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Ex vivo applications of porcine ocular surface tissues: Advancing eye research and alternatives to animal studies

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Summary. The use of tissues of porcine origin has gained significant momentum in the scientific community due to their anatomical and physiological resemblance to human tissues.

This review provides a comprehensive overview of the key biological features of porcine ocular structures, including the cornea, conjunctiva, and associated tissues, in comparison to their human counterparts. Additionally, this review outlined the *ex vivo* applications of these tissues in the study of different biological processes and the simulation of pathological conditions. By highlighting the potential of porcine ocular surface tissues as cost-effective, ethically appropriate, and reliable models, this review underscores their value in advancing eye and vision research.

Key words: Alternative methods, Anterior eye, Ocular surface, *Ex vivo* models, Porcine tissues

Introduction

The anterior segment of the eye is in direct contact with the external environment and is equipped with highly specialized tissues to cope with that situation and protect the intraocular tissues from harm. The conjunctiva is the complex mucosal tissue that lines the inner surface of the eyelids and the anterior surface of the eyeball, reaching the cornea, the transparent and avascular tissue that allows light to enter the eye. The boundary between the conjunctiva and the cornea is the limbus, a transition epithelium in whose subepithelial stroma corneal stem cells reside in specialized and protected niche structures. These three components, the conjunctiva, cornea, and limbus, along with the tear film that moisturizes and nourishes them, form the ocular surface. However, the ocular surface is more than a collection of tissues; it functions as an integrated anatomical and pathophysiological unit (Stern et al., 1998) with supporting secretory glands, a local immune system, and a neuro-hormonal network regulating all involved elements.

The ocular surface can experience diverse insults that lead to different pathological conditions, many of them quite complex and poorly understood. In addition, there is a great need for curative treatments for these as, often, only palliative treatments are available. Clinical and preclinical research is pivotal to better understanding and developing novel treatments for ocular surface pathology. The information gathered from tissue biopsies and cytology under *in vitro* or *ex vivo* conditions is essential for understanding the morphological and molecular changes that occur in diseased ocular surface tissues. However, there is an important limitation in the availability of human tissues that restricts the performance of this kind of preclinical study.

Within this context, pigs are a recurrent source of biological material for scientific research, including eye-related research. Tissues from the white domestic pig (*Sus scrofa domestica*) are the most widely used due to several advantages. These animals can be easily obtained from registered slaughterhouses at little cost, with full sanitary, regulatory, and ethical assurances, as they are already being sacrificed for human consumption. While the increased variability in experimental outcomes compared with more genetically homogeneous laboratory animals could be seen as a disadvantage, it can also be argued that this better represents the actual biological processes.

Aside from the domestic pig, there are miniature swine breeds, such as the Göttingen minipig, developed at the University of Göttingen (Germany) in the 1960s, that have become popular in recent years. There are

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several publications using these minipigs in the field of ocular research (Shrader and Greentree, 2018), one of them related to the histological description of the eyeball (Schrader and Mowry, 2019) and some other studies related to corneal damage (Chen et al., 2015; Telinius et al., 2020). However, as far as we know, this swine breed is preferred for *in vivo* surgery or toxicology studies requiring an animal model closely resembling humans rather than for obtaining ocular surface tissue for *ex vivo* culture.

The aim of this review is two-fold: first, to provide the interested reader with an overview of the key biological features of porcine ocular surface structures compared with those of their human counterparts. Second, to summarize how these tissues have been used *ex vivo* to study alterations of these structures or to simulate pathological situations. Porcine *in vivo* models are outside the scope of this review, however, interested readers can obtain information about this topic elsewhere (Shrader and Greentree, 2018).

Characteristics of porcine ocular surface structures

Figure 1 shows the macroscopic aspect of an exenterated porcine eyeball with the eyelids highlighting the cornea and the conjunctiva. Next, we describe in detail what is known about the porcine ocular surface components.

Cornea

There is abundant published information regarding the porcine cornea, which is described as asymmetrically oval (Faber et al., 2008). Different authors reported detailed measurements of different porcine eyeball parameters, including corneal diameter, thickness, and endothelial cell density (Sánchez et al., 2011; Menduni et al., 2018; Crespo-Moral et al., 2020). Porcine corneal thickness is almost twice that of the human cornea, although measurements vary depending on the method used. Table 1 summarizes the comparison between values measured in pig and human corneas using different techniques. Pig corneal diameter is larger (Faber et al., 2008) with a larger corneal radius than that of the human cornea (Ma et al., 2021).

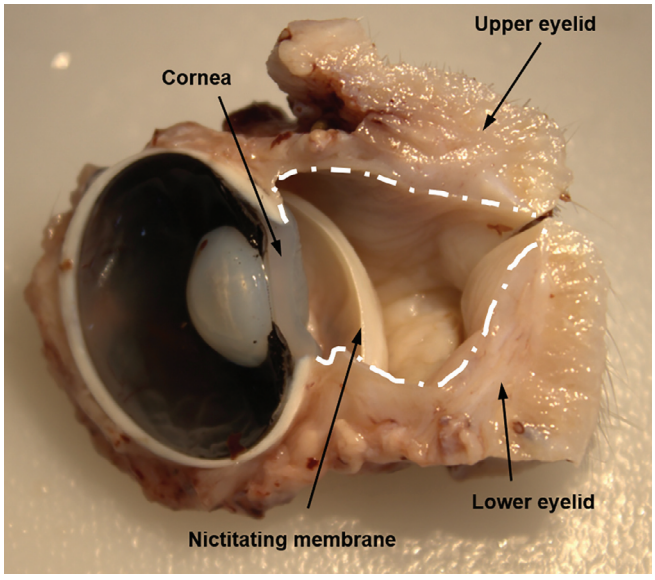

Fig. 1. Macroscopic photograph of a fixed and sectioned porcine eyeball with the eyelids. The white lines demarcate the conjunctiva. This figure was originally published in Crespo-Moral et al. (2020) under the terms of the Creative Commons Attribution License.

