Organoids are promising tools for species-specific in vitro toxicological studies

Review

Organoids are promising tools to species-specific *in vitro* toxicological studies

Short title (up to 70 characters): Organoids in species-specific veterinary toxicology

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Abstract (<250 words)

Organoids are three-dimensional self-aggregating structures generated from stem cells (SCs) or progenitor cells (PCs) in the process that recapitulate molecular and cellular stages of early organ development. The differentiation process is leading to appearance of specialized mature cells and is connected with changes in the organoid internal structure rearrangement and self-organization. The formation of organ-specific structures *in vitro* with highly-ordered architecture is also strongly influenced by the extracellular matrix (ECM). These features make organoids as a powerful model for *in vitro* veterinary toxicology. Nowadays this technology is developing very quickly. In this review we present the state of the art of veterinary organoid generation from adult stem cells (ASCs) and pluripotent stem cells (PSCs): ESCs (embryonic stem cells) or iPSCs (induced pluripotent stem cells). The current culture organoid techniques are discussed for their main advantages, disadvantages and limitations. In the second part of the review, we concentrated on the characterization of species-specific organoids generated from tissue-specific stem cells (SCs) from different sources: mammary (bovine), epidermis (canine), intestinal (porcine, bovine, canine, chicken), and liver (feline, canine).

Short abstract (<80 words)

Organoids are three-dimensional aggregates obtained from stem cells (SCs) or progenitor cells (PCs) which are able to generate organ-specific structures *in vitro*, thus representing a powerful model for *in vitro* veterinary toxicology. In this review we summarized the state of the art of veterinary organoid generation and culture techniques, with their main advantages, disadvantages and limitations. We also described species-specific organoids obtained from different stem cells (SCs) sources: mammary (bovine), epidermis (canine), intestinal (porcine, bovine, canine, chicken), and liver (feline, canine).

Key words: organoids, spheroids, 3D models, iPCS, ESC, stem cells, toxicology

Abbreviations:

2D - in vitro two-dimensional cell culture; 3D - in vitro three-dimensional cell culture; ASCs - adult stem cells; EBs- embryoid bodies; ECM - extracellular matrix; EGF - epidermal growth factor; ESCs - embryonic stem cells; FGF - fibroblast growth factor; HFs - hair follicles; HGF - hepatocyte growth factor; HTS - High-Throughput Screening; HCS - High-Content Screening; IFE- interfollicular epidermis; IGF-1 - insulin growth factors 1; iPSCs - induced pluripotent stem cells; PCs - progenitor cells; PSCs - pluripotent stem cells; SCs - stem cells;

1. Introduction

Organoids are three-dimensional (3D) multi-cellular *in vitro* tissue constructs that mimic the corresponding *in vivo* organ, thus they can be described as lab-grown miniature models of organs and used to study aspects of that particular organ in the *in vitro* environment (de Souza, 2018). The organoids can be grown in different types of 3D gels generating an organ-like structure. The ability to mimic the *in vivo* architecture of organs is due to the application of extracellular matrix (ECM) gels and special signaling molecules which act on the cell signaling networks. Nowadays, many small-molecule modulators of key signaling pathways and specific hormones in the culture organoids of various tissues are unknown (for example, the ovary tissue), so their role as potential *in vitro* culture components should be evaluated to enhance the efficiency of organoid generation (Fatehullah et al., 2016).

These 3D *in vitro* models of tissues are built from many types of cells which, compared to 2D cultures, response to the tested agents in different ways depending on their architectural organization which is influenced by many factors, including the extracellular matrix (ECM) composition, and the cell-cell and cell-ECM communication (Fatehullah et al., 2016). Moreover, the physical attributes of ECM, such as composition, porosity and stiffness, shape to a large extent the usefulness of organoids in toxicology and pharmacology research (Fatehullah et al., 2016) because these properties strongly influence the xenobiotic penetration into the organoids. It is important to remember that the most often used ECM is a mouse-sarcoma-derived Matrigel, whose components cannot be standardized, and this can affect the *in vitro* toxicological results and their repeatability (Fatehullah et al., 2016). Matrigel is a fundamental component in organoid formation and development, since it supports cell attachment and survival (Xu et al., 2001). Moreover, Matrigel plays a scaffold role and provides signaling cues via basement membrane ligands which is important for cell polarization, retention, and mobilization (Peerani & Zandstra, 2010; Xu et al., 2001). Matrigel is the most popular ECM, but other proteins like collagen, laminin, and fibronectin are also used to support organoid (Peerani & Zandstra, 2010; Yin et al., 2016). Organoids can be used as 3D *in vitro* models for organ development, drug screening, disease modeling, and toxicity testing. Compared to cell monolayers, they better represent, the *in vivo* structure of organs, which has a profound influence on the cell signaling networks. In 2D cultures the cells grow adherent to a substrate. This type of the cell culture is very popular, but a lot of *in vitro* studies have shown that it does not represent the *in vivo* situation, as is still far from the tissue organization and the cellular connections observed *in vivo* (Fatehullah et al., 2016). The organoids are more complex *in vitro* models compared to monolayer cultures, but still they cannot accurately mimic the mechanical forces that the stem cells encounter *in vivo*. Moreover these *in vitro* models cannot reproduce the interaction occurring between the stem cells grown in the 3D aggregates and stem cell niches, immune cells, *etc* (Fatehullah et al., 2016). These problems have been partially solved using organotypic culture systems or co-cultures with other cell types and searching for novel substances and ECM factors which better mimic the *in vivo* situation (Fatehullah et al., 2016).

