Species-specific models in toxicology: in vitro epithelial barriers

In vitro species-specific epithelial barriers in toxicology

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Abstract

Species-specific *in vitro* epithelial barriers represent interesting predictive tools for risk assessment evaluation in toxicological studies. Moreover, these models could be applied either as stand-alone methods for the study of absorption, bioavailability, excretion, transport, effects of xenobiotics, or through an Integrated Testing Strategy. The aim of this review is to give a comprehensive overview of *in vitro* species-specific epithelial barrier models from bovine, dog and swine. Bovine mammary epithelial barrier as a fundamental instrument for the evaluation of the toxicant excretion, the blood brain barrier as a useful first approach in toxicological and pharmacological studies, the porcine intestinal barrier, the canine skin barrier, and finally the pulmonary barrier from bovine and swine species are described in this review.

Keywords

Barriers, *in vitro*, species-specific, toxicology

1. Introduction

Epithelial barrier models represent an interesting predictive tool for risk assessment evaluation, in terms of absorption, bioavailability and transport of xenobiotics, alone or in mixture (Gordon, 2015).

In vitro models of epithelial barriers are often cultured on the trans-well chambers. With this system, the cells grow in monolayer on semi-permeable membrane filter (which can be coated with extra cellular matrix to promote cell adhesion) and two compartments are obtained: the apical and basal chamber (Gordon, 2015; Thomsen et al., 2015). After seeding, the cells differentiate to form a polarized epithelial cell monolayer that plays a physical and biochemical

barrier role. The cells can grow in monolayer or in co-culture with other cell types to generate more complex *in vitro* models (Gordon, 2015; Thomsen et al., 2015).

Different *in vitro* species-specific epithelial barriers are being successfully applied in toxicology studies (Bertero et al., 2018) but, despite this, these models and their relative applications have not been organically summarized yet.

Thus, this review aims to provide a comprehensive view on the topic, focusing on the analysis of *in vitro* epithelial barrier models from bovine, dog and swine, as an *in vitro* predictive tool in toxicology, considering differences in their genome, epigenome, proteome and metabolome.

In this paper, we describe the bovine mammary epithelial barrier, the brain barrier, the intestinal barrier, the skin barrier and finally the pulmonary barrier, summarizing their salient characteristics and main applications.

2. Bovine mammary epithelial barrier

The mammary barrier has two main functions: the secretion of milk and the maintenance of the separation between milk and blood, hence preserving the osmotic gradient necessary for the mammary secretion (Nguyen and Neville, 1998). The development of *in vitro* models mimicking the bovine mammary epithelial barrier function is a useful tool to understand the pathophysiological changes, which can lead to a loss of barrier integrity, occurring for example in case of exposure to toxicants. Both epithelial cell damage (Akers and Nickerson, 2011) and opening of the tight junctions (Burton and Erskine, 2003), structures that prevent paracellular transport of ions and molecules, can contribute to the blood-milk barrier impairment. Moreover, models of the mammary barriers can be valuable for the *in vitro* investigation of toxicant excretion in milk.

To obtain the barrier formation, bovine mammary epithelial (BME) cells are seeded on permeable tissue culture supports and maintained in culture until the cells reach confluence and complete polarization with the formation of an apical and a basolateral compartment. To verify the functional integrity of the epithelial monolayer, and thus the occurred polarization, the transepithelial electrical resistance (TEER) is evaluated (Hernandez et al., 2011; Wellnitz et al., 2016). It is well known that the TEER value is inversely proportional to the barrier permeability, thus it represents a good measure for the intercellular tight junction integrity (Srinivasan et al., 2015). Some BME cell lines have been cultivated on permeable supports (Wellnitz et al., 2016), even if the majority of BME cell studies have been performed using solid supports, technique that prevent the cell polarization and thus the barrier formation (Ghadiri et al., 2019; Girolami et al., 2015).

2.1. Primary Bovine Mammary Epithelial cell barrier

Primary BME cells have been used to investigate *in vitro* the mammary barrier functions and the effects of different pathogens on the barrier integrity, thus pathogen-specific mechanisms of barrier impairment have been observed (Wellnitz et al., 2013).

In a study performed by Wellnitz and colleagues (Wellnitz et al., 2016), primary BME cells were cultivated on polyester membrane inserts for 4-5 days, using the standard medium for BME cells both in the apical and basolateral compartments, then the cells were fixed to verify the tight junction formation using transmission electron microscopy. The cells showed the specific structure of the epithelial tissue, which is characterized by a continuous monolayer of polarized cells (apico-basal polarization), with the basal cell membranes firmly attached to the insert membranes and the apical cell membranes (luminal compartment) displaying numerous microvilli. Moreover, the presence of intercellular tight junctions and desmosomes between adjacent cells was demonstrated. Immunofluorescence staining of the tight junction protein zona occludens-1 was also performed, revealing that zona occludens-1 was expressed on the cell membranes in the areas of intercellular contact. To confirm the barrier integrity and function, TEER measurement was performed as well as diffusion test with fluorescent dye (Lucifer yellow). Lucifer yellow staining and TEER evaluation (mean value 1531 ± 75 Ωcm^2), demonstrated the ability of primary BME cells to form an epithelial monolayer characterized by functional integrity when cultured on inserts.

