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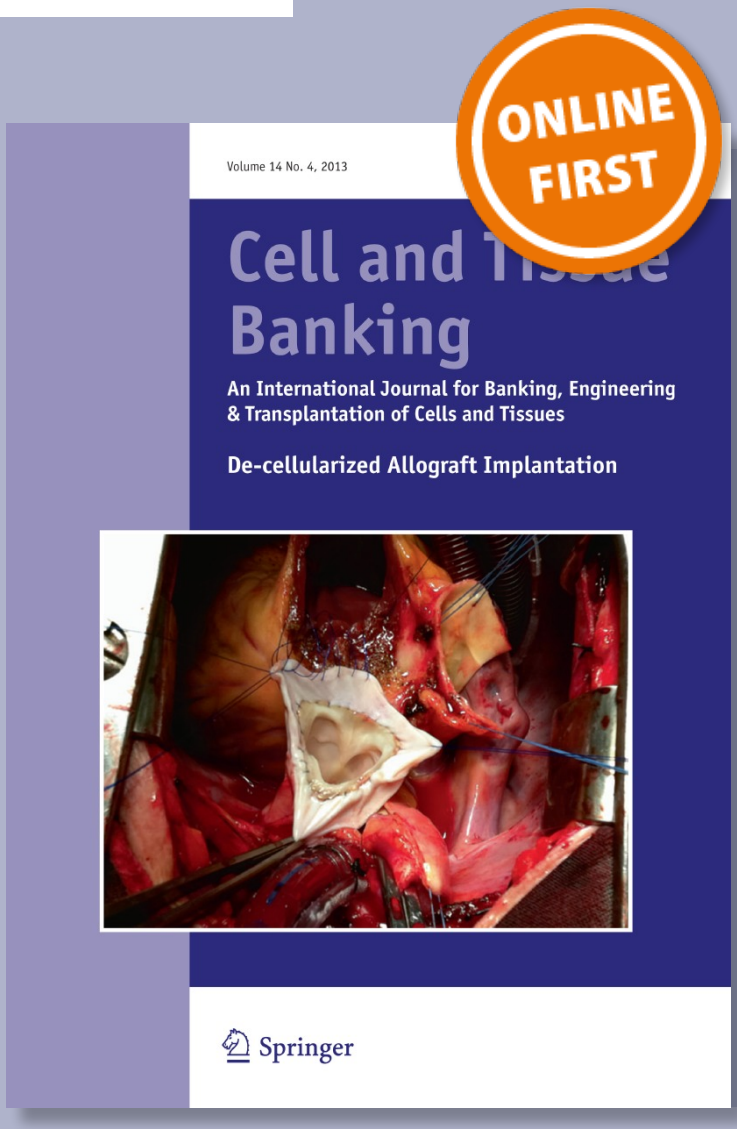

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ORIGINAL PAPER

Amniotic membrane properties and current practice of amniotic membrane use in ophthalmology in Slovenia

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Abstract Amniotic membrane (AM) is the innermost, multilayered part of the placenta. When harvested, processed and stored properly, its properties, stemming from AM biological composition, make it a useful tissue for ophthalmic surgery. AM was shown to have several beneficial effects: it promotes epithelization, has antimicrobial effects, decreases inflammation, fibrosis and neovascularization. Many case reports and case series as well as practical experience (e.g. reconstruction of conjunctival and corneal defects, treatment of corneal ulcers) demonstrated the beneficial effect of AM for different ophthalmological indications. The combination of the above mentioned beneficial effects and reasonable mechanical properties are also the reason why AM is used as a substrate for ex vivo expansion of epithelial progenitor cells. Recently, amnion-derived cells, which also have stem cell characteristics, have been proposed as potential contributors to cell-based treatment of ocular surface disease. However, the use of AM remains one of the least standardized methods in ophthalmic surgery. In this review, the various properties of AM and its current clinical use in ophthalmology in Slovenia are discussed.

Keywords Amniotic membrane · Ophthalmology · Regenerative medicine · Cornea · Conjunctiva · Amniotic stem cells

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Molecular composition, ultrastructure and biological properties of AM

Human amniotic membrane (AM) is the innermost layer of the placenta that contributes to the homeostasis of amniotic fluid (Dua et al. 2004). AM is avascular and contains no nerves (Niknejad et al. 2008). Its thickness is 0.02–0.5 mm and is composed of metabolically active (a) epithelial layer, which consists of a single layer of AM epithelium attached to the (b) basement membrane (BM), one of the thickest basement membranes found in human tissues (Fig. 1a; Danforth and Hull 1958; Bourne 1962; Niknejad et al. 2008). The apical surface of epithelial cells is covered with microvilli (Fig. 1b; Bourne 1962). The third

