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SESSION 20: *IN VITRO* MODELS IN PHARMACOLOGY AND TOXICOLOGY

O20.1 | Canine organoids for drug efficacy and safety testing: an innovative preclinical model for drug development

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Introduction: The high attrition rates in drug development emphasize the need for alternative screening systems for the evaluation of candidate therapeutics at the early stage of the R&D lifecycle. Intestinal stem cell (ISC)-derived organoids constitute an innovative model to identify new molecular pathways that could lead to therapeutic breakthroughs. Of the large animal species used in translational research, the dog is highly relevant because canine gut physiology and microbiota are comparable to that of humans. Also, dogs are a spontaneous model for human chronic enteropathies, such as Inflammatory Bowel Disease (IBD). The aim of this proof-of-concept study was to develop protocols for the culture and growth of canine organoids, using ISCs from healthy dogs and dogs with IBD.
Materials and Methods: 10 endoscopic biopsies of intestinal (duodenum, jejunum, ileum and colon) mucosa from 23 healthy dogs and 12 dogs with IBD were collected for crypt isolation. Optimized ISC growth media contained rho-associated kinase inhibitor Y27632, glycogen synthase kinase 3β inhibitor CHIR99021, and wnt-3a. Organoids were passaged weekly over 9 months. Characterization of organoids was performed using brightfield imaging, transmission electron microscopy, immunohistochemistry and RNA *in situ* hybridization. Functional assays were developed using 2D transwell cultures and optical metabolic imaging (OMI).
Results: Our results showed that organoids from primarily stem cell spheroids (D3) can differentiate into enterocytes, goblet cells, enteroendocrine cells, Paneth-like cells and Tuft cells mosaics (D6-D8). Ultrastructural characterization confirmed increasing density of tight junction proteins from D1 to D9. Functional assays, using LPS stimulation, showed increased growth rate of small vs. large intestinal organoids, as well as differential mRNA gene expression profiles. Preliminary OMI results on a few organoids (n = 7) suggested a trend for greater metabolic activity (+60%, $p > 0.05$) in dogs with IBD compared with healthy subjects.
Conclusions: This is the first report of successful propagation of organoids from dogs with IBD. The ability to grow organoids from the main GI targets for drug-related toxicity opens up new avenues for complementary animal-based toxicology studies. By providing an *ex vivo* screening platform to evaluate the toxicity and efficacy of drug candidates, we expect to reduce the number and associated costs of dogs used in preclinical studies.

O20.2 | 3D printing and the future of development of animal health products

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Introduction: 3D printing has numerous applications and has gained much interest in the medical world. The current medical and veterinary medicine uses of 3D printing can be organized into several broad categories: tissue and organs fabrication; creating of implants, prosthetics and anatomical models; and pharmaceutical research concerning drug discovery, delivery, and dosage forms. The purpose of our work was to gain a better perspective of how 3D printing technology works by getting a first-hand look at this process and then strategize ways that this technology can potentially be used in development of animal health products.
What Is 3D Printing and How Does It Work?: 3D printing is a type of additive manufacturing. It is a process that creates a three-dimensional object by building successive layers of raw material. Objects are produced from a digital 3D file, such as a computer-aided design (CAD) drawing or a Magnetic Resonance Image. 3D printing can create a solid object of virtually any shape and can use an assortment of starting materials, including plastic, metal, ceramic, tissue and organ cells.
Exploring the Possibilities of 3D Printing in Development of Animal Health Products: The purpose of veterinary drug development in the future should be production of personalized animal health products that can be achieved through the application of 3D printing. The application of 3D printing in veterinary medicine, including tablets,

holds promise for made-to-order drugs, and removes mass product manufacturing from the production line, although the technology in pharmaceutical industry is still in infancy. 3D printing promises a future of drugs printed on demand, to custom doses, especially in companion animals.