Moreover, to assess impairment of the barrier integrity caused by different mastitis pathogens, the polarized cells were treated with endotoxins (0.2-0.5 mg/ml of *E. coli* lipopolysaccharide -LPS- and 2-20 mg/ml of *S. aureus* lipoteichoic acid -LTA- added into the apical compartment) for 24 h (Wellnitz et al., 2016). Permeability test evaluating the paracellular diffusion of Lucifer yellow showed a significant alteration of the barrier function from 7 h of exposure to 20 mg/ml LTA and from 3 h of exposure to 0.5 mg/ mL LPS, whereas 2 mg/ml LTA and 0.2 mg/ml LPS did not show any significant effects on barrier permeability. Lactate dehydrogenase (LDH) release into the cell culture (apical compartment) was used as a marker of cytotoxicity as it is well known that the alteration of the epithelial barrier permeability is related not only to tight junction impairment but also to epithelial cell damage (Akers and Nickerson, 2011). A cytotoxic effect, revealed by an increased release of LDH, was demonstrated on the cells treated with 0.5 mg/ml LPS, whereas cells treated with 20 mg/mL LTA did not show any cytotoxic actions (even if this treatment showed a negative effect on the barrier function, increasing barrier permeability). Hence cell damage is responsible, together with tight junction impairment, for the loss of barrier function induced by LPS, whereas LTA acted on the barrier function just through tight junction alteration.

Primary BME cells were also used to evaluate the effects of a selective serotonin reuptake inhibitors (SSRI) on mammary barrier permeability (Hernandez et al., 2011). It has been demonstrated that a serotonin system is responsible for the homeostatic regulation of lactation and that the mammary gland express serotonin receptors which are species-

specific (Hernandez et al., 2009). The tryptophan hydroxylase-1, a rate-limiting enzyme expressed in the mammary gland of all the animals, is responsible for the synthesis of the serotonin and the serotonin transporter (SERT) that re-uptake the serotonin into the cell starting the degradation cascade of the neurotransmitter. It has been showed that serotonin acts increasing tight junction permeability during the mammary gland involution binding to serotonin receptor 7 (5-HT_7) and thus accelerating the involution process (Pai and Horseman, 2008), and that the tight junction dynamic is mainly regulated by the cycle of milk stasis and removal: tight junction permeability decreases when milk is frequently removed and increases in case of milk stasis (Nguyen and Neville, 1998). More specifically, serotonin has a biphasic action on tight junction permeability, causing initially a decrease (via protein kinase A) and then an increase (via p38 MAP kinase) (Pai and Horseman, 2008). SSRI agents act inhibiting the membrane protein SERT and thus increasing the serotonin concentration around the cell, causing the activation of serotonin signaling pathways. In a study performed by Hernandez and colleagues (Hernandez et al., 2011), primary BME cells were cultured on polyester membrane inserts in standard medium until the TEER plateau was achieved (namely about after 6 days of culture) and then treated with different concentrations (40, 100, 200 and 400 μM) of fluoxetine (FLX, a SSRI) applied in the apical compartment for 72 h. Immunostaining was performed to confirm the presence of SERT on the apical membrane of the BME cells. FLX induced a concentration-dependent decrease in TEER values at 24 and 48 h, with dosages of 100-400 μM causing a decrease in TEER to about 100 Ωcm^2 after 24 h of exposure ($P < 0.0001$). Moreover, immunofluorescent staining of the tight junction protein ZO-1 (protein that is necessary for the assembly of the tight junctions and represents a morphological marker for these structures (Paris et al., 2008) was performed after 48 h of treatment with different concentration FLX for 48 h. Concentrations of 40 and 100 μM FLX caused a decrease of the intensity of ZO-1 staining in the cells compared with the untreated control cells, whereas concentrations of 200 μM resulted in a complete loss of the ZO-1 proteins as proven by the complete absence of peripheral immunostaining. In addition, with the higher doses of FLX, cell death and fragmented nuclei were observed. Tight junction disruption in the mammary gland is a sign of mammary gland involution and the FLX property to accelerate this process could have interesting application in dairy cow medicine. These results proved that polarized BME cells represent a consistent and accurate model for *in vitro* studies on the mammary gland barrier function and an excellent tool to investigate the pathophysiological mechanisms related to the mammary gland as well as a useful starting point to design eventual subsequent *in vivo* studies.

2.2. BME UV1

BME-UV1 is an immortalized (by transfection with the SV40 T-antigen) bovine mammary cell line, responsive to EGF and IGF-I which was established by Zavizion et al. in 1996 (Zavizion et al., 1996). BME-UV1 cells were cultured on membrane inserts coated with a bovine extracellular matrix (BEM) (Wicha et al., 1982) and confluence and complete polarization were assessed via TEER measurement. After confluence (which was achieved in