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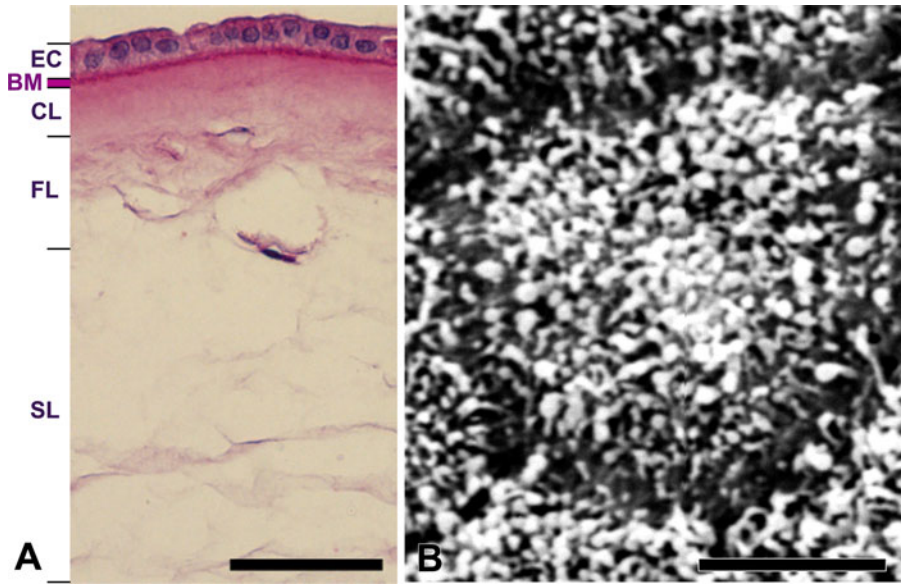

Fig. 1 The amniotic membrane histology (**a**) and the apical surface of amniotic epithelial cells (**b**). **a** Periodic Acid Schiff reaction marks thick amniotic basement membrane (BM) and neutral proteoglycans (*purple–red*) in avascular AM stroma. AM stroma can further be subdivided into compact (CL), fibroblast (FL) and spongy layers (SL). Nuclei of epithelial cells (EC) and fibroblasts in FL are stained with hematoxylin. **b** Scanning electron microscopy reveals microvilli at the apical surface of amniotic epithelial cells. *Bars* 50 μm in **a** and 5 μm in **b**

component of AM is (c) an avascular stroma, which is subdivided into compact, fibroblast and spongy layer (Dua et al. 2004; Niknejad et al. 2008; Thomasen et al. 2009; Kreft and Dragin 2010).

The major components that contribute to the integrity of AM and its biochemical properties are: (1) cytoskeletal proteins of AM epithelial and stromal cells, e.g. actin, tubulin, different cytokeratins, vimentin, desmin; (2) junctional proteins between AM epithelial cells, e.g. occludin, claudin-3 and -4 and desmoplakin and (3) different types of collagen—I, III, IV, V, VI, hyaluronan and proteoglycans, which are abundant in AM stroma (Malak et al. 1993; Ockleford et al. 1993; Kobayashi et al. 2010; Fukuda et al. 1999; Meinert et al. 2001; Wolf et al. 1991).

Several cytokines are associated with AM: interleukins-6 and -8 were found in the media of cultured amnion cells in response to different stimuli. It was proposed they play a role during infection-induced preterm labor (Fortunato et al. 1996). Since they were also detected in amniotic fluid at term, their role during normal human delivery was suggested (Keelan et al. 1997). AM (fresh or preserved at −80 °C for 6 months) showed the presence of epidermal growth factor (EGF), transforming growth factor-α (TGF-α), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), hepatocyte growth factor receptor (HGFR), basic fibroblast growth factor (bFGF) and transforming growth factor (TGF)-β1 and -β2, suggesting the AM epithelium as the source of various growth factors (GFs) (Koizumi et al. 2000; Li et al. 2005; Gicquel et al. 2009). The amounts of GFs in preserved tissue were in the range of several picograms per milligrams or picograms per gram of fresh tissue (Koizumi et al. 2000; Russo et al. 2012). GFs, produced by AM, were demonstrated to participate in the facilitation of corneal re-epithelization and reduction of corneal scarring and inflammation (Riau et al. 2010).

There are conflicting reports on the presence of human leukocyte antigens (HLA) A, B, C and DR on cells of AM. Some authors suggest that there is no expression (Akle et al. 1981), while others oppose these findings (Hunt et al. 1988; Hammer et al. 1997). The first option is more likely since the acute immune rejection did not occur in human volunteers after the transplantation of human amniotic epithelial cells (Akle et al. 1981), nor was there a significant immuno response in a xenotransplantation model when human AM was transplanted to rat eyes (Kubo et al. 2001). It was suggested that AM is an immuno-privileged tissue

and that it secretes an inhibitory factor for the suppression of allogenic response (Kubo et al. 2001). It was shown that human amnion can express HLA-G, the major factor that prevents rejection of trophoblasts by mediating immune tolerance (Hammer et al. 1997). When AM was used for culturing limbal and conjunctival cells, upregulation of HLA-G was observed in the conjunctival epithelial cell line CCL20.2 (Higa et al. 2006).

