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Comparison of the preparation and preservation techniques of amniotic membrane used in the treatment of ocular surface diseases

Comparação dos meios de preparação e preservação de membrana amniótica humana para uso no tratamento de doenças da superfície ocular

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ABSTRACT

Currently, the amniotic membrane (AM) has obtained importance due to its ability to reduce inflammation, helping in the healing and epithelialization processes, having antimicrobial and antiviral properties and low immunogenicity. Its indications in ophthalmology have increased considerably in the past two decades. Objective: To describe the basic structure and biological properties of the AM, the components of the extracellular matrix and growth factors, the consequences of different techniques used in its preservation, and sterilization methods for the epithelium removal. To compare the costs of the different preservation solutions currently employed. Study design: literature review. Methods: Research in BVS databases, PubMed, Cochrane, Scielo and Lilacs with keywords: amniotic membrane transplantation, corneal reconstruction, conjunctival diseases. Results: The literature is vast in describing the effects of different agents and techniques used in the preparation of MA, including its preservation, sterilization and desepithelization. The naked membrane is the choice to reconstruct the ocular surface, as it facilitates the healing course. Regarding the preservatives, glycerol is the most used worldwide due its low cost and easy handling. Conclusion: Comparing different techniques guides us in developing a MA preparation protocol for ophthalmic use. The naked membrane facilitates the healing process compared with the presence of epithelial cells. The glycerol is the most used preservation method because of its low cost and easy handling.

Keywords: *Transplantation; Amniotic membrane; Corneal diseases; Conjunctival diseases*

RESUMO

Atualmente a membra amniótica (MA) tem obtido importância devido à comprovada capacidade de reduzir inflamação, auxiliar a cicatrização e epitelização, possuindo propriedades antimicrobianas e antivirais, além de baixa imunogenicidade. As indicações de seu uso na oftalmologia têm aumentado muito nas duas últimas décadas. Objetivo: Descrever a estrutura básica e as propriedades biológicas da MA em relação aos componentes da sua matriz extracelular e fatores de crescimento, as consequências de diferentes técnicas empregadas na sua preservação e esterilização, métodos para remoção do epitélio e a comparação dos custos dos diferentes meios de conservação atualmente empregados. Métodos: Pesquisa nas bases de dados do Portal da Biblioteca Virtual em Saúde (BVS), Pubmed, Cochrane, Scielo e Lilacs com as palavras-chave: membrana amniótica, transplante, reconstrução da córnea, doenças da conjuntiva. Resultados: A literatura é vasta na descrição dos efeitos de diversos agentes e técnicas na preparação da MA, dentre elas sua preservação, esterilização e desepitelização. A membrana desnuda tem sido a escolha para a reconstrução da superfície ocular, pois facilita a cicatrização. Em relação aos agentes conservantes, o glicerol é o meio mais utilizado mundialmente pelo baixo custo e facilidade de manuseio. Conclusão: A comparação das diversas técnicas nos guia na elaboração de protocolos de preparo da MA para uso oftalmológico. A membrana desnuda facilita a cicatrização em relação a com células epiteliais. O glicerol é o meio de conservação mais utilizado pelo baixo custo e facilidade de manuseio.

Descritores: Transplante; Membrana amniótica; Doenças da córnea; Doenças da túnica conjuntiva

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Introduction

The amniotic membrane (AM) is the innermost of the three layers composing the fetal membranes. Back in 1910, a skin grafting case was the first record of its use to treat diseases.[1] Since then, it has also been used in surgical procedures associated with genitourinary tract, brain, head and neck issues, among others. [2,3] The first documented use of AM in the ophthalmology field dates back to 1940, when it was used to treat eye burns.[4] Kim and Tseng (1990) have spread AM use in the ophthalmic practice due to its success in repairing corneal defects. [5] Nowadays, AM has gained importance given its proven ability to reduce inflammation and to enable healing and epithelialization processes, as well as because of its antimicrobial and antiviral properties, and low immunogenicity.[6]

Amniotic membrane use recommendations to treat persistent corneal epithelial defect, neurotrophic ulcers, corneal perforations, shield ulcers, infectious keratitis, bullous keratopathy, ocular surface reconstruction after tumor excision, Stevens Johnson Syndrome, seidel repair in trabeculectomies, pterygium surgery, band keratopathy and chemical burns, have increased over the past two decades. [4] More recently, AM has been used as substrate for the transplantation of ocular surface epithelial stem and corneal endothelial cells, and as retinal pigment epithelial substrate. [7-10]

The process encompassing the period from donor placenta removal to AM transplantation is complex. It is necessary conducting the appropriate selection of potential donors prior to placenta removal. De-epithelialization is another important step in the aforementioned process. Naked AM enables better cell proliferation and differentiation, better structural integrity, as well as more consistent cell growth pattern than those of non-deepithelialized AM. [11,12]

Several studies available in the literature have described the effects of different agents and AM preparation techniques associated with AM preservation, sterilization and de-epithelialization. The current review addresses AM basic structure and biological properties associated with its extracellular matrix components and growth factors, as well as the consequences of different AM preservation and sterilization techniques, epithelium removal methods and the comparison of costs with different conservation means adopted nowadays.

