Decellularization and recellularization of cornea: Progress towards a donor alternative

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Abstract

The global shortage of donor corneas for transplantation has led to corneal bioengineering being investigated as a method to generate transplantable tissues. Decellularized corneas are among the most promising materials for engineering corneal tissue since they replicate the complex structure and composition of real corneas. Decellularization is a process that aims to remove cells from organs or tissues resulting in a cell-free scaffold consisting of the tissues extracellular matrix. Here different decellularization techniques are described, including physical, chemical and biological methods. Analytical techniques to confirm decellularization efficiency are also discussed. Different cell sources for the recellularization of the three layers of the cornea, recellularization methods used in the literature and techniques used to assess the outcome of the implantation of such scaffolds are examined. Studies involving the application of decellularized corneas in animal models and human clinical studies are discussed. Finally, challenges for this technology are explored involving scalability, automatization and regulatory affairs.

Key words: decellularization, cornea, tissue engineering, keratoplasty, eye

1. Introduction

The cornea is the outer most structure of the eye. It is comprised of three main cellularized layers, the epithelium, the stroma, and the endothelium. The corneal epithelium consists of stratified epithelial cells whose main function is to act as a barrier to protect the inner corneal layers [1]. Limbal epithelial stem cells that reside in the Palisades of Vogt, between the sclera and the cornea, replenish the epithelial cell population [2]. The stroma is the thickest part of the cornea and is comprised of extracellular matrix (ECM) populated by keratocytes and consisting of collagen type I and lesser amounts of collagen type V, glycosaminoglycans (GAGs) and proteoglycans, such as decorin, lumican and keratocan [3–7]. The endothelium consists of a monolayer of hexagonal cells with limited regenerative capabilities. These cells pump fluids from the stroma to the aqueous humour to maintain water homeostasis and are responsible for transporting nutrients to the avascular cornea [8,9].

It is estimated that 10 million people worldwide have bilateral corneal blindness [10]. Severe cases can only be treated by a corneal transplant, or keratoplasty. Despite cornea being an immune privileged organ due to its avascular nature, corneal transplants can undergo graft rejection, which is the most common cause of transplant failure for corneas [11,12]. In a recent global survey it was determined that there is only one cornea available for every 70 needed, which highlights the severe donor shortage of such a tissue [13]. It is for these reasons that there has been a lot of interest in corneal bioengineering as an approach to generate transplantable tissues.

Tissue bioengineering aims to overcome donation shortages and immune rejection by fabricating organs in the lab with patient-specific cells. Traditional tissue engineering is based on the interplay between cells, the fabrication of biocompatible scaffolds and the application of external stimuli including mechanical, chemical or biological stimuli. In the field of corneal tissue engineering, multiple biomaterials are being investigated [14]. Collagen-based materials are the most common as collagen is the most abundant component of the corneal stroma. These have been fabricated as highly-hydrated hydrogels [15,16], plastically compressed matrices [17,18] and membranes by vitrification [19]. Other natural polymers such as chitosan [20,21], silk fibroin [22–24], fibrin [25,26] and self-assembling peptides [27] have also been investigated. 3D bioprinting, a revolutionary technique, has also been used to fabricate corneas using bioinks [28,29]. While these studies have produced some promising results, these materials lack the biochemical composition of the real cornea and fail to replicate the cornea

fibril arrangements and ECM architecture. Techniques such as 3D bioprinting [28,29] or electrospinning [30,31] have the potential to generate scaffolds with similar shape and structure to real cornea but these still do not replicate the composition. Hydrogels derived from corneal ECM have also been developed that maintain much of the cornea's ECM but often lack its fibril organization [32–34]. Scaffold-free approaches where cells secrete their own ECM and create a tissue *in vitro* have been reported too [35–37] but these are costly and take months to obtain. Therefore the use of decellularized corneas as a scaffold for corneal engineering is attractive since both the fibril architecture and corneal composition can be accurately mimicked.

2. Decellularization of the cornea

Decellularization is a process that aims to remove cells from organs or tissues resulting in a cell-free scaffold consisting of the tissue's own ECM. Cell removal reduces graft rejection by depleting the scaffolds from major histocompatibility complexes. Insufficient decellularization has been shown to illicit polarization of macrophages to an M1-phenotype *in vivo* and *in vitro* [38]. Furthermore, cellular components that have not been removed can be bound by immunoglobulins and complement proteins which will activate macrophages and B lymphocytes [39]. In the last decade a wide range of organs have been decellularized including heart [40,41], kidney [42], liver [43–45], lung [46–49] or pancreas [50]. In this section different techniques used to remove cells and cellular components from the cornea will be explored. All decellularization techniques can be categorized into three types of methods: physical, chemical and biological methods. However, it should be noted that most decellularization protocols combine several methods and techniques. A summary of these methods can be found in Table 1.