Mechanism of action of AM

Although the exact mechanism of many AM actions is still unclear, several beneficial effects of AM have been suggested on the basis of its biological composition. It was suggested that AM promotes epithelization and decreases inflammation, neovascularization and fibrosis (Sippel et al. 2001; Dua et al. 2004). It also provides a substrate for cell growth, has antimicrobial effects and functions as a biological bandage (Dua et al. 2004). Reduced pain after AM transplantation was also observed, although it is possible that this is due to anti-inflammatory action of AM (Liu et al. 2010). It was suggested that removing the epithelial layer of AM promotes cell proliferation, differentiation, and structural integrity better than intact AM (Riau et al. 2010).

Promotion of epithelization and cell differentiation

Laminin and fibronectin are potent chemoattractants for epithelial cells. They serve as a basement membrane, facilitating migration of epithelial cells by acting as a substrate for epithelization (Ohshima et al. 2003; Meller and Tseng 1999; Velez et al. 2010; Choi et al. 2009; Dua and Azuara-Blanco 1999). AM is a good support for human retinal pigment epithelium (Capeáns et al. 2003). AM was also shown to support growth of conjunctival or limbal cells in culture (Cho et al. 1999; Meller et al. 2002a, b; Ang et al. 2005; Tsai et al. 2000; Sudha et al. 2009; Solomon et al. 2001; Mariappan et al. 2010). AM may be an ideal biological substrate that can help maintain and support the expansion of limbal epithelial stem cells by serving as a stem cell niche (Grueterich et al. 2003).

It was proposed that AM can promote cellular differentiation, maintain normal morphology of epithelial cells, prevent apoptosis and enhance adhesion of different cells (Meller and Tseng 1999; Ohno-Matsui et al. 2005; Kurpakus et al. 1992, 1999; Jerman et al. 2013; Cho et al. 1999; Boudreau et al. 1995; Velez et al. 2010). When used as an overlay patch, AM protects cells from being washed away by the movement of eyelids (Letko et al. 2001; Azuara-Blanco et al. 1999).

Certain proteins (i.e. collagen I, III, IV, V, VI, VII, laminin, fibronectin) are present in different layers of AM stroma as well as conjunctiva and cornea, thus partially explaining the usefulness of AM as a substrate for epithelial cell organization (Fukuda et al. 1999; Malak et al. 1993; Kolega et al. 1989; Gipson et al. 1987; Endo et al. 2004; Riau et al. 2010).

Inhibition of inflammation, fibrosis and apoptosis

Several studies indicated that AM has anti-inflammatory properties, showing expression of anti-inflammatory proteins interleukin-1 receptor antagonist (IL-1ra) and interleukin-10 (IL-10) in epithelial cells of human AM (Fidel et al. 1994; Hao et al. 2000). When conjunctival, corneal or limbal cells were cultured with AM, in the AM treated eye or when monocyte/macrophage cell line was treated with AM extract, the expression of proinflammatory cytokines IL-1α, -1β, IL-2, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and IL-6 was reduced (Solomon et al. 2001; Tseng et al. 1999; Heiligenhaus et al. 2001; He et al. 2008; Kreft and Dragin 2010; Sippel et al. 2001).

Anti-inflammatory properties can also contribute to the decrease in fibrosis and neovascularization, although some anti-scarring effects were contributed directly to the action of AM. Culturing corneal or limbal cells on preserved AM suppressed myofibroblast differentiation (Tseng et al. 1999). The expression of transforming growth factor (TGF)-β2, -β3 and all three types of TGF-β receptors was suppressed when fibroblasts were grown on AM (Lee et al. 2000). TGF-ß expression was also suppressed—along with IL-8—when conjunctival fibroblasts were grown on AM (Solomon et al. 2005).

It was demonstrated that AM can modulate T cell response, suggesting that the anti-inflammatory action of AM is due to its local effects (Heiligenhaus et al. 2003). Shimmura et al. (2001) demonstrated that inflammatory cells (i.e. cells of monocyte/macrophage lineage, T and B lymphocytes) infiltrated the AM that was transplanted to the ocular surface of patients.

Infiltration of AM with lymphocyte cell lines and peripheral blood mononuclear cells was contributed to the interaction of CD44 on the cells with hyaluronic acid (HA) of AM (Higa et al. 2005). Application of human AM as a graft also reduced the infiltration of polymorphonuclear leukocytes (PMN) while promoting apoptosis of PMNs (Wang et al. 2001; Park and Tseng 2000). PMN infiltration was reduced in AM-covered rabbit corneas with alkali wounds (Kim et al. 2000).

One of the main functions of AM is to maintain the integrity of fetal membranes until term (Hao et al. 2000). Since it was shown that premature proteolysis of AM is caused by endogenous tissue proteases or proteases, excreted from leucocytes, the identification of several protease inhibitors is not surprising (Main et al. 1985). α1-antichymotrypsin, α2-macroglobulin, α1-antitrypsin, α2-antiplasmin, secretory leukocyte protease inhibitor and plasminogen activator inhibitor type-2 were all identified in the AM (Anon 1999; Izumi-Yoneda et al. 2009; Takashima et al. 2004; Zhang et al. 2001; Tsatas et al. 1998). Expression of tissue inhibitors of metalloproteases (TIMPs), major endogenous regulators of matrix metalloproteases (MMPs), was demonstrated in epithelial cells of human AM (Hao et al. 2000; Rowe et al. 1997; Fortunato et al. 1998). Herpes simplex virus type-1 (HSV-1)-induced ulcerative keratitis was rapidly improved in mice after the AM transplantation. Reduced expression of some matrix metalloproteases, increased expression of TIMP-1 and reduced levels of IL-1α, IL-2 and tumor necrosis factor-α (TNF-α) was demonstrated as well (Heiligenhaus et al. 2004, 2005; Bauer et al. 2007). It was also demonstrated that alkali wounded rabbit corneas covered with AM had much lower protease activity when compared to the uncovered ones (Kim et al. 2000).