3-4 days of culture, with TEER values of 1200-1500 Ωcm^2) the cells were maintained in culture for 8 days and samples of media from the apical and basolateral compartments were collected for proteins and α-casein content analysis and for plasminogen activator (PA) activity assessment. The effect of calcium on α_{s1}-casein and plasminogen activator production were evaluated using different concentration of calcium (0, 0.5, 5 mM) added into the culture medium. The protein content analysis showed that the BME cells reached functional polarity characterized by the secretion of α_{s1}-casein and PA from the apical membrane of the cells into the apical compartment (the alveolar side), and the concentration of α_{s1}-casein was 15-fold higher in the apical compared to the basolateral compartment ($P < 0.01$). The addition of calcium into the culture medium increased total proteins and α_{s1}-casein content: concentration of 0.5 and 5 mM calcium caused a 3-fold and 4-fold increase in α_{s1}-casein, respectively ($P < 0.01$) in comparison with the control cells, whereas the ratio α_{s1}-casein/total proteins of the control cells and that of the calcium-treated cells were similar. Thus, calcium probably acted as a non-specific up-regulator of protein production. Conversely, calcium had no effect ($P > 0.1$) on PA activity of these cells, indicating that the up-regulation produced by this ion does not affect all the proteins. Moreover, it is likely that the calcium mitogenic effects are not related to the expression of plasminogen activator associated genes.

BME-UV cells were also used to study the P-glycoprotein. This protein, an efflux pump of to the ATP-binding cassette super-family that effects the bioavailability a of many substances and drugs, is expressed by mammary epithelial cells.

In a work by Al-Bataineh et al. (Al-Bataineh et al., 2010), the effect of TNF-α on P-glycoprotein regulation was evaluated. Indeed, during pathological states of the mammary gland, such as an inflammatory status, cytokines (i.e. TNF-α) increase in milk and blood and can lead to the impairment of the mammary epithelial barrier function. The BME-UV cells were cultivated on polyester inserts and treated with TNF-α (200 ng/ml for 24, 72 and 120 h), then the P-glycoprotein was determined as well as the mRNA expression of bABCB1 (gene encoding for P-glycoprotein). They found that the production of P-glycoprotein its mRNA expression increased after 24 hours of TNF-α exposure, with a maximum effect after 72 h of exposure. An increase was also observed after 120 h.

A significant decrease in the apical to basolateral flux of digoxin (a P-glycoprotein substrate) was also described in the treated cells, probably because of occurred upregulation of P-glycoprotein. Moreover, the mannitol flux across the BME-UV cells increased after exposure to TNF-α, indicating in impairment of the barrier function.

All these data suggest that the BME-UV cells are a powerful model to investigate the role of P-glycoprotein in the barrier function of the mammary epithelium, since this protein influences the accumulation and removal of specific molecules from milk and blood.

3. Brain Barrier *in vitro* model

The blood–brain barrier (BBB) is a dynamic and highly selective interface between blood and the central nervous system, consisting of three different cell types (endothelial cells, astrocytes and pericytes) that constitute the neurovascular unit (Abbott et al., 2010). Among other several functions, this structure is responsible for the maintenance of the brain homeostasis controlling the exchanges between the two compartments and protecting the central nervous system (CNS) from toxic compounds (endogenous or exogenous) and pathogen agents from systemic circulation (Cardoso et al., 2010).

The BBB includes two barriers which can be considered as a single unit or as independent barriers (Blood-Brain Barrier and Blood Cerebro-Spinal-Fluid Barrier, BCSFB). The BBB is composed by an endothelial layer of cerebral capillaries, while BCSFB is made of the epithelium from the choroid plexuses (Begley, 2004; Eyal et al., 2009).

BBBs used in *in vitro* toxicology studies included primary (subpassaged) cultured cells, immortalized brain endothelial cells, cell lines of non-cerebral origin and co/multi-cultures (Prieto et al., 2004).

Even though cell lines and primary cell cultures have been used as BBB models, the latter are described as more representative of the *in vivo* condition with morphological characteristics of the brain endothelium more similar to those observed *in vivo*, even if the culture techniques for primary cells are more complex and time consuming (Cardoso et al., 2010). Despite these drawbacks, the use of *in vitro* models for the BBB is convenient since it allows to avoid invasive procedures on animals, with a reduction of costs and ethical issues, and a decrease of the interindividual differences with more controllable experimental conditions. Moreover a large number of physiological and pathological processes can be studied using this model which can represent a first approach in toxicological and pharmacological studies (Cardoso et al., 2010).

3.1. Primary BBB cell cultures

Primary cultured brain vascular endothelial cells have been derived from microvessels of the grey matter of the cerebral cortex as heterogeneous cells population (endothelial cells with pericytes) of some animal species, such as bovine and swine. After isolation and selection, the primary culture showed about 95% of microvascular endothelial cells maintaining the characteristics typical of the BBB, but, on the other hand, the primary cells gradually lose BBB properties during long term culture and an increase in the pericyte number during subpassaging was also detected. Méresse and co-workers (Méresse et al., 1989) developed a protocol for the isolation of a 100% pure population of capillary endothelial cells from bovine brain. To obtain the cell polarization and the barrier formation, the cells are cultivated on polycarbonate filter inserts and the differentiation is proved usually by measuring the TEER (Benson et al., 2013). With regard to the porcine species, two main BBB based on primary cultures have been developed. One is a

barrier formed by a primary culture of porcine brain endothelial cells, characterized by the presence of complex intercellular tight junctions and an apical-basal polarized expression of transporters and receptors. This barrier has the advantage to be relatively simple to grow, robust and reliable, showing high TEER values (mean~800 Ω cm^2) and a good expression of intercellular junctions, transporters, receptors and enzyme proteins (Patabendige et al., 2013). The other main swine *in vitro* BBB model is based on primary porcine brain endothelial cells maintained in co-culture with primary astrocytes and pericytes (Thomsen et al., 2015).