2.1. Physical methods

Some form of mechanical agitation is present in most decellularization protocols and mostly carried out using orbital shakers or rotators to promote the flow of decellularizing agents through the ECM. For example, Nara and colleagues have described the use of a direct perfusion chamber that applied detergents in a continuous unidirectional flow [51]. The use of automatized devices that allow both mechanical agitation and fluid exchange, such as the one

described by Pellegata and colleagues to decellularize blood vessels, offers great promise to speed up the process and ensure sterility [52].

A simple method of decellularization is using repeated freeze-thaw cycles where cells are lysed due to the formation of ice crystals. This is usually followed by rinsing or the addition of another decellularization method to ensure removal of cell debris [34,53–56]. Temperatures used for the freeze-thawing cycles range from -20 °C to -197 °C with the use of liquid nitrogen. Another method is the use of electrophoresis, which has been reported in the literature as a final step to remove debris [57,58] but is not a widespread method. The use of high hydrostatic pressure has been shown to reduce DNA and maintain glycosaminoglycan (GAG) levels in decellularized porcine corneas [59–61]. This method is not used commonly as the equipment needed is expensive [62]. Li and colleagues have recently reported on the use of ultrasound in conjunction with freeze-thawing and nucleases for the decellularization of porcine corneas [56].

Decellularization using supercritical CO₂ was first used by Sawada and colleagues for vascular tissues [63]. This technique is based on the high permeability and transfer rate characteristic of supercritical fluids. Carbon dioxide can be used as a critical fluid under moderate conditions ($T_C = 32\text{ °C}$, $P_C = 7.38\text{ MPa}$) and after the procedure there are no remnants as the gas can diffuse out easily. Bovine [64] and porcine [65] corneas have also been recently decellularized using this method with good initial results. Furthermore, this technique can be used to sterilize the tissue simultaneously [66].

While not strictly a decellularization method, lyophilisation has been used by researchers to obtain more porous scaffolds as water in the decellularized cornea becomes frozen and then removed by sublimation once a vacuum is applied. With this approach the repopulation by neighbouring cells is thought to increase although the process would likely disrupt the cornea's collagen architecture [67–69].

2.2. Chemical agents

The use of detergents is the most common method for corneal decellularization. The most widely used is the ionic detergent sodium dodecyl sulphate (SDS) [69–75]. While extremely effective, SDS can disrupt the ECM as it is a denaturing agent. Therefore other milder detergents are being explored such as sodium deoxycholate [55,76]. Non-ionic detergents, such

as Triton X-100, are less effective but in turn less disruptive [77–81]. Zwitterionic detergents have characteristics of ionic detergents, as they have positive and negative functional groups, and non-ionic detergents, as their net charge is zero. However, these detergents have been used unsuccessfully for cornea decellularization [78].

Organic acids have also been used to decellularize corneas. Peracetic acid has widely been used to decellularize and terminally sterilize urinary bladder and small intestine submucosa by the Badylak lab [82–84], and Ponce Márquez reported the use of it for the cornea with suboptimal results [85]. Mild acids commonly found in nature, such as acetic, formic and citric acids, have been recently employed for porcine cornea decellularization. Formic acid at a 30% concentration showed the best results in terms of DNA removal, and sulfated GAG (sGAG) and collagen retention [86]. Bases are less used than acids and only ammonium hydroxide appears in the literature for corneal decellularization [77,87] while sodium hydroxide has been used to decellularize lungs and heart valves [88,89].

Hypertonic solutions, especially sodium chloride at concentrations of 1.5 or 2 M, have been used by multiple research groups with positive outcomes in terms of transparency, cell removal and retention of ECM components [53,65,73,81,90–94]. When combined with a washing step using 0.2 % Triton X-100 the best results were achieved [81,94].

Other chemicals used to decellularize corneas include alcohols, such as ethanol, and chelating agents, such as Ethylenediaminetetraacetic acid (EDTA). Ponce Márquez and colleagues used 75% ethanol following a protocol originally described to decellularize arteries [85,95]. EDTA is commonly used in conjunction with trypsin as it helps disrupt cell-cell interactions by sequestering calcium present in cadherin junctions [53,75,80].

2.3. Biological agents

Several enzymes have been used in the literature to decellularize corneas. Trypsin and Dispase II are cell dissociating proteins commonly used for cell isolation. If used for longer periods or at higher concentrations, they can result in cell lysis [53,68,80,92,96–98]. Phospholipase A₂ (PLA₂) is an esterase that breaks the phospholipids present in the cell membrane and has been shown to remove cell components successfully with minimal ECM disruption [67,99–101]. Another biological agent is human serum which has been used to decellularize blood vessels [102] and corneas alongside the use of electrophoresis [57,58]. Most studies include an

additional step of incubation with nucleases to assist with the degradation of DNA released following cell lysis [55,72,76,103–106].