Regarding corneal layers, the porcine epithelium has 6-9 layers of differentiated cells expressing the specific cytokeratin pair 3 and 12 (K3/K12), with an average thickness of 80 µm that can vary by 25 µm (Sanchez et al., 2011; Guindolet et al., 2017). The most superficial epithelial cells also express zonula occludens-1 (ZO-1), and the basement membrane is positive for laminin-5 (Guindolet et al., 2017). The stromal thickness is 900 µm, almost twice the human stromal thickness, and Descemet's membrane with the endothelium has a thickness of approximately 30 µm (Sanchez et al., 2011). The stroma has a large amount of collagen type I organized in lamellae (Jay et al., 2008) with a mainly circumferential orientation, unlike humans, in which it is mainly orthogonal (Hayes et al., 2007). It is noteworthy that the porcine cornea lacks the Bowman's layer (Jay et al., 2008), although this aspect is still controversial. Our results support the absence of the Bowman´s layer (Crespo-Moral et al., 2020); however, a recent work using histochemical analysis reported the presence of a rudimentary Bowman's layer, which the authors hypothesize may be evolutionarily vestigial (Barhaiya and Kumar, 2024).

It is interesting to note that several authors published similarities in refractive power and biomechanical properties between porcine and human corneas (Zeng et al., 2001; Faber et al., 2008), which further supports the functional resemblance of the cornea between the two species.

Limbus

The limbal tissue area represents the transition between the cornea and the conjunctiva. Histologically, the porcine limbus has epithelial folds like the Palisades of Vogt, characteristically described in the human limbus as the anatomical location of corneal stem cells and their niche (Notara et al., 2011; Crespo-Moral et al., 2020). The porcine limbal epithelium has 6-9 layers of differentiated epithelial cells, including basal cells that express putative stem cell markers such as ABCB5, K14, and p75 and are negative for the K3/K12 pair, fairly similar to the human limbal epithelium (Guindolet et al., 2017).

The three-dimensional structure of the limbal niche was further analyzed by Grieve et al. in 2015 using full-field optical coherence microscopy. The porcine limbal

niche is characterized by the presence of limbal crypts with an average volume of 9% of the limbal volume, close to the 13% measured in the human limbus. Porcine and human limbal niche architecture share a radial orientation of the crypts that extend beneath the scleral roof, which provides protection from light irradiation (Grieve et al., 2015).

Not only is porcine limbal niche anatomy similar to that of the human limbus, but also highly proliferative cells with progenitor-like phenotypes, including the expression of the putative stem cell markers p63-α and integrin β1, were identified in the porcine limbal crypts (Notara et al., 2011). Also, limbal epithelial cells harvested from crypt regions show high colony-forming efficiency when cultured (Grieve et al., 2015). This resemblance is of great interest for further functional studies of the stem cell niche. Interestingly, porcine limbal stromal cells with mesenchymal-like properties were identified and characterized a few years ago (Fernández-Pérez et al., 2017).

Conjunctiva

The resemblance between porcine and human conjunctival tissue structure is remarkable, except for the presence of the nictitating membrane in the porcine eye, which makes the surface area of conjunctival tissue greater in pigs than in humans. The nictitating membrane, informally known as "the third lid", is a semilunar fold of conjunctival tissue that extends from the medial canthus in most vertebrate species and moves horizontally as a blink across the eyeball, partially covering the cornea during its movement. It has well-established lubricant and protective roles (Arends and Schramm, 2004), and in the pig, the nictitating membrane displays a substantial percentage of the total goblet cell number present in the whole conjunctival area (Crespo-Moral et al., 2020).

Another point in common of the conjunctiva in the two species is the presence of an abundant, well-organized conjunctiva-associated lymphoid tissue (CALT) (Crespo-Moral et al., 2020), whose characteristics and topographical distribution are quite similar to that described for humans (Knop and Knop, 2000). CALT is responsible for the immune protection of the ocular surface. Its presence in the porcine conjunctiva makes it more suitable to study immune-based ocular surface diseases than that from other species, such as the commonly used rodents, which lack CALT. Besides, IgA and secretory component immunoreactivity is clearly observed in the porcine nictitating membrane, analogous to the findings observed in the conjunctival epithelium and the main and accessory lacrimal glands in humans (Schlegel et al., 2003).

Lacrimal and Meibomian glands

The comprehensive study published by Henker et al. (2013) on the morphological features of the porcine lacrimal gland revealed a similar anatomical location of the gland within the orbit as in humans, as well as its arterial blood supply. The authors described that the porcine lacrimal gland has approximately seven main excretory ducts with a mean diameter of 200 µm responsible for transporting lacrimal fluid to the ocular surface, which corresponds to the analogous human gland that usually shows six to twelve excretory lacrimal ducts. A previous morphological and histological study of the lacrimal gland of fetal pigs (Klećkowska-Nawrot and Dzięgiel, 2008) showed that its shape is triangular in the prenatal period and that this shape is maintained in the postnatal period and adulthood, while the human gland

Table 1. Comparison between values measured in pig and human corneas.

