Decellularization methods for developing porcine corneal xenografts and future perspectives

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Abstract

Corneal transplantation is the only option to cure corneal opacities. However, there is an imbalance between supply and demand of corneal tissues in the world. To solve the problem of corneal shortage, corneal xenotransplantation studies have been implemented in the past years using porcine corneas. The corneal xenografts could come from 1) wild type pigs, 2) genetically-engineered pigs, 3) decellularized porcine corneas, and 4) decellularized porcine corneas that are recellularized with human corneal cells, eventually with patients' own cells, as in all type of xenografts. All approaches except, the former would reduce or mitigate recipient immune responses. Although several techniques in decellularization have been reported, there is still no standardized protocol for the complete decellularization of corneal tissue. Herein, we reviewed different decellularization methods for porcine corneas based on the mechanism of action, decellularization efficacy, biocompatibility, and the undesirable effects on corneal ultrastructure. We compared 9 decellularization methods including: (i) sodium dodecyl sulfate, (ii) triton x-100, (iii) hypertonic saline, (iv) human serum with electrophoresis, (v) high hydrostatic pressure, (vi) freeze-thaw, (vii) nitrogen gas, (viii) phospholipase A_2, and (ix) glycerol with chemical crosslinking methods. It appears that combined methods could be more useful to perform efficient corneal decellularization.

Keywords

Cornea transplantation; Decellularization; Porcine xenograft; Tissue engineering

1. INTRODUCTION

According to data from the Global Vision Database, 36 million people worldwide are blind.[1] The World Health Organization indicates that corneal opacities account for 5% of all causes of blindness.[2] When corneal transparency becomes defective, corneal transplantation is the only curative option.[3] Currently, deceased human donors are the only source of corneal grafts. Despite the existence of countries that export corneas, e.g., the United States (that accounts for 85% of the world's corneal 'exports'), Sri Lanka (9%), and Italy (3%), there is a major imbalance between supply and demand.[4,5] In the last decade, tissue engineering has become a more prominent method of potentially solving the problem of corneal shortage.

Tissue-engineered corneal scaffolds should have complex features that are essential to corneal function. These features, which include (i) light transparency, (ii) resistance to ultraviolet light, (iii) insensitivity to photons, and (iv) specific tensile strength, make the replication of a corneal scaffold from biomaterials much more difficult.[6,7] To combat this issue, decellularized corneas have been successfully tested in pre-clinical animal studies. [4,8–10] The most significant advantage of a decellularization process is conservation of the extracellular matrix (ECM) proteins and mechanical properties of the cornea.[8,11] For these reasons, a decellularized corneal scaffold could be a good option for creating tissue-engineered corneal xenografts.

Normally, the cornea is an immune-privileged site, but in some pathological conditions, especially infections and after transplantation, this privilege can be diminished.[12,13] In addition, decellularized porcine corneal xenografts (DPCXs) offer another important advantage in that they exhibit a decreased risk of an inflammatory reaction compared to the fresh porcine corneal xenografts.[14,15] Therefore, DPCXs may provide a basis for successful corneal transplantation.[8]

Decellularization of a three-dimensional matrix scaffold (i.e. organ or tissue, cornea) enables the removal of cellular and immunogenic components from an organ/tissue while preserving ECM with its native micro- and macroscopic anatomy.[16] Many different protocols for decellularizing human and porcine corneal tissues have been reported using biological, physical and chemical compounds.[17–20] However, there is still no standardized protocol. An ideal decellularization method should maximize the elimination of cellular components and nuclear materials, while limiting the possible detrimental effect on histo-architecture and mechanical properties of the ECM. In addition, being able to repopulate host cells (e.g., keratocytes, epithelial cells) should be an important factor after decellularization.

In the present review, we will assess favorable, promising, and new decellularization protocols and agents for developing DPCXs.

2. DECELLULARIZATION METHODS

To date, there has been no documented method that can achieve complete decellularization while maintaining the principal properties of the cornea (Table 1). Moreover, the term "complete decellularization" is currently not well-defined. Crapo et al.[21] suggested that it is impossible to remove 100% of cellular material by decellularization techniques and defined the term of "sufficient decellularization" by conditioning minimal criteria those are following (i) ECM should contain <50ng dsDNA per mg dry weight, (ii) DNA fragments should be shorter than 200 bp, and (iii) there should be no visible nuclear component within the ECM. Besides decellularization, reduced antigenicity in the collagen tissues of cornea is also essential if a recipient immune response is to be prevented. Additional conditions must be implemented to reduce edema, maintain or achieve transparency, and increase mechanical strength. Albeit, there are numerous procedures and agents to reverse undesirable effects. However, the balance between decellularization and damage must be established well in advance in order to achieve effective decellularization.

