmetic safety assessment there is still more to do. A new collaboration between Humane Society International, industry partners and other interested groups intends to share the decision-making approaches which are being applied to assess safety without animals to help shape future cosmetics legislation. This activity contributes to the investment in education and training to ensure that there is the ongoing ability to meet the needs of regulations which require non-animal safety approaches.

The collaboration will focus on delivering three primary objectives:
1) Global harmonization of non-animal cosmetic safety assessment legislation
2) Sharing information on decision-making approaches without new animal testing
3) Investment in education & training

Structured around the International Cooperation on Cosmetics Regulation (ICCR) principles (Principles underpinning the use of new methodologies in the risk assessment of cosmetic ingredients) [1], the education and training will provide insights into the tools and approaches applied in risk assessment with a specific focus on how they are applied to reach a safety decision.

In this presentation, the NACSA project will be introduced and an industry perspective shared on the opportunities it brings. An example of an integrating non-animal methods towards a cosmetic risk assessment will be shared through a case study.

Reference

[1] Dent et al. (2018). *Comput Toxicol 7*, 20-26. doi:10.1016/j.comtox.2018.06.001

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The importance of data quality and governance in the acceptance of Next Generation Risk Assessment (NGRA) approaches

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Chemical safety assessment is undergoing a paradigm shift from assessments based on a structured and defined animal testing approach to more bespoke, ab initio problem solving. Next Generation Risk Assessments (NGRA) use novel, human-relevant, toxicity pathway-based *in vitro* approaches coupled with *in silico* models. NGRA, being hypothesis driven, requires a variety of tools and technologies to address data gaps and build a coherent risk assessment for a given exposure scenario.

Good Laboratory Practice (GLP) has traditionally been the required standard for generation of experimental data in support of studies conducted to assure the safety of chemicals. However, many of the approaches used to support an NGRA are not currently available to GLP or due to their bespoke nature do not fit easily within many existing structures (e.g. 'omics panels and computational modelling). For modelling, limited accepted guidance exists and certainly there are no regulatory accepted frameworks analogous to GLP against which compliance can be claimed [1,2].

Recent publications and workshops on the topic [3,4] have highlighted the need for a more flexible approach to assuring quality and robustness from data and information that may be used in NGRA. With this, the emphasis shifts to the experimentalists and modellers to continue to ensure that data integrity is upheld to high standards as new approaches evolve and are evaluated.

The overarching principles of ensuring that experiments are planned, performed, monitored, recorded, reported and retained such that they are transparent and reproducible is fundamental to building confidence in new and bespoke approaches. Here we describe the challenges of gaining acceptance for NGRA approaches and define the key principles transferable between experimental and computational approaches. An example NGRA case study (coumarin) will be used to present the challenges and highlight the opportunities for development of robust data integrity approaches.

References
[1] Calder, M. et al. (2018). *R Soc Open Sci 5*, 172096.
[2] Loizou, G. et al. (2008). *Regul Toxicol Pharmacol 50*, 400-411.
[3] NC3Rs workshop: Improving Science, Advancing Animal Welfare: Towards better In Vitro Practices; Eindhoven, The Netherlands (June 2018).
[4] OECD Guidance Document on Good In Vitro Method Practices (GIVIMP), ENV/JM/MONO(2018)19.

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The combination of 3D human cancer tissues and fluid-dynamic drug administration as winning strategy for *in vitro* drug efficacy testing

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Recently, 3D *in vitro* cancer models are becoming important alternatives to *in vivo* animal models for establishing the efficacy of potential anticancer therapeutics treatments [1]. The integration of 3D human tumor tissues with fluidic cell culture chambers allows to mimic the *in vivo* organ-organ fluidic connections, finally offering a reliable and fast approach for prescreening tests.

In this work, 3D cell-laden hydrogels have been properly realized as ovarian tumor models and cultured within a fluidic Multi In vitro Organ-MIVO®-device for resembling *in vitro* the cancer environment and carry out drug efficacy assays, alternative to animal preclinical models, by monitoring and measuring the cancer regression over time.

Human ovarian cancer cell line (SKOV-3) has been expanded in petri dishes and then used to realize 3D cell-laden hydrogel-based tumor models as previously reported [2]. 3D tumor tissues have been cultured within MIVO® chamber under fluid flow resembling the capillary blood flow feeding the tumor; 3D organoids cultured under static conditions have been used as controls.

The effect of cisplatin (CIS) chemotherapy at different concentrations (100 μM and 10 μM) was assessed. SKOV-3 viability was quantitatively assessed through Alamar Blue Assay at different time points (days 2, 4, 7) and the tumor regression was derived as percentage of alive cells normalized to the control without drug treatment. Immunostaining for apoptosis, cell proliferation and hypoxia was also carried out.

The SKOV-3 cell laden hydrogels were initially more responsive to 10 μM drug under static conditions (cell viability of 81.8%) than under fluid within MIVO® (cell viability of 94.2%), as effect of the different drug diffusion kinetics in the two conditions.

Interestingly, for prolonged days of culture (i.e. 4, 7 days) the viability of SKOV-3 cell laden hydrogels cultured within MIVO® was decreasing (67.8% and 49.2% after 4 and 7 days) and always lower than under static conditions, where the cell viability was pretty constant (95.1% and 85.2% after 4 and 7 days). These data are in line with *in vivo* results: mice with SKOV3 tumours (0.1 ml) were treated with 6 mg/kg Cisplatin intravenously (i.v.) every 7 days for 3 weeks (q7dx3). Tumour growth inhibition of 50% was achieved on average 30 days from the first treatment with Cisplatin.

Moreover, the drug testing carried out within MIVO® displayed a drug dose effect *in vitro*, with an increasing cell viability reduction when drug dose was 100 μM (42.3%, 22.5%, 8.0% after 2, 4 and 7 days).

In conclusion, we have shown that the cell-laden hydrogels within MIVO® device better resemble the fluid dynamic stimuli of in the cancer environment, finally representing a highly reliable platform for preclinical drug efficacy tests.

References

[1] Stock, K., Estrada, M. F., Vidic, S. et al. (2016). *Sci Rep 6*, 28951. doi:10.1038/srep28951
[2] Cavo, M., Caria, M., Pulsoni, I. et al. (2018). *Sci Rep 8*, 5333. doi:10.1038/s41598-018-23250-4

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Demonstration of different intestinal absorption mechanisms of lactulose and mannitol by using 3D tissue inserts within fluidic multi-chamber devices

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Intestinal permeability is crucial in regulating the bioavailability and consecutively the biological effects of drugs and compounds in our body. However, systematic and quantitative studies of absorption of molecules are quite limited due to the lack of reliable experimental models. Generally, standard *in vitro* models do not take into account the 3D intestinal tissue heterogenicity and/or the dynamic stimuli of the intestinal environment, fundamental in regulating absorption processes; on the other side *in vivo* models, either in animal or in human being, are far to be reproducible and standardized. In this work, we present a novel platform as *in vitro* fluidic model of healthy and pathological small intestinal barrier, composed of a 3D *in vitro* tissue organoid of intestinal epithelium integrated with a fluidic bioreactor resembling the physiological stimuli of the bloodstream. The platform has been validated, both in healthy and calcium depletion induced pathological conditions, by monitoring the absorption of two non-metabolized sugars, lactulose and mannitol, involved in two different routes of drug absorption (transcellular and paracellular), and by assessing the capability of the damaged tissue to undergoes a self-wound healing process, re-establishing its integrity.

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Marine sponge collagen-derived bioactive products and biomaterials for the development of cellular supports for tissue engineering and oxidative stress protection of human skin cells

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Collagen is involved in the formation of complex fibrillar networks able to provide the tridimensional support and to ensure the structural integrity of many tissues. Its low immunogenicity and mechanical properties allow the use of this molecule as a biomaterial suitable as support for *in vitro* monolayer cell cultures mimicking *in vivo* basal membranes, for the development of cell culture 3D matrices as well as for tissue engineering and regenerative medicine (TERM) strategies in human health issues.

Here, we used four different methods to obtain sponge collagenous fibrillar suspensions (FSs) from *C. reniformis* demosponge which were then chemico-physically and biologically characterized, in terms of collagen and glycosaminoglycans content, viscous properties, biocompatibility and antioxidant activity [1]. These four FSs were then tested for their capability to generate thin sponge collagenous membranes (SCMs) suitable for TERM and *in vitro* cell monolayer culture purposes. Two types of FSs were able to generate SCMs and showed good mechanical properties, enzymatic degradation resistance, water binding capacity and antioxidant activity. Interestingly, they showed good biocompatibility for both fibroblast and keratinocyte cells cultured onto the various SCMs opening the possibility for the use of these thin membranes for TERM purposes and for the setup of *in vitro* assays of human skin cells alternative to animal testing.

Furthermore, besides their role as structural supports and scaffolds, marine collagen and marine collagen hydrolysates may also possess interesting bioactive properties, which to date, in some organisms like sponges, have been poorly investigated. Thus, starting from the abovementioned *C. reniformis* collagen FSs,

we obtained four HPLC-purified fractions of trypsin-digested extracts (MCHs) of which we studied the *in vitro* toxicity, antioxidant, wound healing and photo-protective properties by use of cellular *in vitro* assays alternative to animal testing [2]. The four MCHs had no degree of toxicity and were able to stimulate cell growth. They showed a significant antioxidant activity in H2O2- or quartz-stimulated macrophages, they stimulated wound healing in a 2D *in vitro* cell assay and they promoted significant cell survival in an *in vitro* assay reproducing damages from UV-radiation on skin cells. Overall, our data, obtained by the sole use of alternative methods to animal testing, open the way to the use of *C. reniformis* MCHs in drug and cosmetic formulations for damaged or photo-aged skin repair.

References

[1] Pozzolini, M., Scarfi, S., Gallus, L. et al. (2018). *Mar Drugs 16*, 111. doi:10.3390/md16040111

[2] Pozzolini, M., Millo, E., Oliveri, C. et al. (2018). *Mar Drugs 16*, 465. doi:10.3390/md16120465

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Improving drug toxicity assays through the oxygen-dependent induction of metabolic zonation in primary rat hepatocytes

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The development of new pharmaceutical compounds requires strict scrutiny over potential adverse effects caused by the drug. Especially drug-related cytotoxicity during hepatic metabolization poses a critical risk to the patient. Assessing the possibility of unwanted drug interactions before trials in human begin, heavily relies on *in vitro* hepatocyte cultures as a model of *in vivo* metabolism. While these cultures account for a large portion of pre-clinical assays in terms of metabolic functions and toxicity, they often do not represent *in vivo* situations accurately, resulting in the need for further trials in living animals.

In order to improve the relevance of these cell culture models, critical environmental parameters of the hepatic microarchitecture, like ambient Oxygen tension, need to be included in *in vitro* systems. Tissue-specific Oxygen levels are supplied to resident cells by the influx of blood from both the portal venule and the hepatic artery. Due to cellular respiration, sinusoidal Oxygen tension then drops towards the central vein [1]. As a result, cells along the sinusoid assume location-specific roles. This leads to the formation of heterogeneous phenotypes performing specialized tasks, in order to allow the efficient compartmentalization of overlapping pathways in the same tissue. Most notably, this leads to some hepatocytes being specialized in drug metabolism. While this phenomenon has been well described for a long time [2], general application in *in vitro* systems has been lacking, which are typically performed in atmospheric Oxygen conditions.

In order to investigate the induction of zonal phenotypes *in vitro*, we modulate pericellular oxygen levels by culturing primary rat hepatocytes on polydimethylsiloxane (PDMS) culture plates in different ambient Oxygen concentrations. Here, Oxygen is supplied through the gas-permeable membrane, leading to higher Oxygen supply rates compared to conventional tissue culture plates [3], which limit the supply to diffusion through the medium. Furthermore, a comparison between different Oxygen supply methods is achieved by selectively blocking the gas-permeable membrane with a polyester seal, mimicking conventional polystyrene tissue culture plates.

As expected, cellular oxygen consumption depends on ambient Oxygen concentration and supply method. Comparison of metabolic profiles in high flux conditions reveals changes according to previously reported zonal phenotypes [4] when exposed to different ambient Oxygen tension. These conditions have shown to be able to perform more energy-intensive tasks, like gluconeogenesis and serum protein synthesis. Moreover, a typical increase in drug metabolism genes can be observed, leading to a distinctive increase in cellular injury after Paracetamol administration. Conversely, we have observed a general unspecific downregulation of gene expression in conventional culture setups, highlighting the importance of biomimetic Oxygen supply rates in *in vitro* culture systems. Taken together, we have shown that the consideration of Oxygen conditions improves hepatocellular culture systems and allows to mimic *in vivo* situations more closely. By incorporating zonal effects into *in vitro* systems, a more accurate model is available for pre-clinical trials, which should ultimately enable a reduction of required *in vivo* testing.

References
[1] Kietzmann, T. (2017). *Redox Biology 11*, 622-630. doi:10.1016/j.redox.2017.01.012
[2] Jungermann, K. and Kietzmann, T. (1996). *Annu Rev Nutr 16*, 179-203. doi:10.1146/annurev.nu.16.070196.001143
[3] Place, T., Domann, F. and Case, A. (2017). *Free Radic Biol Med 113*, 311-322. doi:10.1016/j.freeradbiomed.2017.10.003
[4] Halpern, K. B. et al. (2017). *Nature 542*, 352-356. doi:10.1038/nature21065

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Differentiation of motor neurons for *in vitro* potency testing of botulinum neurotoxins

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Botulinum Neurotoxins (BoNTs) are potent inhibitors of neurotransmission, which can lead to a flaccid paralysis and result in death by asphyxia. Nevertheless, these toxins are used for medical and aesthetical applications. Due to the varying activity and high toxicity of this bacterial toxin, a potency assessment needs to be performed for every batch of toxin produced for which the mouse bioassay is the gold standard. BoNTs show a high selectivity for motor neurons (MNs), where they bind to specific receptor proteins and are then endocytosed. Intracellularly, the small subunit of the toxin cleaves SNARE proteins required for synaptic vesicle fusion and subsequently inhibits the release of neurotransmitters. Each BoNT serotype binds to a specific receptor and cleaves its substrate at a specific site.