Parameter	Values	
	Pig cornea	Human cornea
Corneal keratometry (steep radius of curvature)	7.85±0.32 mm (Menduni et al., 2018)	7.7±0.26 mm (Ma et al., 2021)
Corneal keratometry (flat radius of curvature)	8.28±0.32 mm (Menduni et al., 2018)	7.9±0.25 mm (Ma et al., 2021)
Vertical corneal diameter	12.09 mm (Bartholomew et al.1997) 12.4±0.7 mm (Faber et al., 2018) 12.69±0.58 mm (Menduni et al., 2018)	10-11 mm (Yanoff and Duker, 2019)
Horizontal corneal diameter	14.23 mm (Bartholomew et al.1997) 14.9±0.5 mm (Faber et al., 2018) 14.88±0.66 mm (Menduni et al., 2018)	11-12 mm (Yanoff and Duker, 2019)
Corneal thickness	980-1,190 µm[A] (Bartholomew et al., 1997) 534-797 µm[B] (Faber et al., 2018) 1009±1 µm[B] (Menduni et al., 2018) 1,248±144 µm[C] (Menduni et al., 2018) 1,131-1,497 µm[D] (Crespo-Moral et al., 2020)	520-590 µm[B] (Doughty and Jonuscheit, 2007) 542 µm[B] (Doughty and Zaman, 2000) 542-640 µm[B] (Ma et al., 2021)
Corneal endothelial cell density	3,250±172 cells/mm^2 (Menduni et al., 2018)	2,700-2,900 cells/mm^2 (Yee et al., 1985) (Middle-aged adults)

[A]: Measured using ultrasound biomicroscopy; [B]: Measured using ultrasound pachymetry; [C]: Measured using optic coherence tomography (OCT); [D]: Measured using the Automated Cellular Imaging System III (ACIS III; Dako, Glostrup, Denmark).

shape is classically defined as concavo-convex (Noyes, 1894), and more recently as almond or oval-shaped (Lorber, 2007). Gland structure is lobular, as in humans, arranged into alveoli and tubules, whose ultrastructure is composed of mucous and serous cell types with a predominance of mucoserous cells. The intercalated ducts have a stratified cuboidal epithelium, the striated duct epithelial cells are columnar, and the excretory ducts are composed of a simple cuboidal epithelium with myoepithelial cells surrounding them (Kühnel and Scheele, 1979; Klećkowska- Nawrot and Dzięgiel, 2008). Regarding dimensions, glands in adult pigs seem to be 50% larger in width and 90% larger in length than the average human equivalent (Tamboli et al., 2011).

Numerous Meibomian glands are located in the tarsal plate of porcine eyelids (Crespo-Moral et al., 2020). Glands are organized as large ducts connected by ductules to numerous acini in which lipid-loaded meibocytes can be observed. To the best of our knowledge, no additional anatomical and histological description of porcine Meibomian glands has been published, although they have been analyzed in some articles that study their innervation and the localization of neuropeptides such as VIP (Hartschuh et al., 1984) and the feasibility of a rapid examination of ocular surface structures using a high-resolution 3D ultrasonic system (Peyman et al., 2012).

Tear film

There is scarce information on the physiology of porcine tear film. We know the reference values for basal and reflex tear production using the Schirmer test without anesthesia (STT I) and basal tear production after topical anesthesia (STT II) in normal Landrace pigs (Trbolova and Ghaffari, 2012). In this work, the mean STT I value was 15.6±3.7 mm/minute (range, 10-22 mm/minute), while the mean STT II value was 12.4±3.8 mm/minute (range, 5-18 mm/minute). Another study in normal Landrace pigs showed that the α2-adrenoceptor agonist drug medetomidine, which is widely used in veterinary medicine, reduced tear production (Kanda et al., 2019). It has also been reported that the average blink interval in pigs is 3.8 seconds (Hayes et al., 2007) compared with 2.8 seconds in humans (Ponder and Kennedy, 1927). The difference in the blink interval might be related to behavioral differences between the two species.

Apart from these three articles, we could not find any study on the structure of porcine tear film and its molecular characteristics, not even in Göttingen minipigs. Interestingly, the porcine conjunctival epithelium displays a rich pattern of carbohydrate-binding sites (Lange et al., 1989; Kirkeby et al., 2011; Crespo-Moral et al., 2020). Also, a comparative study identified MUC1 expression in porcine conjunctival epithelium and the lacrimal gland, while MUC5AC was detected in the eyelid gland duct lumen (Kirkeby et al., 2011). The demonstrated similarities between porcine and human mucin and lectin binding sites strongly suggest the presence of glycoproteins, such as mucins, in porcine tear film. On the other hand, the meibocytes identified in some Meibomian gland areas can be seen secreting their lipid content into the gland ductules (Crespo-Moral et al., 2020), presumably indicating a lipid component in the porcine tear film.

Finally, it is worth mentioning that the porcine ocular surface microbiome has recently been analyzed and compared with that of humans (Leis et al., 2021). Using standard swab sampling and Schirmer test strips, authors identified regional bacterial communities associated with the corneal surface, the conjunctival fornices, and the tear film that exhibited different compositions. The main conclusion of this work was that the porcine corneal surface harbors a specific bacterial community with a higher proportion of Proteobacteria compared with the conjunctiva and tear film. The authors attributed this to the unique growth conditions for colonizing bacteria present in different ocular surface tissues.