Methods

A literature review was carried out after a search for studies was conducted at Virtual Health Library (VHL), Pubmed, Cochrane, Scielo and Lilacs databases based on the following meshes: amniotic membrane, transplantation, corneal reconstruction and conjunctival diseases.

Results

Basic structure

In histological terms, AM is a five-layer, 0.02 to 0.5mm-thick avascular membrane that delimits the innermost of the three layers forming the fetal membranes. The amniotic fluid bathes the inner apical surface, whereas the outer surface is attached to the chorion. The deciduous layer, which is composed of modified endometrium and is the only component of maternal-origin fetal membranes, is the outermost layer of fetal membranes. [13]

The epithelial amniotic layer is a simple layer comprising cuboidal and columnar cells with microvilli on the apical surface. The epithelium is located over the basement membrane (BM), which strongly resembles the conjunctival BM. Substantia propria has a compact collagen layer that promotes tensile strength, as well as a fibroblastic layer, which is the thickest AM layer that consists of fibroblasts embedded in a loose reticular network. The mucin-rich spongy layer is the outermost MA layer and the closest to the chorion.[13] The last 3 layers may be hard to be distinguished in case of histological examination applied to cryopreserved tissues (Figure 1).

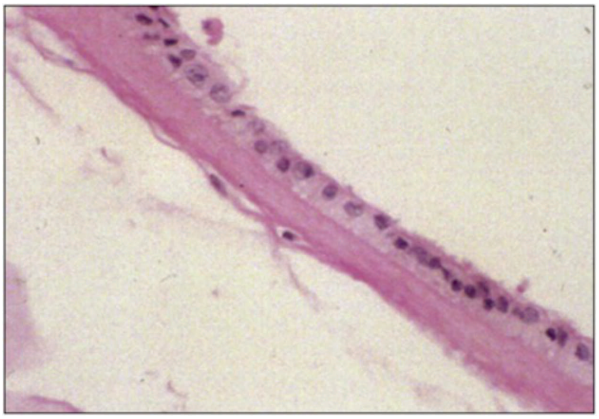

Figure 1: Amniotic membrane sample stained with hematoxylin-eosin. It is possible seeing a single layer of cuboid epithelial cells covering the basement membrane and the loose stromal connective tissue. (14).
Source: Krachmer JH, Mannis MJ, Holland EJ. Cornea. 3rd ed. New York: Elsevier; 2011.

Biological properties

Mesenchymal Components of Extracellular Matrix (ECM)

The extracellular matrix (ECM) has several functions, such as providing cell support and anchoring, and regulating cell's dynamic behavior. AM's substantia propria encompasses a whole variety of growth factors and acts as local storage. [15]

Collagen types 1 and 3 are the main structural fibrillar components of substantia propria. Immunohistochemistry reveals type 1 collagen prevalence in stromal layers. Type 3 distribution is similar to that of type 1; however, type 3 staining intensity is higher than that of type 1, near the amniotic BM. Type 4 collagen is a common component to all basement membranes; it appears in AM as a dense band of approximately 0.2 microns. Compact and fibroblastic layers show poor type 4 collagen fluorescence. Collagen types 5 and 6 are filamentous and they connect fibrillar collagens to surrounding connective tissue structures and to BM. Type 6 collagen presents equivalent intensity between compact and fibroblastic layers. Its concentration gets higher near the amniotic basement membrane, whereas the spongy layer shows less intense immunostaining. [16-19]

Another fundamental structural property in clinical application lies on the supporting role it plays as substrate for epithelial cell organization, since basement membranes overall act as biological support. Fukuda et al reported similarities among laminin, fibronectin and collagen types 4, 5 and 7 of conjunctival BM, cornea and AM. However, it was possible observing that the amniotic BM collagen type 4 alpha sub-chain was more similar to the conjunctival BM than to the corneal one. [19-21]

Endostatin is another important BM component; it is a proteoglycan heparan sulfate that has been proven to be a powerful anti-angiogenic factor capable of inhibiting endothelial cell proliferation, angiogenesis and tumor growth. Thrombospondin-1 (TSP-1) is a multifunctional protein matrix secreted by cells and it also has anti-angiogenic property. Based on immunohistochemical studies conducted by Riau et al, TSP-1 is found in amniotic epithelial cells with minimal expression in the stroma. [22]