Our aim is to develop a serotype-independent assay based on human MNs for the testing of the most common BoNT serotypes. MNs were generated *in vitro* from human induced pluripotent stem cells (iPSCs) transfected with a plasmid coding for a Luciferase reporter enzyme, which is trafficked to the synaptic vesicles. The release of neurotransmitters, whose inhibition is the toxicological endpoint of all BoNT serotypes at the cellular level can then be quantified. For the neuroblastoma cell line SIMA, a BoNT-dose dependent inhibition of Luciferase release has been shown [1].

MNs were generated from human iPSCs with three different protocols. The MN yields of the resulting cell populations were quantified by immunocytochemistry utilizing the neuronal marker TUJ1 and the MN marker Islet1. Furthermore, the expression levels of receptors (SYTI/II, SV2A/B/C) and substrates (STX-1A/B, VAMP1/2/3/4, SNAP25, YKT6) of the BoNT serotypes

A-G and X, as far as they have been identified, were quantified with RT-qPCR. The yield of MNs and the expression levels of the individual genes varied significantly between the differentiation protocols, while the sum of the substrate or receptor expression levels were similar. The presence of the analyzed receptors and substrates indicates that the cells generated *in vitro* should be suitable for detecting the corresponding BoNT serotypes.

First steps to assay establishment were already taken. However, a Luciferase release by the MNs by usage of a potassium-containing buffer could not be stimulated reproducibly up to now. Instead, a high amount of the reporter seems to be released into the cell culture supernatant stimulation-independently. Further steps are currently being taken to optimize the assay principle, in order to determine the BoNT sensitivity of human MNs generated *in vitro*.

Reference

[1] Pathe-Neuschäfer-Rube, A., Neuschäfer-Rube, F., Genz, L. et al. (2015). *ALTEX 32*, 297-306. doi:10.14573/altex.1503061

Submission declaration:

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A reconstructed human skin model containing macrophages to set up a delayed wound healing model of cutaneous leishmaniasis

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Cutaneous leishmaniasis (CL) is a vector-borne neglected disease caused by protozoan parasites of the genus Leishmania. Disfiguring and socially stigmatizing skin lesions develop at the bite site of the parasite-infected female sand fly [1]. Tissue damage and disease in CL are primarily caused by an excessive host immune response against the intracellular infection of dermal macrophages [2]. The dermal lesions persist for months or even years, but eventually heal on their own [3]. Treatment of CL is problematic, as long series of painful injections with the toxic pentavalent antimonials remain the standard therapy [1] and lesions are left alone to self-cure with the risk of secondary bacterial or fungal infection. New therapies for CL and CL lesions are urgently needed. Therefore, realistic CL lesion models are essential as a predictive experimental platform to identify more effective topical strategies.

To that aim we integrated for the first time *in vitro*-generated M1 polarized macrophages differentiated from the human monocytic THP-1 cell line into reconstructed human skin (RHS).

THP-1 derived macrophages were localized in the RHS dermal compartment and distributed homogenously in accordance with native human skin. Standardized circular wounds were made with a 18 gauge blunt tip needle or by punch biopsy. In order to impair wound healing, wounded RHS was stimulated with intradermal application (for needles) or drops (for punch wounds) of IFN-γ in combination with LPS and/or hydrocortisone.

Wound healing was monitored on days 1, 3 and 7 after wounding by histological examination of RHS. Immunohistochemical (Ki67, K14, tenascin-C, laminin 5, α-SMA) and pro-inflammatory cytokine analyses were performed pre- and post-skin wound and stimulation, to increase the characterization of the model and to assess the effects of IFN-γ, LPS and hydrocortisone in wound healing RHS models.

Early in healing, IFN-γ-LPS-hydrocortisone wounds displayed reduced proliferation and re-epithelialisation and heightened inflammatory response compared with control wounds. H&E-stained sections showed increased epidermal thickness and a lack of dermal epidermal junction in the wound zone.

In summary, we integrated functional THP-1 derived macrophages into RHS and induced a delayed wound healing to provide a unique experimental test platform to evaluate the effects of new topical treatments.

References

[1] Murray, H. W., Berman, J. D., Davies, C. R. et al. (2005). *Lancet 366*, 1561-1577. doi:10.1016/S0140-6736(05)67629-5

[2] Wijnant, G. J., Van Bocxlaer, K., Fortes Francisco, A. et al. (2018). *Antimicrob Agents Chemother 62*, e00631-18. doi:10.1128/AAC.00631-18

[3] WHO (2010). Control of Leishmaniasis. *WHO Technical Report Series 949*, 1-187.

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Viva3R!

Stefanie Schindler

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The presentation provides a summary on 60 years of the 3Rs, focussing on successes and on possible ways forward. Since the publication of Russell and Burch, the scientific community has seen huge progress in alternative methods, but also regards on how we perceive laboratory animals and *in vivo* research. One major remaining challenge is the communication of all these developments to the general public, and implementation of alternative methods as well as effective networking between all relevant stakeholders, such as animal welfare bodies, 3R Centers, members of approving authorities and researchers. Viva3R offers to provide support on these and other 3R-relevant issues, promoting positive animal welfare and a culture of care which improves both the well-being of animals and the people performing the procedures.

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Tissue-on-a-chip

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In this study we demonstrate a microphysiometric system based on IMOLA-IVD device that will allow real-time measurement of multiple parameters in human small intestine tissue model (EpiIntestinal™). The parameters are measured non-invasively, while the system provides fresh nutrients and allows programmable repeated dosing of test articles. In the presented set of experiments, we have concentrated on trans-epithelial electric resistance (TEER) as this parameter was previously established to reflect an integrity of EpiIntestinal tissue model [1]. The performance of the system was verified and baselines were established by long term incubation of EpiIntestinal tissue in IMOLA-IVD, followed by single application of 2% SDS. In the subsequent experiments, EpiIntestinal tissue was pre-incubated in the system for 8 hours and then treated with a single dose of 0.2% SDS. TEER data was continuously collected during the whole experiment up to 16 hours post application. As expected, we have demonstrated gradual decrease of TEER values following the detergent application. As IMOLA-IVD device allows monitoring multiple parameters and programming of variable substance application protocols, in the subsequent experiments we are planning to simulate more complex treatment schemes and parallel monitoring of TEER, medium pH and dissolved oxygen concentration [2].

All in all, the IMOLA-IVD device has proven itself to be a flexible and customizable system for analysis of various cell cultures. These include models such as immortalized 2D cell lines, 3D spheroids and tissue-on-a-chip for the reconstructed human epidermis and intestine [2,3,4]. Ongoing work includes investigation [5] and the optimization of the fluidic cycles (e.g. by increasing the stabilization time before the test substance is added) to create a versatile tissue-on-a-chip tool, which can also be applied to lung or other mucous cell models.

References

[1] Peters, M., Landry, T., Pin, C. et al. (2019). *Toxicol Sci 168*, 3-17. doi:10.1093/toxsci/kfy268

[2] Brischwein, M. and Wiest, J. (2018). Microphysiometry. In *Bioanalytical Reviews*. Berlin, Heidelberg: Springer. doi:10.1007/11663_2018_2

[3] Alexander, F., Eggert, S. and Wiest, J. (2018). *Cytotechnology 70*, 375-386. doi:10.1007/s10616-017-0152-x

[4] Alexander, F., Eggert, S., Wiest, J. (2018). *Genes 9*, 114. doi:10.3390/genes9020114

[5] Gilbert, D. F., Mofrad, S. A., Friedrich, O. et al. (2019). *Cytotechnology 71*, 443-452. doi:10.1007/s10616-018-0263-z

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When the co-culture does not do what it is supposed to do – a step "back" to ex *vivo* models

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Inflammatory bowel disease (IBD) is a chronic disease of the GI-tract which affects the quality of the patient's life in a strong manner. The number of the affected individuals increased over the past decade especially in newly industrialised countries caused by the expansion of western lifestyle [1]. Immunosuppressants like Azathioprine and Mercaptopurine are permanently used for the treatment of the symptoms. This leads to strong side-effects including infections due to the weakened immune system. In addition, these patients have a higher risk for the development of lymphomas and melanomas [2]. New drugs and therapies for the treatment of IBD are required urgently.

In order to test new drugs and following the 3-R principle (Replacement, Reduction, Refinement) we investigated a co-culture based on macrophages (differentiated from THP, dTHP) and Caco-2 cells in more detail. Effects of cytokines (such as TNF) on barrier properties were examined with the aim to set up a robust inflammable *in vitro* tool. However, the co-culture model suffered from low barrier properties even in a non-inflamed state. We assume that this observation is due to a signal secreted by dTHP, as the supernatant from dTHP had the same effect on the Caco-2 monoculture.

Future steps will focus on *ex vivo* models and the combination with an automated measuring system to recognize the (anti)-inflammatory reaction of the biological barrier (e.g. intestinal tissue). We will investigate whether the inflammation of an *ex vivo* model can be observed by measuring the electrical resistance. This would be a first step to set up a useful and fast *in vitro* tool for testing safety and efficacy of new drugs targeting IBD.

References

[1] Ng, S. C., Shi, H. Y., Hamidi, N. et al. (2017). *Lancet 390*, 2769-2778. doi:10.1016/S0140-6736(17)32448-0
[2] Wehkamp, J., Götz, M., Herrlinger, K. et al. (2016). *Dtsch Arztebl Int 113*, 72-82. doi:10.3238/arztebl.2016.0072

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The novel BFH12 cell line: An alternative model of bovine biotransformation

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Introduction

There are growing concerns about the widespread use of pesticides and veterinary drugs in livestock farming. Given their tendency to accumulate in milk and meat, these substances represent a significant risk to animal and human health. The novel hepatocyte-derived cell line BFH12 [1] is a promising model to identify underlying toxicity mechanisms by investigating the metabolism and clearance of xenobiotic substances.

In this study, we examined the gene expression of drug-metabolizing enzymes (DME), efflux transporters and the inducibility of CYP1A1 and CYP1A2 in BFH12.

Methods

BFH12 were cultured in Williams' Medium E containing 5% heat-inactivated FBS, 1% penicillin/streptomycin, 2 mM L-alanyl-L-glutamine, 100 nM dexamethasone and 0.2 U/mL insulin. Total RNA from BFH12, fetal hepatocytes and adult liver tissue was isolated, reverse transcribed and amplified by PCR using gene-specific primers. Several genes of phase I - III enzymes/transporters were selected for validation. Benzo[a]pyrene was used as inductor of CYP1A activity, determined by the ethoxyresorufin-O-deethylase (EROD) assay. Efflux transporters were identified by indirect immunofluorescence using transporter-specific antibodies.

Results

BFH12 and fetal hepatocytes exhibited a similar pattern of gene expression. Phase I and II enzymes CYPs 1A1, 2C19, 3A4, UDP glucuronosyltransferase 1 family polypeptide A1 (UGT1A1), UGT1A6, glutathione S-transferase M1 (GSTM1), as well as phase III efflux transporters P-glycoprotein (PGP), sodium-taurocholate cotransporting polypeptide (NTCP), ATP-binding cassette sub-family G member 2 (ABCG2) and multidrug resistance-associated protein 1 (MRP1) were found to be expressed in BFH12. Immunofluorescence analysis revealed strong expression of ABCG2 and MRP1. Dose-dependent induction of CYP1A1 and 1A2 gene expression and activity was confirmed.

Conclusion

The BFH12 cell line that shows induction of DME and expresses several efflux transporters is a promising *in vitro* model to study bovine biotransformation and therefore provides an alternative to current methods.

Reference

[1] Gleich, A., Kaiser, B., Schumann, J. et al. (2016*). In Vitro Cell Dev Biol Anim 52*, 662-672. doi:10.1007/s11626-016-0018-0

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Cultivation of primary melanoma cells with human platelet lysate or bovine serum show different phenotypic and genotypic characteristics

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Malignant melanoma is one of the fastest growing cancers and, if it occurs during pregnancy, can often cause metastases in the placenta and fetus. However, the literature is very contradictory. It is claimed that women who are pregnant at the time of diagnosis have a worse prognosis than women who are not pregnant and, in addition, the recurrence rate increases with each further pregnancy. Conversely, the most recent studies show that the prognosis of melanoma during pregnancy is similar to that of non-pregnant women. In addition, melanomas are also characterized by heterogeneity in the tumor, in order to understand the biology of melanoma especially during pregnancy and to establish new treatment options, meaningful *in vitro* models must be established.

We received two lymph nodes, one pigmented and one unpigmented, from a young pregnant woman. The isolation of the BRAF mutated tumor cells was performed mechanically and cells were cultured with fetal bovine serum (FBS) medium or human platelet lysate (hPL) medium. Differences in cell morphology, growth behavior, growth rate, and degree of expression of typical melanoma markers such as MelanA, HMB45, Sox10, and S100 were observed. Chromosomal aberration was detected by copy number variation analysis. By comparing the non-pigmented nodules under hPL and FBS growth conditions, differences are found in chromosome 3, 6, 8, 12, 14, 17, 18 and 19. Identification was measured by short tandem repeat analyses. In addition, cancer associated fibroblasts are isolated from the same patient to establish adequate 3D models.