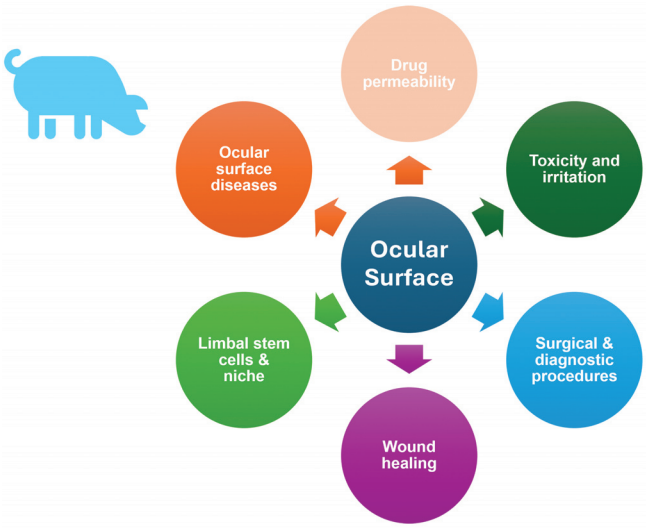

Fig. 2. Schematic summarizing the reported *ex vivo* utilization of porcine ocular surface tissues in different fields of vision research.

Porcine ocular surface *ex vivo* models

In this section, we revise the reported *ex vivo* utilization of porcine ocular surface tissues to test ophthalmic drugs, surgical procedures, and diagnostic tools. Also, several *ex vivo* models of different ocular surface diseases are discussed. Figure 2 summarizes our findings.

Ex vivo porcine ocular surface to study permeability to topical drugs

In the field of pharmacology, there has been a long-

standing interest in ocular tissue permeability, with a particular focus on corneal permeability (Prausnitz and Noonan, 1998; Loch et al., 2012). *Ex vivo* preparations of porcine cornea facilitate the testing of potential novel treatments either directly or using Franz-type diffusion chambers. Franz chambers are devices traditionally used to study transdermal drug penetration (Ng et al., 2010). They have donor (top) and receiving (bottom) compartments separated by a membrane in which different tissues and/or solutions are placed for permeation measurement. Usually, samples are taken from the receiving compartment at regular intervals to quantify the number of active molecules under study that has permeated the membrane at each time point. Currently, Franz-type diffusion chambers are employed for transcorneal and transscleral permeation of drugs, predominantly within the context of toxicological experimentation (Van den Berghe et al., 2005; Pescina et al., 2015). For instance, Pescina et al. (2015) developed a de-epithelization procedure for porcine cornea to simulate corneal permeability in pathological conditions using the Franz-type diffusion chamber. This *ex vivo* model allowed the characterization of the barrier properties of epithelium and stroma and highlighted the barrier function played by epithelium, showing that positively charged compounds had a significantly higher transcorneal permeability than negatively charged ones. This kind of setup is also frequently used to test novel experimental pharmaceutical formulations in their initial phases. There is a recent publication (Samarkanova et al., 2021) in which the bioavailability of two novel eye drops, formulated from placental tissues (cord blood platelet lysate and amniotic membrane extract), was assayed in an *ex vivo* porcine corneal model using a Franz chamber.

It is well known that conjunctival tissue also has a relevant role in ocular drug permeation (Urtti, 2006). Because of its vasculature and more permeable nature than the cornea, topically administered ocular drugs can reach the retina and choroid (Shikamura et al., 2016). The impact of drug absorption throughout the transconjunctival route is an important issue in topical ocular administration, and for that reason, there are multiple examples of using porcine conjunctival tissues removed from the eyeball to study the permeability of clinically used drugs. For instance, Ramsay et al. (2017) used *ex vivo* porcine conjunctiva to study the permeability of thirty-two drugs using Ussing chambers, which are similar to the Franz diffusion chambers mentioned above. Interestingly, this work allowed the development of a predictive quantitative structure-property relationship (QSPR) model of conjunctival drug permeability, which is useful for developing new ocular drugs. These same authors additionally performed a comparative permeability study of the thirty-two drugs previously reported between *ex vivo* cornea and conjunctival tissues, showing that the porcine cornea is an 8.6±4.4 times tighter barrier than the conjunctiva, similar to that described in other species (Ramsay et al., 2018).

Other authors have also used either *ex vivo* porcine cornea or conjunctiva, or even *ex vivo* whole eye, to study the permeability of different therapeutic molecules, such as erythropoietin for its tissue-protective and regenerative properties (Resende et al., 2017), the antibiotic azithromycin to manage ocular infections (Agarwal et al., 2019), and papaverine, an opium alkaloid with anti-inflammatory properties (Agarwal et al., 2021). In addition, the corneal and/or conjunctival penetration of drug formulations prepared using different delivery systems is classically studied using *ex vivo* porcine tissues. From 1996 to the present, there are abundant reported examples of the use of these *ex vivo* models to evaluate formulation penetration and drug release. For instance, liposomes loaded with a thrombospondin-1-derived peptide (Soriano-Romaní et al., 2018), everolimus microemulsions (Baspinar et al., 2008), cyclosporine-loaded sorbitan ester nanoparticles coated with hyaluronic acid (Álvarez-Trabado et al., 2018), or erythropoietin-loaded chitosan nanoparticles (Silva et al., 2020). Considering the impossibility of citing all publications dealing with this topic, we recommend two review articles for interested readers (Mazet et al., 2020; Yang and Lockwood, 2022).