Primary Porcine Brain Capillary Endothelial Cells (PBCECs) is a well-known, flexible and validated *in vitro* predictive model that represents an effective tool for neurotoxicity and transport studies between the blood and the brain (Behrens et al., 2015; Franke et al., 2000; Weidner et al., 2013). The PBCEC barrier has several advantages including handling and good *in vitro/in vivo* correlation (Behrens et al., 2015). Studies on fusariotoxins have been performed using PBCECs. The T-2 fusariotoxin (T2) and its main metabolite HT-2 (HT2) showed very high cytotoxicity at low (nanomolar) concentrations (24 or 48 h of exposure), and both T-2 toxin and HT-2 were able to cross the PBCEC barrier, after application from the apical as well as from the basolateral site, suggesting a translocation in brain with a direct effect on the barrier. A complete loss of the barrier function was detected with dosages of 75 nM T-2 toxin and 200 nM HT-2 toxin. Thus T-2 showed a more potent action and a faster penetration (already after 2 h of exposure) in comparison to HT-2 whose penetration required a longer exposure period (Weidner et al., 2013). On the other hand, deoxynivalenol (DON) was slowly transferred through the barrier (even if it showed a permeability comparable with morphine, that is a well-known CNS-active drug able to cross the BBB), but impaired drastically the barrier integrity causing a decrease in the TEER at 10 μM concentration (from 6.6 to 48 h of exposure), whereas 3-acetyldeoxynivalenol was transferred very fast through the barrier but produced just mild alterations of the barrier integrity compared to DON. However, 10 μM 3-AcDON caused a TEER decrease but the action on the barrier function was, in contrast to 10 μM DON, not detrimental enough to provoke an increase of sucrose permeability (used as a negative permeability marker in case of intact barrier function). Moreover, neither T2 and HT2 nor DON were found enriched in one of the two compartments during active transfer evaluations, so it could be hypothesized that their transport did not involve an efflux protein (Behrens et al., 2015). Moniliform (MON) exposure for 48 h (10 μM) did not exert any detrimental effects on cellular viability, integrity of cytoplasmic membranes and permeability of PBCEC monolayer. Due to its small molecular size it is likely to cross the cellular membranes, but its high polarity represents an important factor that can limit this passage, thus MON showed a transfer rate across the BBB similar to that of DON, which was 3-4 times more rapid that ^{14}C sucrose that was taken as negative control (Behrens et al., 2015). This rate indicates that MON is able to cross BBB but in a limited extent. Moreover an active transfer study was performed and MON was not found more concentrated in one of the two compartments, so efflux protein are not likely to be involved (Behrens et al., 2015).

The effect of enniatin B (ENN B) and enniatin B1 (ENN B1) on cell viability of PBCECs was also evaluated, demonstrating a significant cytotoxic effect for ENN B and ENNB1 with exposure concentrations above 5 μM and 2.5 μM for 48 h, respectively (Krug et al. 2018). Moreover passive transport studies, from the apical to the basolateral side were performed, exposing PBCECs to 1 μM ENNB and ENN B1 in the apical compartment. At this concentration (which did not show any cytotoxic effects on PBCECs and did not cause changes in TEER values during a 48 h exposure) the permeability coefficient for ENN B was of 14.9×10^{-6} cm/s, similar to that of ENN B1 (14.6×10^{-6} cm/s), thus they both showed a transport kinetic characterized by a very rapid and high transfer rate, similar to those of molecules that are known cross the BBB (Krug et al., 2018). Active transport studies have also been performed, adding ENN B and ENN B1 both in the apical and in the basolateral compartment of the PBCEC barrier at an equimolar concentration of 200 nM (dosage that did not cause any detrimental effect on the barrier integrity and permeability). A slight enrichment in the apical side was found for the two mycotoxins, suggesting just a weak efflux of ENNs from the basolateral to the apical side (Krug et al., 2018) .

A study has been performed on PBCECs to evaluate the effects of exposure to a concentration of 15 μg/ml of different metallic nanoparticles (silver nanoparticles: 25, 40 and 80 nm; copper-oxide nanoparticles: 40 and 60 nm; gold nanoparticles: 3 and 5 nm) in terms of release of pro-inflammatory mediators (IL-1β, TNFα and PGE_2) and effects on barrier permeability. Exposure to copper-oxide nanoparticles or silver nanoparticles resulted in a significant increase of the barrier permeability (permeability ratio), whereas gold nanoparticles appear least likely to cause an increased permeability. Smaller silver nanoparticles produced an increased barrier permeability and a strong inflammatory response, on the contrary the effects induced by larger silver nanoparticles were less intense. PGE_2 release after copper-oxide nanoparticles and silver nanoparticles exposure was significantly increased but not after gold nanoparticles exposure. Similarly, release of TNFα and IL-1β was observed after copper-oxide nanoparticles and silver nanoparticles treatments while gold nanoparticles did not cause any secretions (Trickler et al., 2014).

PBCECs have also been used in a work performed by Müller and colleagues to evaluate the toxicity of arsenolipids, finding that these compounds were able to increase the permeability of the BBB barrier (Müller et al., 2018).