The 3D *in vitro* cultures can be divided in two groups, namely organoids and spheroids. Some of the authors use the term “organoids” to indicate both. Other authors suggest that organoids, unlike spheroids, are obtained from the culture of PSCs or directly from tissues with self-renewal and self-organization capacity while spheroids are obtained from a single cell clones or aggregates of cell line cultured in monolayer (Moreira et al., 2018). In the early stage of organoid research, the terms “spheroids” and “organoids” were used as synonyms. Now spheroids can be defined as clumps of

cells which are obtained from differentiated cells that aggregate exhibiting some tissue-like structures. In comparison to organoids, the spheroids do not show any relevant tissue structure or, in other words, their structure presents low similarity to the original tissue (Simian & Bissell, 2017). The "spheroid" term can also be used to describe an intermediate stage of organoid development as done by Arora and colleagues (Arora et al., 2017) who classified the hindgut spheroids as an intermediate step between endoderm and mature intestinal organoids.

Stem cells (SCs) play an important role in the 3D organoid culture due to their self-renewal and differentiation abilities. Organoids derived from stem cell bioengineering capture a lot of the anatomical and even functional hallmarks of the real organs at a micrometer to millimeter scale and these properties could give them a key role in toxicology studies because of the easier sample accessibility and the absence of ethical concerns in comparison to *in vivo* animal studies (Rossi et al., 2018).

1.1. Main advantages, disadvantages and limitations of organoids in species-specific *in vitro* toxicology studies

Organoids are built to generate near-physiological *in vitro* models for the study of adult stem cells and tissues in a variety of different experiments regarding nutrient and xenobiotic bioavailability, toxicology, pharmacology, *etc*. (Fatehullah et al., 2016; Rossi et al., 2018) allowing the reduction of animals needed in veterinary research. Moreover these 3D cultures represent an important tool for the *ex vivo* modelling in tissue morphogenesis and organogenesis studies (Kretzschmar & Clevers, 2016). Organoids as *in vitro* models have some advantages but also a lot of limitations which are related with the organoid technology. The advantages, as well as disadvantages, are typical for all the organoid models independently from their tissue phenotype (Fatehullah et al., 2016). Organoids are generated from stem cells, thus they have the ability to propagate for a long time with low predisposition to genomic alterations (Moreira et al., 2018) and a limited amount of starting material is needed at the beginning of the experiments (Fatehullah et al., 2016). Organoids in animal science rarely come directly from the tissues but those obtained directly from the tissues are mostly epithelial and originated by mechanical or enzymatic digestion. After this step, small pieces of the tissue are embedded in different types of ECM (extracellular matrix) to produce an organ-like structure.

With regard to other limitations, organoids growth is limited to low-adhesion plates, their structure can be variable, they may lack key cell types and they may have difficulties in achieving maturity (Fang & Eglen, 2017). The presence of components such as fibroblasts, endothelial cells, immune cells and neural cells can be seen both as an advantage and as a disadvantage depending on the experimental design. If more complex models are needed, the organoid co-culture with other cell types allows to study cell-cell interactions and to better mimic physiological microenvironmental conditions (Moreira et al., 2018). On the other hand, the lack of some important cell components typical of the *in vivo* tissues can be observed. A fundamental difference between organoids and *in vivo* tissues is the lack of vasculature (Fang & Eglen, 2017). However, researches have shown that organoids transplanted into mice have become an integral part of the *in vivo* tissue and have been vascularized. The organoids have also the capacity to develop into replacement tissue and possibly whole organs (Mansour et al., 2018). All the organoid characteristics make these models less amenable to High-Throughput Screening (HTS)/High-Content Screening (HCS) and are responsible for complication in *in vitro* assays (Fang & Eglen, 2017). On the contrary, spheroids better compliant with HTS/HCS because they are characterized by easy-to-use protocols and scalable culture methods (co-culture or monoculture) with high reproducibility (Fang & Eglen, 2017).

Another limitation concerns the use of these models for the study of the inflammatory response (for example in response to xenobiotic exposure), which is an important endpoint in animal research. The inflammation response is hard to be evaluated using *in vitro* models including organoids due to absence of immune cells in these culture systems.