Ex vivo porcine cornea as an alternative method to study eye toxicity and irritation

The suitability of porcine eyes for the enucleated eye test (EET) was studied long ago (Prinsen and Koëter, 1993). This *ex vivo* assay is considered a valuable alternative to the classical Draize Eye Irritation Test performed *in vivo* in rabbits to evaluate the potential adverse effects induced in the eye by irritant compounds. Generally, the EET uses postmortem isolated rabbit whole eyes with the cornea *in situ* and measures three corneal parameters: corneal swelling, corneal opacity, and fluorescein retention by corneal epithelial cells. In the work by Prinsen and Koëter, the porcine eye resulted in a less practical evaluation of corneal swelling than rabbit or chicken eyes because the cornea is thicker than that of rabbits or chickens, which makes swelling measurements difficult and less precise. However, other authors have reported using the whole porcine eyeball to evaluate corneal toxicity using a more simplistic approach. For instance, the toxicity of two nitric oxide donors in terms of epithelial erosions/perforation appearance, corneal opacity, or other gross damage was evaluated under a surgical microscope, and findings were confirmed by histological analysis (Cariello et al., 2013). The non-invasive measurement of corneal swelling is still a challenge and very relevant at the same time because of the pathophysiological impact of an edematous cornea on the eye. Interestingly, a study published in 2010 reported the use of reflective terahertz technology as an *ex vivo* tool to measure the hydration level of porcine corneal flaps prepared using a microkeratome (Singh et al., 2010). This technology

exploits the fact that water has a very high dielectric constant in the terahertz region of the electromagnetic spectrum. For that reason, terahertz radiation measured in reflection can be used to sense small changes in water content in biological samples (Singh et al., 2010). The promising results obtained using that technology moved the field forward, and there are now improvements in the application of terahertz to medical imaging, always using *ex vivo* porcine cornea to test them (Taylor et al., 2011; Joshi et al., 2015).

More recent studies showed the feasibility of maintaining *ex vivo* excised porcine corneas in culture for 21 days without experiencing significant epithelial alteration (Piehl et al., 2010). The *ex vivo* Porcine Corneal Opacity Reversibility Assay (PorCORA) is performed using *ex vivo* excised corneas filled with agar/gelatin on the endothelial side, which is allowed to gel to support the maintenance of the natural corneal curvature on laboratory cell plates. The epithelium is directly exposed to potential irritants, and epithelial damage is evaluated by using sodium fluorescein, which is a standard indicator of the integrity of corneal epithelial barrier function *in vivo*. The PorCORA assay was used to test five benchmark chemicals and successfully predicted ocular injury reversibility, i.e., natural corneal re-epithelialization, as determined by correlating data from sodium fluorescein corneal retention over 21 days of *ex vivo* culture with confocal microscopy and histopathology analysis (Piehl et al., 2010). The PorCORA protocol was later used to evaluate three personal care products (Donahue et al., 2011) and a laundry detergent (Vij et al., 2024), showing again that this test can discriminate between reversible and irreversible damage to corneal tissues. Despite these promising results, the PorCORA test is not yet formally validated as an alternative method to evaluate ocular irritation.

Ex vivo porcine cornea and conjunctiva to evaluate surgical and diagnostic procedures

The use of porcine corneas from enucleated eyeballs is a common practice for testing surgical procedures and ophthalmic imaging methodologies, as reported in the literature. Usually, the cornea is not dissected from the eyeball; rather, it is kept for easier manipulation. The eye maintains its shape using different support systems, and the cornea receives the procedure under study. For instance, Optical Coherence Tomography (OCT), a widely used diagnostic tool in ophthalmology, has been tested on *ex vivo* porcine corneas to evaluate different contrast agents (Ehlers et al., 2010). Similarly, other ophthalmic imaging methodologies have also been tested in *ex vivo* porcine corneas to better understand corneal tissue properties such as elastic anisotropy (Nguyen et al., 2014), its topography (Heichel et al., 2016), or its biomechanical properties (Kling et al., 2014). Also, porcine bulbar conjunctiva was visualized using OCT, although thickness measurements were not reported (Zhang et al., 2011). Multiphoton autofluorescence and second-harmonic generation microscopy is another combined imaging modality that has been tested in *ex vivo* whole porcine eyes to obtain spectrally resolved morphologic features of the cornea, limbus, and conjunctiva (Teng et al., 2006). Interestingly, porcine corneas have been used to develop a non-contact tonometer based on the photoelasticity of the cornea with the purpose of being used as a non-contact device for intraocular pressure measurement in glaucoma patients (Hwang et al., 2011).

Sánchez et al. (2011) indicated that pig cornea would not be a suitable model to study corneal refractive surgery due to the reported larger corneal thickness and topographical differences compared with the human cornea; nevertheless, corneas of enucleated porcine eyeballs have also been frequently used to study safety and tissue effects of different corneal photorefractive surgical techniques. For instance, *ex vivo* corneas were used to evaluate the effect of using femtosecond laser technology for penetrating keratoplasty in terms of corneal graft endothelial ultrastructure and viability (Kim et al., 2009), corneal shape and ablation performance (Vengris et al., 2010), corneal tissue histopathology (Sumioka et al., 2014), or self-sealing features and dimensional stability (Kojima et al., 2018), among many other examples. More recently, *ex vivo* porcine corneas have also aided in understanding corneal distortion artifacts when using advanced computer-assisted eye surgery (Peter et al., 2025).