This experimental approach shows the importance of the choice of media additives, reflected the heterogeneity of the cells and finally provides an ideal model system for the investigation of malignant melanoma in pregnant women.

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Proteomic analysis of canine leptospira vaccines

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Background

In the recent past, very promising techniques have been developed that have reduced or refined animal testing in batch testing of veterinary vaccines. However, there has been less progress in completely replacing *in-vivo* testing. The goal of this study is a demonstrated proof of concept of the consistency approach for batch release testing. The consistency approach implies the use of a set of *in-vitro* parameters to constitute a product profile that can replace classical *in-vivo* animal tests. Therefore, we use mass spectrometry (MS) to develop a physicochemical method for the *in-vitro* characterization and quantification of canine *Leptospira interrogans* antigen intermediate products (pre- and/or post inactivation material) or final batches to link either the entire quantitative antigen profile or the amount of selected antigens to demonstrate batch to batch consistency. Leptospira vaccines are also considered to be model vaccines to assess the MS suitability as a platform technology that could successfully be applied to other vaccines.

Methods

Analytical liquid chromatography (LC)-ESI-MSE method was applied for recording protein profile of *Leptospira interrogans* serogroups icterohaemorrhagiae and canicola.

Results

In total, 1074 and 849 proteins were identified in Leptospira icterohaemorrhagiae and canicola samples, respectively. Seven out of ten most abundant proteins were reported as known or potential antigens [1-5]. Out of 231 proteins assigned to *Leptospira interrogans* serogroup canicola in the final product, 164 were also identified in pre- and post-inactivation products. On the other hand, 162 proteins from *Leptospira interrogans* serogroup icterohaemorrhagiae were identified in all three samples – the bivalent final product and both monovalent intermediate products. Only one protein was identified that could be used to distinguish between two serogroups.

Conclusions

These results show that MSE is suitable to record semi-quantitative protein profiles for batch to batch consistency testing. OmpL1, OmpA, LipL32, LipL41, 60 kDa Chaperonin, Flagellin, LipL45, LipL21, LipL36 and the protein M6RB32/A0A1B9FL represent the proteins with the greatest immunological relevance and the best MS performance. They are candidates for targeted, absolute quantification of selected relevant antigens in Leptospira vaccines.

References

[1] Humphryes, P. C., Weeks, M. E. and Coldham, N. G. (2014). *Int J Proteomics 2014*, 572901. doi:10.1155/2014/572901
[2] Humphryes, P. C., Weeks, M. E., Gielbert, A. et al. (2012). *Clin Vaccine Immunol 19*, 587-593. doi:10.1128/CVI.05622-11
[3] Oliveira, T. L., Grassmann, A. A., Schuch, R. A. et al. (2015). *PLoS One 10*, e0142821. doi:10.1371/journal.pone.0142821
[4] Hartwig, D. D., Forster, K. M., Oliveira, T. L. et al. (2013). *Clin Vaccine Immunol 20*, 747-752. doi:10.1128/CVI.00034-13
[5] Haake, D. A., Mazel, M. K., McCoy, A. M. et al. (1999). *Infect Immun 67*, 6572-6582.

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Optimization of gene silencing in a cell-laden 3D organ-like model by means of RNA interference

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Three dimensional (3D) cell cultures which mimic the complex environment and natural physiological conditions in native tissues, not only provide a better model for *in vitro* responses in comparison to the conventional monolayer two-dimensional (2D) cell cultures, but can also eliminate the need for animal models. Recent studies have shown the potential of bioprinted 3D tissue models for viral infection and viral gene delivery studies [1,2]. However, the suitability of cell-laden 3D organ-like structures for non-viral gene delivery as a safe alternative to viral vectors has not yet been fully realized. The goal of this study was the optimization of the non-viral gene delivery and gene silencing in 3D cell-laden constructs by means of RNA interference (RNAi).

For this purpose, alginate- and gelatin-based 3D constructs, supplemented with Matrigel (20%), containing HEK293T cells were generated using extrusion printing. The 3D structures were transfected with siRNA against human cyclophilin B (hCycB), a cyanine-labelled (Cy3)-siRNA, or a green fluorescent protein (GFP)-encoding plasmid, with different transfection reagents (Isifect and Lipofectamin 2000). Subsequently, the RNAi mediated by transfection with hCycB siRNA was examined by quantitative polymerase chain reaction (qPCR). In addition, the expressions of GFP as well as the fluorescence of the Cy3-tagged siRNA within the generated 3D constructs were analyzed by fluorescence microscopy. The results of this examination showed a distinct hCycB gene silencing in 3D construct transfected with both Isifect and Lipofectamin 2000. Moreover, in order to improve the transfection, the 3D constructs were modified to reduce the alginate-induced cell encapsulation within the 3D constructs. For this purpose, the alginate-based 3D cell-laden constructs were treated with sodium citrate, which partially degraded the alginate cross-links within the constructs.

The subsequent fluorescence microscopic examinations and qPCR analysis showed that the addition of sodium citrate favored the transfection of the generated 3D constructs.

References

[1] Hiller, T., Berg, J., Elomaa, L. et al. (2018). *Int J Mol Sci 19*, 3129. doi:10.3390/ijms19103129

[2] Berg, J., Hiller, T., Kissner, M. S. et al. (2018). *Sci Rep 1*, 13877. doi:10.1038/s41598-018-31880-x

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HepaRG™: An integrated model for studying bile canalicular functions and dysfunctions for studying cholestasis toxicity

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Drug-induced cholestasis (DIC) is primarily associated with impaired bile acid (BA) homeostasis, leading to the intrahepatic retention and accumulation of toxic BAs [1]. However, the dynamics of DIC is poorly understood and is mainly limited to assessing the compound's potential to inhibit the bile salt export pump (BSEP) [1]. HepaRG™ cells can be used to predict DIC states in patients treated with drugs, resulting from the accumulation of Bile acids (BA). HepaRG™ cells allow to measure the impact of cholestatic drugs by measuring their effects on bile canaliculi dynamics, Rho kinase (ROCK)/myosin light chain kinase (MLCK) pathway implication, efflux inhibition of taurocholate [a predominant bile salt export pump (BSEP) substrate], and expression of the major canalicular and basolateral bile acid transporters [2]. HepaRG™ cells clearly shown the dose dependent effect cyclosporine A (CsA) on Inhibition of efflux and uptake of taurocholate [3]. HepaRG™ cells predicted the effect of bosentan in bile canaliculi dilation and chlorpromazine (CPZ) in Bile canaliculi constriction by difference in BA accumulation [4]. Therefore, HepaRG™ cells can be used as integrated model for studying bile canalicular functions, dynamics and dysfunctions.

References

[1] Brouwer, K. L. et al. (2013). *J Pharm Sci 102*, 3037-3057.
[2] Guillouzo A. et al. (2016). *Drug Metab Dispos 44*, 1780-1793.
[3] Guillouzo A. et al. (2014). *Toxicol Sci 141*, 244-253.
[4] Guillouzo A. et al. (2016). *Sci Rep 12*, 6.

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An overview of Toxicity Testing 21st Century (TT21C) *in vitro* methods to assess next generation products

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The utility of TT21C *in vitro* methods have been demonstrated when used as part of an overall assessment framework for Next Generation Products (NGP).

TT21C methodologies can be used to assess harm reduction potential of NGPs using human-derived cellular systems and biological responses. The potential harm reduction potential of three different NGP products were compared to the 3R4F reference cigarette in a series of *in vitro* assays. The products investigated were the Kentucky reference cigarette (3R4F), a tobacco heated product (THP), a hybrid product (HYB) and a myblu e-cigarette (Tobacco Flavour 1.6% Nicotine). The 3R4F and THP were smoked using the Health Canada Intense smoking regime. HYB and myblu vaped were according to CORESTA Recommended Method N°81. Four puffs/ml of PBS was used for all NGPs and 1.8 puffs/ml for 3R4F.

Smoke/aerosols were captured in PBS to enable the use of *in vitro* systems where direct exposure to smoke/aerosol was not possible. Chemical characterisation was conducted on the aerosol/smoke PBS solutions to measure nicotine and 8 carbonyls.

Regulatory accepted *in vitro* assays (neutral red uptake, Ames assay and the *in vitro* micronucleus test) were employed for testing PBS trapped smoke/aerosol. All regulatory accepted assays indicated reduced cytotoxic and genotoxic activity of the THP compared to 3R4F. Additionally, there were limited to no effects observed in each assay for HYB and myblu™. The test samples were also analysed in several TT21C assays to provide a wider mechanistic understanding, including endothelial cell migration (scratch wound assay), tumour promotion (Bhas cellular transformation assay), cellular health (high content screening), 3D reconstituted bronchial epithelia with repeated exposure and developmental toxicity (devTOX quickPredict assay). The results of all assays indicated limited to no toxicity for myblu™ aerosol or extracts. Based on the tests conducted the overall ranking in terms of the biological response was 3R4F > THP > HYB ≥ myblu™.

We believe regulatory bodies should actively encourage the use of TT21C methodologies as an alternative to *in vivo* animal data.

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Gluconeogenic processes in a bovine liver cell line offered short chain fatty acids

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Gluconeogenic processes play an important role in the provision of energy in ruminants, especially in dairy cows. Most carbohydrates feed to ruminants are metabolized by microorganism in the forestomach to short chain fatty acids (SCFA). The prominent SCFA are acetate, propionate and butyrate. Another important energy metabolite is β-hydroxybutyrate (βOH), which is produced in the rumen wall and in the liver. The key enzymes of gluconeogenesis in the liver are pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase 1 and 2 (PEPCK1+2), and glucose-6-phosphatase (G6P). Of the SCFA, propionate is the only substrate for gluconeogenesis in dairy cows. Therefore, propionyl-CoA-carboxylase (PCCB) is the key enzyme for gluconeogenesis from propionate. Free Fatty Acid Receptors 2 and 3 (FFAR2/3) have recently been identified as receptors for SCFA. βOH is the endogenous ligand for the Hydroxycarboxylic Acid Receptor 2 (HCA2). In our investigations, we address the question whether SCFA and βOH affect the gene expression of their specific receptors and key enzymes of gluconeogenesis in the bovine liver cell line BFH12 [1].

Methods
First, we ran conventional polymerase chain reaction (PCR) with primer sets for all genes of interest and different housekeeping genes (β-Actin, GAPDH, HPRT, RPL13, SDHA). In a next step we looked at basic conditions of quantitative PCR (qPCR), like primer efficiency. BFH12 cells were cultured for 4 hours in a medium containing 3.3 mmol/l glucose without insulin and beside a control group they were treated with 1000 µmol/l acetate, 250 µmol/l propionate, 20 µmol/l butyrate or 1500 µmol/l β-hydroxybutyrate in different experimental approaches. qPCR was performed using the qPCRBIO SyGreen Mix Separate-ROX kit

from PCRBiosystems. Statistical analyses were performed with the REST© software [2].

Results
PCR analysis showed that the genes for PCCB, PC, PEPCK2 and the housekeeping genes are expressed in untreated BFH12. In qPCR studies, GAPDH and SDHA were identified as suitable reference genes. All genes of interest were expressed in untreated and treated cells. Under the experimental conditions chosen, SCFA and βOH treatment resulted in higher gene expression levels. FFAR3 expression was induced by all tested treatments while PC was up-regulated by acetate and butyrate.

Conclusion
In our study, we demonstrate that the expression of gluconeogenic enzymes and specific receptors is induced by SCFA and βOH in BFH12. These results suggest that the cell line BFH12 is a suitable *in vitro* model for the bovine liver.

References
[1] Gleich et al. (2016). *In Vitro Cell Dev Biol – Animal 52*, 662-672. doi:10.1007/s11626-016-0018-0
[2] Pfaffl et al. (2002). *Nucleic Acids Res 30*, e36. doi:10.1093/nar/30.9.e36

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Norecopa: Working to advance harmonisation and dissemination of best practice in animal research and testing

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The 2018 meeting in Linz will be remembered for the participants' enthusiasm in acknowledging the creation of new 3R centres in Europe, and for the initiative taken by EUSAAT to start closer collaboration between them. There is now a large number of European 3R centres, and it is important to get a clear view of their functions and goals, to avoid unnecessary duplication of effort, aid collaboration where that would be helpful, and to learn from the experiences of the older centres.

Norecopa has since 1991 had experience in maintaining databases of 3R resources. Its website (https://norecopa.no) currently contains over 8,000 pages with over 20,000 unique links. It serves as host for several initiatives, including the International Culture of Care Network and the EU Commission's 3Rs Knowledge Sources data set. Its own resources include an interactive map of European 3R Centres, with information on the function of each centre. Norecopa also hosts the website of the PREPARE guidelines for planning animal research and testing (https://norecopa.no/PREPARE). Interest in PREPARE has grown rapidly since its launch in 2017, since it provides guidelines for planning from day one, thereby complementing existing guidelines for reporting animal experiments. Other resources on Norecopa's website include: a database of alternatives to animal use in education and training; a database of literature within Laboratory Animal Science; a collection of global guidelines, 3R information centres, databases and other 3R resources; and presentations from international consensus meetings on the care and use of fish, wildlife and farm animals in research.

The aim of this presentation is to describe the experiences gained in networking, and the milestones achieved in establishing a global source of 3R resources.