2.4. Confirmation of decellularization

There is no current consensus to validate the success of a decellularization protocol. In general, the decellularization procedure should remove exogenous cells, and cell debris, including DNA, RNA and other cell remnants such as mitochondria, while minimally disrupting the ECM. To verify the removal of nuclei and cellular components three parameters should be examined [107]. The first parameter involves staining with Haematoxylin and Eosin (H&E) and/or 4',6-diamidino-2-phenylindole (DAPI) to demonstrate absence of intact cell nuclei. Next dsDNA is quantified via Hoechst 33342 binding or via PicoGreenTM (Invitrogen) assay. This should give values of below 50 ng of dsDNA per mg of dry weight [108]. Finally the maximum length of DNA remnants should be 200 bp determined via agarose gel electrophoresis [39,108].

While these parameters will determine the presence of cellular components, it is important that the decellularization process also maintains the cornea's structure, composition and transparency to allow the scaffold to retain its function. ECM can be analysed using standard histological stains such as Picrosirius Red, to stain collagen fibers, or Alcian Blue, to visualize GAGs. These parameters can also be quantified using biochemical assays such as hydroxyproline reagent to quantify collagen [109,110] or dimethylmethylene blue assay to quantify GAGs [111]. Immunohistochemistry can be used to identify specific ECM components such as the type of collagen present or specific proteoglycans and GAGs. Second harmonic generation imaging can be used as a non-destructive technique to give information on collagen fiber orientation and identify damage to the collagen during decellularization [112]. Electron microscopy offers a great deal of ultrastructure detail, especially in the cornea where the precise arrangement of collagen bundles determines the optical properties of the tissue. Both SEM and TEM require highly specialized and expensive equipment, only available in big universities and research centres.

Unlike most other tissues and organs, the transparency of scaffolds is an important parameter that needs to be considered for corneal tissue engineering. The optical characteristics of decellularized corneal scaffolds can be easily quantified using a simple spectrophotometer (or plate reader). The absorbance at different wavelengths in the visual spectrum of light provides

information on the clarity or opacity of a scaffold. The mechanical properties of decellularized cornea also need to be sufficient to allow suturing and withstand any applied forces. Minimizing disruption to the ECM during decellularization should allow the mechanical characteristics of the cornea to be maintained. Furthermore, the use of osmoregulators such as dextran or glycerol can aid on palliating swelling and thus recovering transparency and increasing tissue stiffness and strength [104,113].

2.5. Species used for corneal decellularization

Porcine corneas have been the most extensively studied for decellularization mainly due to their availability and anatomical similarities with the human cornea. However, porcine cells present several epitopes that are extremely immunogenic to humans such as Galactose- α -1,3-galactose (α -Gal) and N-glycolylneuraminic acid (Neu5Gc). Insufficient cell removal could elicit an immune response that could lead to graft rejection. It is therefore recommended to perform immunostaining against these epitopes to ensure their absence in the scaffolds. As reviewed by Kim and Hara [114], the thickness of the porcine cornea varies greatly with age and breed, which highlights the need of standardisation. By breeding animals specifically for organ decellularization and transplantation in quarantined animal facilities, heterogeneity from batches can be monitored and brought to a minimum. In addition, with the advent of genetic engineering, pure xenogenic transplantation could become a reality, with the use of so-called “humanized” pigs. Two main hurdles have to be overcome in order to use pig corneas for transplantation as if they were human: the removal of multiple xenoreactive cell surface molecules and porcine endogenous retroviruses (PERV). The use of CRISPR-Cas9 has been shown to be successful in obtaining triple knockout strains of pigs lacking GGTA1, CMAH, and β 4GalNT2, genes important for the presence of immunogenic surface glycans [115–117]. This same genome editing technique has been used to remove PERVs *in vitro* to obtain PERV-inactivated pigs [118]. Other species that have been used to fabricate decellularized corneal scaffolds include cat [101], ostrich [119], bovine [85,120] goat [51] or dog [121].

Human corneas have been used in decellularization experiments [72–74,87]. While the use of human tissue does not have to face the same challenges of xenogenic tissues, the availability of such tissues is scarce. However, corneas deemed unsuitable for corneal transplantation due to low endothelial cell count could be used to obtain decellularized scaffolds [122]. Furthermore, recent studies have focused on the use of discarded tissue after refractive

surgeries as an alternative source [73,123]. In this case, thinner tissues are decellularized which facilitate the removal of cellular components, compared to whole corneas.

2.6. Optimal parameters for decellularization

It is difficult to determine the optimal technique for decellularizing cornea due to the different researchers obtaining differing results despite using similar protocols. A number of papers have directly compared different decellularization techniques on cornea to determine the optimal technique. Shafiq et al., (2011) compared several different techniques and found that a combination of sodium chloride and nucleases was the best option for removing cellular components while still supporting the growth of new cells [103]. Wilson et al., (2016) compared hyperionic, ionic and non-ionic detergents and concluded that increasing the efficiency of cell removal led to increased ECM damage [91]. For this reason it is still difficult to identify one specific methods that is closer to use in clinical practice.