Moreover, *ex vivo* porcine corneas were used to test other surgery-related aspects, such as the effectiveness of a fibrin sealant to stabilize temporarily sutured keratoprostheses (Uhlig and Gerding, 2003), the impact of topical anesthetics and ethanol on ocular surface wound healing after excimer laser application (Tappeiner et al., 2012), the recommended size of incisions made in cataract surgery, and the intraoperative enlargement caused by different intraocular lens injector systems (Friedrich et al., 2023), or even for cornea surgeons to practice complex surgical techniques under wet lab conditions before adapting them to clinical practice (Droutsas et al., 2014). It is interesting to note that there are about 50 reported examples investigating the effect of corneal collagen cross-linking with riboflavin and ultraviolet A irradiation on the mechanical and morphological properties of the porcine cornea. Cross-linking is a frequently used treatment to stabilize the mechanical properties of the cornea and stop the progression of different human degenerative pathologies such as keratoconus (Wittig-Silva et al., 2008). To mention but one example, *ex vivo* porcine corneas helped to model the photochemical kinetics of corneal cross-linking with riboflavin and monitor oxygen consumption in the cornea during the procedure, whose role seems to be key to modulating the photochemical kinetic mechanisms that occur (Kamaev et al., 2012).

Finally, *ex vivo* porcine corneas have been classically used to study the effect of different storage

solutions on endothelial cells. Early on, in 1992, Ellis et al. reported the percentage of endothelial cell disruption and loss in porcine corneas stored in 2.5% chondroitin sulfate at 3, 5, 8, and 10 days (Ellis et al., 1992). Also, the effect of temporary exposure to hypothermia on endothelial cell density in porcine corneas maintained in a dextran-containing organ-culture medium was analyzed to evaluate the potential influence of low temperatures during the transport of corneal tissues from the tissue bank to the operating room (Schroeter et al., 2007). More recently, an experimental study compared corneal tissue preservation in two different preservation solutions at 4°C and 36°C for 30 days (Koulouri et al., 2020). In addition, a new preparation method that allows for the maintenance of porcine corneal tissues in organ culture for 15 days without the need to add de-swelling additives to the preservation medium was reported (Kunzmann et al., 2018). Using this simple model, endothelial cell death rates displayed were comparable to those of cultured human corneas, which is interesting in studying corneal endothelium changes during storage.

Interestingly, there is a published work on the compatibility of Seoul National University miniature pig corneas for use for xenocorneal transplantation in human clinical trials, in which miniature pig corneas were preserved using the same preservation protocol as that used for human corneas, and the endothelial cell density loss after seven days was comparable to that of human corneas maintained under the same conditions (Kim et al., 2016).

Additional examples of the *ex vivo* use of porcine corneas are the study of potential applications of tissue equivalents, such as 3D-bioprinted corneal stroma for regeneration after surgery (Boix-Lemonche et al., 2023), or even the use of porcine corneal tissues for different kinds of xenotransplants (Lee et al., 2014; Liu et al., 2018; Fernández-Pérez et al., 2020).

The porcine conjunctiva is another tissue brought to organ culture to practice surgical procedures, such as laser soldering for closing conjunctival incisions instead of suturing (Norman et al., 2009), tissue adhesives for tumor brachytherapy (Zloto et al., 2016), near-infrared laser for conjunctivoplasty (Yang et al., 2018), and several cryotherapy methods to evaluate the immediate effects on tissues after application (Barachetti et al., 2020). As with the cornea, the conjunctiva usually remains attached to the eyeball to test the procedures, and most of the time, it is the conjunctiva from the nictitating membrane that is used.

Ex vivo porcine cornea to study corneal wound healing

There is increasing interest in testing active molecules or formulations with healing potential to accelerate corneal wound healing. Any injury in the cornea can put its transparency at risk and consequently threaten vision. There are many different pathological situations that can lead to the development of corneal opacity, which supports the interest of the eye research community in understanding why defective healing occurs and how to prevent or reverse it to avoid opacification.

Different biological aspects of corneal wound healing, as well as potential accelerating therapies, have been studied *ex vivo* in recent years using porcine corneas, either dissected from the eyeball or maintaining the eyeball in toto in the experimental laboratory. Epithelial and stromal injuries can usually be produced mechanically with a trephine or using an excimer laser (Flueckiger et al., 2005), or else chemically induced using n-heptanol. However, there is no gold-standard corneal wounding model established for porcine corneas. There is not even a standard corneal lesion diameter that can vary from 4 to 8 mm. Our own results highlight the importance of selecting an appropriate method to injure the porcine corneal tissues and the time period of organ culture according to the pursued goal. For instance, we demonstrated that 7-mm epithelial mechanical wounds are more repeatable and heal faster than chemical wounds, however, there are more apoptotic keratocytes in the corneal stroma when the corneal epithelium is chemically damaged than when mechanically damaged (Crespo-Moral, 2022). This can be relevant depending on whether partial or full healing is intended and the desired duration of the follow-up period. In our hands, five days in organ culture should be the maximum follow-up time to avoid the natural decay of *ex vivo* tissues placed in culture (Crespo-Moral, 2022).

Ex vivo-cultured injured porcine corneas have largely contributed in the last twenty years to the understanding of different molecular mechanisms involved in corneal wound healing. For instance, the molecular mechanism in which erbB2, a member of the erbB family of receptor tyrosine kinases, is involved during wounding was clarified using this model (Xu et al., 2004). The impact of high glucose levels on attenuating corneal epithelial healing was also studied in *ex vivo* injured porcine corneas, demonstrating that high glucose suppressed Akt phosphorylation in a reactive oxygen species-sensitive manner and decreased intracellular glutathione (Xu et al., 2009). This is relevant to understanding the delayed corneal epithelial healing that occurs in diabetic individuals. Another example related to diabetes is the elucidation of the role played by advanced glycation end products in the mechanism of delayed corneal epithelial healing (Shi et al., 2013). Also, *ex vivo* porcine corneas were used to model host-pathogen interactions where bacteria inhibit wound healing and provided evidence that secreted bacterial lipopolysaccharide has a key role in that mechanism (Brothers et al., 2015).