A problem is related to the control of the factors/ signalling gradients that are present in the ECM where the organoids are drowned during their generation and that cannot be controlled without

the application of microfluidic technologies (Fatehullah et al., 2016). In this regard, the biggest challenge for the organoid application in toxicology and pharmacology is their heterogeneity (also in the same culture) in terms of viability, size, and shape. These characteristics make essential to individually track by live or time-lapse imaging the single organoids (Fatehullah et al., 2016). Nowadays, although the 3D organoid systems and their potential applications are attractive, this technology is still expensive and time-consuming (Moreira et al., 2018). Cultured organoids require different approaches in terms of sample handling, manipulation, and functional assays. New imaging and quantitative analysis techniques must be implemented to expand and improve the use of organoids in *in vitro* toxicology studies. Despite these limitations, progress in this area is fast. So far, protocols for organoid generation from large animals have been developed. Moreover published results regarding successful genetic modification of organoids (Koo et al., 2012), high-throughput screening (van de Wetering et al., 2015), and 3D imaging and analysis techniques (Nantasanti et al., 2015) demonstrate that they may have the potential to become in the future one of the most powerful tools for *in vitro* toxicology studies.

1.2. Species-specific induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) organoids generation

Kretzschmar and Clevers (Kretzschmar & Clevers, 2016) defined organoids as "3D *in vitro* grown structures derived either from PSCs or ASCs that self-organize into a near-native microanatomy with organ-specific differentiated cell types and tissue compartmentalization". Generally, to obtain organoids, pluripotent stem cells (ESCs, iPSCs) as well as adult stem cells (ASCs) (Fatehullah et al., 2016) can be used. Organoids generated from pluripotent stem cells (ESCs, iPSCs) present heterogenic cell populations, whereas organoids obtained from ASCs differentiate into more tissue-specific 3D models (Fatehullah et al., 2016).

In comparison to the organoids generated from ASCs derived from adult tissues, protocols for organoid generation from iPSCs and ESCs contain two important steps at the beginning of the procedure. In first step, the formation of 3D aggregates of cells from the 2D cultures of pluripotent stem cells (iPSCs, ESCs) is described. The second stage is the induction of differentiation into embryoid bodies (EBs). This phase ends when the EBs are transferred into the ECM gel. At the end of this procedure the organoids are differentiated into specialized mature cells (the endpoint is connected with the organoid characterization). Depending on the organoid type, they can be cultured in adhesion (Sato et al., 2009) or in suspension as floating (Lancaster & Knoblich, 2014) organoid culture or .

ESCs and iPSCs are obtained from many animal species: bovine (Bogliotti et al., 2018; Han et al., 2011; Talluri et al., 2015); swine (Chakritbudsabong et al., 2017; Ezashi et al., 2009; Siriboon et al., 2015); horses (Quattrocelli et al., 2016; Tecirlioglu & Trounson, 2007); sheep (Liu et al., 2012); dogs (Betts & Tobias, 2015; Tecirlioglu & Trounson, 2007); cats (Ezashi et al., 2016; Tecirlioglu & Trounson, 2007); chickens (Fuet & Pain, 2017; Zhang et al., 2013); *etc*., but, to our knowledge, up to date organoids technology based on the PSCs has not been used in animals like pigs, bovines, dogs and cats organoids generation. With the growing popularity of organoid technology, the efficiency of iPSCs generation and differentiation into the specific lineage are likely to be improved in the future, making these technologies very interesting tools in *in vitro* veterinary toxicology.

1.3. Species-specific adult stem cell (ASC) tissue-derived organoid generation

In comparison to the organoids generated from PSCs organoids from ASCs are obtained directly from tissues containing SCs. ASCs are long-living cells which can be found in specialized tissues and are characterized by high multipotency and self-renewal properties. ASCs can divide asymmetrically to generate progenitors that can differentiate into functional cells of the tissue, or remain in quiescent stage in the stem cell niche (Regan & Smalley, 2007). ASC derived organoids can be generated from SCs or from fragments of tissues containing SCs. The procedure to obtain

ASC-derived organoids starts with the homogenization of the tissue fragments followed by mechanical combined with enzymatic fragmentation. Then the 3D cell aggregates are transferred into the ECM where the organoid expansion and differentiation take place (Sato et al., 2009). Organoids from single SCs can be obtained spontaneously or using the hanging drop method.

The main difference between organoids generated from ASCs and PSCs-derived organoids is the absence of the EBs phase in the formation steps. Organoids from PSCs show an embryo phenotype and can be used as models for *in vitro* embryotoxicology, developmental toxicology or teratogenicity studies (Fang & Eglen, 2017).

2. Bovine mammary organoids

Mammary-like organoids can be generated from ASCs (Shackleton et al., 2006; Stingl et al., 2006) and iPSCs (Cravero et al., 2015; Qu et al., 2017) what was previously shown in the case of human species (Cravero et al., 2015; Qu et al., 2017). Qu and colleagues (Qu et al., 2017) have demonstrated that human iPSCs can be directed differentiated *in vitro* toward mammary lineage and the obtained 3D aggregates expressed mammary gland function-associated signaling pathway markers. They have showed also that iPSC-derived mammary organoids expressed common breast tissue, luminal and basal markers, including estrogen receptors, and were able to produce milk proteins (Qu et al., 2017).