In addition to the decellularization efficiency of these techniques, issues associated with gaining regulatory approval for clinical translation of corneas subjected to each technique has to be considered. For example, the use of detergents and nucleases may provide a barrier to the cornea's clinical use since there is a risk that any residual chemical could have a negative effect post-implantation. Non-chemical techniques may be more beneficial for gaining clinical approval but can be more expensive to operate.

3. **Recellularization of the cornea**

Once the removal of exogenous cells has been confirmed, it may be beneficial to repopulate these matrices with human cells to generate a viable cornea. The origin of the cells used for scaffold recellularization is an important issue to take into account. For every cell type found in the cornea, there are several potential sources, with benefits and drawbacks associated to each of them. There are reports of decellularized matrices being repopulated using immortalized cell lines [55,76,93] and while such cell types are acceptable for *in vitro* studies as a proof of concept, these should not be acceptable for transplantation. Cells modified to be passaged *ad infinitum* pose the risk of tumour formation [124]. Therefore, it is advisable to only use primary cultivated cells. This requires the initial isolation of cells from the patient or donor, further expansion in the laboratory, seeding of these cells onto the scaffold, culture/maturation of the newly cellularized organ and implantation into the patient. Recellularization of perfused organs such as the heart or the lungs have used the endogenous

vasculature to reintroduce cells [125], however the cornea, being an avascular organ, relatively thin and containing 3 different cell types needs to be recellularized using other approaches. Potential cell sources for corneal recellularization are discussed in the following sections and are summarized in Table 2.

3.1. Stroma

For the repopulation of the corneal stroma, autologous corneal stromal cells can be obtained from a small biopsy from the contralateral eye. Since these cells are from the patient, they are less likely to illicit an immune response. If both eyes are compromised, alternatives have to be found. Since the cornea is avascular, it is often considered to be an “immune privileged” organ so allogenic cells could be used with less risk of rejection compared to other organs. Alternatively a number of studies have examined the use of autogenic cells but from an extraocular source. Since keratocytes originate from the neural crest mesenchyme, other mesenchymal tissues have the potential to be differentiated into keratocytes. Mesenchymal stem cells derived from adipose tissues have been successfully induced to express corneal keratocyte markers such as ALDH3A1, keratocan, lumican and decorin [126–130]. Adipose-derived MSC have been used clinically as a cell therapy without a scaffolding material [131] and seeded onto decellularized human corneal stroma sheets [74]. The differentiation of induced pluripotent stem cells (iPSC) into keratocyte-like cells has been reported in the literature [132]. iPSCs were differentiated into neural crest cells and cultured on cadaveric human corneas into which the cells migrated and adopted a similar phenotype to keratocytes. This technique offers the opportunity for autogenic cell transplantation. There have been reports of oncogenic transformation of iPSCs [133] so the use of such cells for the treatment of low risk corneal diseases might not be recommended.

The stroma is the thickest layer of the cornea consisting of densely packed collagen fibrils. This structure makes it difficult for cells to penetrate and recellularize after decellularization. One approach to overcome this problem is to inject cells directly into the stroma, however the optimal procedure for doing this is unclear. Cell seeding densities, volume per injection and number of injections vary between publications with values for the final injected volume ranging from 12 μ l in 2 μ l injections [76] to 1 full ml in a single injection [69,79,134]. In addition, during decellularization tissues swell significantly making it difficult to inject more liquid [104]. The number of cells seeded ranges in the literature from only 1200 and 4500 cells

per construct [55,76] up to 1×10^5 , 4×10^5 and 5×10^5 cells [79,103,134]. It is noteworthy that in multiple publications the final number of seeded cells is difficult to determine [72,75]. Furthermore, injections can disrupt the stromal fibril architecture and result in permanent damage [125].

The simplest method to recellularize the stroma is to seed cells directly on the surface of the decellularized scaffold. This approach relies on the capacity of the cells to migrate into deeper regions of the stroma. González-Andrades and colleagues showed repopulation of NaCl-decellularized porcine corneas with human keratocytes which were distributed similarly to the native counterparts [92]. More recently, Alió del Barrio *et al.* reported recellularization of 120 μm thick decellularized lamins with adipose-derived MSCs for 24 hours before implantation [74]. The authors did not shown the distribution of these cells on the scaffold. In addition, freeze-drying have been use to induce the formation of pores to increase the depth that cells can penetrate and repopulated the corneas *in vitro* [69].

While not commonly used for cornea, there are reports of bioreactors being used to aid in repopulating decellularized corneas with cells or as a culture method after initial seeding. Fu and colleagues used a magnetic stirrer to keep the construct in suspension during the culture period [79]. The use of a rotary cell culture system for repopulating purposes has also been described in the literature, whereby the cells are encouraged to colonize the scaffold as they cannot attach elsewhere [135]. A more sophisticated bioreactor system has been reported for the repopulation of the epithelium which mimicked and *in vivo* air-liquid interface [100].