Regarding the study of scarring, that is, the anomalous stromal fibroblast response during wound healing that results in scar formation, *ex vivo* porcine corneas have also been extensively used and provided relevant information. For that purpose, the corneal wound can be made solely in the epithelium (Schumann et al., 2021) or by also incising the upper portion of the

corneal stroma (Castro et al., 2019). These models permit the analysis of myofibroblast activation in the anterior stroma, the detection of apoptotic cells and fibrotic markers, and the silencing of certain molecules, such as deubiquitylating enzymes that are known to be overexpressed in myofibroblasts (Gillespie et al., 2017; Castro et al., 2019). For instance, the important role played by insulin-like growth factor 2 receptor (IGF2R) in regulating myofibroblast differentiation from stromal fibroblasts during wound healing was elucidated using wounded porcine corneas (Bohnsack et al., 2014).

There is also abundant literature that reports the *ex vivo* use of porcine corneas to evaluate the efficacy of drugs designed to accelerate epithelial or endothelial repair and stimulate the biosynthesis of extracellular matrix components (Schumann et al., 2021), including advanced therapeutical proposals, such as eyedrop formulations from placental tissues (Samarkanova et al., 2021), hepatocyte growth factor-loaded transparent hydrogels (Jumelle et al., 2021), mesenchymal stromal cell secretomes (Rouhbakhshzaeri et al., 2019), or more recently, a liposomal formulation of ascorbic acid to enhance its bioavailability in corneal stroma (Csorba et al., 2023).

Ex vivo porcine limbus to study limbal stem cells and the stem cell niche

It was already mentioned that the porcine limbus possesses highly proliferative cells with progenitor-like phenotypes similar to that of the human limbus arranged in a limbal niche that is anatomically equivalent to that of humans (Notara et al., 2011). This discovery prompted the authors to use the porcine limbal niche as a new model to study the performance of transplanted tissue-engineered human limbal epithelial cells. This is clinically relevant to studying limbal epithelial stem cell deficiency, a severe problem that arises from the genetic disorder aniridia or secondary to different situations that can lead to corneal failure and progress to vision loss. Patients with limbal epithelial stem cell deficiency require a surgical supply of healthy stem cells to maintain normal corneal epithelium renewal. The fate and function of limbal epithelial stem cells post-transplantation cannot be studied in transplanted patients, so the *ex vivo* use of the porcine limbal niche appears as a good laboratory tool to study the biology of transplanted cells. The work published by Vattulainen et al. (2019) is another interesting example of the use of the *ex vivo* porcine model to transplant human stem cells and study their biology. In this work, a limbal stem cell deficiency was created *ex vivo* in porcine corneas, and three limbal stem cell lines differentiated in culture from human pluripotent stem cells were transplanted. This experimental setup allowed a better understanding of the functional roles of limbal progenitors in corneal regeneration and an in-depth molecular characterization of their different phenotypes.

There is also an interesting study in which acellular porcine cornea stroma was used to construct a tissue-engineered limbal graft (Zhu et al., 2013). For that purpose, the authors differentiated human limbal stem cells (LSCs) from human embryonic stem cells and seeded them onto decellularized porcine cornea stroma. Porcine corneas had been previously brought to culture for one day, then the stromal matrix was dissected and maintained in culture for an additional day. After 14 days in culture, there was clear cell stratification on the porcine matrix, and the basal cells still kept the LSC characteristics. One of the conclusions of this work is that engineered construction helps to reconstruct a functional ocular surface in a rabbit limbal stem cell deficiency model, which suggests another potential *ex vivo* use of porcine ocular surface tissues. One year later, another group published a similar work using decellularized porcine conjunctiva rather than corneal stroma to engineer a graft for the reconstruction of the ocular surface in rabbits with limbal stem cell deficiency (Zhao et al., 2014).

Ex vivo porcine cornea as a model of ocular surface diseases

In addition to that revised in previous sections of this article, the porcine cornea was also used to further explore different human ocular surface diseases, always looking for a reliable model in which potential pharmacological treatments can be tested. Several disease models were established using *ex vivo* porcine corneas. For instance, Yadavalli et al. (2021) inoculated Herpes simplex virus type-1 (HSV-1) on porcine corneas after epithelial debridement to study HSV-1 infection-induced cornea wound progress. In humans, recurrent viral eye infections can result in corneal scarring, neovascularization, and even stromal keratitis. With this model, it is possible to evaluate corneal damage due to HSV-1 infection, the cell and molecular mechanisms involved, and also test drugs to treat the herpetic wound.

Another example is dry eye disease, which is an inflammatory immune-based pathology of the ocular surface with a notable impact on the worldwide human population over fifty years old (Stapleton et al., 2017). *Ex vivo* porcine corneas *in situ* were used by Choy et al. (2004) to simulate the exposure keratitis that appears in humans because of inadequate blinking associated with dry eye. The authors evaluated the condition of the porcine corneal epithelium immediately after enucleation and 4 and 6 hours after air exposure using Trypan blue staining and counting stained (damaged) cells. A few years later, the same authors reported a more complex porcine tissue model in which *ex vivo* lacrimation and blinking intervals were adjusted to simulate different severity levels of desiccation-induced dry eye (Choy et al., 2008). Interestingly, the porcine nictitating membrane, the lacrimal gland, and the connecting bulbar conjunctiva were maintained in the eye culture that was placed onto a holder. An infusion set helped to apply Dulbecco's Phosphate-Buffered Saline onto the eye surface in a controlled fashion,