However, as for other organoids described in this review, ASCs, which are directly obtained from mammary gland tissue, and they play main role in the mammary organoid generation (Shackleton et al., 2006; Stingl et al., 2006). If compared to iPSCs, ASCs require gland tissue biopsies, since they can be generated from fibroblasts and cultured *in vitro* for a long time. Mammary stem cells provide opportunities for modeling of animal mammary glands *in vitro*: the mammary gland has a peculiar development during different stages of the life of the animal, and this characteristic makes difficult to mimic its phenotype using 2D *in vitro* cultures (Shackleton et al., 2006; Stingl et al., 2006). During the whole life of the animal, an ASCs population typical of the mammary tissue resides into the mammary gland. This cell population is activated at specific stages of animal's life (pregnancy, lactation) by steroid signaling pathways (Shackleton et al., 2006; Stingl et al., 2006).

In 1994 successful harvesting of bovine mammary organoids consisting of alveolar-like and duct-like clumps of few single cells from Holstein and Angus was described (Furumura et al., 1994). Furumura *et al.* analyzed the role of serum and also combinations of mammogenic hormones in media without serum for the development of collagen-embedded organoids generated from the mammary tissue of these two breeds. Immediately after slaughter the udders were removed and transported into the laboratory, where the mammary tissue was aseptically minced and then subjected to enzymatic fragmentation with trypsin, collagenase, hyaluronidase, and bINS. After digestion, the obtained material was filtered, centrifuged and resuspended three times. At the end of the procedure the suspension was filtered again using a Natex filter (100 μm) in order to collect the organoids that were then transferred into a collagen solution obtained from rat tail tendons. As we previously described, the ECM gel embedding is a critical step also in bovine organoid generation. The aggregates were transferred into the ECM gel (consisting of Matrigel/Collagen I) and treated with mammary related hormones and growth factors (Qu et al., 2017). The use of collagen gel as an ECM for bovine mammary epithelial cells and mammary organoids development was described very early in the history of organoid generation (Baumrucker et al., 1988; Mackenzie et al., 1982). The use of collagen gel as ECM for mammary organoids is considered to be superior to the majority of the culture methods because of its ability to mimic the *in vivo* conditions and to allow relatively long-term studies (Furumura et al., 1994). In the study of Furumura *et al.* (Furumura et al., 1994) organoid morphologic changes were observed after 24h of collagen culture: at this time, the growth and development of alveolar-like and duct-like type organoids was evident. Results of mammary organoid collagen cultures indicate that FCS contains mammogenic hormones together with growth factors (Furumura et al., 1994). During organoid development, a synergism between EGF, mammogenic hormones and other growth factors in FCS has been described as crucial for the stimulation of mammary cell

proliferation (Forsyth, 1991; Furumura et al., 1994). In this regard, Shamay and colleagues (Shamay et al., 1988; Shamay & Gertler, 1986) pointed out that insulin, insulin growth factors (IGF-1, *etc.*), epidermal growth factor (EGF) and other components of serum are critical for the stimulation of mammary organoid proliferation in collagen gel culture, because maturation and differentiation of mammary epithelial cells *in vitro* is controlled by many factors among others an IGF-1, epidermal growth factor (EGF), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF) (Gurusamy et al., 2014; Howard & Lu, 2014; Zhang et al., 2014).

The differentiation of ASCs was observed during organoid cultures (Furumura et al., 1994) but this process was not completely understood until ASCs were isolated from mammary gland tissue in 2006. Indeed, mammary stem cells were first isolated from the murine species in 2006 (Shackleton et al., 2006; Stingl et al., 2006). Two group in 2006 (Shackleton et al., 2006; Stingl et al., 2006) showed independently that pure population of ASCs isolated from mammary gland tissue is characterized by high differentiation potency which is responsible for the ability to regenerate *in vivo* a functional mammary gland after transplantation of ASCs into the fat pad of a recipient mice. In the mammary organoids, different committed progenitors are present, and they are fundamental for the construction of the 3D structure and the control of the functional activity of specialized somatic cells that constitute the mammary tissue *in vivo*. There are two primary epithelial cell lineages and two distinct cellular progenitors (myoepithelial and luminal) for ductal and lobular structures into the mammary gland and they are presumed to arise from a common progenitor cell (Shackleton et al., 2006; Stingl et al., 2006). In this regard, in 2009 Martignani and colleagues (Martignani et al., 2009) identified bovine adult mammary stem cells which were able to recreate alveoli that secreted milk when transplanted into a immunodeficient mice (Martignani et al., 2014; Martignani et al., 2010). Their experiments were important for the future generation of bovine organoids because clarified the hierarchical stem cell organization of the mammary tissue. In the present days, bovine mammary organoids obtained by mechanical and enzymatic dissociation of mammary gland tissue, are described as a useful and manageable model for mammary epithelial cells. Indeed bovine mammary organoid generation is a good method to obtain a pure cell population of mammary epithelial cells, moreover the culture is relatively easy to manage since it can be frozen during the intermediate dissociation steps (Martignani et al., 2018).