2.1. Sodium Dodecyl Sulfate (SDS)

SDS is a strong ionic detergent and is the most widely used agent in corneal decellularization practices (either alone or in combination with other agents) due to its extreme efficiency in removing cells and solubilizing cellular membranes.[22–30] Numerous studies have reported 'complete' or 'sufficient' decellularization at different concentrations and exposure times.[19,22–24] However, Sasaki et al. was not able to replicate the findings despite using higher concentrations and the same or prolonged time-points.[30] These results indicate that other conditions, besides exposure time and concentration, may be important to reach sufficient corneal decellularization. In this regard, studies have suggested different optimal conditions. For instance, Zhou et al. recommended 0.1% (wt/vol) SDS for 7 hours at 37°C[24], Gonzalez-Andrades et al. recommended 0.1% (vol/vol) SDS for 48 hours at room temperature with 300 rpm continuous shaking[17], and Du and Wu recommended 0.5% (wt/vol) SDS for 24 hours at 4°C with protease inhibition and continuous shaking[22].

Despite its effectiveness in decellularization, SDS has undesirable effects on the cornea (especially in high concentrations) including matrix edema, disruption of collagen layers, disordered fibrils, and reduced transparency. Nonetheless, these effects can be managed by decreasing the concentration and/or exposure time. There is a delicate balance between decellularization and damage. Therefore, determining the best conditions with the least possible damage to the cornea is of upmost importance.[24] To restore transparency (the effect of edema, and/or disrupted collagen conformation), glycerol and dextran can be utilized.[20,31,32] In some animal studies, reduced transparency, edema, and disrupted histo-architecture gradually returned to normality after corneal transplantation.[22–24,26]

2.2. Triton X-100 (TX)

TX is a non-ionic detergent and the second most commonly-used detergent in corneal decellularization experiments.[17–19] Its decellularization activity is based on the disruption of lipid-to-lipid and lipid-to-protein interactions, but not protein-to-protein interactions.[33] The efficacy of decellularization agents greatly fluctuates with the tissue being treated. For

instance, while TX is able to decellularize cartilage, corneal decellularization studies have shown great variability.[34] Du et al. found that decellularization of pig corneas using TX was unachievable, regardless of high concentrations (5% w/v) and prolonged time (96h).[17] Others have also failed to obtain satisfactory decellularization on pig and human corneas. [17,30,32]

These studies suggest that TX alone is not a strong enough detergent to sufficiently decellularize corneas. On the other hand, since TX is not able to break protein-to-protein interactions, disruption of the histo-architecture is not as complete as with SDS, and there remains reduced transparency and edema after use of TX.[30,35]

2.3. Hypertonic Saline (HS)

HS has been used to decellularize human, porcine, rabbit, and ostrich corneas.[3,8,35,36] HS is an effective decellularization agent due to its ability to disrupt DNA-to-protein interactions and to induce osmotic lysis.[21] HS is able to 'completely' decellularize corneal tissue on its own or in combination with TX, though its effects on natural ultrastructure are variable. [20,29,37] For instance, Lee et al. obtained decellularized opaque corneas by using 1.5 mol/L HS for 24 hours, but Luo et al. obtained decellularized transparent corneas by using 2 mol/L for 30 min.[20,38] HS protocol treated porcine corneas resulted in well-maintained collagen fibrils after the transplantation into the rabbit corneal bed.[37]

Choi et al. transplanted HS-treated and fresh porcine corneas to nonhuman primates. The decellularized corneas showed encouraging results over the fresh corneas with less rejection rate and longer graft survival.[14] On the other hand, TX-assisted HS is one of the methods that has been used in a clinical trial.[39] A different approach to decellularize corneas was implemented in which corneas were first soaked in ultrapure water to induce swelling, after which 2 mol/L HS was used to decellularize, followed by a wash with 0.2% TX. Dehydration and sterilization has been achieved by using glycerol and cobalt-60 irradiation, respectively.[39]