Another strategy is to recellularize the scaffolds *in situ* during transplantation. Ma and colleagues describe a method by which cells were seeded on thin sheets of decellularized porcine cornea as they were being placed on the bed of a lamellar keratoplasty [90]. The procedure was repeated so that five layers had been deposited. When compared to the sheets without cells or a thicker acellular tissue, the recellularized sheets showed better results in terms of transparency and overall transplant success.

3.2. Epithelium

The corneal epithelium is constantly undergoing renewal from limbal derived stem cells [2]. If the limbus is damaged and the stem cells are lost, the patient can develop Limbal Stem Cell Deficiency (LSCD), resulting in conjunctivalization and neovascularization of the cornea

[136]. If the contralateral eye is not affected, a small biopsy can be taken and limbal stem cells can be isolated and expanded. These cells can then be transplanted onto the diseased cornea and replenish the stem cell niche [137]. If both eyes are affected, allogenic cells or cells from the same patient but from an extraocular source, such as the oral mucosa, can be used [138,139]. An alternative autogenic cell source are iPSCs differentiated into limbal epithelial stem cell-like cells [140].

Decellularized corneas have been repopulated *in vitro* with epithelial cells, which rapidly attach and form a multi-layered epithelium, typically expressing cytokeratin 3 and 12 [69,80,134]. Better results have been reported when using a construct based on a decellularized porcine cornea repopulated with stromal and epithelial cells compared to the acellular construct in a one year anterior corneal transplant model in dogs [76]. However, in this study a group with only stromal cells was not included and the implanted cells were not labelled, therefore the healthy epithelium was probably from the host. It is noteworthy, however, that results in the literature are inconclusive as to *in vitro* re-epithelialization of decellularized matrices before implantation in defects of the central cornea. Luo and colleagues seeded amniotic epithelial cells onto decellularized scaffolds and implanted them into an alkali burn rabbit model [81]. When compared to an acellular scaffold, the pre-epithelialized construct was accepted better, probably due to the anti-angiogenic and anti-inflammatory factors secreted by the amniotic epithelial cells.

3.3. Endothelium

Corneal endothelial cells do not proliferate *in vivo* as they are arrested in the G₁ phase [141,142]. *In vitro*, however, endothelial cells can be isolated and expanded, and seeded onto carrier materials for transplantation. Multiple studies have investigated the optimization of culture conditions to promote proliferation and avoid endothelial-to-mesenchymal transition [143]. The use of these cells relies on donor corneas and can only be done with allogenic cells. As for keratocytes and epithelial cells, human corneal endothelial-like cells have been obtained from iPSCs that could potentially be used for implantation [144].

Attempts have been made to use decellularized corneas as carriers for endothelial cell transplantation [54,77,80,93,145,146]. Choi and colleagues decellularized 110 µm-thick sections of human corneas with Triton X-100 and NH₄OH and seeded them with human

endothelial cells for 14 days [77]. The constructs expressed zonula occludens-1 (ZO-1), gap junction protein connexin-43 and Na⁺/K⁺-ATPase, markers of mature and functional corneal endothelium. Another study obtained decellularized sections using a femtosecond laser, seeded an endothelial cell-line and performed an *ex vivo* transplantation, demonstrating the potential for translation into the clinic [93]. An alternative approach has been to use trypsin-decellularized crystalline lens capsules as it is a tissue usually discarded during cataract surgeries [147].

3.4. Further remarks

While one would think that *in vitro* recellularization prior to implantation would be essential for positive outcome, there are several examples where the implantation of an acellular cornea has been successful [60,65,68,69,71,73,80,94,99,106,148]. Most of these studies evaluate their success in terms of low immune reaction, little or no vascularization and transparency recovery. It is important to note that the surgical procedure in these studies was an intrastromal pocket or an anterior lamellar keratoplasty, i.e. the endothelium remained undamaged. The repopulation of the acellular graft by host stromal cells is usually only assessed by simple histological staining and claims of infiltrating keratocytes are often inconclusive. Generally the epithelium is able to grow over the scaffolds with no major differences to the control, especially at the longer time-points. Other studies, however, show better results when comparing cell-containing scaffolds versus their acellular counterparts [55,72,76,81,90]. More studies need to be done to determine the necessity of recellularization, although eliminating the need for cells makes its potential translation to the clinic easier, with reduced costs and decreased risk of infection and disease transmission.

4. *In vivo* studies

While there has been considerable *in vitro* research undertaken to demonstrate the potential of decellularized and recellularized corneas as an alternative donor corneas for keratoplasties, the ultimate goal is to translate this research into the clinic to benefit patients. Prior to their use with patients, the cornea need to be tested in animal models *in vivo* to evaluate the host response to the implants. Following the successful outcome of animal models, a number of studies have

recently entered clinical trials. This section will focus on the progress of decellularized corneas as transplants with the most up-to-date results of animal experiments and clinical data, which are summarized in Table 3.