3. Canine Keratinocyte Organoids

The epidermis of mammalian is composed by a multilayered epithelium, the interfollicular epidermis (IFE), with associated appendages such as hair follicles (HFs), sebaceous and eccrine sweat glands. All these epidermal compartments are generated by keratinocytes which represent the main epidermal cell type in the mammalian epidermis (Hsu et al., 2014; Kretzschmar & Watt, 2014; Pavletic, 1991; Watt, 2014). The homeostasis of keratinocyte compartment is maintained because of the presence of stem cells in the basal layer of the epidermis. These cells express some typical markers, such as keratins: KRT5 and KRT14 (Kretzschmar & Watt, 2014), whereas in the hair follicles the stem cells are characterized by other markers such as Lgr5, Krt15, CD34 and Sox9 (Jaks et al., 2008; Liu et al., 2003; Nowak et al., 2008; Trempus et al., 2003). Indeed, ASCs of the epidermis are divided into multiple stem cell populations which maintain the tissue turnover and are involved in tissue repair. The canine keratinocyte organoids can be generated from adult canine hair follicles (HFs) and interfollicular epidermis (IFE). HFs and IFE, after isolation from canine skin tissue by microdissection, were enzymatically digested using collagenase 1A, II, hyaluronidase and then trypsin/Versene solution (Wiener et al., 2018). The canine keratinocyte organoids were cultured sequentially in the expansion and differentiation conditions (Wiener et al., 2018). In the expansion conditions canine keratinocyte organoids expressed the undifferentiated keratinocyte markers KRT5 and KRT14 (Wiener et al., 2018). Even if the canine HFs organoids did not show a distinct HF gene expression, in the future the *in vitro* generation of HFs organoids with specific markers may be possible acting on culture conditions (such as growth factors, co-cultures with other cell types, *etc*.)

in order to induce the expression of a distinct HF signature. Likewise, organoids may potentially differentiate (using specific growth factors) towards mature IFE structures.

Moreover, it is important to indicate that, since cell to cell adhesion molecules as well as markers of blistering diseases are expressed in IFE organoids, the canine keratinocyte organoids may become a powerful tool to investigate the physio-pathological alteration of the epidermidis. Thus, canine keratinocyte 3D models have the potential to be successfully used in *in vitro* toxicology studies, but culture conditions should be better optimized for the canine species. Moreover more researches are needed to better identify and define canine stem cell markers and differentiated cell markers.

4. Intestinal organoids

The 2D intestinal epithelial cultures traditionally used in *in vitro* toxicological studies are composed of a single cells type and grown in monolayer, and poorly represent the *in vivo* physiological and pathological intestinal processes (Chopra et al., 2010). This 2D *in vitro* models are, in fact, well-established and relatively cheap but they are characterized by the lack of 3D architectural details (Clevers, 2016; Sato & Clevers, 2013). To better reflect the *in vivo* conditions, 3D culture of intestinal enteroids (Sato et al., 2009) or organoids (Lahar et al., 2011; Spence et al., 2011) in the ECM have been developed in many species such as. pig, cat, dog, cow, horse, pig, sheep and chicken. Some authors use the term intestinal "organoids" and "enteroids" as synonyms. Others define the organoids as 3D aggregates containing both epithelial and mesenchymal cells built of intestinal tissue, whereas the enteroids are intended as structures constituted only of epithelial cells which are obtained from isolated intestinal epithelium of lumen, crypt and villi domains or single intestinal epithelial cells (Finkbeiner & Spence, 2013). The enteroids are also called "mini guts" because they show high similarity to the small intestine. 3D *in vitro* models are built using specific epithelial cell types of the intestine, thus they present the typical functionality of the original tissue (Clevers, 2016; Sato & Clevers, 2013). The enteroids are generated from ASCs present in the intestinal tissue (Clevers, 2016; Sato & Clevers, 2013), Eteroids 3D structure is formed by enterocytes, enteroendocrine cells, goblet cells, tuft cells, Paneth cells and stem cells (Clevers, 2016; Sato & Clevers, 2013).

Organoids are a valuable resource for the study of the intestinal function and also for the investigation of stem cell ability to regenerate and produce differentiated epithelium. Large animal organoids have not been deeply analyzed as the 3D *in vitro* models derived from humans and rodents had. Using bovine organoids in *in vitro* toxicology evaluations is difficult due to the lack of knowledge about the types of differentiated cells which are present in the bovine intestinal epithelium and their similarities to the murine/human epithelium. Until now, researches connected with the morphology of bovine intestine were mostly related to food production and consumption, *e.g.*, sausage casings (Wijnker et al., 2008) and the effects of animal diet on the mucosal architecture (Montanholi et al., 2013). Livestock species-specific intestinal organoids, which nowadays are successfully generated (Derricott et al., 2018; Powell & Behnke, 2017), have little use in *in vitro* toxicology, since this technology is mostly applied for insights into large animal (swine, bovine) host-pathogen interactions and disease responses (Derricott et al., 2018).

4.1. Swine intestinal organoids

Intestinal stem cells, which are located into the intestinal crypts and are characterized by the expression of the LGR5+ marker gene, possess the ability to differentiate into more specialized intestinal villus cell types, such as Paneth, goblet and epithelial cells (Biswas et al., 2015; Sato & Clevers, 2013).

Porcine intestinal crypts obtained from 1-14 days old wild type Yorkshire piglets can be cultured *in vitro* as 3D aggregates (Gonzalez et al., 2013). 2D and 3D models can also be combined as did van der Hee *et al.* that created a system where a primary epithelial monolayer was generated from porcine intestinal ileum organoids which were obtained from the intestinal tissue of two 5-month-old pigs

(van der Hee et al., 2018). Nowadays a lot of methods for culturing animal organoids, derived from intestinal crypts, have been optimized (Sato et al., 2009).