3.2. Immortalized BBB cell lines

These type of cell lines present the advantage to be ready to use, not requiring the periodic isolation from brain material thus reducing the costs and the labor. On the other hand, immortalized cell lines are characterized by a paracellular leakiness with consequently low TEER values, <500 Ω cm2 (Lauer et al., 2004). The co-culture with astrocytes (usually from newborn rats or rat glioma cells) or the addition of astrocyte-conditioned medium (ACM) to the culture can increase paracellular tightness and also stimulate the expression of many transporters (Gaillard et al.,

2000). But, in this way, the BBB system will be made of a heterologous co-culture and/or it will be added with a tumor-type phenotype, in either case increasing the variability of the model and losing on species-specificity.

Immortalized porcine brain microvessel endothelial cells (PBMEC/C1-2), generated by the immortalization of PBMEC isolated from fresh brains with pRNS-1 encoding for the small and large T-antigens of simian virus 40 (Teifel and Friedl, 1996), has been used to develop a model for the evaluation of the BBB permeation for drugs acting on the CNS (Lauer et al., 2004). In this work inserts with a pore width of 1 μm were used, since they demonstrated that this type of filters is optimal for permeation studies of lipophilic substances.

Recently, endothelial-like bEnd.3 murine immortalized cell line and U87 human glioblastoma cell line were used to develop realistic model of BBB, where 1:1 scale of 3D biomimetic, microfluidic microenvironment is proposed (Marino et al., 2018). In this model the biohybrid porous tubular structures are scaffolding biological barrier of endothelial and glioblastoma cells. The technological elaboration of reliable *in vitro* models of the BBB is presented in a recent review paper (Sivandzade and Cucullo, 2018).

4. Intestinal barrier *in vitro* model

The intestinal epithelium is a cell monolayer that constitutes the largest and most important barrier against the external environment. The villi are the main anatomical and functional unit for xenobiotic absorption in the intestinal mucosa (Pácha, 2000). The intestinal barrier includes the epithelial layer, the lamina propria, and the wall of vessel, which are the layers that any molecule that has to cross to enter the bloodstream (Le Ferrec et al., 2001). The selective barrier function of the epithelium is due to a protein network which is responsible for the cohesion of adjacent cells, closing the intercellular space. The connection between the epithelial cells is possible thanks to three adhesive complexes: desmosomes, adherens junctions, and tight junctions (Groschwitz and Hogan, 2009).

According to the ECVAM classification, *in vitro* models of the intestinal barrier are divided into few groups: organotypic models, Ussing chambers, everted gut sac, isolated and perfused intestinal segments and cell models (Le Ferrec et al., 2001). Most recent toxicology *in vitro* models are based on three-dimensional structures including biomaterial cultures, spheroids and organoids (Augustyniak et al., 2019) obtained by spontaneous differentiation and bioprinting, and micro-flow organ-on-a-chip models. Nowadays, to our knowledge, none of these systems are based on cells other than murine or human (i.e. swine, bovine or dog origin).

Some of the most used *in vitro* toxicology tests are based on monolayer cell culture systems. The main parameters evaluated to assess the occurred differentiation and thus the barrier formation, in order to use the system in toxicology studies, are: the transepithelial electrical resistance (TEER, which allows the assessment of the cell

monolayer integrity), differentiation markers, morphological differentiation, permeability glycoprotein (Pgp) expression, permeability to certain molecules such as mannitol, Lucifer Yellow, etc. (Le Ferrec et al., 2001).

Comparing *in vitro* epithelial intestinal barrier studies with *in vivo* studies, the latter present many disadvantages, such as an uncontrolled environment, microbiome modulation, immune response and individual variation which can mask the mechanism of the xenobiotic toxicity.

The intestinal epithelial cell lines can be generated from a cancer tissue and from normal tissue. The intestinal barrier *in vitro* toxicology studies are mainly based on cell cultures of primary (sub-passaged) cells, immortalized intestinal epithelial cells, cell lines of non-intestinal origin and co/multi-cultures.

Petto and colleagues (Petto et al., 2011) developed a method for culturing **primary porcine colonic epithelial cells** obtained from the proximal colon of piglets. They found that primary cultures of porcine colonic epithelial cells show characteristics similar to those of the polarized colonic epithelium *in vivo* and that they are a suitable model to investigate the epithelial barrier function, particularly the *in vitro* epithelial tightness by the analysis of the transepithelial electrical resistance (TEER).

The **spontaneously immortalized intestinal epithelial cell lines 1 and J2** (IPEC-1, IPEC-J2) are swine cell lines obtained from piglets less than 24 hours old and are considered important tools for the study of the intestinal function (Nossol et al., 2015).

IPEC-1 are a non-transformed, non-carcinoma, polarized epithelial cells of porcine small intestinal origin (Diesing et al., 2011) isolated from the small intestine of (Arce et al., 2010; Koh et al., 2008) one day old piglets (Gonzalez-Vallina et al., 1996).