Elastin is another poorly investigated component mainly found in amniotic epithelial cells presenting regular expression in the stroma. The major importance of elastin lies on its functional ability to enable tissue elasticity. [23]

Growth factors

The release of growth factors capable of enabling corneal reepithelialization and the reduction of scars and inflammation processes stand out among the mechanisms of action of AM transplantation. Several growth factors are found in AM, namely: epidermal growth factor (EGF); transforming growth factors (TGF) -α, -β1, -β2 and β3; keratinocyte growth factor (KGF), KGF receptor (KGFR), hepatocyte growth factor (HGF), HGF receptor (HGFR), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). [24,25] ELISA-based quantification has shown higher EGF, HGF, KGF and bFGF levels (and potent mitogens) in AM presenting epithelium than in naked AM. EGF (EGFR), KGF and bFGF receptors were mostly concentrated in the amniotic epithelium (Figure 2). EGF is a potent mitogen for epithelial cell growth; thus, high EGF expression levels may help explaining ocular surface healing after transplantation. PDGF also accounts for enabling cell responses such as proliferation, survival, migration, as well as for the deposition of remodeling and ECM factors. [25,26]

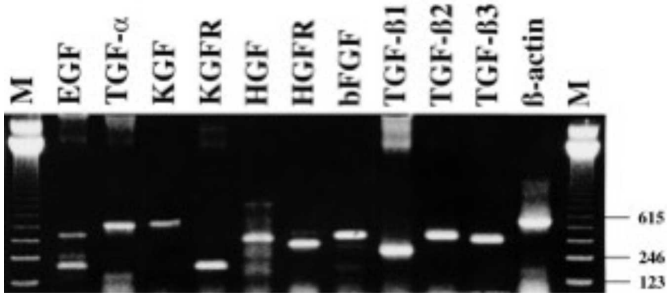

Figure 2: Gel stained with ethidium bromide deriving from RT-PCR products for eight growth factors (epidermal growth factor (EGF), transforming growth factor (TGF), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF) and transforming growth factors (TGF) -Fβ 1, -Fβ2 and Fβ3); and two growth factor receptors (KGF receptor (KGFR) and HGF receptor (HGFR)) in preserved human amniotic membrane.

Source: Koizumi NJ, Inatomi TJ, Sotozono CJ, Fullwood NJ, Quantock AJ, Kinoshita S. Growth factor mRNA and protein in preserved human amniotic membrane. Curr Eye Res. 2000;20(3):173–7.[25]

Donor membrane selection and uptake

Disease transmission to recipient patients is always a risk factor in human organ and tissue transplants. The AM donor should be a patient with healthy, non-drug-using medical history, who underwent elective cesarean section. [31]

Based on recommendations by the Food and Drug Administration (FDA), blood samples collected at prenatal visits should be subjected to a minimum series of serological tests, including HIV-1/2, hepatitis B and C, and syphilis. (4,32) Additional tests such as cytomegalovirus and HTLV screening should be performed. These tests, even the negative ones, should be repeated 6 months after delivery given the possibility that donors may be in immunological window period. Meanwhile, AM is often preserved in glycerol until results get confirmed. (31) Since it is a tissue donation process, AM uptake documentation is mandatory.

Membrane processing and preparation is carried out under sterile conditions. An antibiotic cocktail is added to washing and storage solutions and used to cover Gram-negative and Gram-positive bacteria and fungi. Two different protocols are very popular nowadays. The first one was popularized by the Tsuboto group. According to such protocol, the membrane is cut into 10 cm x 10 cm pieces, which are sequentially rinsed with 0.5 M dimethyl sulfoxide (DMSO) (4% w/v in phosphate-buffered saline solution 0.01M PBS), 1.0 M DMSO (8% w/v in 0.01M PBS) and 1.5 M DMSO (12% w/v in 0.01M PBS) for five minutes (each).(33) The second protocol was popularized by Tseng et al.; it consists in storing the membrane pieces in 50% glycerol in Dulbecco's Modified Eagle Medium (DMEM, Gibco) or TC-199. (5) Membrane pieces are often spread on the epithelial side, on nitrocellulose paper, prior to be stored in medium. The tissue is frozen-stored at -80°C and only released for use after the second serological screening test, which is performed six months after cesarean delivery - it can be stored and used for up to 2 years after delivery. The tissue must be thawed at room temperature and rinsed with normal buffered saline solution right before use. (4)