2.4. Human Serum with Electrophoresis

Galactose-α−1,3-galactose (α-Gal) is the major carbohydrate antigen to porcine cells.[40] Cohen et al. found that α-Gal expression exists in the limbal and stromal areas of porcine corneal tissue but not in the endothelium and epithelium.[41] However, Lee et al. demonstrated increased α-Gal expression in all layers of cornea after xenotransplantation.[42] The protocol (originally reported by Shao et al.[43]) is based on the cytotoxic effect of human serum to α-Gal antigenic wild-type porcine cells through binding of anti-Gal IgM and IgG antibodies, which induce complement-mediated cell destruction.[43] This method equates with the effect of pig corneas in which α-Gal gene-knockout has been achieved by genetic engineering. Anterior and posterior lamellas have been used separately after decellularization, and the results showed a decreasing number of cells in the anterior, but not posterior, stroma.[43] These findings indicate heterogeneity of corneal stromal structure and keratocytes.

The aim of using electrophoresis after exposure to human serum is to enhance the removal of dead cells. Evaluation of the biomechanical properties and collagen fibril interactions of

DPCX demonstrated ultrastructural similarity to native corneas, with remarkable transparency.[44]

2.5. High Hydrostatic Pressure (HHP)

The main decellularization mechanism of the HHP method is the disruption of cellular membranes. In this method, a pressure of 980 MPa or 10.000 kPa is applied to the cornea for 10 min at 10°C or 30°C. In order to achieve sufficient decellularization and maintain corneal histoarchitecture, corneas must then be washed with PBS or EGM-2 medium containing dextran and DNase I.[10,18,30] Applying the HHP method at 10°C instead of 30°C maintained collagen fibrils and interactions to a greater extent.[45] When compared to TX and SDS, HHP preserved more glycosaminoglycans, removed more DNA content, and decellularization was achieved in a shorter time-frame.[45,46] Hashimoto et al. have performed anterior lamellar and intra-lamellar keratoplasties in animals, and showed that corneal transparency was preserved without any immune reaction for 6 months.[10,18] HHP has many advantages, especially maintaining the native histo-architecture of the cornea, but the high expense of this technology is seen as a disadvantage.

2.6. Freeze-Thaw

Freezing and thawing lyses and removes the cells to produce a decellularized graft. Freezing can be carried out at −80°C (freezer) or −196°C (liquid nitrogen), and thawing should be carried out at a biological temperature of 37°C.[47] Li et al. achieved sufficient decellularization through a freeze-thaw method by using different adjuvants, including DNase, RNase, and Tris hydrochloride, resulting in corneas that were transparent.[48] Subsequent transplantation in rabbits showed consistent transparency, with no immune reaction for 3 months. However, neovascularization and inflammation was observed in amniotic membrane transplanted group during the same period.[48] Lin et al. applied the freeze-thaw method in a different way, and were unable to obtain transparent corneas.[49] However, after transplantation into rabbits, the corneas gradually regained transparency and the angiogenesis that developed immediately after transplantation started to regress after the first two weeks.[49] Van den Bogerd et al. have successfully used the freeze-thaw method on anterior capsule of the lens to make a useful scaffold for corneal endothelial cells (CECs).[50]

2.7. Nitrogen (N_2) Gas

The decellularization mechanism of N_2 gas is by inducing apoptosis through the creation of a hypoxic environment. Amano et al. reported decellularization with complete transparency after treatment of porcine corneas with N_2 gas for 7 days at room temperature. Furthermore, after transplantation in rabbits, N_2 gas-treated corneas maintained transparency for 6 months.[51] The maintenance of transparency could be a major advantage of the N_2 gas method. However, future studies need to demonstrate the effects of decellularization on the mechanical strength and ultrastructure of N_2 gas-treated corneal stroma. Lee et al. compared modified and classic N_2 gas methods on porcine corneas, including methods using 2800 centi-gray irradiation and 4% TX. The modified methods were associated with 'complete' decellularization only when N_2 gas treatment was combined with TX treatment.[20]