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Apparent permeability coefficients of ciprofloxacin in human lung epithelial cells appropriately predict bioavailability after inhalation exposure

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Inhalative application of antibiotics is often considered in patients with pulmonary infections in order to achieve higher drug concentrations at the site of infection and to minimize systemic side effects. For the development of inhalable drug formulations, it is necessary to understand the pulmonary uptake mechanisms and to estimate the extent of systemic absorption. This study aimed at the determination of apparent permeability coefficients (Papp) of the inhalable antibiotic ciprofloxacin hydrochloride monohydrate (CHM) in human lung barrier models and to utilize these coefficients as input parameters for a lung PBPK model. Two different cell models were used to account for different lung regions: The Calu-3 cell line is a well-established immortalized cell line producing features of differentiated small airway epithelial cells. Functionally immortalized human alveolar epithelial cells (CI-hAELVi), resembling alveolar type 1 cells were used as model for the alveolar region.

Papp values were determined for submerged cultures and under air-liquid interface (ALI) exposure conditions. In both cases, the cells were cultured on permeable membrane inserts. For the submerged exposure, the antibiotic CHM was dissolved and added into the apical medium prepared for exposure. The cells exposed under ALI conditions were exposed to CHM aerosol generated using the PreciseInhale® device. For efficient and precise aerosol exposures, the P.R.I.T.®ExpoCube® was used. During the ALI exposure there was no liquid on the cells except for the lining fluid produced by the cells themselves.

After exposure the samples of the individual experiments were analysed by LC-MS/MS and the Papp values determined. The cells under submerged conditions showed Papp values of $6.34*10^{-7}$ cm/sec for AT-1 cells and $7.24*10^{-7}$ cm/sec for Calu-3 cells. If the same thickness is assumed for the lining fluid layer for Calu-3 and AT-1 cells (2 µm) the resulting Papp coefficients of $1.99*10^{-8}$ cm/sec (AT-1) and $1.09*10^{-8}$ cm/sec (Calu-3) for the experiments under ALI conditions are nearly identical.

The Papp values obtained from *in vitro* transport experiments were used as input parameters for the physiologically based pharmacokinetic model (PBPK model) to simulate the absorption and distribution processes in the human body. The results obtained from the simulation were finally compared to human literature data. Blood level concentrations of Ciprofloxacin calculated by the model, using Papp coefficients from ALI exposure, showed the same concentration-time course and concentration range as measured in human patients. These findings suggest that a combined approach utilizing *in vitro* data and *in silico* modelling is able to predict the bioavailable dose after inhalation exposure in humans.

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Biology-inspired micro-physiological system approaches to solve the prediction dilemma of substance testing

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Currently, compounds under preclinical development are being discarded owing to the use of inappropriate animal models. Progress in investigative toxicology could lead to humanized *in vitro* test systems and the development of medicines less reliant on animal tests. To advance this field, a group of 14 European-based leaders from the pharmaceutical industry founded the Investigative Toxicology Leaders Forum (ITLF), an open, non-exclusive, and pre-competitive group that shares knowledge and experience. The ITLF collaborated with the Centre for Alternatives to Animal Testing Europe (CAAT-Europe) to organize an "Investigative Toxicology Think Tank" (t[4]), a collaboration of the toxicologically oriented chairs in Baltimore, Konstanz and Utrecht sponsored by the Doerenkamp-Zbinden Foundation.

At a first meeting in Berlin in 2015 t[4] reviewed the status quo of "Microfluidic micro-physiological systems" (MPS) (also referred to as tissues-on-a-chip, organ-on-a-chip, multi-organ-chip, human-on-a-chip, body-on-a-chip or patient-on-a-chip tools), since the MPS technology holds promise for the development of approaches to reliably predict the safety and efficacy of novel drug candidates prior to their use in humans [1]. In 2017 a t[4] workshop, which was held in Ranco (Italy), discussed "how to optimize drug discovery by investigative toxicology" and published its analysis and recommendations earlier this year [2].

In June of 2019 the t[4] experts met again in Berlin with international experts from academia and regulators for drug efficacy and safety from around the world to discuss the roadmap towards the reduction and replacement of animals by MPS tools for the benefit of patients more precisely. An overview will be given on of the discussions and on the consensus reached.

References

[1] Marx, U., Andersson, T. B., Bahinski, A. et al. (2016). *ALTEX 33*, 272-321.
[2] Beilmann, M., Boonen, H., Czich, A. et al. (2019). *ALTEX 36*, 289-313.

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Human urine as a non-invasive source of kidney progenitor cells amenable for nephrotoxicity studies

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The prevalence for kidney-associated diseases such as acute kidney injury or chronic kidney diseases is on the rise. By effecting ca. 10% of the population worldwide it is an enormous challenge for the health and economic system. Due to these facts the demand for good quality human kidney-derived cellular models for research is increasing. Besides animal models, kidney biopsies are used to obtain cells for *in vitro* drug testing, toxicology studies and research purposes. However, this is an invasive process associated with risks for the patient.

Recently, amniotic fluid (composed of fetal urine) has been shown to be a reliable source of kidney-derived stem cells [1]. Obtaining amniotic fluid at term is also an invasive process. We have isolated and characterized UdRPCs (urine-derived renal progenitor cells) from 34 individuals (ages 1 to 79 years) of both genders.

Our protocol is non-invasive and free of ethical concerns and risks. As urine is an unlimited and easy accessible source, this method is very cost-effective. Over 20 million UdRPCs can be expanded in one month starting with 50 ml of urine.

UdRPCs have features of mesenchymal stem cells (MSCs)-they express Vimentin and the typical MSC cell surface markers-CD73, CD90 and CD105. They can be induced to differentiate into osteoblasts, chondrocytes and adipocytes, and have a cytokine profile similar to that of fetal MSCs by secreting molecules known to support tissue regeneration (Angiogenin, PDGFAA, VEGF, Osteopontin) and modulate the immune system (IL-6, IL-8).

Although not-pluripotent, UdRPCs express TRA-1-60, TRA-1-81, SSEA4, C-KIT and CD133, thus they can be reprogrammed very efficiently into induced pluripotent stem cells [2].

UdRPCs express the well-established renal progenitor self-renewal regulating transcription factors-SIX2, CITED1, OSR1 and WT1. They also express the typical bipotential renal progenitor cell surface markers CD106, CD24, and CD133. Unlike pluripotent stem cells, UdRPCs can be easily and efficiently differentiated into mature podocytes and renal epithelial tubular cells which exhibit transporter function as shown by Albumin uptake and the expression of numerous transporters such as Na-K-ATPase. Transcriptome analysis revealed a correlation co-efficient of 0.95 between a commercially bought kidney-derived renal epithelial tubular cells (hREPS) and UdRPC-differentiated renal epithelial tubular cells. To closer mimic the *in vivo* situation, UdRPCs can be used to generate an isogenic 3D nephron-progenitor cell model [3].

In summary, UdRPCs are a valuable source of human kidney derived cells, thus obviating the need for induced pluripotent stem cells. Furthermore, in combination with HLA and CYP2D6 analysis these cells will be better suited for research on nephrogenesis, nephrotoxicity tests and, drug screening thus enabling the reduction of animal experiments.

References

[1] Rahman, M. S., Spitzhorn, L. S., Wruck, W. et al. (2018). *Stem Cell Res Ther 9*, 113.
[2] Bohndorf, M., Ncube, A., Spitzhorn, L. S. et al. (2017). *Stem Cell Res 25*, 18-21.
[3] Nguyen, L., Spitzhorn, L. S. and Adjaye, J. (2019). *Stem Cells Int 2019*, 3298432.

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Development of a human pancreatic cell line with optogenetically controllable receptor for high content screening

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Pancreatic adenocarcinoma is a devastating disease that accounts for 7% of all cancer deaths in the US. The 5-year survival rate for people with pancreatic cancer is about 8% in average and depending on whether the cancer is detected at an early (32%) or late stage (3%) [1].

In humans, Toll like receptors (TLRs) are expressed in the pancreatic cancer tissue and in several cancer cell lines, whereas they are not expressed in the normal pancreas tissue [2]. Accumulating evidences indicate that TLRs are also involved in pathogenesis of autoimmune, chronic inflammatory and infectious diseases that often cause tumor development in consequence [3]. One of the reasons is, for example, that the activated form of TLR4 is able to stimulate NF-κB and inflammatory cytokine production, which is not only responsible for activating the immune system but also tumor development and progression [4].

Therefore, predictive cellular models are needed to i) understand the underlying molecular mechanisms of TLR4/NF-κB and carcinogenesis, ii) to identify new drug targets for the development of diagnostic tools and/or diagnostic profiles and drug design and iii) to discover new specific therapeutic agents that specifically inhibit the TLR4/NF-κB signaling pathway.

Optogenetics is an innovative technique where light-sensitive protein domains of photoreceptors are integrated into effector proteins to direct them with light stimuli. Consequently, light induction allows activation, inactivation, localization, or stabilization/destabilization of signaling pathways, depending on the protein type and setup This method has enriched the target and assay portfolio with a myriad of new approaches and was therefore chosen as the "Method of the Year 2010" by the journal *Nature Methods* [5].

We therefore engineered a new highly standardized opto-TLR4-Panc-1 cell lines (PANC-1; pancreatic carcinoma of ductal origin) using optogenetic manipulation, in which the TLR4 can be switched on by light (470 nm) and off in the dark. TLR4 activation and real-time detection of the underlying signaling pathways can be confirmed with the stable integrated NF-κB-Gaussia Luciferase reporter system.

The newly established opto-TLR4-Panc-1 Cell Line enables screening without additives but minimal operation steps and increased information and is thus an ideal tool for phenotypic and signal-specific drug discovery.

References

[1] American Cancer Society (2018). Cancer Facts & Figures 2018.
[2] Morse, D. L. and Gillies, R. J. (2010). *Biochem Pharmacol 80*, 748-754. doi:10.1016/j.bcp.2010.04.01
[3] Korneev, K. V., Atretkhany, K. N., Drutskaya, M. S. et al. (2017). *Cytokine 89*, 127-135. doi:10.1016/j.cyto.2016.01.021
[4] Plantinga, T. S., Ioana, M., Alonso, S. et al. (2012). *J Innate Immun 4*, 168-175. doi:10.1159/000329492
[5] Pastrana, E. (2010). *Nat Methods 8*, 24-25. doi:10.1038/nmeth.f.323

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Safety testing of adult novelties using methods *in vitro*

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The first adult novelties (or so-called sex toys) in history appeared in ancient times and are widely used particularly in the last decades. Despite their widespread and prolonged usage (e.g. Kegel balls recommended for strengthening of pelvic floor muscles) their health safety is not regularly tested or legally regulated. The aim of our pilot study was to subject extracts of 20 adult novelty products, i.e. complex test items, purchased on the Czech market to toxicological testing by means of selected bioassays, including tests for cytotoxicity, sensitization and endocrine potential. The NRU Cytotoxicity Assay was performed according to standard ISO EN 10993-5:2009 for testing of medical devices. Two *in vitro* tests for determination of sensitization potential were performed, namely DPRA (OECD TG 442C), *in chemico* procedure addressing the molecular initiating event leading to skin sensitization, namely protein binding reactivity, by quantifying the reactivity of test chemicals towards model synthetic peptides containing lysine or cysteine, and LuSens (ARE-Nrf2 luciferase test method, OECD TG 442D), covering the second key event on the adverse outcome pathway leading to skin sensitization utilizing human keratinocytes transfected with stable reporter gene for luciferase. Estrogen and androgen activity of the tested extracts was assessed using YES/YAS assay (Xenometrix, CH) based on genetically modified Saccharomyces strains. Cytotoxicity effects were elicited by of the tested samples by NRU cytotox-

icity assay and also by LuSens assay (mainly samples from latex and PVC). Sensitization potential was recorded for several of the tested sample extracts by both methods (DPRA, LuSens). As the most sensitization material show latex and some kinds of plastic (PVC, ABS). Regarding endocrine disruption, some sample elicited antiestrogen and antiandrogen effects and some sample exhibited androgen potential. Positive results suggesting possible adverse health effects were recorded repeatedly for few low-cost nonbranded adult novelties. The study has confirmed promising usefulness of the test method battery with regard to safety testing of this type of consumer products and the study is planned to continue in a larger set of products.

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Refinement in education

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The principles of 3Rs should not be the only basis of the planning of an animal experiment but also a highlighted part of the education. The usage of experimental animals cannot be replaced in 100% by alternative methods. Thus, consequently we should focus on reduction and refinement. Efforts must be put into the education to reduce the number of used animals in courses and to refine the learning procedures. The teachers must find the good balance between good practice and animal welfare. In our courses we endeavour to reduce the number of used animals and to minimize their suffering. If we set the good example it will result in more responsible researchers and animal caretakers. We make the best of refinement year by year to improve the animal welfare.

How can we apply the refinement in the education? The animals arrive at least 2 weeks before the practices. The assistants handle them every day to get them accustomed to the procedures.

As a first step we teach the proper handling and treatment of the animals. If somebody has never held animals, it results in a stressful situation not only for him/her but also for the animals. In a short time the animals become nervous, the continuous untrained handling causes stress. The repeated treatments results in aggression. To avoid this we use slight analgesia. The animals are more patient, do not feel stress and pain, do not attack and the participants and become stress less as well.

The treatment volume in practices should be reduced as less as possible. We have more animals than basically necessary. The animals are changed frequently during the practices meaning less stress each of them.

For invasive interventions, including blood sampling, abdominal operation, we use surgical platform integrated warming. For gavage we can use polypropylene feeding tubes, which can reduce the risk of trauma. Participants should be experienced using metal tubes before gavaging with plastic tubes.

To reduce the stress of the rabbits, we use a diaper-like textile instead of using simple surgical towel fixed around the animal body by clamps. This restrain-textile is made of softshell, that is soft but strong, washable and it can be fixed by velcro fastener.

We introduce two solutions for measuring the body temperature. One is with rectal-probes and the other is a non-invasive method with infrared light. For blood sampling from tail veins we use restraint tubes. The animals do not like the tube with strong light. Our novelty is to use red restrain-tube. We could easily follow-up the reaction of the animals and the red colour calm down them.