It was suggested that some of the anti-inflammatory and anti-scarring effect of AM could be due to its effect on apoptosis (Wang et al. 2001; Shimmura et al. 2001). mRNAs of proapoptotic TNF-α, TNF-related apoptosis-inducing ligand (TRAIL), and Fas ligand (FasL) were detected in cultured amniotic epithelial cells and cells of the amniotic epithelium were found to express Fas and FasL (Li et al. 2005; Runić et al. 1998; Harirah et al. 2002). Supernatant of cultured amniotic epithelial cells could increase apoptosis of T and B lymphocytes (Li et al. 2005). AM also induced apoptosis and inhibited cell proliferation of peripheral blood mononuclear cells (PBMC) and abolished the synthesis and the secretion of pro-inflammatory cytokines by PBMC (Garfias et al. 2011). It was demonstrated that AM stromal matrix induced apoptosis not through the generation of NO, but by downregulating anti-apoptotic NF-κB and Akt-FKHR signalling pathways (Li et al. 2006).

In animal models, herpetic stromal keratitis (HSK) in murine corneas improved after AM transplantation through reduced local T-helper cell immune responses by inducing apoptosis in T lymphocytes (Bauer et al. 2009). In HSV-1-induced ulcerative keratitis model increased apoptosis of PMN-like cells and increased removal of apoptotic PMNs by macrophages was observed (Bauer et al. 2007). Cells of monocyte/macrophage lineage, T and B lymphocytes, that were entrapped in AM, used for ocular surface disorders, underwent apoptosis (Shimmura et al. 2001). However, there is some conflicting data on the effect of AM on apoptosis, with AM-covered rabbit corneas displaying less inflammation and keratocyte apoptosis (Wang et al. 2001).

Inhibition of vascularization

It is essential that cornea and vitreous are avascular—so anti-angiogenic molecules are essential in ocular tissues. Being avascular tissue itself, it is not surprising that several anti-angiogenic proteins were identified in the AM as well (Hao et al. 2000).

Pigment epithelium-derived factor (PEDF), an anti-angiogenic molecule, was detected in the eye and could also be released from cells of human AM (Shao et al. 2004; Spranger et al. 2001). Other anti-angiogenic molecules, detected in the AM in the study of Hao et al. (2000), were precursor of endostatin, thrombospondin-1 (TSP-1), TIMP-1 and -2. TSP-1 was also detected in the proteomic study of cryopreserved AM (Hopkinson et al. 2006b). It was postulated that part of AM's anti-angiogenic action could be mediated by a covalent complex of HA and the heavy chain (HC) of inter-α-inhibitor (Shay et al. 2011). The complex was also found to exert a potent anti-inflammatory effect by suppressing TGF-β promoter (He et al. 2009). However, not all studies support the anti-angiogenic role of AM. For example, neither fresh or preserved AM directly inhibited angiogenic process in vitro, despite high endostatin content (Ma et al. 2004).

Clinical data demonstrates that the anti-angiogenic effect of AM was observed in corneas with removed corneal epithelium and total limbal keratectomy (Kim and Tseng 1995a). Graft vascularization was delayed after pterygium excision and AM transplantation and neovascularization of corneal ulcer in dogs was decreased after the application of supernatant from human amniotic epithelial cell culture (Küçükerdönmez et al. 2007; Wichayacoop et al. 2009). Extract of AM inhibited alkali burn-induced corneal neovascularization, the effect that was not associated with PEDF (Jiang et al. 2006).

Anti-microbial properties

Since microbial colonization and infection of placental tissues can happen during the pregnancy, it is not surprising that these tissues have anti-microbial properties. It was demonstrated that cytoplasm of amnion epithelial cells expresses histones H2A and H2B that display antimicrobial and endotoxin-neutralizing activity (Kim et al. 2002). Several antimicrobial factors like bactericidin, beta-lysine, lysozyme, transferrin, peroxidase and others were measured in amniotic fluid (reviewed in Dua et al. 2004; Talmi et al. 1991). Expression of antimicrobial molecules elafin and β-defensin was detected in AM with β3-defensin being the predominant form (Buhimschi et al. 2004; King et al. 2012). A cDNA for cystatin E was found in amnion cell and fetal skin epithelial cell cDNA library (Ni et al. 1997). In addition to functioning as a cysteine protease inhibitor, cystatin E also has antiviral properties (Sangwan and Basu 2011). It was also demonstrated that the growth of a few bacterial species was completely inhibited when they were in direct contact with AM (Talmi et al. 1991).