De-epithelialization

The amniotic membrane (AM) has a variety of proteins and growth factors. It is not yet clear how these proteins and growth factors are affected by different procedures adopted to prepare AM. In addition, it is yet to be determined what extracellular matrix factors and components are the most determinant in promoting epithelial growth and adhesion. Several techniques and reagents have been described to perform epithelial cell removal as the attempt to produce a biological substrate capable of being used to grow different cell types. [34] There are doubts about whether intact or naked AM is the best substrate for ex vivo expansion of epithelial cells for ocular surface construction. Some scholars have reported the potential benefits of intact AM. In addition, AM epithelial cells have been reported to produce neurotransmitters, neuropeptides, neurotrophic factors and pigment-derived growth factors. [32-38]

On the other hand, other groups of researchers have shown that naked AM can promote better cell proliferation and differentiation, better cell adhesion, as well as more consistent cell growth patterns than intact AM.[11,39] The presence of amniotic epithelium can also impair the uniform growth of membrane-grown explants, as well as delay the formation of resistant hemidesmosomal bonds. [40] These factors translate into a better healing process. One of the most important AM properties lies on its ability to make healing easier, a fact that determines most of its clinical recommendations. Therefore, naked AM has been one of the options made for ocular surface reconstruction.

Several methods have been described for AM de-epithelialization. Each method has revealed different effects on membrane structure and biological properties. Ideally, the de-epithelialization method would be efficient and not change

AM structural integrity (mainly, the basement membrane) or its biological function.

Dispase Method

The idea of using dispase to remove amniotic epithelial cells was adapted from the technique used to obtain intact corneal epithelium.[41,42] Dispase acts enzymatically through the proteolytic action that reaches BM proteins such as laminin, collagen types IV and VII, and fibronectin.[43,44]

Studies available in the literature have investigated the clinical use of naked AM based on 1.2 U / mL of dispase II, at treatment time ranging from 5 minutes to 2 hours, at room temperature, followed by delicate scraping procedure. Treatment with dispase II resulted in BM rupture, mainly in lamina densa. There was irreversible physical and biological damage after incubation with dispase II. The underlying stromal arrangement became loose, whereas collagen type VI, fibronectin and several growth factors (TGF-α, β1 and β2 receptors, PDGF-A, VEGF, and EGFR) decreased after prolonged incubation. Complete BM removal was observed only after 30 minutes of treatment.[45]

Li et al have investigated limbal epithelial explants grown in intact and de-epithelialized AM.[42] After basement membrane disaggregation in AM samples, it was possible observing BM component formation, even in the de-epithelialized sample. This outcome suggests that intact membranes may not be of paramount importance for epithelial cell expansion. It also explains the high success rate recorded for the use of dispase-treated AM, even when BM is fully removed, in ocular surface reconstruction.

EDTA Method

Ethylenediaminetetraacetic acid (EDTA) is a chelating agent that influences calcium ion-regulated intercellular contact. Studies have investigated EDTA use at concentrations ranging from 0.02% to 0.25%, for 10 minutes to 2 hours, at 37°C.[45-47] Such use is often followed by mechanical scraping of the remaining epithelial cells.

The literature has described the use of this technique to enable wider epithelial cell removal without breaking the underlying BM.[46,48] Extracellular membrane (ECM) components of AM subjected to EDTA-based de-epithelialization - without causing BM damage - were similar to those of intact AM, whereas growth factors were scarcer in naked AM due to vigorous mechanical scraping after EDTA chelation. Amniotic membrane components (collagen types IV and VII, laminin-5 and fibronectin) also remained after de-epithelialization.[49] However, the association between de-epithelialization and EDTA treatment has led to impaired membrane integrity.[50] More aggressive scraping was necessary to reach full epithelial cell removal, a fact that led to further membrane damage.

There was not statistically significant difference in resistance between intact and de-epithelialized AMs (based on the 0.02% EDTA method).[51]

Trypsin-EDTA Method

Trypsin-EDTA is often used to separate seeded cells from the culture flask. This method has been used to remove epithelial cells from AM at concentrations ranging from 0.1% to 0.25%, at 37ºC, for 30 minutes. In most cases, amniotic epithelial cells were removed without scraping, although some groups required scraping after incubation. Mehta et al have treated the amniotic membrane with 0.25% trypsin-EDTA and reported the presence of collagen types IV and VII, laminin-5 and lamina densa.[24] However, many ECM components and growth factors were not detected through the immunostaining technique. It may have happened due to the aggressive nature of trypsin-EDTA treatment, which likely degraded the ECM components and growth factors.