Refinement is the result of complex planning and implementation. We take the opportunity of the innovations, good ideas and our many years of experience and apply them in our course and introduce them to our participants. Nowadays we have many possibilities of refining methods. All ways that can support the animal welfare should be encouraged because many tiny steps can drive closer to the 3Rs.

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Uric acid metabolism and excretion of translucent skin mutants of 5th instar larvae of the silkworm "o06" strain as an animal model of hyperuricemia

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Mutant silkworms, called "Aburako", have decreased ability to store uric acid (UA) in the epidermis, which then builds up in the hemolymph and causes the skin of the mutants to become translucent. Our study explored the use of the mutants of the silkworm "o06" strain as a possible substitute for the mouse hyperuricemia model in medical studies [1]. Here, we discuss the o06 strain as a possible substitute and examine its UA metabolism and excretion.

Methods
UA and allantoin (AL) were measured in the o06 strain (3 to 6 days of the 5th instar) from the National BioResource Project (NBRP) silkworm collection. The UA content was analyzed using improved high-performance liquid chromatography with ultraviolet detection [2], and the quantity of AL was determined of hydrophilic interaction liquid chromatography. The silkworm mutants were then treated with Febuxostat (a xanthine oxidase inhibitor) for hyperuricemia.

Results
There was no difference in the total amount of UA excreted (mg/individual) between normal and diet groups. The UA level in the hemolymph of the diet group was lower than that of the normal group. Because 5th instar larva of strain o06 contain less AL, they do not express uricase, an enzyme that degrades UA to AL, as

Hayashi showed *in vitro* [3]. Purine metabolism is similar in silkworms and humans and the end product of both is UA. Our results indicate that strain o06 is a potential substitute for the mouse hyperuricemia model.

References
[1] Tanaka, R. et al. (2016). The use of translucent larval skin mutants of the silkworm "o06" as animal models of hyperuricemia. XXV International Congress of Entomology 2016, Proceedings.
[2] Tanaka, R., Miyata, Y. and Fumitoshi Sakazaki, F. (2013). *Gout and Nucleic Acid Metabolism 37*, 117-125.
[3] Hayashi, Y. (1960). *Nature 186*, 1053-1054.

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Drivers for the pharmaceutical industry to adopt human stem-cell based models

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For many years, the pharmaceutical industry has successfully applied a gamut of *in vitro* and *in vivo* models to develop chemical entities into therapies for a vast range of diseases. Although this strategy was successfully applied for decades within pharma, to-date, now that the treatment opportunities target more complex disease-states and the intervention has to surpass that of the existing therapies, there is a growing call for models that closer resemble human physiology and complex human diseases. In addition to this, the timeless 3R-objective to replace research using animals has motivated scientists to further explore alternative opportunities, not only for efficacy – but also in safety pharmacology. To meet these objectives, the rapid evolution of human induced pluripotent stem-cell (hiPSC) based models seems to be an attractive and tangible option. Within safety pharmacology, we explored different methods using human stem-cells both in the area of cardiovascular and neuronal safety research (since 2009). These objectives subsequently dovetailed into regulatory incentives (like CIPA in 2012 https://cipaproject.org/) to explore the use of stem-cell based technology. We extensively characterised cardiomyocytes, using a large set of clinically positive and negative proarrhythmic compounds to explore the proarrhyth-

mic potential, both after acute and chronic dosing, using calcium transient - and impedance approaches, respectively. In a similar fashion, we are developing and characterising a human stem-cell based neuronal model for detection of seizure liabilities using micro-electrode array technology. After internal method validation, we participated in several cross-company consortia (e.g. HESI Neutox) to grow confidence in the reproducibility and predictive value of these models. Currently, we have implemented the hiPSC-cardiomyocyte model in our derisking flow chart, thereby totally replacing a classical Guinea Pig right-atrium and rabbit Langendorff organ-bath models. In addition, we've implemented a rat primary neuron cell-based assay for detection of neuronal tox, that will hopefully be quickly replaced by hiPSC neurons, ones some technical limitations can be overcome.

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Ethical justification of animal experiments in Germany

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All animal ethical positions are largely in agreement that animals – as beings capable of suffering – must be morally considered for their own sake and that certain consequences for one's own actions must be derived from this. This insight has been incorporated into animal protection legislation based on the EU Directive 2010/63.

German legislation requires a reasonable justification of the pain, suffering, and harm inflicted on animals. For this reason, every scientist must demonstrate ethical justifiability of the intended experiment in accordance with the principle of proportionality within the framework of the approval procedure of animal experiments.

More specifically, it must be demonstrated that no alternative method in reaching the project's aims exists. Furthermore, the project's indispensability must be scientifically explained and it must be assigned to a permissible purpose. Study planning must be carried out by implementing statistical methods to reduce the number of animals and their burden to the indispensable level. Animal keeping and medical care must be ensured by the permission to keep and breed animals in the context of a culture of care. Ultimately, the expected gain in knowledge must be set in relation to the burden inflicted on the animals and must be ethically justifiable or may even be considered an ethical imperative.

The scientist's proposal and declarations are then revised by the animal welfare officer and, if applicable, by the ethics committee of respective institution. It is then further examined by the local authorities and the §15 Commission, in which ethics experts and animal welfare organizations are actively involved. After this revision process, also involving the responsible scientist, the final examination and approval is carried out by the local authorities.

It must be considered that ethical concepts and attitudes of society may be subject to change in the course of time. Hence, a high degree of transparency is necessary in order to maintain public approval.

References

Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes (2010). *Off J Eur Union L276*, 33-79.

Animal Welfare Act in the version published on 18 May 2006 (Federal Law Gazette I p. 1206, 1313) amended by article 4 section 90 of the Act of 7 August 2013 (Federal Law Gazette I p. 3154).

Animal Welfare/Laboratory Animal Ordinance of 1 August 2013 (Federal Law Gazette I p. 3126).

Workshop report on guidance on determining indispensability and balancing potential benefit of animal experiments with costs to the animals with specific consideration of EU Directive 2010/63 EU.

RSPCA and LASA (2015). Guiding Principles on Good Practice for Animal Welfare and Ethical Review Bodies. A report by the RSPCA Research Animals Department and LASA Education, Training and Ethics Section. (M. Jennings, ed.).

Nuffield Council on Bioethics (2005). The ethics of research involving animals.

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Set-up of an *in vitro* physiological relevant 3D-model of human ocular trabecular meshwork to verify therapeutic strategies for glaucoma

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The second major cause of blindness in the world is considered to be Glaucoma, a progressive optic neuropathy related to the death of the retinal ganglion cells and impairment of the visual field. The main features of this neuropathy are an elevated intraocular pressure (IOP) due to trabecular meshwork (TM) damage and extracellular matrix (ECM) turnover failure. Oxidative stress (OS) plays a key role in the onset of Primary Open Angle Glaucoma and consequently of the IOP increase. Therefore, OS represents a relevant target for both prevention and therapy [1-3]. To date, the etiology of the disease is partly still unknown and the lack of human-based glaucoma models has caused one of the main constraints for prevention and treatment therapies since current therapies are focused on lowering IOP values, without preventing blindness.

Starting from these observations, for the past year or so, our research team has put a substantial effort into the design and development of an *in vitro* physiological relevant 3D-model of human ocular Trabecular Meshwork cells (HTMC) based on millifluidic technology in order to define the key elements associated with glaucoma onset. Briefly, HTMC (Cell Application INC) cultures in 3D by Matrigel® Matrix were performed under dynamic conditions by using millifluidic bioreactors connected to a peristaltic pump (Live Box 1 and Live Flow, IV-Tech srl) with a constant flow rate [4]. Prolonged OS was induced by a 2-hour daily exposure to 500 μM H_2O_2, and the increased pressure flow was obtained by a dedicated device that was applied to the millifluidic circuit (Live PA, IvTech) for 10 h/day. Both stressor conditions were performed up to 7 days. Our preliminary results showed a good healthy state in terms of Alamar blue assay and confocal images during prolonged OS. Moreover, under these experimental conditions, the HTMCs evidenced an NF-κB and TNF-α activation without triggering an apoptosis program, and it is conceivable that these cells had adopted an adaptive cellular response. After 48 hrs of FR boost, the HTMCs showed an increase in several ECM profile genes, such COL1A1, SPARC and MYOC. These findings suggest that both OS and FR can be reproduced in our 3D-HTMC model and, therefore, our model can be considered a tool to mimic the crucial stages of glaucoma. Further signaling pathways of cellular adaptive response are presently being investigated. Our 3D-HTMC dynamic platform can be further improved by its millifluidic feature that allows the cross talk among different cell types to verify how the TM damage could pass onto the optic nerve. Based on these reported observations, this *in vitro* platform can provide a new and useful tool to identify the warning signs as well to test drugs for glaucoma therapy.

References

[1] Quigley, H. A. (1996). *Br J Ophthalmol 80*, 389-393.
[2] Acott, T. S. and Kelley, M. J. (2008). *Exp Eye Res 86*, 543-561.
[3] Awai-Kasaoka, N., Inoue, T., Kameda, T. et al. (2013). *Mol Vis 19*, 1332-1340.
[4] Ucciferri, N., Sbrana, T. and Ahluwalia, A. (2014). *Front Bioeng Biotechnol 2*, 74.

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Learning from negative results in animal based research! Critical Incident Reporting System in Laboratory Animal Science (CIRS-LAS)

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The European Commission announced that around 11.5 million animals were used for scientific purposes in EU Member States in 2011 [1].

In view of this number, the relevance of the question of consistent implementation of the 3R principle in the planning, application, and performance of animal experiments is increasing. There is still a great lack of transparency in dealing with critical incidents in animal research. Negative experiences from animal experiments are often lost and rarely mentioned in publications.

For this purpose, the Central Experimental Animal Facility of the University Hospital Jena established a "Critical Incident Reporting System in Laboratory Animal Science" (CIRS-LAS) in 2015 [2], based on established CIRS in human medicine. Such reporting systems have long been standard in German hospitals and are mandatory under the Patient Act since 2013 [3]. In animal experiments, on the other hand, there have been no comparable systems with open access for error detection in the sense of modern risk management.

At this point the reporting system CIRS-LAS helps to clarify negative events and enables the development of error avoidance strategies by means of a cause analysis. The aim of CIRS-LAS is to contribute to the refinement of animal research practice and to the reduction of laboratory animals in accordance to the guiding 3Rprinciple in the field of laboratory animal science.

The CIRS-LAS project is supported by the German Federal Ministry of Education and Research (BMBF) [4] since 2017. CIRS-LAS makes it possible to report critical incidents online within a few minutes using an input mask and to submit them completely anonymously without prior registration or login. All registered users have the possibility to log in with a password to access the contents of the report archive and to directly exchange information about an incident with other scientists. In addition, a panel of experts provides support on possible causes of the critical event.

The high potential of a CIRS for laboratory animal science is proven by the ever faster growing number of registrations of new users and shows that CIRS-LAS is increasingly used as a tool for minimizing sources of error in the different areas of laboratory animal science. Currently, more than 100 people from Europe are already registered users of the CIRS-LAS portal and more than 50 critical incidents have already been entered.

In conclusion, the realization of 3R can be achieved by using the world's first published CIRS-LAS portal. It serves as a valuable tool to monitor the quality of animal care and give insight into the nature of critical incidents. Anonymously shared negative experiences from animal experiments can thus improve the experimental setup of animal based research and leads to an increased safety (refinement) of the laboratory animals and reduces their number by avoiding unsuccessful experiments (reduction). Furthermore, it is conceivable that the implementation of CIRS-LAS serves to enhance the trust in laboratory animal science of both, public and scientific community. The time has been achieved - to learn from negative results in animal based research!

References

[1] https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52013DC0859&from=EN
[2] Bischoff, S. J., Trietschel., D., Enkelmann, A. et al. (2018). *J Anim Res Vet Sci 2*, 009. doi:10.24966/ARVS-3751/100009
[3] http://www.bgbl.de/xaver/bgbl/start.xav?startbk=Bundesanzeiger_BGBl&jumpTo=bgbl113s0277.pdf
[4] https://www.bmbf.de/

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Serum-free media contribute to better reproducibility in *in vitro* research

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Reproducibility has become a major issue in the discussion of validity of scientific results. The recently drafted OECD "Guidance Document on Good In Vitro Method Practices (GIVIMP) for the development and implementation of *in vitro* methods for regulatory use in human safety assessment" [1], identifies and discusses several factors that contribute to this lack of reproducibility in *in vitro* methods.

One of the factors identified is the use of foetal bovine serum (FBS), which is still being applied as the universal medium supplement to grow and maintain cells and tissues. But, the use of FBS has also been regarded critically for decades. The use of FBS presents four significant issues:

(i) the degree of suffering experienced by the calf during blood collection [2];

(ii) inappropriate cellular growth profiles and physiological responses of cells;

(iii) FBS contamination with viruses, prions, etc.;

(iii) the large variability of FBS such that it is very difficult to even ensure consistent and well controlled *in vitro* cell culture between batches;

(iv) the fraud-problem [3].

Nevertheless, to date, FBS is used at a large scale, despite several attempts to develop FBS-free media. Recent years showed that human platelet lysates (HPLs) can be a valuable alternative to FBS as cell culture supplement. In addition, for several applications, there is large interest in chemically-defined media [4].

As HPLs work for most cell types but are undefined, chemically-defined media, on the other hand, work only for a specific cell type. To facilitate the search for serum-free media, the *fcs-free. org* database was recently established [5]. Not for every cell type is yet a serum-free medium available. It will be discussed how a serum-free medium for a specific cell type can be developed [6].