4.1. Techniques used to assess outcome

Multiple techniques are used in *in vivo* experiments to assess the progress of an implant. These can be mainly divided into two groups: non-disruptive techniques used during the experiment and while the animal or patient is still alive, and terminal techniques, performed after the sacrifice of the animal (or in case of failure and the patient is re-transplanted).

Most of the techniques used during experiments to assess the implanted decellularized corneas are the same of those routinely by ophthalmologists after a real corneal transplant. Intraocular pressure (IOP) is routinely measured using a tonometer since increased IOP post transplantation can lead to glaucoma. Slit lamp biomicroscopy can be used to evaluate the thickness and transparency of the cornea. Optical coherence tomography (OCT) can also be used to measure thickness for all the cornea and obtain a pachymetric map, i.e. a topographical map [149]. To assess health of the epithelium, the anterior surface is stained with fluorescein and imaged using blue light. Areas of debrided or damaged epithelium will appear fluorescent. Other parameters such as neovascularization, inflammation, infection are usually determined visually by trained personnel using a grading scheme [150]. A technique that is becoming popular in the recent times is laser scanning *in vivo* confocal microscopy. As it is a contact technique, in patients it is done under local topical anaesthesia while in animal models it is performed under total anaesthesia. This method allows the visualization with high resolution at all depths of the cornea without the aid of any enhancing contrast agents. It is the only technique that can be used to quantify nerve regeneration [151,152].

Terminal techniques require the excision of the cornea. Despite being quite an old technique, histological staining with H&E provides useful information about the implant. This technique can assess epithelial health by allowing the number of cell layers and their morphology to be visualized, quantifying the repopulation of the implant by the surrounding stromal cells and identifying the presence of inflammatory cells in addition to examining other parameters. Other standard histological stains can also provide information about the ECM composition, such as Alcian Blue for GAGs and Picrosirius Red for collagens. Another method to obtain more information on the corneas is immunohistochemistry. The phenotype of quiescent keratocytes

can be determined by positive staining of crystalline protein aldehyde dehydrogenase 3A1 (ALDH3A1) or transmembrane phosphoglycoprotein CD34. Presence of myofibroblasts, typical of fibrosis, can be detected by positive staining for alpha smooth muscle actin (α -SMA). Blood vessels can be identified staining for Platelet endothelial cell adhesion molecule (PECAM-1), also known as CD31, or for von Willebrand factor (vWF). The presence of Integrin alpha M ($\alpha_M\beta_2$), also known as CD11b, is a hallmark of the activity of the innate immune system. The fate of implanted cells can be determined by several different methods. Cells can be fluorescently labelled before implantation and this staining is still visible even after 12 weeks, as reported by Alió del Barrio and colleagues [72]. Another approach is to stain for species-specific markers when implanting cells from one species into a recipient of a different species. An example of this is the human nuclear antigen, which is located in human nucleoli but not in rodent nucleoli. The possibility of implanted cells undergoing apoptosis can be determined by the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay which detects DNA fragmentation. On the other hand, proliferating cells can be detected by Bromodeoxyuridine (BrdU) labelling one hour prior to animal sacrifice. Cells synthesising new DNA will incorporate this synthetic nucleoside which can be detected with specific antibodies. By using electron microscopy more detailed information about the arrangement of the collagen fibrils can be obtained. Serial block face-scanning electron microscopy (SBF-SEM) and 3D reconstruction of the sections allows the visualization of cell distribution and lamellae arrangement with great resolution [153]. X-ray scattering is another method to analyse the structure of the cornea and the orientation of fibrillar collagens [154].

4.2. Selection of animal models

The most common used animal model in research is the mouse. This is due to the relative low cost, short gestation period, large litter size and overall ease of housing. Furthermore, the abundance of genetically defined strains offers the possibility of studying immune responses in a mechanistic way [155]. However, the size of the mouse ocular globe is rather small, hence it is rarely used for corneal studies. Rat models have been developed to study corneal rejection and immunomodulation with cell therapy, such as MSCs infusion [156–158]. Shafiq and colleagues have described the use of decellularized human cornea as a limbal graft in a rat laser induced limbal injury model [159].