Urea Method

Urea is well-known for its protein denaturation property, besides its ability to solubilize them. Amniotic membrane de-epithelialization can be achieved through the application of a treatment with refrigerated urea (5 M) for 5 minutes, which is followed by mild de-epithelization. The advantages of using this method lie on its relatively short incubation period and on the fact that the used agent (urea) is readily available. Amniotic membrane de-epithelialized with this method presented smooth surface and presence of basal lamina. ECM components remained in the de-epithelialized membrane.[52]

Ethanol Method

Ethanol has been used to separate the epithelial layer from BM for flap making.[53] Ethanol-based de-epithelialization comprises AM incubation in 20% ethanol for 30 seconds, and further mechanical de-epithelialization. Similar to the urea technique, the benefits of the ethanol technique comprise short incubation period and easy ethanol availability. This method requires more aggressive scraping due to low ethanol denaturing power. Although it is usual observing BMB injuries due to aggressive scraping, as shown by many studies, few researchers have found the presence of intact BM under electron microscopy examinations.[24] The existence of cell remnants may be beneficial to cultured–cell growth; similarly, it has been suggested that micro or nano surface irregularities may enhance cell growth in other biomaterials.[54] ECM components such as collagen types I, II, IV, VI and VII, laminin-5, fibronectin, elastin and thrombospondin, as well as growth factors such as TGF-α, β1 and β2 receptors, EGFR, KGF, bFGF, VEGF and PDGF are expressed in naked AM. (24)

Thermolysin Method

The use of thermolysin for amniotic epithelium removal is relatively new. Thermolysin is a thermostable metalloproteinase isolated from bacillus stearothermophilus, which was previously used to isolate epidermal cells.[55,56] However, based on the thermolysin method, a single washing procedure can remove amniotic epithelial cells without the need of adopting any mechanical scraping procedure. Amniotic membrane treatment with 125μg/mL of thermolysin for 9 minutes can generate a fully naked membrane, which has consistent morphological appearance and intact basal lamina.[50]

Basement membrane (BM) components such as collagen types IV and VII, α6 and β4 integrins, and laminin-5 are distinctly and consistently expressed in AM treated through Thermolysin method.[50] The identification of α6 and β4 integrins in the absence of cells suggested that thermolysin has specifically cleaved the desmosomal complex.[57] This outcome also suggested that the proteolytic activity of thermolysin was more specific than that of other agents used to isolate epithelial cells. These observations were corroborated by Perreault et al, who used thermolysin to isolate and generate viable healthy human intestinal epithelial cell cultures.[58]

Hypotonic Buffer, SDS and Nuclease Method

Wilshaw et al were pioneers in using this method; they have successfully transplanted AM into the subcutaneous region in a murine model.[59,60] The use of this method in ocular surface

reconstruction processes has not been reported so far. However, Shortt et al performed the expansion ex vivo of limbal epithelial stem cells in de-epithelialized AM, based on the technique described by Wilshaw et al, and found larger cell proliferation and number of cells expressing ΔN-p63α and ABCG2 than the intact AM.[61]

Based on the aforementioned method, AM is incubated in 10mM hypotonic buffer added with 0.1% EDTA and 10 KIU/mL aprotinin, at 4°C, for 16 hours. It is done to induce lysis through cellular edema by using sodium dodecyl sulfate (SDS), which is an ionic detergent used to treat AM at room temperature for 24 hours. The detergent binds to the cell membrane, separates the lipid bilayer and disintegrates it. Next, AM is incubated with 50 U/mL of DNase and 1 U/mL of RNase for 3 hours, at 37°C, to enable removing residual DNA and RNA from the matrix. Amniotic epithelial cells can be isolated without mechanical scraping. There was no evidence of type IV collagen, glycosaminoglycans or elastin loss in AM; moreover, there was no significant difference between the amount of denatured collagen found in naked AM and in its intact form. Electron microscopy revealed similar collagen fibril arrangement in naked and intact AMs.[59]

Membrane Preservation and Sterilization

Fresh membrane or the membrane processed through several preservation methods such as freezing, lyophilization or cryopreservation in glycerol have been the AM used in ophthalmic surgeries. Recent studies have used the combination between preservation and gamma irradiation sterilization techniques to help minimizing the risk of infections that can be transmitted through AM. New agents such as peracetic acid and trehalose have also been recently used for AM preservation and sterilization purposes. (Figure 3)

Cryopreservation in Glycerol

Cryopreservation in glycerol is the most used method worldwide. Storage in glycerol was introduced in the Netherlands, in 1984, to help preserving transplant donor skin. Excellent results obtained in the last decades have led to its clinical acceptance at global level, which includes its use in AM preservation processes. Although glycerol is known to have some antiviral effect, its sterilizing action remains unknown. Viruses and bacteria can remain viable even after preservation for several months. High glycerol concentration use compromises the viability of AM cells.[62-66]