2.8. Phospholipase A$_2$ (PLA)

PLA is a specific type of esterase which breaks down the ester bonds between the second fatty acid and the glycerol molecule of membrane glycerophospholipids. PLA activation requires a significant Ca^{2+} concentration.[52] Wu et al. accomplished the sufficient decellularization of porcine corneas using PLA and sodium deoxycholate. Staining with hematoxylin and eosin demonstrated no cells or cellular components, but a small amount of nucleonic acid debris was detected on transmission electron microscopy. No significant change in mechanical properties and ultrastructure was observed. Lamellar keratoplasty performed in rabbits showed maintained transparency and significant biocompatibility, such that no immunological reaction occurred in the 12 months after transplantation.[9]

2.9. Glycerol with Chemical Crosslinking

Chemical crosslinking is defined as the intermolecular or intramolecular joining of two or more molecules by a covalent bond.[53] The general purpose of using crosslinkers is to modify the mechanical, biological, and degradation properties of hydrogels.[54] Lin et al. used glycerol to decellularize porcine corneas by creating a high osmotic pressure.[55] Thereafter, decellularized corneas were treated with a crosslinker (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysulfosuccinimide) to improve biomechanical and biocompatibility properties, while also maintaining transparency. Additionally, this group used γ-irradiation to reduce immunogenicity. Following completion of these procedures, transparent and mechanically-stable corneas were obtained. Lamellar keratoplasty was performed in rabbits to treat fungal keratitis, and demonstrated that corneal grafts effectively controlled the infection while also maintaining transparency.[55]

2.10. Other Decellularization Methods

Sodium deoxycholate is an ionic detergent that can sufficiently decellularize corneal tissue, but only if treated for an extended period of time. This protocol has negative effects which result in the disruption of corneal histo-architecture, including Descement's membrane and Bowman's layer.[19]

'Complete' decellularization was not achieved by 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS, a zwitterionic detergent). In certain conditions, it resulted in the destruction of the corneal histo-architecture.[18] Benzalkonium chloride is a cationic surfactant that was unable to 'completely' decellularize corneal tissue. Undesired effects occurred, including collagen damage and alteration of the fibrillary pattern.[17] Octylphenoxypolyethoxyethanol (Igepal) is a non-ionic detergent that has been previously used for heart valve, pericardial, and vein decellularization.[17] Although Igepal preserved the proteoglycans and fibrils very well, it was unable to achieve a significant percentage of decellularization.[17]

Trypsin is a protease that hydrolyses peptide bonds, and ethylenediaminetetraacetic acid (EDTA) is a chelating agent; the trypsin-EDTA combination has only been used in human corneal tissue engineering once, and was demonstrated to be a more effective way to decellularize corneas than SDS and TX. Furthermore, the trypsin-EDTA method preserved natural corneal features to a greater extent than did SDS and TX.[23]

3.   RECELLULARIZATION

Ideally, decellularized porcine corneas should have suitable stroma and surface features that allow for repopulation by multiple human corneal cell lines, including endothelial cells, keratocytes, and epithelial cells. However, creating an endothelial sheet (ES) might be the most important challenge to overcome. Unlike murine, rabbit, and bovine cells, human CECs have limited proliferative capability *in vivo*.[56] Although decellularized and layered human corneal lamellae have shown biocompatibility with human CECs, making endothelialized human lamellae a feasible option for endothelial keratoplasty in the future. Currently, re-endothelialization by human CECs is only possible *in vitro*.[57,58] This is a limitation of decellularized corneas that cannot be directly used in many corneal diseases with endothelial cell damage.

A comparative study found that the acellular human lens capsule is a better scaffold to create ES when compared with human Descemet's membranes and human amniotic membranes.[50] Lee et al. demonstrated a porcine model of corneal endothelialization, and successfully obtained an ES on DPCXs with porcine CECs.[20] Recellularization of the posterior portion of the DPCXs with human CECs is necessary in order to perform endothelium included keratoplasties. Yoeruek et al. completed an DPCX endothelialization with hCECs by using a modified SDS method with EDTA and aprotinin for decellularization. However, *in vitro* studies are needed to investigate the biocompatibility of other decellularization methods.[23]