AM as a substrate for the cell proliferation

Several studies showed that AM can be a good substrate for cell proliferation. It was suggested that AM acts as a substrate for epithelization by acting as a transplanted basement membrane (Dua and Azuara-Blanco 1999). It was shown that AM may be an ideal biological substrate that can help maintain and support the expansion of limbal epithelial stem cells by serving as a stem cell niche (Grueterich et al. 2003). AM was suggested to serve as a chondrocyte carrier in cartilage repair (Jin et al. 2007), support for human retinal pigment epithelium (Capeáns et al. 2003) or limbal epithelial stem cells (reviewed in Grueterich et al. 2003; Tsai et al. 2000; Shortt et al. 2007). Expansion of limbal epithelial stem cells on AM was mediated by PI3 K/Akt/FKHRL1 and MAPK pathways, which are known to govern cell survival and cell mitosis, respectively (Sudha et al. 2009). Corneal epithelial cells were able to attach to different laminins, molecules that are part of basement membrane and stroma of AM (Kurpakus et al. 1999; Fukuda et al. 1999; Takashima et al. 2008; Cooper et al. 2005).

Anti-cancer properties

The latest findings of Magatti et al. (2012) demonstrated that amniotic mesenchymal cells significantly reduce the proliferation of cancer cell lines of hematopoietic and non-hematopoietic origin, in both cell–cell contact and transwell co-cultures, therefore suggesting the involvement of yet unknown inhibitory soluble factor(s) in this "cell growth restraint". They provided evidence that the anti-proliferative effect of amniotic mesenchymal cells is associated to induction of cell cycle arrest in G0/G1 phase. Gene expression analyses demonstrated that amniotic mesenchymal cells can down-regulate cancer cells' mRNA expression of genes associated with cell cycle progression, such as cyclins (cyclin D2, cyclin E1, cyclin H) and cyclin-dependent kinase (CDK4, CDK6 and CDK2), whilst they upregulate cell cycle negative regulators such as p15 and p21, consistent with a block in G0/G1 phase with no progression to S phase. These are very promising results, however, further studies are needed to investigate the applicability of AM cells for controlling cancer cell proliferation in vivo (Magatti et al. 2012).

AM as a source of stem cells

In recent years AM fragments as well as AM-derived cells are being used in plethora of preclinical studies, not only for ophthalmological, but other diseases as well: neurological, pancreatic, muscle, vascular, heart, etc. (Parolini and Caruso 2011). Cells derived from the amniotic fetal membrane of human term placenta have drawn particular attention mainly for their plasticity and immunological properties, which render them interesting for stem cell research and cell-based therapeutic applications. Recently, amnion-derived

cells have been proposed as potential contributors to cell-based treatment of ocular surface disease (Parolini et al. 2010).

The human amniotic membrane contains two cell types from different embryological origins. Amnion epithelial cells are derived from the embryonic ectoderm, while human amnion mesenchymal stromal cells are derived from the embryonic mesoderm. Immunophenotypic characterization of both cell types demonstrated the presence of the common, well-defined human mesenchymal stem cell markers CD90, CD44, CD73, CD166, CD105, CD29, CD13, CD49e, CD54, as well as the embryonic stem-cell markers TRA-1-60, TRA-1-81, SSEA-3 and -4 and STRO-1 (Bilic et al. 2008; Díaz-Prado et al. 2010; Stadler et al. 2008). In addition, amniotic epithelial cells express Oct-4 and Nanog, two transcription factors known to be required for self-renewal and pluripotency (Niwa et al. 2002; Chambers et al. 2003). Unlike human embryonic stem cells, amnion epithelial cells do not express telomerase and are nontumorigenic upon transplantation. It has been shown that these cells do not form teratomas when transplanted into the testes of SCID mice, a strain of mice with severe combined immunodeficiency, nor when using amnion epithelial cells to repair damaged ocular surfaces (Tseng et al. 1998; Uçakhan et al. 2002).

Stem cell phenotypes of amnion epithelial cells and mesenchymal stromal cells could be maintained in culture from passages P0 to P9 and both cell populations had the ability to retain their capacity for differentiation during culture passages from P0 to P4 (Díaz-Prado et al. 2010). Bilic et al. (2008) optimized protocol for amniotic stem cell culturing and demonstrated that primary yields are 6.3×10^6 amnion epithelial cells and 1.7×10^6 amnion mesenchymal stromal cells per gram of human amnion. Based on immunohistochemical and genetic analysis, amniotic epithelial cells have the potential to differentiate to all three germ layers; endoderm (liver, pancreas), mesoderm (cardiomyocyte), and ectoderm (neural cells) in vitro (Miki et al. 2005). They also have the potential to differentiate into corneal epithelial cells (Fatimah et al. 2010). The functional differentiation studies demonstrated that human amnion mesenchymal stromal cells possess a much greater mesodermal differentiation capacity than human amnion epithelial cells (Díaz-Prado et al. 2010). These considerations are important for use of these cells for cell therapy. When subsequent research will reveal methods to efficiently propagate and differentiate amnion stem cells towards cell types useful in clinical transplantation, amnion from discarded placenta may be therefore an abundant, noncontroversial source of cells to treat damaged or diseased tissues.