While corneal transplants can be performed using mice and rats as experimental animal models, they are difficult surgeries and poorly model human corneas. For this reason, the rabbit remains the most commonly used animal model for corneal studies [160]. Rabbit eyes have a very similar anatomy to human eyes and are big enough to be operated with standard human surgical techniques. As a proof of concept many early studies using decellularized corneal tissue for transplantation were used in intrastromal pocket surgeries [60,61,69–73,80,90,105,148]. This technique can give a good insight into the immune response generated by the scaffold and some information about the neighbouring cells' behaviour and migration into the implanted matrix. However, these surgeries are not routinely performed in the clinic, so for a more translational approach anterior or deep anterior lamellar keratoplasties are more suitable. One of the first experiments in rabbits using decellularized corneal tissues was performed by Wu and colleagues in 2009 [99]. They reported the use of PLA₂ decellularized porcine corneas on an anterior lamellar keratoplasty model in rabbits. The acellular grafts were re-epithelialized in one week, transparency recovered in less than 3 months and with no presence of neovascularization. Luo and colleagues decellularized corneas using 2 M NaCl and seeded amniotic epithelial cells [81]. Tissue-engineered constructs implanted in rabbits showed better integration and transparency than the acellular counterparts. Detergent decellularized corneas were implanted intrastromally with no major signs of rejection or neovascularization and recovering transparency before 6 months post-implantation [69]. Hashimoto and colleagues decellularized porcine corneas with high hydrostatic pressure and implanted them in a deep anterior lamellar keratoplasty model [106]. Scaffolds integrated completely into the host cornea, recovered transparency and showed no signs of inflammation or neovascularization. Re-epithelialization was slow and some keratocytes migrated into the scaffold. Porcine corneas decellularized using supercritical carbon dioxide have shown very promising results after grafting into rabbits [65]. Transparency was achieved within 2 weeks and epithelium re-grew in a month. While these studies demonstrate the promise of decellularized corneas for use in transplantations, differences in the regenerative capacities of rabbit and human corneas should also be considered.

In addition to rabbits, other species have been used as animal models for corneal transplantation such as mini-pigs [161,162], cats [37] and dogs [76]. However, only one study has used the dog as a model for implantation of decellularized matrices as an alternative to human cadaveric donor corneas. Porcine corneas were decellularized using sodium deoxycholate and sodium orthovanadate and seeded with stromal and epithelial cells. Constructs were implanted in dogs

and followed during a one year period. Recellularized scaffolds showed improved re-innervation, epithelial integrity and central corneal thickness.

4.3. Human studies

Despite being a recent approach, clinical studies have been carried out with decellularized corneal tissues. Alió del Barrio and colleagues recently reported the use of thin sections (laminas) of decellularized human corneas as intrastromal implants for advanced keratoconus patients [74]. Some of the laminas were seeded with autologous adipose-derived MSCs. Initial reports show improved visual acuity, corneal shape and topography, and patients recovered normal corneal thickness. The implanted tissue remained visible with OCT after 6 months and some signs of recellularization by host keratocytes detected by confocal biomicroscopy. The authors identified no positive effect from the recellularized tissues versus the acellular tissues.

Chinese company China Regenerative Medicine International Limited (CRMI) was granted a medical device registration certificate by the China Food and Drug Administration (CFDA) in 2015. Their product consists of a porcine cornea decellularized with 2 M NaCl, washed with 0.2% Triton X-100, dehydrated with glycerol, and irradiated with Co⁶⁰ to ensure sterility [81]. In a first study in 2015, Zhang and colleagues reported the outcome of 47 patients with fungal corneal ulcers treated with such product [94]. Irritation and neovascularization scores improved as did the transparency of the graft, originally hazy. Another short-term study using this product has been reported for the treatment of herpes simplex keratitis with generally positive results, albeit some patients needing re-grafting of human allograft transplantation due to scaffold dissolution [163]. While more than 1000 scaffolds have been implanted, no long term follow up studies have been published and it is still to be determined if this product would be suitable for corneal diseases other than fungal infections.

Being a relatively new field, there is still much research and optimization needed before these corneas can be accepted as valid alternatives to real corneas for keratoplasty. Most of the decellularization techniques discussed can only be done in small batches, at a laboratory scale. Efforts are required to automate the optimized decellularization protocols under sterile conditions at a larger scale. Furthermore, an international consensus is required to assess the quality of these scaffolds in terms of xenogenic or allogenic cell removal and ECM preservation. It would be beneficial to identify the most appropriate cell source for specific corneal transplants and how best to deliver these cells to recellularize the scaffolds. Good

manufacturing practices (GMP) should be ensured in all steps and correct storage and transportation organized in a similar fashion as in current eye banking. If recellularization is not necessary for a specific application, then off-the-shelf scaffolds can be developed. In all cases, these alternative scaffolds should show similar if not improved efficacy to keratoplasties.

5. Conclusions

While multiples issues still have to be overcome in the field of corneal decellularization, such as identifying the optimal decellularization and recellularization protocols (if required) and assessing their performance *in vivo* in patients, decellularization research has advanced substantially in recent years. Decellularized corneas have enormous potential to be used as alternatives to traditional donor corneas and could help alleviate the shortage of donor corneas suitable for transplantation worldwide.

Table 1. Summary of the decellularization methods reviewed.