IPEC-J2 is an intestinal porcine epithelial cell line derived from the jejunum of neonatal unsuckled pig (Arce et al., 2010; Koh et al., 2008), established from normal intestinal epithelium cells and thus belonging to the group of non-transformed cell lines (Diesing et al., 2011; Schierack et al., 2006). The *in vitro* models using porcine intestinal epithelial cells (IPEC-1 and IPEC-J2) are well established and predictive tools for toxicological studies. Acute and chronic toxicity, due to a long-term exposure to toxicants, have been studied using these cell lines including many fusariotoxins (Bertero et al., 2018): in particular, a recent study performed by Ying and colleagues (Ying et al., 2019), evaluated the effects on the intestinal barrier function of DON and ochratoxin A, alone and in combination, using polarized IPEC-J2 cells, whereas Gu and colleagues (Gu et al., 2019) used a co-culture of polarized IPEC-J2 and porcine peripheral blood mononuclear cells (PBMCs) to evaluate the effects of fumonisin B1 (FB1) and its hydrolyzed metabolite (HFB1) on the intestinal barrier function and immunity.

Another porcine intestinal cell line is the IPI-2I, which was isolated from ileal tissue (Arce et al., 2010; Koh et al., 2008) and transformed with an SV40 plasmid (Kaeffer et al., 1993) . The IPEC-J2 and IPI-2I represent two porcine

epithelial cell lines from different regions: IPEC-J2 from jejunum and IPI-2I from ileum (Arce et al., 2010). Moreover, IPI-2I cells were isolated from adult boar (Kaeffer et al., 1993), while IPEC-J2 cells were isolated from neonatal piglets (Diesing et al., 2011). Kaeffer and co-authors (Kaeffer et al., 1993) demonstrated that IPI-2I cells show characteristics of both epithelial and mesenchymal cell, expressing the keratin 18 filament marker for the epithelia and the vimentin filament which is a typical mesoderm marker. The IPI-2I cell line is characterized by poor differentiation abilities demonstrated by a lack of villin expression and of trans-epithelial resistance. Stable epithelioid phenotypes of IPI-2I cell line were created by multiple subcloning (Kaeffer et al., 1993). IPI-2I cells were used for evaluating the effect of zinc (zinc oxide -ZnO- and zinc chloride -ZnCl2-, additives used in feedstuffs). In a work by Rossi and colleagues (Rossi et al., 2014), the IPI-2I cells were exposed to different concentrations of zinc oxide and chloride (namely 50, 200, 1000 and 4000 μM) for 3 and 24 h, then the effect of zinc on cell viability was assessed by MTT test. After 3 h of exposure, ZnO concentration of 50, 200 and 1000 μM did not cause any significant effect on the cell viability, whereas the highest concentration (4000 μM) caused a significant reduction ($p<0.05$). After 24 h, all the tested concentrations significantly affected the cell viability, causing a reduction ($p<0.05$). A similar pattern of action (time and dose dependent) was observed with ZnCl2.

Another cell line, characterized by epithelial phenotype and isolated from swine species, that has been used as a model of intestinal barrier is the mature pig small intestinal (PSI-1) cell line.

Trapecar et al. (Trapecar et al. 2011) used PSI cell to evaluate the toxicity of Bacillus cereus probiotics. More in details, the attachment, cytotoxicity and the ability to cause nitric oxide production of various toxigenic strains of B. cereus and two probiotic strains (Paciflor and Toyocerin) were evaluated.

Wild-type toxigenic strains and the Paciflor probiotic were able to induce cytotoxicity and nitric oxide production, whereas attachment was low for all the strains. In the same experiment, 10^8–10^9 spores of the toxigenic B. cereus NVH75／95 were administered to weaned piglets without inducing diarrhea or intestinal lesions, thus confirming that this cell line is a good and sensible tool for the evaluation of the potential enterotoxicity of B. cereus strains.

Regarding the type of culture (monoculture *vs* co-culture), cells maintained in monoculture may have different behavior compared to those cultured in co-culture. The cell behavior depends on the cell-cell interaction and the chemical communication (i.e. cytokine release) (Langerholc et al., 2011). Because the *in vitro*-based assays that use cells in monoculture are just a partial representation of the complexity of the interactions occurring between the cells and the extracellular environment and between the different cell types of specific organs (Adler et al., 2011), tests based on multi-cultures are often preferred. Multi-culture *in vitro* systems include models which are built from untransformed pig small intestinal epithelial cell lines and pig macrophage cell lines that can be co-cultured in a functional multi-culture *in vitro* model of the intestine. The use of co-culture systems (i.e co-culture of white cells, muscle cells,

adipocytes, hepatocytes etc.) shows a great potential for applications in bioavailability studies, since these systems can offer a more complete representation of the intestinal barrier (Trapecar and Cencic, 2012).

In addition to "traditional" 2D cultures (monolayer), 3D *in vitro* intestinal models (enteroids) have been developed; these systems are obtained from intestinal monolayer epithelium, and are very similar (from a morphological point of view) to the small intestine thus they are also known as "mini guts". These structures have lumen, crypts and villi domains and they are obtained from enterocytes, enteroendocrine cells, goblet cells, tuft cells, Paneth cells and stem cells (Clevers, 2016; Sato and Clevers, 2013). The enteroids, compared to the 2D *in vitro* models, present all the specific epithelial cell types of the intestine, as well as the functions of the tissue of origin, are self-renewal and self-organized, but these models do not have immune or stromal cells and are complex to obtain, thus requiring expertise. The 2D *in vitro* models, on the other hand, are well-established are relatively cheap but they lack 3D architectural details (Clevers, 2016; Sato and Clevers, 2013).