Placenta is often washed in balanced saline solution added with antibiotics such as streptomycin, penicillin, neomycin and amphotericin. The amniotic membrane (AM) is stored in glycerol, which is overall mixed to Dulbecco's Modified Eagle Medium (DMEM) at ratio 1: 1 (vol/vol). Next, AM can be stored at -80ºC for up to 2 years.[22] This preservation method was introduced by Lee and Tseng[67] and, since then, it has shown high rates of successful AM transplantation.[47,68-70] Almost all studies available in the literature about AM use for ocular surface reconstruction have evidenced its adoption for preservation purposes. The components and biological structure of AM cryopreserved in glycerol resemble the fresh (non-preserved) AM.[71]

The main obstacle to this process lies on the need of an expensive, heavy and hard-to-be-found freezer, mainly in less developed countries. Moreover, it is relatively hard to keep a stable temperature during membrane transportation.

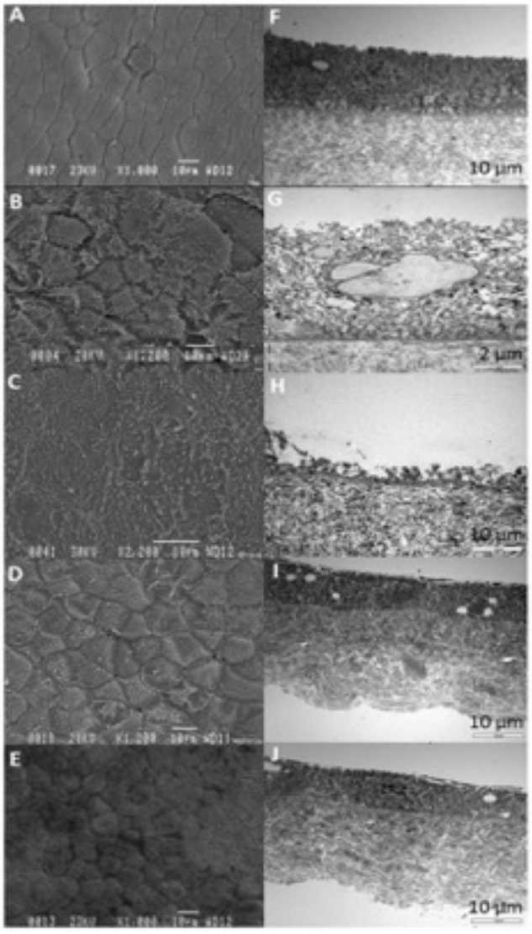

Figure 3: Scanning electron microscopy and transmission electron microscopy (TEM) micrographs of epithelial and stromal layers on preserved AM substrates. Fresh, (A, F); cryopreserved, (B, G); naked (C, H); dry (D, I) and after treatment with trehalose (E, J). Photomicrographs show extensive damage to the CEA layer and post-cryopreservation microvilli in comparison to fresh, dried and trehalose-treated substrates.

Source: Allen CL, Clare G, Stewart EA, Branch MJ, McIntosh OD, Dadhwal M, Dua HS, Hopkinson A. Augmented dried versus cryopreserved amniotic membrane as an ocular surface dressing. PLoS One. 2013;8(10):e78441.

Cryopreservation in Dimethyl Sulfoxide

Dimethyl sulfoxide (DMSO) has been used as an alternative to glycerol-DMEM. Increased DMSO concentration was used to wash AM after placenta removal; it replaced the use of saline solution added with antibiotics. AM was stored in 10% solution or in 0.15M of DMSO at -80°C, for several months. Several studies have shown clinical success after using this preservation technique in ocular surface surgery.[33,72,73] The effect of this technique on AM structural integrity and biological components has not been extensively investigated so far; however, based on data deriving from tests already performed, it is possible assuming that AM subjected to cryopreservation in DMSO has similar quality to the one cryopreserved in glycerol.

Lyophilization and Gamma Radiation Sterilization

Lyophilization is a method used to remove water from tissues through sublimation. It inhibits destructive chemical

reactions capable of changing tissues. The lyophilized amniotic membrane can be stored at room temperature for a long period of time without facing any deterioration risk. This membrane is easily transported and avoids cryopreservation issues. Some studies have investigated the association between lyophilization and gamma irradiation sterilization.[51,74]

Adequate radiation doses are effective against bacteria, fungi and viruses; however, radiation affects the biological properties and integrity of human tissues.[75,76] There are few studies in the literature focused on evaluating the effectiveness of using the combination between lyophilization and gamma irradiation as AM sterilization technique.