It has been demonstrated that many factors (*i.e.* R-spondin, Noggin) can induce the propagation of intestinal cells and the expansion of the intestinal crypt cells into organoid structures (Jung et al., 2011; Sato et al., 2011; VanDussen et al., 2015). Other groups investigated the role of factors such as TGFβ, p38 MAPK, GSK inhibitors in porcine organoids growth and survival (Date & Sato, 2015; Gonzalez et al., 2013). Powell and Behnke demonstrated that 3D porcine aggregates cultured *in vitro* in WRN conditioned media (a media characterized by the presence of the signaling factors Wnt3a, R-spondin-3, and Noggin) were able to propagate, expand and express crypt stem cell marker LGR5 and mesenchymal marker VIM (Powell & Behnke, 2017). Moreover, when cultured in differentiation medium, porcine enteroids started to express chromogranin A, mucin 2 and E-cadherin, markers typical of enteroendocrine cells, goblet cells, and adherens junctions, respectively (Derricott et al., 2018).

Niche-regulated stem cell renewal could be altered in many pathological statuses and also in response to toxicant exposure. Thus, the possibility to recreate *in vitro* those niches, obtaining epithelial organoids characterized by the presence of Lgr5(+) stem cells and all types of differentiated lineages from a single intestinal stem cell, could be very useful in toxicology studies (Date & Sato, 2015).

4.2. Bovine intestinal organoids

3D *in vitro* intestinal bovine models can be obtained from bovine jejunum in which intestinal crypts were isolated. In the next step, the bovine intestinal crypts were suspended and cultured in Matrigel (Derricott et al., 2018; Powell & Behnke, 2017). Powell and Behnke (Powell & Behnke, 2017) showed that bovine intestinal organoids (enteroids) could be generated in an expansion media containing Wnt3a, R-spondin-3, and Noggin (WRN) signaling factors. They demonstrated that those organoids contained proliferating crypt-like regions and expressed stem cell marker (LGR5) and mesenchymal marker (VIM). Derricott and collogues (Derricott et al., 2018) demonstrated the presence, in bovine intestinal organoids, of different types of differentiated epithelial cells showing the expression of chromogranin A, mucin 2 and E-cadherin, thus proving that the isolated crypts were capable of regeneration and differentiation (Derricott et al., 2018). Moreover, the organoid epithelium polarized, acquiring a morphology similar to those of the *in vivo* situation (Derricott et al., 2018). This research has shown that bovine intestinal organoids can be used to obtain bovine differentiated epithelial cells (Derricott et al., 2018). Bovine crypt isolation and organoid derivation required modifications from murine and bovine protocols: the bovine enteroid media was supplemented with R-spondin (Wnt agonist), Noggin (TGFβ superfamily inactivator) and EGF (to enhance epithelial proliferation) and inhibitors such as A83-01 (TGFβ type 1 receptor), SB202190 (MAP 3K), Y27632 (ROCK) and CHIR99021 (GSK3) (Derricott et al., 2018).

4.3. Canine intestinal organoids

Canine gastrointestinal organoids (enteroids and colonoids, obtained from small and large intestine, respectively) have been recently developed (Chandra et al., 2018). In a study performed by Chandra and colleagues, organoids were generated from intestinal adult stem cells isolated from whole jejunal tissue and from duodenal, ileal and colonic biopsy samples of dogs, demonstrating that the isolation of crypt epithelium can be performed from different canine intestinal regions (duodenum, jejunum, ileum and colon) (Chandra et al., 2018). The canine enteroids and colonoids have shown good colony forming efficiency when cultured in human organoid defined media including Wnt3a, R-spondin-1, and Noggin (Powell & Behnke, 2017). Two methods for canine enteroid culture are described in literature: one method use collagenase digestion for crypt isolation (Powell & Behnke, 2017), while the other apply the EDTA chelation method (Chandra et al., 2018). The use of EDTA for crypt isolation significantly enhance the purity of the crypt epithelium and decrease contamination with other cell types (Saxena et al., 2016), but its concentration and the time of incubation should be

specific for each animal species because these parameters are related to length, numbers and size of villi which are species-specific (Mahe et al., 2013). Canine jejunal enteroids are capable of reproducing the anatomical and physiological features of native jejunal tissue (Chandra et al., 2018). In order to comprehend if the stemness of whole jejunal tissue is maintained in enteroids, and to understand the possible effect of *in vitro* culture on intestinal stem cells, the identification and study of stem and progenitor cell populations in enteroids is fundamental. Chandra *et al.* (Chandra et al., 2018) used a canine specific LGR5 probe to identify crypt base columnar stem cells, since the presence of LGR5-positive adult ISCs (namely the crypt base columnar stem cells) is essential to develop the peculiar crypt-villus enteroid structure if a non-epithelial cell niche is not present. They found LGR5 positive cells in the crypt region of both canine intestinal enteroids and jejunal tissue while Sox9 (another marker of ISCs, also expressed in secretory-lineage epithelial cells) was found in the intestinal crypts and also, to a lesser extent, in the villus region (Chandra et al., 2018). Whole intestinal tissue contains epithelial, mesenchymal and immune cells, but enteroids and colonoids obtained from adult ISCs showed only epithelial cell populations. Epithelial markers (keratin, chromogranin A, and PAS) expressed in whole jejunal tissue were found in canine enteroids, but mesenchymal (vimentin, actin) and immune cell (c-kit and CD3 T) markers were expressed just in the whole jejunal tissues, demonstrating that canine enteroids consist only of epithelial populations without other cell types (Chandra et al., 2018). These findings proved that canine gastrointestinal organoids, being a good representation of the canine intestine, could be successfully used as innovative *in vitro* models in toxicology studies.