Conclusions: Prototype projects demonstrated that it is possible to use 3D printing techniques to generate and manufacture chemical compounds. In applying this technology to animal health products, the first generation technology is already a reality. Proof of this is recently approved 3D-printed oral drug Spritam (levetiracetam) by US FDA in human medicine. The second-generation efforts will involve getting a digital prescription, buying the "blueprint" and chemical "materials" needed, and then printing the drug with the software and a 3D molecular printer. Hence, 3D drug printing will have important repercussions in the realm of distribution of animal health products. However, even more profound are the eventual implications for new drug discovery and personalized therapy for animals. Indeed, third-generation 3D drug printing would entail the creation of new drugs that maximize efficacy and minimize toxicity. But there are a number of questions. Approval of a 3D-printed veterinary drugs opens up a new world of customised medication, but also the possibility of counterfeit drugs, mislabelling and a regulatory vacuum.

O20.3 | Preliminary characterisation of a novel cattle foetal hepatocyte-derived cell line (BFH12)

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Introduction: Actually, the obtainment of cattle hepatocyte primary cultures to be used for drug metabolism studies is still troublesome; therefore, a reliable hepatic *in vitro* model is needed for cattle. To this purpose, we preliminarily characterized a novel bovine foetal hepatocyte-derived (BFH12) cell line, measuring gene expression profiles of principal drug metabolizing enzymes (DMEs) either in control conditions or following the incubation with common DMEs inducers.

Materials and Methods: After the definition of the best conditions for cell culturing, BFH12 cells at different passages were seeded and cultivated either at standard conditions or in presence of some prototypical DMEs inducers, i.e., β-naphthoflavone (βNF), PCB126, phenobarbital, CITCO, FL81, dexamethasone, rifampicin, pregnenolone 16α-carbonitrile (PCN), SR12813 and RU486. Total RNA was extracted, and mRNA levels of main cytochromes P450 (CYPs), glucuronosyl- and glutathione-transferase (UGT and GST, respectively) as well as of nuclear receptors, i.e. aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), constitutive androstane receptor (CAR), retinoid X receptor alpha (RXRα), and hepatocyte nuclear factor (HNF) 1α, 1β and 4α were measured by using quantitative real time PCR (qPCR) assays.

Results and Conclusions: BFH12 cells expressed detectable amounts of AhR, PXR, CAR, RXRα, HNF-1α, -1β and 4α mRNAs as well as main mixed function oxidases, i.e. CYP1A1, 1A2, 1B1, 2B22, 3A28, 3A38, 3A48. Also conjugating DMEs, namely UGT and GST, were constitutively expressed. Among the tested inducers, best results in terms of mRNA up-regulation were observed with PCB126 and βNF (CYP1A), FL81 (CYP2B), and RU486 (CYP3A). This preliminary study confirms that the BFH12 cell line is a promising *in vitro* tool for studying drug metabolism in cattle. Nevertheless, prospective studies are needed to better characterize BFH12 as a useful *in vitro* model for regulation, induction and functional studies.

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O20.4 | Effects of natural antioxidants and pentachlorobiphenyl (PCB126) on the modulation of aflatoxin B1 (AFB1) cytotoxicity and metabolism in a bovine mammary epithelial cell line

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Introduction: Upon bioactivation by drug metabolizing enzymes (DMEs), including CYP1A1, AFB1 can generate highly toxic metabolites like AFB1-exo-8,9-epoxide and AFM1, a dairy milk contaminant. Dioxin-like (DL) compounds and some natural antioxidants (e.g. curcuminoids and flavonoids) have the potential to modulate DMEs involved in AFB1 bioactivation/detoxification, thus influencing AFB1 kinetics and toxicity. As the mammary gland actively participates in the generation of AFB1 metabolites, we evaluated the role of selected natural antioxidants (i.e. curcumin, curcuminoids, quercetin and resveratrol) in the modulation of AFB1 toxicity and metabolism using a bovine mammary epithelial cell line (BME-UV1). The effect of PCB126, a DL-compound highly excreted in dairy milk was investigated, too.