Cell cultures composed of a single cell type are usually grown in monolayer in a 2D culture (Clevers, 2016), while enteroids are grown as 3D structures and are generated from pluripotent stem cells: embryonic stem cells or induced pluripotent stem cells, as well as adult stem cells from intestinal tissue (Clevers, 2016; Sato and Clevers, 2013). Gonzales and colleagues in 2013 described a method to obtain 3D porcine enteroids after intestinal crypt-villi unit isolation from 2-14 days-old wild type Yorkshire piglets (Gonzalez et al., 2013).

Van der Hee and Wells (van der Hee et al., 2018) presented a model that combine 2D and 3D culture system. In this study, the epithelial monolayer is generated from porcine interstinal ileum organoids obtained from the interstinal tissue of two 5 month-old slaughter pigs according to Sato and colleagues' procedure (Sato et al., 2011). In this *in vitro* model the confluent monolayer reached high TEER value, with tight junction expression, in three days and it could be employed in toxicology experiments for up to two weeks. This type of culture, which allow the formation of a polarized intestinal cell monolayer, in comparison with organoids offers an easier access to the apical surface, thus the trans-epithelial permeability and the response to xenobiotic exposure can be measured for example by TEER evaluation, cell count, expression of cytokines, nuclear factors, connexins etc. (van der Hee et al., 2018) with the possibility to apply this system to bioaccessibility, absorption, and biotransformation studies.

5. Skin barrier

In vitro models of skin barrier include 2D and 3D culture systems that are characterized by different properties, among others the degree of complexity and the cell-cell interactions. The 2D skin *in vitro* models are simple static cell cultures grown in monolayer that mimic the epidermis, whereas 3D skin *in vitro* models, which are usually called "full-thickness skin equivalents", are more complex systems that combine epidermis with dermis (Black et al., 1999). Further

improvements of these systems could include the addition of adipose tissue, hair follicles and a functional vascular network, in order to better reflect the complexity and the functionality/characteristics of the skin barrier such as the resilience, cell sheeting, cell layering, immune function, blood perfusion, and innervation (Sakolish et al., 2016). In the 3D organotypic skin equivalents, the keratinocytes are seeded on the surface of a dermal equivalent made of fibroblasts incorporated into a bio-matrix of collagen and other proteins (Serra et al., 2007).

2D and 3D skin *in vitro* models are developed to follow the 3R principles but also to simplify the complexity of the skin biological processes that are, for many reasons, difficult to study *in vivo*.

The study of the skin barrier using 2D and 3D *in vitro* models is mostly based on the evaluation of three skin integrity parameters: Electrical Resistance (ER), Tritiated Water Flux (TWF) and Trans-Epidermal Water Loss (TEWL) (Davies et al., 2004; Heylings et al., 2001).

Serra and colleagues in 2007 presented a method to generate and characterize a 3D dog skin barrier *in vitro* model with an epidermal and a dermal compartment. The protocol developed by Serra et al. demonstrate the possibility to obtain *in vitro* a canine skin equivalent, even if this model still presents some limitations, such as the absence of hair follicles, inflammatory cells, nerves, blood and vessels (Serra et al., 2007). The 3D *in vitro* model of dog skin was developed as an organotypic co-cultures of the two major skin cell populations: epidermal keratinocytes and dermal fibroblasts (Serra et al., 2007). These cell populations were isolated via enzymatic digestion of fresh samples obtained by skin biopsies of heathy dogs. Then the primary culture of keratinocytes (cultured in medium supplemented with EGF to stimulate differentiation and to obtain a more physiological dog skin structure) was seed onto a collagen bio-matrix which was obtained adding mature fibroblasts to a collagen rat tail type I collagen solution. Primary keratinocytes were isolated from different canine skin samples. At the end of the differentiation process, a multilayer epidermis with the four typical layers (stratum basale, stratum spinosum, stratum granulosum and stratum corneum) and the epithelial cells connected by desmosomes, was obtained. The epidermal structure showed by this model is very close to that of healthy dog skin, with a normal cornification process and a dermal–epidermal junction very similar to that observed *in vivo*, thus this model could represent a useful tool for studies on the biology of the canine epidermis and dermis, for the evaluation of the mechanisms of cell–cell and cell–matrix interactions in many fields (i.e. toxicology, pharmacology, nutrition, etc.) (Serra et al., 2007). Another 3D *in vitro* model of dog skin, characterized by the expression of the tight junction proteins zonula occludens-1 and claudin-1 with a disposition similar to those of the *in vivo* canine skin, was developed using canine progenitor epidermal keratinocytes (CPEKs) by Teramoto and colleagues (Teramoto et al., 2018).

6. Pulmonary barrier

The normal alveolar epithelial barrier is composed of alveolar epithelial type I (AT1) and alveolar epithelial type II (AT2) cells and has the peculiarity to be mostly impermeable to proteins and solutes (Bhattacharya and Matthay, 2013). The respiratory tract, together with the gastrointestinal and dermal barrier, is considered an important route for xenobiotic absorption, also because of its peculiarity (fast absorption and absence of first pass metabolism). Permeability and solubility of molecules at this level are useful data to obtain, and, in this context, lung epithelial barrier models are needed (Eixarch et al., 2008).