4.4. Chicken intestinal organoids

Chicken intestinal organoids can be generated from embryonal intestinal epithelium and from adult chicken intestine, showing very similar structure cultured in Matrigel. Differences between intestinal organoids obtained from embryo or adult intestine are connected with the presence of myofibroblasts in the 3D aggregates of the enteroids in case of embryo intestine tissue, which became visible in the second day of the organoid sphere forming. Pure intestine organoids without myofibroblast are obtained from the adult chicken intestine (Pierzchalska et al., 2016). The presence of myofibroblasts is probably connected to the higher plasticity of the embryo, in which epithelial-mesenchymal transition occurred during the development. Chicken embryo intestinal organoids can be generated from intestinal epithelium obtained from chicken embryos at day 3 of development (Pierzchalska et al., 2016). After isolation, the intestinal epithelium is minced into small fragments and embedded in Matrigel matrix on cell culture inserts. Here the organoids form spheres covered by epithelial cells after 24 hours (Pierzchalska et al., 2016). Intestine fragments can also be isolated from chicken embryos at day 18-20 of development. In this case, the fragments embedded in Matrigel can successfully differentiate into intestinal organoids with both epithelial and mesenchymal cells (Pierzchalska et al., 2012). A critical role in chicken intestinal organoid survival and development is played by three factors: R-spondin 1, Noggin and prostaglandin E2 (Pierzchalska et al., 2016). The ECM also acts a fundamental role in the procedure. However researches have showed that intestinal organoids can be generated also in media containing a low (5%) percentage of Matrigel. The hanging drop method with some technique modification is used to establish spheroid *in vitro* culture from various types of cells and tissues (Beauchamp et al., 2015; Kelm et al., 2003; Wang & Yang, 2008). This method could be applied also to generate the suspended culture of intestinal organoids obtained from cells isolated from chicken embryonic intestine. In this case, the media is supplemented with a low amount of Matrigel, thus it has been shown that chicken intestine organoids could be generated without the solid ECM support. In these conditions chicken intestinal organoids express the typical makers for the normal intestinal epithelium (transcripts and protein): Villin (an enterocyte marker) and Sox9 (a stem/progenitor population marker), sucrase-isomaltase (an enterocyte differentiation marker) (Panek et al., 2018; Pierzchalska et al. 2016), Pcna (a proliferating cell marker) and Lgr5 (a stem cell marker). Summarizing, organoids growth in solid ECM (insert cultures) and in low ECM

(hanging drop technique) present similar expression pattern of specific markers (Panek et al., 2018; Pierzchalska et al., 2016), thus they both are a good *in vitro* representation of the chicken intestine.

5. Liver organoids

Hepatic organoids, which can be induced to differentiate *in vitro* into functional hepatocyte-like cells, were first generated by Huch and colleagues (Huch et al., 2013). They obtained organoids from LGR5+ liver stem cells isolated from mice in which a hepatic damage was previously induced in order to stimulate the expression of Lgr5-LacZ gene. This gene is not expressed in healthy adult liver but, in response to a harmful event in the organ, Lgr5-LacZ+ cells appear near bile ducts. Nowadays liver organoids are successfully generated from many species like such as mouse, human, cat and dog (Huch et al., 2013, 2015; Kruitwagen et al., 2017; Nantasanti et al., 2015), but not from swine or bovine. As for the other types of organoids, two stages of liver organoid generation can be distinguished: expansion and differentiation. In each of these stages different markers of hepatocyte maturity are expressed (Roskams et al., 2003, 2004) but, to our knowledge, the generation of fully differentiated hepatocyte from pluripotent stem cells (ESCs, iPSCs) or from primary hepatocytes in *in vitro* culture (Fraczek et al., 2013) has not been described yet. Nowadays, for animal species (dog, cat), liver organoids are obtained using ASCs directly isolated from the liver tissue (Kruitwagen et al., 2017; Nantasanti et al., 2015), but not using iPSCs-derived hepatocyte progenitors. As for other organoid types described in this review (mammary, intestine, keratinocyte, *etc*.) animal liver organoid generation began when 3D cell structures (spheroids, fragments of tissue) started to be embedded in ECM (Kruitwagen et al., 2017; Nantasanti et al., 2015). There are still some challenges connected with liver organoid technology which need to be addressed. One of the main challenges is the low differentiation potential, which is a key factor in order to obtain representative models for adult animal species to be used in *in vitro* toxicology studies (Nantasanti et al., 2016). Improved differentiation methods for generation of fully mature hepatocytes and cholangiocytes are thus required, especially considering that mature hepatocyte generation is problematic not only in 3D but also in the 2D adult hepatic stem/progenitors cells (HPCs) cultures. ECM plays an important role in hepatocyte maturation (Nantasanti et al., 2016). ECM derived from decellularized liver improves primary hepatocyte culture function *in vitro* (Lee et al., 2014), but this technique has not been applied to animal species-derived liver organoids yet. There is also the need to develop methods for the production of feline, canine and other species liver organoid co-cultures with blood vessels, and to develop techniques to generate 3D species-specific liver organoids which could better represent the *in vivo* species-specific organ structure.