References

[1] OECD (2018). Guidance Document on Good In Vitro Method Practices (GIVIMP). *OECD Series on Testing and Assessment No. 286*. OECD Publishing, Paris. doi:10.1787/20777876

[2] van der Valk, J. et al. (2004). *Toxicol In Vitro 18*, 1-12. doi:10.1016/j.tiv.2003.08.009

[3] Gstraunthaler, G., Lindl, T. and van der Valk, J. (2014). *Altern Lab Anim 42*, 207-209. doi:10.1177/026119291404200308

[4] van der Valk, J. et al. (2018). *ALTEX 35*, 99-118. doi:10.14573/altex.1705101

[5] https://fcs-free.org/

[6] van der Valk, J. et al. (2010). *Toxicol In Vitro 24*, 1053-1063. doi:10.1016/j.tiv.2010.03.016

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3Rs databases: FCS-free database, Interspecies Database and Humane Endpoints website

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The availability of databases with specific 3R information may save time within the process of compliance to the 3Rs implementation, as required according to Directive 2010/63/EU. To be successful, these databases should be easily found, accessed, managed and updated. Designing, building and filling a database is a time and money consuming activity. Often, financial support can only be obtained in the development stage, whereas continuous updating and maintenance is essential for the sustainability of the databases. Continuous financial support is therefore crucial.

The 3Rs-Centre Utrecht Life Sciences (ULS) has initiated the 3Rs Database Programme, which aims to provide up-to-date 3Rs information free of charge, thereby contributing to the implementation of the 3Rs in research. The programme currently offers the FCS-free Database, the Interspecies Database and the Humane Endpoints website.

The *FCS-free database* (*FCS-free.org*) was launched in 2017 to raise awareness about the scientific and ethical problems related to the use of foetal calf serum (FCS), and to provide an overview of FCS-free media for specific cell types. Furthermore, the comment function in the database allows researchers to exchange information on the applicability of FCS-free media. This website will increase the reproducibility of *in vitro* methods and contributes to the replacement of animals used for research.

The *Interspecies Database* (*www.interspeciesinfo.com*) provides insight into physiological, anatomical and biochemical parameters of different animal species and humans. With the database, researchers can design their experiments smarter with respect to the choice of an animal model. This could lead to a reduction in the number of experimental animals.

The *Humane Endpoints website* (*www.humane-endpoints.info*) provides information and training modules on how to recognize and apply humane endpoints in laboratory animals. This helps to prevent unnecessary pain and distress in the animals. Therefore, the website contributes to refinement.

To guarantee a sustainable future for these websites and increase their usage, the 3Rs Database Programme is inviting partners who are willing to collaborate and support its activities.

For more information, visit *www.uu.nl/en/3Rsdatabases* or contact *3RsCentreULS@uu.nl*.

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An innovative *in vitro* physiologically relevant model as a tool to test therapeutic strategies for glaucoma

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Glaucoma, a progressive optic neuropathy that leads to irreversible blindness, is caused by increased intraocular pressure (IOP) and reduced optic nerve head vascular perfusion. Furthermore, much clinical evidence shows that increased IOP depends on a dysfunction in the trabecular meshwork cells (TM), implying that TM cells play a crucial role in maintaining normal IOP and could prevent glaucoma [1,2].

Currently, the animal and cell-based models do not fully explain the molecular mechanisms underlying glaucoma onset and its adverse outcome events, and therefore no efficient cure exists for this disease. To date, IOP is the only risk factor modifiable by therapies or surgical techniques [3]. Thus, it is crucial to deepen our knowledge of the early cellular events that underlie the pathogenesis of glaucoma, and to identify new therapeutic approaches for disease treatment and prevention [4].

Recently, our research group has been committed to setting-up and assessing a reliable *in vitro* 3D human-based TM cellular (HTMC) model in a millifluidic system (IV-Tech srl, Italy) in order to mimic the conditions of a prolonged exposure to oxidative stress and pressure flow increase. The performance of this innovative 3D dynamic model, as a useful platform to test chemicals for a therapeutic approach for glaucoma disease, was assessed by analyzing the biological property of a dry extract of a polyphenol mixture (PM), an active compound of commercial eye drops for glaucoma, in counteracting the effects of prolonged oxidative stress (OS) and increased pressure flow (IPF). Briefly, HTMCs (CellAPPLICATION INC) were embedded into a Corning Matrigel™ Matrix and set in a millifluidic bioreactor system connected to a peristaltic pump (Live Box 1 and Live Flow, IV-Tech) with a constant flow rate [5]. Prolonged OS was induced by a 2-hour daily exposure to 500 µM H_2O_2, and the IPF was obtained by a dedicated device that was applied to the millifluidic circuit (Live PA, IvTech) for 10 hrs/day. Both stressor conditions were performed up to 7 days. The PM (0.15%, v/v) was added to the culture media for 2 hrs after daily exposure to oxidative or mechanical stressors.

The effects of the PM were verify by several end-points that included: cell morphology by confocal analysis; healthy metabolic state by Alamar blu assay; ROS production by Dichlorofluorescein assay; inflammatory cytokine (IL-1, IL-6 and TGFβ) and Matrix Metallopeptidase 1 and 9 (MMP1 and MMP9) gene levels by real-time PCR.

Preliminary data evidenced that the PM was able to counteract the effects of OS and IPF after 48 hrs of treatment. Indeed, a significant decrease of ROS production was observed in OS-exposed HTMCs and a significant reduction of IL-1, IL-6, TGFβ, MMP 1 and MMP9 gene expressions were encountered both in OS and in IPF-stressed HTMCs. These results seem very promising since, for each experimental condition, the PM was seen to interfere with the upregulation of pro-inflammatory and pro-fibrotic genes. Taken together, it can be assumed that our *in vitro* model can be exploited as a platform to test the efficiency of therapeutic approaches to glaucoma.

References

[1] Acott, T. S. and Kelley, M. J. (2008). *Exp Eye Res 86*, 543-561. doi:10.1016/j.exer.2008.01.013

[2] Saccà, S. C., Gandolfi, S., Bagnis, A. et al. (2016). *Ageing Res Rev 29*, 26-41. doi:10.1016/j.arr.2016.05.012

[3] Vranka, J. A. and Acott, T. S. (2017). *Exp Eye Res 158*, 67-72. doi:10.1016/j.exer.2016.06.009

[4] Aires, I. D., Ambrósio, A. F. and Santiago, A. R. (2017). *Ophthalmic Res 57*, 77–86. doi:10.1159/000448480

[5] Ucciferri, N., Sbrana, T. and Ahluwalia, A. (2014). *Front Bioeng Biotechnol 2*. doi:10.3389/fbioe.2014.00074

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The Danish 3R-Center

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3R-News

The 3R-Center's website focuses on disseminating research and news relevant to persons interested in or working with 3R and laboratory animal science www.en.3rcenter.dk/

Research funding

Each year the 3R-Center funds 3R related research projects. Funded projects must be of a high scientific quality and relevance. Every year 1.5 million DKK is reserved for research funding.

3R-Symposium

The annual international symposium is a significant event where around 200 researchers, veterinarians, animal caretakers, and other people with an interest in 3R meet and network. The program is composed of Danish and international speakers and covers all three Rs. The 2019 Symposium will take place on November 12-13.

Teaching material

The 3R-Center has prepared teaching material for use in Danish lower and upper secondary classes. The material is free of charge.

Board members

Christine Lydia Nellemann (Chairman of the Board), Adrian Smith, Axel Kornerup Hansen, Erwin L. Roggen, Jan Lund Ottesen, Lisbeth E. Knudsen and Peter Bollen.

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Beating organs-on-chip as advanced tools in drug screening: Engineered *in vitro* models of human organs and diseases

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Drug development continuously faces challenges in efficiently predicting toxicity and verifying efficacy of new compounds during the early pre-clinical phases [1]. Organs-on-Chips (OOCs) have recently emerged as innovative *in vitro* tools holding the potential to improve prediction over human drug responses earlier in the timeline, thus reducing failures and costs of the clinical trials [2].

Here we present new beating OOCs providing cells with a 3D environment and mimicking the mechanical stimulation that tissues sense *in vivo*. This results in improved functionalities or induction of pathological changes. Our technology, named uBeat, relies on specific geometrical structures that modulate the mechanical deformation exerted on 3D microtissues, to achieve different magnitudes of either uniaxial strain or confined compression. Based on uBeat, we developed two platforms: i) uHeart, a spontaneously beating heart-on-chip integrating real-time measurement of cardiac electrophysiological signals [3] and ii) uKnee, the first *in vitro* 3D model of human osteoarthritic (OA) cartilage on chip [4]. We also exploited our models for drug screening purposes, by testing the effect of well-known compounds.

uHeart provides 3D cardiac microtissues with a physiological cyclic uniaxial strain (~10%, 1Hz) and integrates electrodes to specifically measure the field potential (FP) signals online. Cardiomyocytes from human induced pluripotent stem cells (hiPSC-CMs) and human dermal fibroblast (h-DFs) were embedded in fibrin hydrogel in a 3:1 ratio and cultured for 7 days within uHeart. The cardiac model developed a synchronous beating and the electrical activity was recorded through paired electrodes specifically inserted. We preliminary calibrated the system by assessing the pro-arrhythmic effect of known compounds (i.e. Verapamil, Sotalol and Terfenadine) on uHeart electrical activity. Aspirin and DMSO were used as negative control and vehicle, respectively. As expected, Terfenadine and Sotalol prolonged the repolarization time of cardiac microtissues at 100-1000 nM and 10-60 μM, respectively. In contrast, Verapamil decreased the FP duration and both Aspirin (up to 100 μM) and DMSO (up to 0.5% w/v) did not alter the beating properties.

uKnee provides 3D cartilage-like constructs with either physiological (10%, 1Hz), or hyper-physiological compression (30%, 1 Hz). The latter is able to elicit OA pathogenesis by mechanical factors. Primary human articular chondrocytes were embedded in a poly(ethylene-glycol)-based hydrogel and cultured in uKnee under static chondrogenic conditions for 2 weeks. Deposition of a cartilage-like matrix was demonstrated by immunofluorescence staining (i.e. Aggrecan, Collagen II) and a stable cartilage phenotype was evidenced by the increased expression of specific genes (i.e. ACAN, PRG4, ATX, FRZB and GREM1). After maturation, constructs were subjected to additional 7 days of confined compression at both intensity levels. Hyper-physiological compression induced OA-like traits and significantly enhanced catabolic and inflammatory response, as evidenced by MMP13 and IL8 gene expression. Responses to 4 drugs already used in the clinic (i.e. Rapamacyn, Celecoxib, IL-1Ra and dexamethasone) were assessed and resulted consistent with data from animal studies, probing the potential of uKnee as anti-OA drugs screening platform.

Our new technology (uBeat) allows to develop beating OOC as powerful and reliable pre-clinical tools for efficient *in vitro* drug screening and disease modelling.

References
[1] Esch, E. W., Bahinski, A. and Huh, D. (2015). *Nat Rev Drug Discov 14*, 248-260. doi:10.1038/nrd4539
[2] Sutherland, M. L., Fabre, K. M. and Tagle, D. A. (2013). *Stem Cell Res Ther 4, Suppl 1*, I1 doi:10.1186/scrt361
[3] Marsano, A., Conficconi, C., Lemme, M. et al. (2016). *Lab Chip 16*, 599-610. doi:10.1039/C5LC01356A
[4] Occhetta, P., Mainardi, A., Votta, E. et al. (2019). *Nat Biomed Eng 3*, 545-557. doi:10.1038/s41551-019-0406-3

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A novel model for mechanical assessment of biomaterials

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Research for tissue regeneration materials is one of the most prominent topics of current medical research. While the search for optimal biomaterials is flourishing as improved and more sophisticated implants are developed a certain aspect considering the characterization of the implants is still neglected leading to improper choice of animal species for *in vivo* experiments, failed experiments and waste of resources. The assessment of an implant's mechanical properties is crucial as these parameters greatly influence the efficiency of the operative procedure and the short-term postoperative results. Although different approaches are available, what tends to be neglected is the context of the implantation itself, namely the surgical fixation. Suturing is by far the most common method of fixation yet most of the implants are assessed by simple hooks or clumps using uniaxial mechanical testers.

In this regard, we developed two novel models for assessing the mechanical parameters of biomaterials. One model (Simple Running Suture Model) was created to examine the uniaxial loading capacity of sutured membranes for example, surgical meshes used in abdominal hernia repair. A custom-made adapter was used and samples (3.5 x 4.5 cm) were fixated on both sides with a modified simple running suture. Different measuring profiles were created to assess the sutured samples elasticity, durability and strength. Another model was created to further recreate surgical implantation, in this case circular samples of different sizes (D1 = 4 cm, D2 = 8 cm, D3 = 10 cm) were created and sutured along the inner wall of a cylinder. A custom compression head replicating the shape of the abdominal wall applied force on the centre of the samples and indentation was measured. Samples were assessed in air and under liquid (physiological saline) to attempt and recreated the implantation area environment. Investigations were performed on different biomaterials synthesized in our laboratory and commercially bought. Comparative control measurements were also performed using the conventional setup. All examinations were done with an Instron 4952 mechanical tester.

Results were highly conclusive as we observed a definitive difference in the maximal loading capacity when the samples are sutured. In addition, different materials exhibit different properties. Results suggest that samples of viscoelastic nature for example Poly(vinyl alcohol) membranes can better handle suturing compared to more durable yet rigid ones like Polypropylene.