Materials and Methods: WST-1 or Neutral Red Uptake viability assays were performed at 24 and 48 h on cells treated with increasing concentrations of AFB1 (96–750 nM), after the pre-incubation with each antioxidant (5 μM) or PCB126 (10 nM). The modulation of CYP1A1 expression and activity by curcumin was assessed at different time-points (from 4 to 48 h) by qPCR and EROD assay, respectively, on cells incubated with increasing concentrations of the antioxidant (0.6–5 μM). AFM1 generation in cells was assayed by LC/MS-MS on media collected after the incubation with increasing concentrations of AFB1 (375–750–1500 nM) for 4, 8, 12 and 24 h. Data were analyzed by ANOVA followed by Dunnett's or Bonferroni's post hoc tests.

Results and Conclusions: As expected, AFB1 cytotoxicity occurred in a time- and concentration-dependent manner and was enhanced

by PCB 126 to a variable extent ($p \leq 0.001$). By contrast, quercetin ($p \leq 0.0001$) and, to a lesser extent, resveratrol or curcuminoids ($p \leq 0.05$) were able to significantly counteract the effects of AFB1; unexpectedly, curcumin slightly potentiated the impairment of cell viability upon AFB1 treatment. This apparently occurred via a non-AhR pathway, since curcumin failed to induce CYP1A1 at both gene and protein level. Analytical results confirmed that BME-UV1 cells are able to biotransform AFB1 into AFM1, as assessed by the concentration and time-dependent AFM1 increase in the culture medium. In addition, the counteracting effect of resveratrol ($p \leq 0.001$) and quercetin ($p \leq 0.0001$) was confirmed. Further studies are ongoing to test the protective effects of the same natural antioxidants against AFB1 in other tissue cell lines.

O20.5 | Percutaneous absorption in frog species: variability in skin may influence delivery of therapeutics

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Introduction: Transdermal delivery of therapeutics in frogs offers a low-stress alternative to oral or parenteral administration, however little is known about interspecies permeability in amphibian skin. To optimize therapeutic outcomes, an understanding of permeability differences is necessary as this will inform drug, dose and application site selection. Chytridiomycosis is a fungal disease of frogs for which consistently effective transdermal treatments between species remains elusive. Applying a One Health approach may assist in solving this therapeutic conundrum. This study determined the *in vitro* kinetics for a series of model chemicals through the skin of two frog species, to assess interspecies differences in skin permeability.

Materials and Methods: *In vitro* absorption kinetics of caffeine, benzoic acid and ibuprofen were determined utilizing infinite-dosing in Franz-type diffusion cells, with chemical content analyzed using high-performance liquid chromatography. Dorsal and ventral skin samples were excised from two species: the Green Tree Frog (*Litoria caerulea*) and the Cane Toad (*Rhinella marina*).

Results and Conclusion: Absorption kinetics differed significantly between species. Dorsal permeability was consistently higher in *Rh. Marina* compared to *L. caerulea*, with a 10-fold increase seen for the lipophilic chemical ibuprofen ($Kp_{RM} = 3.2 \times 10^{-3}$ cm h^{-1}; $Kp_{LC} = 3.3 \times 10^{-4}$ cm h^{-1}). Many antifungals with activity against chytridiomycosis are highly lipophilic; the permeability differences demonstrated in this study may therefore explain the lack of reproducibility reported across frog species in clinical trials for chytridiomycosis. Differences in ventral permeability were inconsistent between species, being slightly higher in *L. caerulea* for the hydrophilic and moderately lipophilic chemicals, and similar between species for the more lipophilic chemical. Further, the magnitude of difference observed was far less in ventral skin, with no more than a 1.7-fold increase in permeability coefficient (for benzoic acid, $Kp_{LC} = 5.3 \times 10^{-3}$ cm h^{-1}; $Kp_{RM} = 3.2 \times 10^{-3}$ cm h^{-1}). The differences in permeability demonstrated in this study emphasize the importance of considering inter-species differences when selecting drug candidates and dosing regimens in order to ensure safe and efficacious transdermal drug therapy in frogs.