AM as a substrate for ex vivo expansion of ocular surface epithelial progenitor cells

The combination of the aforementioned beneficial effects of AM may explain why AM has been used as an ideal substrate to cultivate epithelial progenitor cells of the conjunctival epithelium, corneal epithelium, limbus, oral mucosa and corneal endothelium.

Amniotic epithelial cells can also function as a surrogate feeder layer to support growth of human limbal stem cells (Tseng et al. 2002; Chen et al. 2007). Mitomycin C-arrested amniotic epithelial cell feeder layers are more effective in promoting clonal growth of human limbal epithelial progenitors than conventional 3T3 murine feeder layers. Cells in amniotic epithelial cell-supported clones are uniformly smaller, sustain more proliferation, and express less CK12 and connexin-43, but higher levels of stem cell-associated markers such as p63, Musashi-1, and ATP-binding cassette subfamily G2 than those of 3T3-supported clones (Chen et al. 2007). These results indicate that amniotic epithelial cells can be efficiently used as a human feeder layer for more effective ex vivo expansion of adult epithelial stem cells from the human limbus. The limbal palisades of Vogt and the interpalisade rete ridges are believed to be repositories of stem cells. The microenvironment of the limbus is considered to be important in maintaining the stemness of stem cells. Under certain conditions, however, the limbal stem cells may be partially or totally depleted, resulting in varying degrees of limbal epithelial stem cell deficiency (LSCD) with resulting abnormalities in the corneal surface. Such deficiency of limbal stem cells leads to conjunctivalization of the cornea with vascularization, appearance of goblet cells, and an irregular and unstable epithelium. This results in ocular discomfort and reduced vision. Partial stem cell deficiency can be managed by removing the abnormal epithelium and allowing the denuded cornea, especially the visual axis, to resurface with cells derived from the remaining intact limbal epithelium. In total stem cell deficiency, autologous limbus from

the opposite normal eye or homologous limbus from living related or cadaveric donors can be transplanted on to the affected eye. With the latter option, systemic immunosuppression is required (Dua and Azuara-Blanco 2000). AM transplantation or amniotic epithelial cells used as a feeder layer for effective ex vivo expansion of adult epithelial stem cells from the human limbus are therefore a useful adjunct to the above procedures.

However, there are also shortcomings of AM as a scaffold for limbal epithelial stem cell expansion and transplantation: its dependency on the availability of suitable donors, variation amongst these, as well as costly screening for infectious risks (Menzel-Severing 2011). In addition, optical properties of AM are not optimal, which is an obvious disadvantage when trying to restore corneal transparency (Notara et al. 2010). For these reasons, research efforts have been directed towards manufacturing an alternative to AM as a carrier for limbal epithelial stem cells. Ideally, this should be transparent, strong enough to allow and withstand manipulation, cytocompatible, easy and cheap to obtain, and it should not induce cell differentiation (Levis and Daniels 2009). A number of different biological or synthetic polymers have been proposed to grow and deliver limbal epithelial stem cells-sheets (overview in Notara et al. 2010). Particularly collagen shields show many favorable properties for the purpose of serving as a replacement to AM: They are cheap, biocompatible, and can be manufactured to show good optical properties and to contain fibroblasts. Limbal epithelial stem cells grown on a plastic compressed collagen scaffold show a phenotype similar to that of central corneal cells (Levis and Daniels 2009). Currently, it still remains unclear which ex vivo expansion protocols are the most effective. AM as a scaffold has a lot of beneficial properties, which are not present or included in the manufactured scaffolds. AM has proved to be very useful in cases of persisting epithelial defects and/or partial LSCD in which conservative measures have failed.

Preparation and storage of AM

An optimal method of preparation and storage of AM should preserve membrane's important features described above. At the same time, the method should also ensure safety and easy handling of AM (Thomasen et al. 2009). Throughout history, several methods of AM preparation were in use such as air drying, glutaraldehyde and polytetrafluoroethylene treatment, or lyophilization (Dua et al. 2004; Riau et al. 2010; Madhavan et al. 2002). Today, two most used methods of processing and preserving of AM are (a) cryopreservation of cleaned fresh AM at −80 °C in either phosphate buffered saline—dimethyl sulfoxide (PBS-DMSO) or minimum essential medium (MEM)-glycerol or (b) freeze-drying of AM and its rehydration before use (Rahman et al. 2009; Riau et al. 2010). AM can also be stored at −196 °C (Alió et al. 2005).

In most of the Western world a strict legislature requires testing for different viral markers of a donor and in some cases a 6-month quarantine to make sure a donor is HIV-negative. Therefore, fresh AM is rarely used (Rahman et al. 2009). However, fresh unpreserved AM is being used, especially in developing countries, where use of expensive and bulky −80 °C freezers required for cryopreservation, is a barrier to the use of AM (Mejía et al. 2000; Uçakhan et al. 2002; Altinok et al. 2010; Nakamura et al. 2004). It was demonstrated that there was no difference in clinical results when fresh (stored at +4 °C for 14 days) versus cryopreserved (in 50 % glycerol at −80 °C for 6 months) AM was used (Adds et al. 2001b), although different processing, storage and sterilisation methods do affect AM properties (von Versen-Höynck et al. 2004; Hennerbichler et al. 2007; Ricci et al. 2013).