Decellularization methods	References
<i>Physical methods</i> Agitation Freeze-thawing Electrophoresis High hydrostatic pressure Supercritical CO ₂ Lyophilization	n.a. [34,53–56] [57,58] [59–61] [63,64] [67–69]
<i>Chemical agents</i> Sodium dodecyl sulphate (SDS) Sodium deoxycholate Triton X-100 Peracetic acid Formic acid Ammonium hydroxide Sodium chloride (hypertonic) Ethylenediaminetetraacetic acid (EDTA)	[69–75] [55,76] [77–81] [85] [86] [77,87] [53,65,73,81,90–94] [53,75,80]
<i>Biological agents</i> Trypsin Dispase Phospholipase A ₂ Human serum Nucleases (DNAse and RNAse)	[53,68,80,97–98] [53,80,92,96] [67,99–101] [57,58] [55,72,76,103–106]

Table 2. Potential cell sources for cornea recellularization.

Cornea layer and cell source	References
<i>Epithelium</i>	
Limbal stem cells from unaffected contralateral eye biopsy	[137]
Oral mucosa	[138,139]
iPSCs	[140]
<i>Stroma</i>	
Keratocytes/Corneal Fibroblasts	[55,76,90,92,103]
Adipose-derived MSC	[74,126–131]
iPSCs	[132]
<i>Endothelium</i>	
Human endothelial cells	[77]
Human immortalized endothelial cells	[93]
iPSCs	[144]

Table 3. Summary of *in vivo* studies using decellularized corneas (SDS: sodium dodecyl sulfate, SDC: sodium deoxycholate, ALK: anterior lamellar keratoplasty, Y: recellularized, N: not recellularized)

Origin of cornea	Decell type	Details of decell	Surgical procedure	Recipient species	Cells (Y/N)	Details about cells	Reference
Porcine	Physical	Nitrogen gas	Intrastromal pocket	Rabbit	N	-	[148]
Porcine	Chemical + Physical	SDS + sodium orthovanadate	ALK	Rabbit	Y	Human immortalized stromal cells injected and cultured for 3 days prior to implantation	[55]
Porcine	Chemical + Biological	Triton + Dispase + Trypsin	Intrastromal pocket	Rabbit	N	-	[80]
Porcine	Physical	High hydrostatic pressure	Intrastromal pocket	Rabbit	N	-	[60]
Porcine	Chemical	SDS	Intrastromal pocket	Rabbit	N	-	[69]
Porcine	Chemical	SDS	Intrastromal pocket	Rabbit	N	-	[70]
Porcine	Chemical	Phospholipase A2	Interlamellar keratoplasty	Rabbit	N	-	[67]
Porcine	Chemical	SDS	Intrastromal pocket	Rabbit	N	-	[71]
Porcine	Chemical	SDS	Intrastromal pocket	Rabbit	N	-	[105]
Porcine	Chemical	NaCl + Triton X-100	Intrastromal pocket and ALK	Rabbit	Y	Rabbit amniotic epithelial cells for ALK cultured for 1 week before implantation	[81]
Human	Chemical	SDS	Intrastromal pocket	Rabbit	Y	Adipose-derived MSC injected into the stroma and cultured 5 days before implantation	[72]

Porcine	Physical	High hydrostatic pressure	ALK	Rabbit	N	-	[106]
Porcine	Chemical	NaCl	Intrastromal pocket	Rabbit	Y	Keratocytes in suspension sandwiched between sheets of decellularized tissue during surgery	[90]
Human	Chemical	SDS	Intrastromal pocket	Rabbit	N	-	[73]
Porcine	Physical	High hydrostatic pressure	Intrastromal pocket	Rabbit	N	-	[59]
Porcine	Chemical	SDC + sodium orthovanadate	ALK	Dog	Y	Human epithelial and stromal cells	[76]
Human	Chemical	SDS	Intrastromal pocket (advanced keratoconus)	Human	Y	Human adipose-derived MSC cultured for 24h prior to implantation	[74]
Porcine	Physical	High hydrostatic pressure	Intrastromal pocket	Rabbit	N	-	[61]
Porcine	Chemical	NaCl + Triton X-100	ALK (fungal ulcers)	Human	N	-	[94]
Porcine	Biological	Phospholipase A2	ALK	Rabbit	N	-	[99]
Cat	Chemical + Biological	Phospholipase A2 + SDC	Tectonic ALK	Rabbit	N	-	[101]
Porcine	Chemical	Glycerol + EDC/NHS crosslinking	ALK in infectious keratitis model	Rabbit	N	-	[164]
Porcine	Biological + Physical	Human serum + electrophoresis	Intrastromal pocket	Rabbit	N	-	[57]
Porcine	Biological + Physical + Chemical	Trypsin + freeze-thawing + NaOH	ALK	Rabbit	N	-	[68]

Porcine	Chemical	NaCl + Triton X-100	ALK (herpes simplex keratitis)	Human	N	-	[163]
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Figure 1. General workflow for the use of decellularized corneas as donor alternatives.

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