simulating lacrimation. Blinking was induced immediately after by means of a mechanical arm that swept the nictitating membrane over the exposed corneal surface. Using this setup, corneal epithelium viability was evaluated by fluorescein staining, and the evaluation was found to be repeatable. The main limitation of this model is the small number of eyes that can be used at the same time. Other authors later improved this porcine desiccation-induced dry eye model and were able to test up to six eyes in culture at the same time (Chan et al., 2014). The eyes were kept in a closed temperature and humidity-controlled chamber and also exposed to a fan to simulate evaporative dry eye. In addition, they used Trypan blue staining instead of fluorescein to assess the viability of corneal epithelial cells, as Choy et al. (2004) demonstrated that fluorescein grading is not sufficiently sensitive to consistently evaluate the condition of epithelial cells in this cornea desiccation model.

Recently, other authors proposed another simplified model in which just the excised cornea is maintained in culture using six-well plates and exposed to reduced humidity percentages (30% instead of the regular 95% humidity) for 12, 24, or 48 hours (Netto et al., 2022). Low humidity induced similar morphological and inflammatory changes in the cultured porcine cornea like those found in human dry eye. Using this simplified and cheaper setup, the authors were also able to analyze whether dexamethasone or hyaluronic acid could reverse the damage caused by the low humidity, showing that both treatments partially counteracted the effects.

Ex vivo porcine conjunctiva as a model of ocular surface diseases

There is much less reported information on the *ex vivo* use of porcine conjunctiva to model ocular surface diseases than on the porcine cornea. The study of conjunctival tissue has historically been eclipsed by that of the cornea. It was not until the last 30 years that the leading role of the conjunctiva in the maintenance of ocular surface homeostasis was acknowledged, leading to an intensification in its study. In this context, the *ex vivo* use of porcine conjunctiva is first documented in an article published in 2011 by Tovell et al. (Tovell et al., 2011). To the best of our knowledge, this is the first example of *ex vivo* culture of conjunctival tissue fragments to model conjunctival scarring. Conjunctival scarring can lead to the failure of several ocular surgical procedures, including those related to glaucoma surgery, and its study is, therefore, of great clinical relevance. These authors demonstrated that it is possible to monitor tissue contraction under different contraction-stimulating molecules and study its real-time kinetics while maintaining porcine conjunctival fragments in organ culture for four weeks in permeable support plates. Subsequently, other authors used this model and determined that it better mimics relevant physiological aspects of conjunctival scarring, including contraction kinetics, than simple 3D fibroblast-loaded collagen matrices (Kozdon et al., 2020). This model is also useful to test the effect of antifibrotic drugs simulating intraoperative drug administration.

Additionally, the *ex vivo* use of porcine conjunctiva to create blebs and study fluid outflow pathways was recently reported (Akiyama et al., 2020). Blebs are fluid-filled blisters located under the conjunctiva that can form naturally or artificially after subconjunctival drug injection or after glaucoma surgery. In these later cases, the bleb's maintenance in place is important for the procedure's success. but at the same time, it must allow slow fluid outflow. These authors studied how fluorescent tracers exit blebs created in the porcine subconjunctival space using lymphangiography and OCT for tissue evaluation to better understand the biology of subconjunctival blebs.

There is also an interesting, published work in which authors simulate transthyretin amyloidosis (ATTR) *ex vivo*, a severe pathological condition related to TTR amyloid deposits in different body tissues that leads to progressive organ toxicity and even death (Pilotte et al., 2024). In one of the most severe forms of this pathology, amyloid is present in the conjunctiva of more than 90% of patients, and for that reason, non-invasive detection of TTR amyloid in the conjunctiva may serve as an early diagnostic of systemic disease. In this context, the authors used a novel fluorescent agent, which binds to amyloid and hyper-fluoresces, and tested it using *ex vivo* porcine conjunctiva. Nitrocellulose discs impregnated with TTR amyloid prepared in the laboratory were placed in the conjunctiva, simulating the presence of amyloid aggregates. It is noteworthy that amyloid deposits were detected using commercially available ophthalmic imaging equipment, which helps as an adjunctive diagnostic test for ATTR.

Finally, although the conjunctiva is also an important element in the development of dry eye disease, there is no reported information using porcine conjunctival tissues. Most of the work to understand the role played by the conjunctiva in this disease has been conducted using mouse models in which dry eye is induced by ocular surface tissue desiccation.

Concluding remarks

The *ex vivo* use of porcine ocular surface tissues for different purposes has increased notably over the last decades, primarily related to surgical training, diagnostic methodology improvement, pharmacokinetic studies, and novel therapy development. The cornea and conjunctiva are the most widely used ocular surface porcine tissues, possibly due to their close anatomical and physiological resemblance to human counterparts. We could not find examples of any *ex vivo* use of porcine ocular glands to model human disease. This may be attributed, at least in part, to the logistical challenges associated with obtaining the entire porcine eyeball, including the lids, from regular slaughterhouses. We must remember that animals are prepared for human

consumption in these facilities, and dedicated eyeball and lid extraction by a veterinarian may delay the overall working process.

As highlighted in this review, porcine ocular surface tissues have helped and still help in eye and vision research. They offer a cost-effective, reliable, and anatomically and physiologically human-comparable model, aligning with the 3Rs. We should keep this in mind and take advantage of the possibilities that the porcine ocular surface tissues can offer when brought to *ex vivo* culture before progressing to *in vivo* studies, maximizing their utility in advancing scientific knowledge.

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