To conclude, we have demonstrated that the current mechanical assessment of biomaterials such as surgical meshes, tissue engineering scaffolds and other materials is currently inadequate and can be indeed improved. Neglect of a comprehensive mechanical assessment can will lead to issues with the surgical handling during operation or inadequate fixation of implants leading to failure of animal experiments. Modifications and alterations have to be made in order to assess the implants in the setting of the implantation itself with considerations regarding the function of the specific implant.

References

Baylon, K., Rodríguez-Camarillo, P. et al. (2017). *Membranes 7*, 47. doi:10.3390/membranes7030047

Xinxin, Li, Kruger, J. A. et al. (2014). *J Mech Behav Biomed Mater 37*, 48-55, doi:10.1016/j.jmbbm.2014.05.005

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Establishment of an organotypic photoreceptor model as an *in vitro* alternative for future retina degeneration studies

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Purpose
The age-related macular degeneration (AMD) is the leading cause of blindness among people over 50 years of age. Since it is a multifactorial disease, its causes are still unknown, and it is difficult to find suitable models. To investigate possible therapeutic approaches, a new organotypic porcine culture model was developed. Therefore, an established porcine degeneration model [1] was modified to enable improved photoreceptor cultivation and to make it applicable for AMD research.

Methods
To establish the AMD model two methods, namely "filter" and "tweezers", were tested to gain porcine neuroretina explants, with the ganglion cell layer facing down. Retinas were cultivated for 4 and 8 days and compared to explants gained via an established method, photoreceptors facing down. To characterize the explants optical coherence tomography (OCT; n = 6/group), H&E staining, immunohistochemistry (n = 9/group), and qRT-PCR (n = 5/group) were performed. More specifically, morphology of cones (opsin), rods (rhodopsin), amacrine (calretinin), bipolar (PKCα) and retinal ganglion cells (RBPMS, β-III-tubulin) was evaluated.

Results
OCT analyses revealed a decrease of retinal thickness to a lower extent in "tweezers" compared to "filter" (p ≤ 0.001) and "established" method (p = 0.04). Moreover, measurements of retinal thickness via H&E staining showed for both new methods (filter: 28.6 ±1.1 μm; tweezers: 28.2 ±0.8 μm) a significantly improved photoreceptor structure compared to the established method (22.6 ± 1.1 μm; filter: p = 0.002; tweezers: p = 0.003). Additionally, the rhodopsin+ area was significantly increased in the "filter" (6.9 ±0.2 area[%]/image; p = 0.0005) and "tweezers" group (6.3 ±0.1 area[%]/image; p = 0.048) in contrast to the established one (5.4 ±0.1 area[%]/image). In contrast, the number of cones was only higher in "tweezers" method (162.0 ±1.3 cells/mm) compared to the established one (141.1 ±2.5 cells/mm; p = 0.035). The amount of amacrine, bipolar and retinal ganglion cells was unaltered. On mRNA level, we revealed an upregulation of Rhodopsin (filter: p = 0.048) and Opsin (tweezers: p = 0.045) in both new methods compared to the established one.

Conclusion
This project aimed to develop a more suitable *in vitro* photoreceptor degeneration model. Both novel methods showed a significantly better retention of the photoreceptors compared to the established degeneration model. The cultivation by the "tweezer" method led to a significantly improved morphology comparable to the *in vivo* situation. Subsequently, to establish the AMD model a co-cultivation of neuroretina and RPE-cells will follow. Hence, this method has the potential to reduce or even replace animal testing in clinical research. Consequently, this system may serve as a first *in vitro* model for AMD drug screening.

Reference
[1] Kuehn, S., Hurst, J., Jashari, A. et al. (2016). *Altern Lab Anim 44*, 557-568. doi:10.1177/026119291604400608

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The kinetic Direct Peptide Reactivity Assay (kDPRA): An *in chemico* method to characterize the skin sensitization potency of chemicals

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While the skin sensitization hazard of substances can readily be identified using non-animal methods, the classification of potency into UN GHS sub-categories 1A and 1B remains challenging. The kinetic direct peptide reactivity assay (kDPRA) is a modification of the DPRA (OECD TG 442C) wherein the reaction kinetics of a test substance towards a synthetic cysteine-containing peptide is evaluated. For this purpose, several concentrations of the test substance are incubated with the synthetic peptide for several incubation times at 25°C. After the respective incubation time, the reaction is stopped by addition of the fluorescent dye monobromobimane (mBBr). The highly reactive and non-fluorescent mBBr rapidly reacts with unbound cysteine moieties of the model peptide to form a fluorescent complex. The remaining non-depleted peptide concentration is determined thereafter by fluorescence measurement at precisely defined time points. Kinetic rates of peptide depletion are then used to distinguish between two levels of skin sensitization potency, i.e. to discriminate between CLP/UN GHS sub-categories 1A and 1B. During an in-house validation [1] 35 of 38 substances with LLNA-based sensitizing potency were correctly assigned to the potency sub-categories, and the predictivity for 14 human data was similarly high. These results warranted the kDPRA for further validation. Here we present the results of a ring trial testing 24 blind-coded chemicals in seven labs[1]. In parallel we present the extension of the kDPRA database to further assess the predictive capacity of the assay. Eventually the kDPRA should be used as a part of defined approach(es) with a quantitative data integration procedure for skin sensitization potency assessment.

Reference

[1] Wareing, B., Urbisch, D., Kolle, S. N. et al. (2017). *Toxicol In Vitro 45*, 134-145. doi:10.1016/j.tiv.2017.08.015

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[1] Upon the submission deadline the ring trial was still in progress and the substance identities remained blind coded. Therefore, no results could be presented in the abstract.

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There is no ethical justification for the use of fetal bovine serum (FBS)

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Fetal bovine serum (FBS), derived from the blood of unborn bovine fetuses, is still a widely used cell culture media supplement. However, both ethical and scientific concerns on the collection and use of FBS are already well-known and continue to be addressed [1]. Despite these concerns, a current article authored by representatives of the serum producing industry negated any ethical concerns in collecting blood for FBS production (when the fetuses were already dead) postulating instead that discarding "available fetal tissues obtained from the slaughter of pregnant cattle" would be "unethical", especially since FBS "contributes greatly to the advancement of the life sciences industry, as well as the replacement and reduction of live animals used in research and testing" [2]. We therefore deem it necessary to show that this line of argument is completely unacceptable from an ethical, legal and animal welfare point of view. It can never be ethically justified to harm a healthy (unborn) animal just for retrieving a product for industrial use if there is a feasible alternative available. Instead of justifying FBS collection as a necessary evil and even denying all ethical and scientific concerns that are already well known and accepted, we should try to change the system that creates the possibility to have FBS collected without any legally binding regulations aimed at avoiding animal harm. In our contribution, also the other arguments are examined that are presented in the article to justify collecting and using FBS (e.g. that the pregnancy status of female cattle is often unknown to the cattle owner before the animals are transported to the slaughterhouse) and show that these arguments are lacking substance and therefore should not be accepted. The current EU legislation that animals in the last 10th of pregnancy should not be transported will be addressed. Some countries have, or will, prolong this period to the last 25% of pregnancy. The current legislation (or lack thereof) concerning the collection of FBS will be examined and demands to put regulations in place that prevent both pregnant cattle and their unborn fetuses from suffering will be presented. Furthermore, the criticized article [2] disregards the scientific disadvantages of FBS as a variable and undefined cell culture medium component with a complex composition which can interfere with phenotypic cell stability and may influence experimental outcomes [1,3]. Instead, the ubiquitous abundance of FBS in cell culture media formulations is described as an achievement *per se* and not as a result of its decade long unquestioned indefeasibility as a standard media supplement. The common use of FBS and its resulting uncertainties in the current scientific reproducibility crisis should instead be an incentive to move to a human-based, xeno-free culture system or even chemically-defined media, e.g. OECD's Guidance Document on Good *In Vitro* Method Practices (GIVIMP) recommends to develop new *in vitro* methods with a serum-free, chemically-defined medium [3] to avoid potential sources of uncertainty that may be introduced by using animal serum [4,5]. For many cell types serum-free media already exist [6].

References

[1] van der Valk, J., Bieback, K., Buta, C. et al. (2018). *ALTEX 35*, 99-118. doi:10.14573/altex.1705101

[2] Nielsen, O. B. and Hawkes, P. W. (2019). *BioProcess J 18*. doi:10.12665/J18OA.hawkes

[3] OECD (2018). *OECD Series on Testing and Assessment No. 286*. OECD Publishing, Paris. doi:10.1787/9789264304796-en

[4] Jochems, C. E. A., van der Valk, J., Stafleu, F. R. et al. (2002). *Altern Lab Anim 30*, 219-227. doi:10.1177/026119290203000208

[5] Pamies, D., Bal-Price, A., Simeonov, A. et al. (2017). *ALTEX 34*, 95-132. doi:10.14573/altex.1607121

[6] Fetal Calf Serum-Free Database. https://fcs-free.org/

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Towards veterinary ethics

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The implementation of Veterinary Medical Ethics in the curricula of veterinary education is not an entirely new phenomenon. But the implementation of this subject in the curricula "has been a creeping progression rather than a huge leap" [1]. This counts especially for the German-speaking area, where the establishment of ethics in curricula and the institutionalization of veterinary ethics is still patchy. The "Netzwerk Tiermedizinische Ethik (NTE)" (Network Veterinary Ethics) has been founded in 2017 in order to promote the upcoming research field. By presenting the networks aims, program and current projects, it will be shown, what veterinary ethics are about and what can be expected from its implementation. Firstly, veterinary ethics are characterized by interdisciplinarity. Current research in Veterinary Ethics thus differs from traditional professional ethics by the integration of concepts and approaches of humanities into veterinary medicine [2]. The NTE aims on breaking down barriers between the humanities and veterinary medicine/science, e.g. by promoting ways to a non-hierarchical cooperation between the disciplines. Veterinary Ethics both differ from and link to animal welfare science and animal research ethics. Both disciplines are strongly related to veterinary ethics and already established veterinary faculties. An ongoing research project of the NTE on the ethics of pain assessment in animal research will serve as an example for interdisciplinary research in veterinary ethics. Concluding, it will be argued, that veterinary ethics shouldn't be mistaken as a mere application of animal ethics on veterinary contexts. Instead, unique concepts and models have become developed in veterinary ethics, in order to grasp the ethical dimensions of veterinary clinics. Ethical reflection on the status of animal as patients clearly exceeds a welfare approach, moreover it is informed not only by animal, but also by medical ethics [3].

References

[1] Gray, C. (2014). *Vet Rec 175*, 590-591. doi:10.1136/vr.g7451
[2] Weich, K. and Grimm, H. (2018). *Food Ethics 1*, 205-208. doi:10.1007/s41055-018-0025-9
[3] Huth, M., Weich, K. and Grimm, H. (2019). *Food Ethics 3*, 91-108. doi:10.1007/s41055-019-00038-4

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In3 Project: A European initiative to evaluate toxicity of chemicals using iPSCs derived *in vitro* models: Focus on blood brain barrier models for neurotoxicity assessment

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Evaluation of chemical toxicity is important for regulatory purposes in order to assess their risks for human health. Human iPSCs-based *in vitro* models represent a promising avenue to limit animal use and achieve better prediction of toxicity in humans.

In the framework of the in3-projet (MSCA-ITN ETN n°721975), we intend to explore the usefulness of iPSC-derived *in vitro* models for toxicity assessment.

Since neurotoxicity of chemicals is an important concern, knowledge on compound distribution through the blood brain barrier (BBB), which is separating brain cells from the peripheral circulation, is required. Located at the level of the brain capillaries, the BBB, acts as a gatekeeper to ensure brain homeostasis, which is essential for optimal brain functioning. Therefore, the deleterious effects of chemicals on BBB endothelial cells would indirectly lead to neurotoxicity.

We first compared the efficiency of various protocols to differentiate iPSCs into BBB like endothelial cells (BLECs). Several characteristics of the BBB have been evaluated: downregulation of pluripotency markers, presence of endothelial markers, formation of a functional tight barrier and functionality of efflux pumps (e.g. ABCB1, ABCG2, ABCC1).

After some protocol optimizations, the results obtained indicate that an iPSC-derived model could be used for assessing the effects of chemicals at the BBB as well as predicting their distribution to the CNS. These data will be integrated.

Several chemicals were selected within the in3 consortium and their effects on the BBB and on other *in vitro* models (e.g. kidney, liver, brain, lung, vasculature) derived from the same iPSCs will be compared based on toxicity assessment and transcriptomic data.

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Adding DIMENSIONS and a COMPLEMENT-ary view on HIV-1 transmission

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The growing spread of resistant HIV strains indicates the need to speed up research on innovative compounds that re-activate the immune system and to reinvent HIV vaccine development using human *ex vivo* models. Clinical trials only illustrated modest protective antiviral immune responses which led to the conclusions that (i) an effective vaccine must induce both humoral and cellular responses against HIV-1, (ii) novel alternative approaches are needed to design improved HIV-1 vaccines and (iii) improved methods for vaccine delivery have to be tested.

My work group recently illustrated the power of complement to boost efficient antiviral humoral and cellular immune responses via DCs – these data were generated in simple 2-dimensional (2D) cell culture models which provide principles in single cellular targets, but they do not reflect the complex interplay *in vivo* during HIV-1 transfer and spread. Novel and rapid developments in high content/high throughput imaging analyses as well as organotypic cultures provide groundbreaking new tools to study HIV-1 transfer at entry sites or to test novel vaccination strategies. Therefore, we design optimized intelligent human barrier models combined with infection-relevant immune cells (DCs, naïve T cells or HIV-specific T cell clones) in order to characterize HIV-1 entry and initial transmission steps within a 3D system. We, too, found within the 3D models, that the opsonization pattern of the virus or pathogenic fungi within a respiratory 3D model markedly modified DC and macrophage function compared to non-opsonized pathogens.