Pulmonary barrier *in vitro* models are very difficult to obtain. It is considered that primary culture cells present cell characteristics and a differentiation status more similar to those of the *in vivo* situation, thus *in vitro* models of pulmonary barrier made of primary cell cultures are more common. Because of the lack of suitable alveolar epithelial cell lines, **primary alveolar epithelial cell cultures** are the most used in the *in vitro* studies regarding the alveolar function (i.e. permeability studies, TEER evaluations, solutes/xenobiotics transport and metabolism analyses). Primary alveolar epithelial cells are obtained by lung resection. However, primary cell cultures are time-consuming, expensive models and, in addition, the monolayer has a lifetime of just few days. With regard to the advantages, the porcine/bovine lungs, which represent the cell sources, are easy to obtain from abattoirs (Steimer et al., 2006). Steimer and colleagues in 2006 (Steimer et al., 2006) presented an evaluation of the main properties of this culture. They analyzed the morphology and the bioelectrical and immunocytochemical characteristics of porcine alveolar epithelial cells in primary culture isolated from pulmonary lobes of 6 months old pigs obtained from slaughterhouse. They demonstrated that primary porcine alveolar epithelial cells (pAEpC) are able to differentiate into a tight monolayer characterized by the presence of two cell types, similar to type I and type II pneumocytes, and that this culture is suitable for studies on barrier and intercell junction properties. The cells formed the epithelial barrier around day 6. Permeability evaluation using sodium fluorescein indicated the need to reach a minimal TEER value of 600 ohmcm2 for transport studies (Steimer et al., 2006). More recently, Sreenivasan and colleagues (Sreenivasan et al., 2019) developed and characterized cultures of swine primary respiratory epithelial cells, finding that primary lung epithelial cells grown on transwell inserts were able to polarize and express tight junction proteins, reaching the highest TEER values (2240 Ωs) after 16 days of culture. Therefore this culture could represent a solid model to evaluate the absorption and the potential detrimental effects on the barrier integrity of toxicants, allowing to perform species-specific *in vitro* inhalation toxicology studies.

McClenahan and colleagues in 2008 used bovine pulmonary epithelial (BPE) cells and bovine lung microvascular endothelial cells (BPMECs) to analyze the consequence of the exposure of an *in vitro* pulmonary barrier model to lipopolysaccharide (LPS), leukotoxin (LKT). These two toxins are produced during infection with *Mannheimia haemolytica* and are described to be associated with extensive alveolar edema due to vascular leakage

(McClenahan et al., 2008). They measured the permeability (via TEER evaluation) in BPE and BPMEC monolayers after exposure to LPS and LKT (McClenahan et al., 2008). Endothelial cell monolayers exposed to LPS showed a significant decrease in the TEER values, increased levels of cytokines, apoptosis, and morphological alterations. Regarding the epithelial cells, neither LPS nor LKT showed a similar action (in terms of morphological alterations and apoptosis) to those exerted on the endothelial cells, just an increase of the secretion of cytokines was recorded (McClenahan et al., 2008).

In 2009 the same group performed the permeability assay after ATP treatment on the BPE and BPMECs *in vitro* models of pulmonary barrier, since it has been found that high levels of ATP are present during pulmonary infections (McClenahan et al., 2009).They observed that the BPE culture underwent noticeable morphological changes after ATP exposure showing also an increased permeability (within the first hour of exposure, assessed via TEER evaluation), which was also noted in the BPMECs (McClenahan et al., 2009).

7. Conclusions

In vitro species-specific blood brain barriers and epithelial barriers of intestine, lung and skin, here described and summarized, result as useful and promising tools in toxicology, that could find huge applicability in different scientific area, through a multidisciplinary approach.

We illustrated the main species-specific *in vitro* barrier models that could be profitably used in toxicological evaluations due to the key role played in relation to xenobiotic absorption, biotransformation, metabolism, and effects. Namely, with regard to the mammary barrier models, they have proven to be a consistent and reliable instrument for *in vitro* studies regarding the mammary gland barrier function and useful systems to analyze the pathophysiological mechanisms linked to xenobiotic exposure that can comprise the loss of barrier integrity with a possible transport/excretion of toxicants into milk. The BBB is also a useful *in vitro* model since it allows the investigation of large number of physiological and pathological processes and represents a first approach in toxicological and pharmacological studies (Cardoso et al., 2010), permitting to reduce the number of animals needed for research and offering more standardized experimental conditions, avoiding the bias of interindividual differences. Another epithelial barrier of great interest is the intestinal one, mimicking *in vitro* oral exposure. Though intestinal organoids (3D self-assembling cell structures) seem to be promising cultures for species-specific toxicological studies (Augustyniak et al., 2019), traditional 2D species-specific intestinal cultures, with apical and basolateral sides, could offer some advantages and additional information, for instance the possibility to perform barrier integrity evaluation with TEER. Finally, the species-specific pulmonary and skin barriers, even if currently their application is limited, could be of interest for inhalation and topic exposure.

Moreover the combination of human epithelial barriers (Gordon, 2015) together with the species-specific ones, could be a starting point for an Integrated Approach for Testing and Assessment of xenobiotics following the One Health concept, connecting animal, human and environment with an *in vitro* strategy.

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9. Bibliography

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