Water removal from AM makes lyophilized membranes seem thinner and macroscopically more fragile than the cryopreserved ones.[71] However, Nakamura et al did not report significant differences in physical resistance between cryopreserved and radiation-associated lyophilized AM. No changes in tissue structure and ECM components (collagens I, III, IV, V and VII, fibronectin and laminin-5) were observed between lyophilized AM subjected to sterilization with 25 kGy of gamma radiation and cryopreserved AM.[51] Total protein level was significantly lower than the one found in non-preserved AM.[77] Lyophilization is likely to cause protein denaturation and changes in tissue structure.[78]

The levels of growth factors such as TGF-α, -β1, β2 and β3, VEGF, PDGF-A and –B, KGF, bFGF, EGFR and TGF-β2 receptor were lower in lyophilized AM treated with irradiation than in cryopreserved AM.[74.79]

Lyophilization based on Trehalose Method

Although lyophilized AM keeps most biological features of non-preserved AM, the freezing process - associated with sublimation-based evaporation inherent to lyophilization processes - affects some of its biophysical properties.

Trehalose is a non-reducing disaccharide found at high concentrations in different organisms able to survive almost 100% dehydration. This disaccharide makes human cells resistant to desiccation due to its ability to replenish part of intracellular water; thus, it helps protecting and stabilizing the amniotic structure during lyophilization processes.[80,81]

Nakamura et al have suggested that the physical properties and biocompatibility of AM treated with 10% trehalose prior to lyophilization are superior to that of lyophilized AM treated without trehalose. There aforementioned study has shown the presence of collagen types I, III, IV, V and VII, fibronectin and laminin-5 in trehalose-treated AM. Although this method is not routinely applied to AM used for ophthalmic purposes, it has been described for other applications such as lung preservation for transplantation purposes, as well as eye drops to treat moderate and severe dry eye.[82-84]

Sterilization in Peracetic Acid

Peracetic acid (PAA), which belongs to the family of organic peroxides, is a highly effective standard sterilizing agent used against bacteria, viruses and spores, given its oxidation potential.[85,86] It is an excellent sterilizing agent, since its metabolites are non-toxic (acetic acid and hydrogen peroxide). The structure of AM sterilized with PAA proved to be well-conserved in comparison to non-sterilized AM. Collagen types I and III were relatively more abundant in PAA-treated AM than in AM sterilized with gamma radiation. Type IV collagen, fibronectin and laminin were also retained in PAA-treated MA; this outcome suggests that BM and its biological contents were not disrupted. [87]

Tables1 and 2 present the comparison between benefits and disadvantages of different conservation means currently used to prepare AM.

Discussion

Both intact and naked AM are substrates suitable to epithelial cell expansion ex vivo for ocular surface reconstruction processes. Some studies have reported the potential benefits of intact AM. According to these studies, AM epithelial cells were capable of producing neurotransmitters, neuropeptides, neurotrophic factors and pigment-derived growth factors. [35-38]

However, other studies have shown that naked AM can promote better cell proliferation and differentiation, better cell adhesion, as well as a more consistent cell growth pattern than intact AM. [11,39] The presence of amniotic epithelium can also prevent the uniform growth of membrane-grown explants and delay the formation of resistant hemidesmosomal bonds.[40] These factors favor the achievement of better healing processes. One of the most important AM properties lies on its ability to make the healing process easier, which is also the determining factor for most of its clinical recommendations. Therefore, naked AM has been the best option for ocular surface reconstruction processes.

Since the first successful grafts performed in the 1960s, AM has been widely used as biodegradable patch in corneal surgeries and as natural anatomical substrate for limbal epithelial stem cell (LESC) culture to treat limbal stem cell deficiency (LSCD). [12,88] Although the epithelium is capable of firmly covering human AM, it is a poorly adhesive substrate for most (if not all) epithelial cells. Limbal epithelial stem cell (LESC) monolayers cannot easily establish in AM if amniotic epithelial cells are not removed from it. Only limbal explants enable substantial growth in intact (not de-epithelialized) AM, likely due to amniotic epithelial cell disruption. Amniotic membrane (AM) cryopreservation in glycerol medium destroys amniotic cells and enable the easy growth of explants containing LESC fragments.[89] The widespread use of this technique is strongly linked to its de-epithelializing effect, since it makes mechanical de-epithelialization optional. Intact membranes show poor LESC growth, in comparison to naked AM, because they inhibit the terminal differentiation of these cells. Cell differentiation stimulation is mainly observed when cultures are exposed to airlifting. [90] In addition, it is preferable using de-epithelialized AM in order to get more transplant-friendly LESC cultures. In addition, it is essential keeping stromal cells away from unwanted agents as much as possible, since they secrete factors necessary to promote cell proliferation and wound healing.[91]

Although de-epithelializing agents such as dispase, EDTA and Trypsin-EDTA are easy to be handled, they enable the obtainment of reasonable-quality naked membrane (they compromise the structural integrity of the matrix) and overall require additional epithelial scraping.