In animal studies, freeze-thaw, N_2 gas, HHP, and SDS-treated DPCXs displayed repopulation of recipient keratocytes in graft stroma after lamellar keratoplasty.[10,22,48,51] However, the recellularization activity of PLA and glycerol combined with chemical crosslinking seems to be a preferred alternative.[9,57] On the other hand, SDS- and HS-treated DPCXs successfully repopulated with human keratocytes *in vitro*.[23] HS has been found to be a convenient protocol for recellularization through its establishment of a more ideal environment for human keratocytes.[23,28] Decellularized human corneas, by using PLA, have also been shown to have sufficient biocompatibility with human keratocytes.[59] Human adipose-derived stem cells have also been used to recellularize decellularized human corneas, and differentiate into functional keratocytes in the stroma.[60] This same technique exhibited functional biocompatibility with thin human corneal stromal laminas (created by femtosecond laser) which were successfully and safely transplanted into patients in a clinical trial.[61] As an alternative to injections and cell cultures, bioreactor platforms can be a useful technique for the recellularization of stroma.

Epithelial repopulation of the cornea is a simpler process than repopulation of the other layers. Adequate graft epithelization has been detected on DPCXs obtained through different decellularization methods, including PLA, HHP, and freeze-thaw.[9,10,48,49] In animal studies, Hashimoto et al. demonstrated the complete epithelialization of DPCXs within 3 months after lamellar keratoplasty.[10] Likewise, the seeding of human limbal corneal epithelial cells onto the anterior surface of DPCXs resulted in successful epithelialization in 5–8 days.[23]

4. FUTURE PERSPECTIVES

In order to reach 'sufficient or complete' decellularization with less damage to the cornea, combinations of a strong and a weak decellularization agent might be useful. For instance, in 2015, Zhang et al. reported the results of the first known clinical trial by using DPCXs. Hypertonic saline was used to prepare the DPCXs with Triton-X 100. Lamellar keratoplasty was performed on 47 patients who had fungal keratitis, and progress was documented over three years.[39] This study showed the safety and efficiency of using DPCXs to treat fungal keratitis, and encouraged more applications with new indications.[39] In a recent clinical trial, Shi et al. reached the sufficient decellularization mainly using high hydrostatic pressure but they also utilized distilled water, glycerol, endonucleases and a protective medium. Lamellar keratoplasty was performed on 23 patients who had different type of corneal ulcers. This study also showed the safety of DPCXs for corneal ulcers with 22/23 graft survival for one year.[62] Therefore, we suggest that combined methodologies should be used for 'sufficient' corneal decellularization.

Besides immune rejection and infection recurrence, xenozoonosis is also one of the most important concerns post-operatively. In the all potential microorganisms, porcine endogenous retrovirus (PERV) is the most significant vector for cornea.[63,64] To date, no evidence has yet shown that PERV can be transmitted to hosts in preclinical and clinical trials.[39,62]

In conclusion, currently DPCXs seem to be the most viable option to use in corneal transplantation with limited indications (Figure 1). However, a Korean group is currently planning to initiate a clinical trial of corneal xenotransplantation using fresh porcine corneas[65], thus bringing corneal xenotransplantation as a clinical therapy one step closer.

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ABBREVIATIONS

CECs	Corneal endothelial cells
DPCX	Decellularized porcine corneal xenograft
ECM	Extracellular matrix
ES	Endothelial sheet
HHP	High hydrostatic pressure
HS	Hypertonic saline

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LKP	Lamellar keratoplasty
PA	Peptide amphiphiles
PERV	Porcine endogenous retrovirus
PLA	Phospholipase A2
SDS	Sodium dodecyl sulfate
TX	Triton X-100
α-Gal	Galactose-α1,3-galactose