Cryopreservation of AM in 50 % glycerol impaired viability and proliferative capacity of AM cells but not AM morphology (Kruse et al. 2000; Adds et al. 2001b; Wolbank et al. 2009) while in other studies, glycerol/MEM stored AM had well preserved epithelium and basement membrane (de Oliveira et al. 2007; Thomasen et al. 2011; Thomasen et al. 2009). Conflicting reports also exist for AM stored in DMSO: in one study it was observed that AM epithelium detached from the basement membrane (de Oliveira et al. 2007), while in another study the epithelium was well preserved, basement membrane intact and stroma homogenous (Rama et al. 2001).

Since data indicates that even after cryopreservation in glycerol AM retains its therapeutic efficacy, it was suggested that AM functions as a matrix and not as a source of functional cells (Kruse et al. 2000). Therapeutic effect can also be contributed to growth factors, present in preserved human AM (Koizumi et al. 2000). Even after 6 months of cryopreservation at −80 °C AM still contained many growth factors and cytokines (Gicquel et al. 2009; Hopkinson et al. 2006a; de Oliveira et al. 2007).

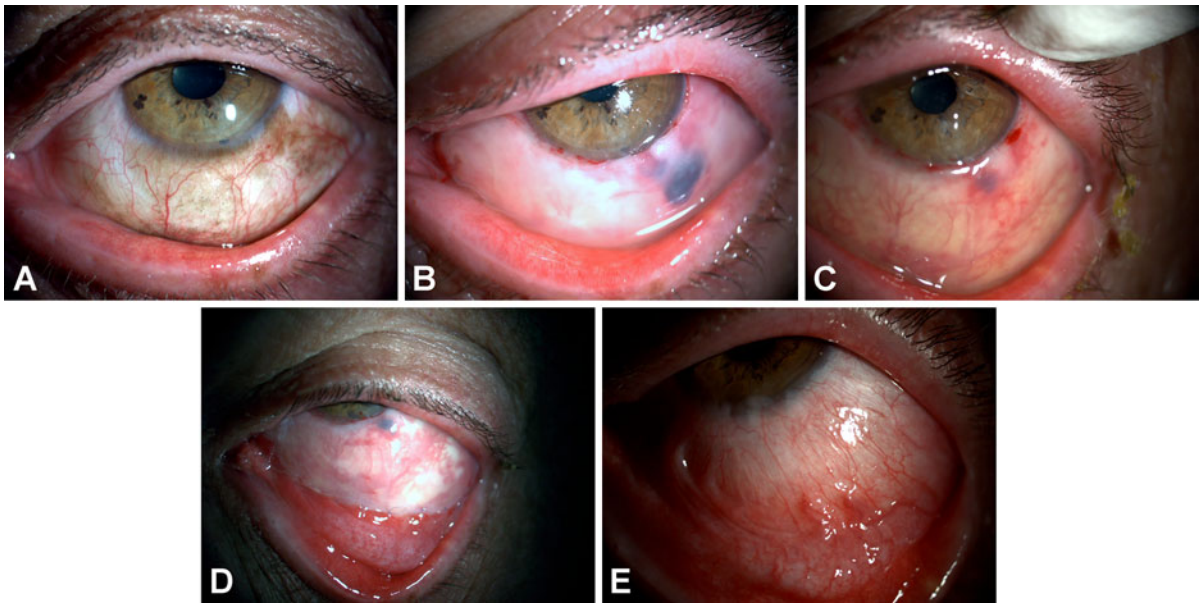

Fig. 2 58-yo male with primary acquired melanosis of the conjunctiva with severe atipia (melanoma malignum in situ) of the left eye. **a** Before treatment: unevenly distributed pigmentation of the conjunctiva covering the entire lower half of the bulbar conjunctiva. **b** First day after large conjunctival excision and reconstruction with AM graft. **c** First week postoperatively. **d** 2 Weeks postoperatively with slight melting of AM. **e** 3 months after complete epithelization of conjunctiva and incorporation of the AM, without scarring or symblepharon formation

Freeze-dried AM membrane was shown to retain most of the physical, biological and morphological characteristics of cryopreserved AM and was successfully used for cultivation of corneal epithelial cells (Nakamura et al. 2004). Other groups showed decrease in release/content of growth factors, changed properties of basement membrane and inability of freeze-dried AM to support growth of limbal cultures (Rodríguez-Ares et al. 2009; Thomasen et al. 2009). No difference in clinical results were shown when freeze-dried versus cryopreserved (in 50 % glycerol) AM was used for reconstruction of ocular surface in rabbit model (Libera et al. 2008). The main disadvantage of using freeze-dried AM is the lack of published data describing its effect and clinical efficiency, which is not the case with cryopreserved AM (Thomasen et al. 2009).