The advantages of using urea as de-epithelializing agent comprise short incubation period and the fact that this agent is readily available. Ethanol is also easy to be obtained, as well as of cheap, fast and simple use.

The thermolysin method allows removing amniotic epithelial cells with a single washing procedure without the need of mechanical scraping. Thermolysin treatment applied to AM is capable of generating a fully naked membrane with consistent morphological appearance and intact basal lamina; however, the membrane becomes very fragile and friable upon handling. Thermolysin and hypotonic buffer allow obtaining excellent naked AM after simple processing,

whereas alcohol increases the risk of structural tissue damage due to the need of aggressive mechanical scraping. It is important emphasizing that hypotonic tampon - SDS - is a time-consuming and laborious method comprising nuclease-based treatment.

All AM de-epithelialization techniques based on EDTA, dispase, trypsin, thermolysin, SDS, alcohol or urea require AM immersion in de-epithelializing solutions from 30 seconds to 24 hours - these solutions affect epithelial and stromal structures. Some drawbacks have been associated with most de-epithelialization methods; thus, it is necessary setting further standardization criteria for these important procedures.

With respect to preservatives, glycerol is the most widely used medium worldwide due to its low cost and easy handling; however, it requires an expensive freezer that is not available in all ophthalmic services.

Conclusion

Nowadays, there is a wide range of agents and techniques focused on amniotic membrane preparation. The naked membrane has been the technique of choice for ocular surface reconstruction processes, since it facilitates healing in comparison to the membrane with epithelial cells. Glycerol is the most widely used preservative worldwide, due to its low cost and easy handling. In addition, glycerol is advantageous for ophthalmic applications when it is used for healing purposes and for stimulating cell differentiation.

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Table 1
Comparative table of de-epithelialization agents used to prepare the amniotic membrane

De-epithelialization agents	Advantages	Disadvantages
DISPASE 1mg/ml 100mL	- Low cost	- It causes MB rupture, mainly of lamina densa - The underlying stromal arrangement becomes loose; and type VI collagen, fibronectin and several growth factors decrease after prolonged incubation
EDTA 100mL(Sigma-Aldrich®)	- Removal of wider epithelial cells without MB rupture	- Impairment in membrane integrity - There is impairment in membrane integrity when it is used in association with de-epithelialization. It requires more aggressive scraping.
EDTA-TRYPSIN 500 mL (Science pro®)	- Epithelial cell removal without scraping	- It degrades ECM components and growth factors
UREIA 100mL (Sigma-Aldrich®)	- Relatively short incubation period - Easily available agent	
ETHANOL 500mL (Sigma-Aldrich®)	- Short incubation period and rapid availability - It keeps the MB intact	- It requires more aggressive scraping due to low denaturation power
TERMOLISIN 25 mg (Sigma-Aldrich®)	- A simple washing procedure can remove amniotic epithelial cells without the need of the mechanical scraping procedure - It specifically cleaves the desmosomal complex (most specific proteolytic activity)	
HYPOTONIC BUFFER, 25g and NUCLEASE (Sigma-Aldrich®)	- The isolation of amniotic epithelial cells an be achieved without mechanical scraping - No collagen loss in the naked AM in comparison to the intact AM	

Table 2
Comparative table of preservatives used to prepare the amniotic membrane

Preservatives	Advantages	Limitations
GLICEROL	- AM components and biological structure resemble the fresh, non-preserved AM - Most used method worldwide.	- Little sterilizing action. - High concentrations of it compromise AM cell viability. - It requires a costly, heavy and hard-to-be freezer. - It is relatively difficult to keep the temperature stable during AM transport.
DMSO 1000mL (Synth®)	- Effective technique AM preservation.	- There are few studies focused on investigating its effect on AM structural integrity and biological components.
LYOPHILIZATION	- Adequate radiation doses are affective against bacteria, fungi and viruses.	- The lyophilized membrane appears to be thinner and, macroscopically, more fragile than the cryopreserved one. - The lyophilized is likely to cause protein denaturation and changes in tissue structure.
GAMMA RADIATION	- No changes in tissue structure and ECM components were observed between the lyophilized AM sterilized with 25-kGy gamma irradiation and the cryopreserved AM.	- Lyophilized naked AM treated with irradiation shows lower growth factor level than the cryopreserved AM.
TREHALOSE (Anhui Elite industrial Co. Ltd®)	- AM treated with 10% trehalose prior to lyophilization presents superior physical properties and biocompatibility.	- Method not often used in ophthalmic environment.
PERACETIC ACID (Lvzhiyuan®)	- Good AM structure conservation in comparison to non-sterilized AM - There is no rupture in MB and MB and in its biological contents.	

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