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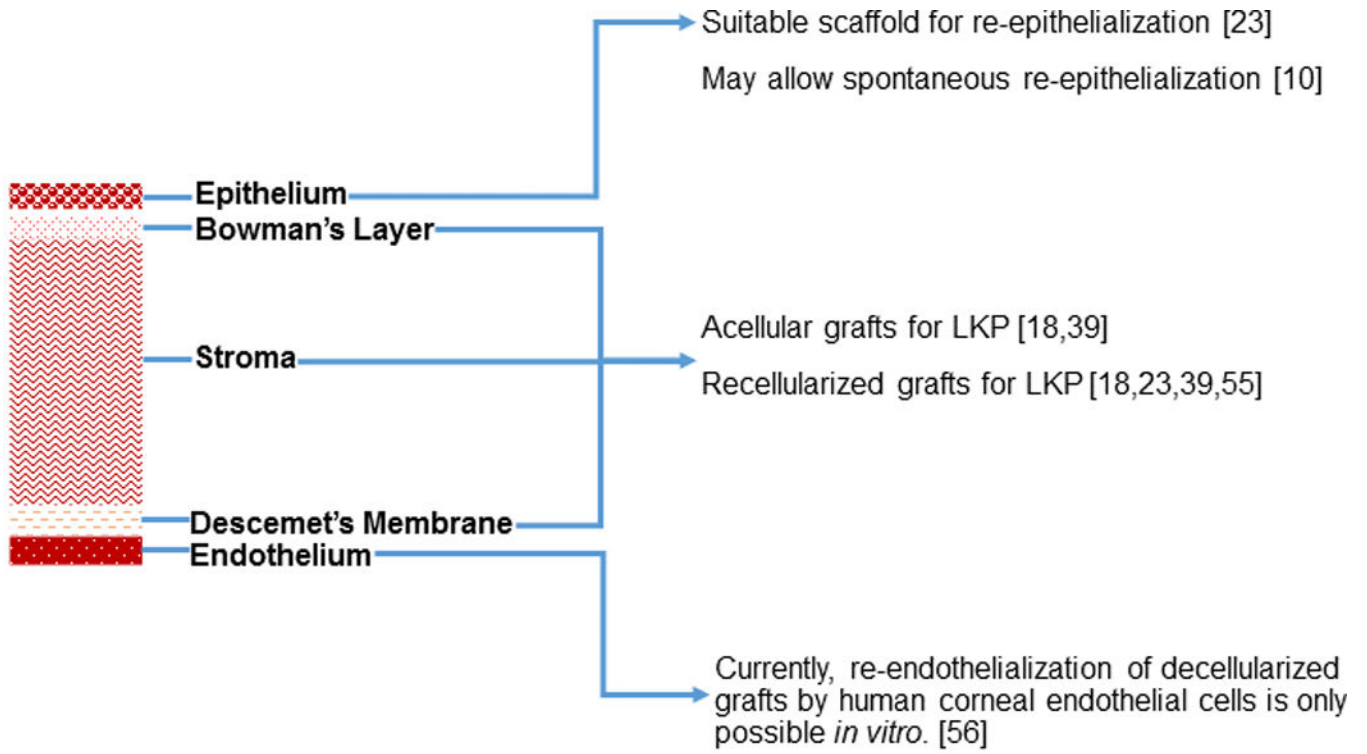
Legend: LKP = lamellar keratoplasty

Figure 1:
What can decellularized porcine corneal xenografts (DPCXs) offer to corneal xenotransplantation?

Table 1:

Advantages and disadvantages of decellularization methods and bioprinting for replacing the cornea

Method	Advantages	Disadvantages	References
Sodium Dodecyl Sulfate	Sufficiently removes cellular and nuclear components.	Harms ECM, lamellar structure. Causes collagen fibril disorganization. Reduces transparency.	17, 22, 23, 24, 26
Triton X-100	Can be useful as an additional treatment with another main decellularization agent.	Cannot remove cellular and nuclear components sufficiently. Damages glycosaminoglycans. Reduces transparency.	17, 30, 32, 35
Hypertonic Saline	Maintains ECM. Removes cellular components sufficiently. Used in a clinical trial.	Mixed reports on transparency. Causes edema.	20, 21, 29, 37, 38, 39
Human Serum with Electrophoresis	Maintains ECM and transparency.	Cannot remove cellular components sufficiently and homogenously.	43, 44
High Hydrostatic Pressure	Maintains lamellar and collagenous structure. Short time-frame. Used in a clinical trial.	Expensive. Disorganizes collagen fibrils and causes edema. Reduces transparency.	45, 46, 62
Freeze-Thaw	Removes cellular components sufficiently.	Harms ECM. Reduces transparency. Reduces mechanical strength.	48, 49, 50
N_2 Gas	Maintains transparency.	Causes edema. Studies needed on mechanical strength.	20, 51
Phospholipase A_2	Maintains ECM, transparency, and collagenous structure. Biocompatible.	Additional treatment needed to achieve sufficient decellularization.	9
Glycerol with Chemical Crosslinking	Maintains corneal transparency and mechanical properties.	Small amount of residual nucleic acids.	54

Legend: ECM = Extracellular matrix