5.1. Feline liver organoids

Feline liver organoids were successfully obtained from cat fresh, frozen and fine-needle aspirate liver samples and then short- and long-term cultured. Spherical structures are formed by biliary duct fragments after digestion but also by undigested samples from fine-needle aspirate seeded in ECM (Matrigel) (Kruitwagen et al., 2017). Feline liver organoids are constituted of cells characterized by a long genetic stability and, within their structure, as for other types of organoids, cells at different maturation stages can be found (Sato et al., 2009). The liver adult stem cell populations play an important role in feline organoid generation. Kruitwagen and colleagues (Kruitwagen et al., 2017) described a method to generate and culture 3D liver organoids that is, to our knowledge, the only one available primary non-transformed long-term cell culture system for feline species. The feline liver organoids displayed a single-layered cubical epithelium and were positive for E-cadherin marker (an epithelial marker) (Kruitwagen et al., 2017). Moreover they were found to express the following markers: adult stem cell markers (LGR5, PROM1, BMI1); hepatic progenitor cell/biliary markers (KRT7, KRT19, HNF1β); early hepatocyte (HNF4α, TBX3, ALB) as well as mature hepatocyte (PROX1, PC, HMGCL, TTR, FAH, CYP3A132) markers, but the expression of the latter, in comparison with normal cat liver *in vivo,* were lower. During differentiation, the expression of gene of mature hepatocytes increased (FAH, CYP3A132, and TTR),

marker for adult stem cells (BMI1) was down-regulated, hepatocyte marker (ZO1) was up-regulated, while mature hepatocyte marker (HepPar-1) was not detected. Kruitwagen and colleagues (Kruitwagen et al., 2017) have succeeded in improving the hepatocyte function (up-regulation of aminotransferase levels, albumin secretion and CYP450 activity) and intermediate hepatocyte phenotype during organoid differentiation. These studies showed that species-specific liver organoid *in vitro* models should be developed, since each species presents some similarities but also peculiarities which are fundamental to investigate in *in vitro* toxicology studies. For instance, feline liver organoids exhibit a specific cellular lipid handling capacity as they tend to accumulate more lipids than it has been observed in human liver organoids after free fatty acid (FFA) treatment; moreover differences in transcriptional activation between human and feline organoids, leading to the activation of different pathways involved in lipid metabolism, have been found (Kruitwagen et al., 2017).

5.2. Canine liver organoids

Several canine adult hepatocyte cell lines, such as canine bile duct epithelial cell line, from gall bladder epithelial cells (Oda et al., 1991); hepatocellular tumor cell line, from a spontaneous hepatocellular carcinoma (HCC) in a dog (Boomkens et al., 2004) and a primary nonparenchymal cell fraction enriched with small hepatocytes and small epithelial cells obtained from normal, mature dog livers (Arends et al., 2009) have been used for decades, but long-term, genetically stable cultures of HPCs were difficult to obtain. Liver organoids can be a potential system for the long-term, genetically stable culture of canine HPCs, which are important models in canine *in vitro* toxicology (Nantasanti et al., 2016). To our knowledge, just two publications illustrated methods that led to the successful generation of canine liver organoids (Nantasanti et al., 2016, 2015). In these works, Nantasanti *et al.* underlined the importance of Notch and Wnt signaling pathways to induce the hepatocyte maturation of canine hepatic organoids. However, more researches are needed to improve the chance to use canine liver organoids in *in vitro* toxicology studies.

6. Conclusion

The technology based on organoid generation plays a fundamental role in human research but, to date, the development of animal species-specific organoids is still in progress. In the human, organoids generated from iPSC are the mostly used for research, whereas in animals ASCs-derived organoids are more popular. Organoids obtained from different species are characterized by specific stem cells and differentiated cell marker patterns which are typical for each stage of development. These markers could be used as *in vitro* endpoints in developmental toxicology studies, thus their identification for each species would be very useful. Moreover, studies have shown that the culture conditions used for human or mouse organoids should be optimized for each species independently. To sum up, to date many challenges concerning the species-specific organoid culture need to be met in order to develop an effective *in vitro* toxicology tool and, among them, the final differentiation of the organoids is probably the most important. Nevertheless, organoid systems seem to be very promising in *in vitro* toxicology and also in different scientific areas since this technology has shown the potential to allow an innovative and also multidisciplinary as well as species-specific approach to research.

7. Conflict of interest

The authors did not report any conflict of interest.

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