These human systems taking into account innate immune mechanisms, such as complement opsonization, offer improved power to test delivery methods, adjuvants, shock-and-kill compounds or novel vaccination approaches and will be an important challenge with broad interest.

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Automated analysis of developmental and reproductive effects using the nematode C. *elegans*

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The soil nematode *Caenorhabditis elegans* (*C. elegans*) is a well-characterized biological model organism that has been successfully employed in research in many fields. As a whole organism, *C. elegans* can be used to study complex endpoints under simplified conditions to either identify molecular mechanisms or to estimate vertebrate toxicity. Because of its small size, simple anatomy, transparency, short generation time, and large brood size, it is an ideal model for medium or even high throughput toxicity testing. Thus, it combines many advantages of whole animal approaches with those of *in vitro* methods and could be used to bridge the intrinsic gap between *in vitro* and *in vivo* approaches. Particularly complex developmental and reproduction endpoints, which are hardly accessible *in vitro* and laborious in vertebrate studies, are much easier and faster feasible in invertebrate model organisms.

Using *C. elegans*, the transfer of growth and offspring count as two substantial developmental and reproductive toxicological endpoints to an automated medium throughput test system was achieved. The test includes a fully automated image acquisition and software-based parameter survey under standardized conditions. In contrast to typical rodent *in vivo* studies, testing in *C. elegans* provides rapid and comprehensive toxicological data acquisition including well-defined concentration-response relationships.

Considering animal welfare issues as well as the many conserved molecular pathways *C. elegans* possesses, we suggest that this model organism can be a valuable tool to identify and prioritize relevant chemical substances or mixtures of concern for humans, animals and the environment.

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Transitioning from mammalian animal testing to non-animal testing using the zebrafish (*Brachydanio rerio*) embryo as a whole organism model for regulatory decision-making in chemical risk assessments

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In order to make the timely transition from animal testing to non-animal testing in chemical risk assessments, the global regulatory community has identified the need for a whole organism predictive tool that is an alternative to the rodent model and facilitates the three R's of reduction, replacement and refinement of animal testing. This transition requires a model that will serve to validate non-animal assays for their ability to robustly predict human health and environmental health hazard in response to chemical exposure. To this end, the Government of Canada initiated research in 2018 to investigate the potential of the zebrafish (ZF) embryo model as a robust alternative to the rodent model for regulatory decision-making. The ZF embryo model has been developed over the last four decades as a high-throughput screening tool for assessing developmental toxicity [1,2]. The strength of the model stems from its high gene homology to humans (70% sequence identity), high fecundity, rapid maturation, transparency, small size and significantly lower maintenance cost when compared with rodent models (< 1% of the cost for rodent assays). Beyond its application as a screening tool, the development of the ZF model as an alternative to the rodent model for use in quantitative risk assessment of repeated dose exposures will lend further to transitioning from animal testing to non-animal testing. Thus, the objective of this research is to refine the existing ZF models in order to optimize their ability to predict general toxicity and endocrine disruption in human health and environmental quantitative risk assessments.

Standardized ZF protocols that assess developmental, general and behavioral toxicity that have been developed by the NRC are being further refined in order to incorporate toxicokinetic and transcriptomics platforms. Toxicokinetic assessment has been prioritized by the international ZF research community as a means to investigate the role of the chorion and the role of static vs. daily renewal of test solutions over the 5-day exposure period on chemical bioavailability. Transcriptomic profiling is being investigated as a means to predict the chronic toxicity potential of test chemicals. To date, 20 substances, (industrial, cosmetic, pharmaceutical and pesticides) have been evaluated for their phenotypic and behavioral effects on ZF embryos. Toxicokinetic and transcriptomic evaluations are currently being conducted on a selection of the 20 substances.

The Government of Canada is collaborating with the US National Toxicology Program (NTP) Systematic Evaluation of the Zebrafish in Toxicology (SEAZIT) project [3] and intends to cross-validate results with those of the SEAZIT ZF inter-lab validation study once completed.

References

[1] Padilla, S., Corum, D., Padnos, B. et al. (2012). *Reprod Toxicol 33*, 174-187. doi:10.1016/j.reprotox.2011.10.018

[2] Horzmann, K. A. and Freeman, J. L. (2018). *Toxicol Sci 163*, 5-12. doi:10.1093/toxsci/kfy044

[3] National Toxicology Program SEAZIT. https://ntp.niehs.nih.gov/pubhealth/evalatm/test-method-evaluations/dev-tox/seazit/index.html

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Flipping co-cultures upside-down for improved imaging of chronic respiratory challenges within a human 3D model

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We established a completely animal free and three-dimensional (3D) respiratory/immune cell system to monitor host-pathogen interactions, especially concerning the first encounter of the epithelial/immune cell barrier.

Different microscopic approaches, as confocal analyses and high content screening, help to examine 3D respiratory samples, resulting in high-resolution pictures and enabling quantitative analyses of a high number of cells. A major problem arising employing these techniques is related to the disposable instead of reusable use due to the way of seeding cells. Usually cells are seeded to the inner field of Transwell inserts, what redound to the fact that the membranes carrying the epithelium have to be cut out and flipped for live cell imaging. To optimizing the process for monitoring differentiation of cells or repeated dose experiments over a long period of time, we flipped the inserts and seeded cells upside-down within an animal-free cellulose hydrogel to the bottom side of membranes. This optimized handling process now offers the possibility of long-time observations as differentiation or mucociliary clearance using the same cells, because the complete insert can be easily transferred to glass bottom dishes for live cell imaging analyses. This technique allows long-term culture of the primary cells and we were able to culture primary respiratory cells of the bronchial and small airway epithelial tract over a period of more than two years without losing the epithelial integrity. Long-term culture over two years also did not show any impact on the mitochondrial fitness of the cells and goblet cells continuously produced mucus.

Mucociliary clearance was monitored in such cultures after addition of either fluorescent beads or inactivated Aspergillus fumigatus conidia. Therefore, here we provide a novel innovative tool to study pathogen interactions with a native human mucus barrier allowing repeated dose experiments on the same 3D layer of epithelial cells within a completely animal-free setting.

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Implementation status of non-invasive methods in wildlife genetic sampling

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The Earth's biodiversity is currently experiencing immense pressure from habitat loss, overexploitation, global climate change, and invasive species, which escalate the global extinction crisis [1]. Comprehensive knowledge of the extent and impact of biodiversity loss is therefore critical for determining species vulnerability and prioritizing conservation goals. An integral part of wildlife conservation research and management has become genetic sampling. Animal DNA has been traditionally obtained invasively, from blood or tissues, however public concerns over animal welfare require that animals are affected as little as possible during research [2]. One of the ways to minimize the impact on animal welfare is to use non-invasive genetic sampling [3,4]. Even though non-invasive genetic sampling techniques have been developed for many animal species, it is however not clear how often they are being implemented. Here, we present an overview of recently published articles on genetics in amphibians, birds, carnivores, molluscs and rodents, for which we examined whether they used a lethal, invasive or non-invasive DNA sampling technique. Disappointingly, only 22% of the identified relevant studies implemented the available non-invasive genetic sampling method. With this review we highlight the need for better implementation of non-invasive DNA collection methods in wildlife research.

References
[1] Pimm, S. L., Jenkins, C. N., Abell, R. et al. (2014). *Science 344*, 1246752. doi:10.1126/science.1246752
[2] Lund, T. B., Morkbak, M. R., Lassen, J. et al. (2014). *Public Understand Sci 23*, 428-444. doi:10.1177/0963662512451402
[3] Beja-Pereira, A., Oliveira, R., Alves, P. C. et al. (2009). *Mol Ecol Resour 9*, 1279-1301. doi:10.1111/j.1755-0998.2009.02699.x
[4] Zarzoso-Lacoste, D., Jan, P. L., Lehnen, L. et al. (2018). *Mol Ecol Resour 18*, 217-228. doi:10.1111/1755-0998.12727

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Integrating organ-on-a-chip devices on a multimodal, microfluidic platform

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Given the constant advances in chemical synthesis allowing the rapid and divers creation of new compounds and substance libraries, the research field of Tissue Engineering becomes increasingly important for drug discovery. Especially Organ-on-a-Chip devices can be regarded as a powerful tool to improve *in vitro* studies and reduce animal tests for high-throughput screenings. The use of human cells and extracellular matrix material and thus, reproducing organotypic functional units of the human organism, is a major advantage of these models. Yet, due to a lack of automatization handling often is difficult and time consuming. We therefore present a multimodal microfluidic platform that was designed to cultivate the Organ-on-a-Chip device, established at KIT, the so-called *vasQchips*. Center of these chips is a curved, porous micro channel lined with endothelium and connected to microfluidic flow representing the blood stream. The pores of the scaffold can be adapted individually to each organ model and allow for the supply of nutrients and gases as well as for the exchange of growth factors or immune cells with the surrounding compartment. Several organ-models are being established and validated in this chip, including liver, blood-brain-barrier, skin or tumor environment and can be used for various applications. Although, the *vasQchips* are compatible to most standard pumps, cultivation and analysis was to be further improved by the development of the stated microfluidic platform. It will allow straightforward setups and standardized conditions for the experiments as well as a constant observation in the connected software. The platform itself consists of a micro annular gear pump, several miniature valves and is controlled via a connected touch display. This allows various microfluidic circuits and enables automated exchange of the culture medium controlled. Additionally, fluidic connections will allow sampling as well as the integration and exchange of sensors (e.g. O_2, pH) via (mini) Luer locks. Having the dimensions of a multi-well plate, the platform can easily be combined with standard devices such as microscopes or fluorescence plate readers. With all these properties, we achieved to design a multimodal platform that both makes the cultivation of microfluidic tissue culture more convenient and accurate, and is also suitable for many ways of analysis to easily acquire all the data from the executed experiments.

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Testing compound transportation *in vitro* within a microfluidic blood vessel model

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Constant advances in chemical synthesis allow the rapid and divers creation of new compounds and substance libraries. Yet, in order to select potential drugs, available screening methods still need to be improved to make them faster, less expensive and safer. While common methods consist of either very simplified cell cultures or animal experiments, the field of Tissue Engineering offers new appropriate models to enhance the spectrum of test systems. Human cell material and ECM are used in more complex micro environments, creating a more *in vivo* like surrounding for cell culturing and thus, representing tissues, organs and eventually the human body *in vitro* as closely as possible. During the evaluation of drugs, their transportation within an organism and towards the target is a crucial process. As the interplay between blood vessel and adjacent organ tissue has an essential role here, our group at KIT developed a microfluidic chip device, the *vasQchip*, mimicking exactly this area of interest. Center of the chip is a curved, porous micro channel lined with endothelium and connected to microfluidic flow representing the blood stream. The round shape of this channel enables an evenly spread shear stress within the system which can be adjusted via different flow rates for the individual conditions needed. In the surrounding compartment, an organotypic 3D culture can be established, allowing interaction between these two tissues. These include different cells layers, embedded spheroids or hydrogel cell cultures as well as 3D bio-printed cellular scaffolds. During our investigations with different drug carriers, we found that the 3D alignment especially of the endothelial cells in the chip has a major impact on their capability of transport and incorporation of drugs. While other setups do not recapitulate such specific micro environments, the cells within our chip form their natural morphology and exhibit *in vivo* like behavior. Like this, the introduced *vasQchip* presents a versatile platform for the screening of many kinds of substances in different specific tissues, as it allows the incorporation of various organotypic endothelial and epithelial cells. In our projects, we therefore constantly work on the establishment and validation of different organ models including liver, blood-brain-barrier, skin or tumor environment, as well as various testing setups concerning permeability, transendothelial modification or influence of shear forces.

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Fully-humanized skin-on-a-chip with a modular architecture for biomedical applications

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In vitro human skin models are gaining attention for their importance as tools for basic research and for the pharmaceutical and cosmetic industries. However, current commercially available human skin models are only suitable for short-term studies due to the time-dependent contraction of their collagen gel-based matrix [1]. Other limitations include the use of non-human extracellular matrix components and lack of dynamical flow systems and mechanical forces [2].

In recent years, advances in biomaterials and microfluidics technology made it possible for the culture of artificial skin to move a step ahead giving rise to the development of microfluidic skin-on-chip platforms. These systems are able to reproduce key aspects of the *in vivo* cellular microenvironment by including fluid flow and finely tuned forces.

This project aims at developing an innovative microfluidic system to grow and sustain a physiologically relevant human skin model. This approach begins with the production of a fully-humanized skin model by combining the production of a fibroblast derived matrix and the use of an inert porous scaffolds for long-term, stable cultivation, without using animal components. This technique is then combined with the use of a biomimetic "organ-on-a-chip" system which includes dynamic perfusion for continuous supply of nutrients and metabolites. Also, we present a reversibly sealed chip with a module-based architecture that provides an easy to use workflow, an efficient and precise cell seeding and a removable culture insert that can be transferred between modules. Finally, to characterize the barrier integrity and permeability of the produced skin, electrodes are integrated on the chip to directly quantify the transepithelial electrical resistance (TEER).

In the future, this innovative platform could reduce the dependence on animal models and provide a new *in vitro* tissue system compatible with long-term studies to study skin diseases and evaluate the safety and efficacy of novel drugs and technologies.

References

[1] Groeber, F., Holeiter, M., Hampel, M. et al. (2011). *Adv Drug Deliv Rev 63*, 352-366. doi:10.1016/j.addr.2011.01.005
[2] Ackermann, K., Borgia, S. L., Korting, H. C. et al. (2010). *Skin Pharmacol Physiol 23*, 105-112. doi:10.1159/000265681

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