AM in ophthalmic surgery: surgical principles of current practice in Slovenia

The first historical reports on the use of AM in ophthalmology date back to the 1940s, when de Rotth (1940) used AM for the reconstruction of the ocular surface and after him Sorsby, who adapted the use of AM in the treatment of chemical injuries of the eye (Sorsby and Symons 1946; Sorsby et al. 1947). Both authors used fresh AM. In the West, these reports were not followed by widespread clinical use of AM in ophthalmology and its use was forgotten for many decades. On the other hand, in the former Soviet Union, the use of AM continued, but its export was banned. This lyophilized AM was traded under the name of 'Alloplant' and most notably promoted by Muldashev et al. (1994). The renaissance of use of AM in ophthalmology in the West was started by Battle and Perdomo in 1992 (Dua et al. 2004), followed by studies from Kim and Tseng (1995b).

Soon after, in 2000, but more than 50 years after the first reports by de Rotth (1940), the regular use of AM began at the Eye Hospital of the University Medical Centre in Ljubljana (UMCL), Slovenia. This is the result of tight collaboration between Blood Transfusion Centre of Slovenia and Women's Hospital and Eye Hospital of the UMCL. The method of AM preparation, first described in Lee and Tseng's paper (1997) is used. Local and European regulations and restrictions are followed and in 2010 our institutions

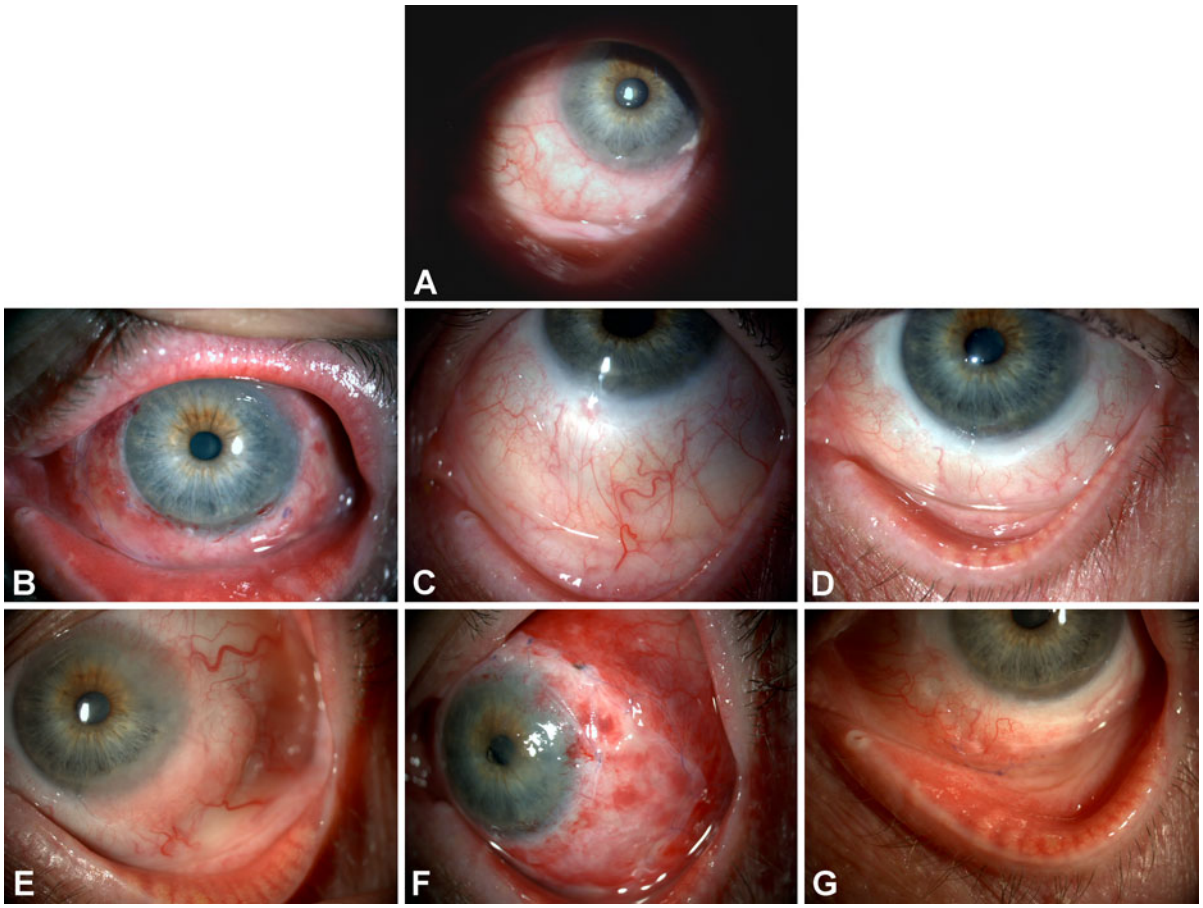

Fig. 3 64-yo male with ocular surface squamous neoplasia (OSSN) of the left eye, extending 9 clock hours from III to XII. **a** Patient at presentation with greyish white growth of conjunctival tissue extending over the limbus and covering the bulbar surface from V to XI o'clock. **b** 1 day after excision and reconstruction of the conjunctival surface with a large graft of AM extending from III–XII o'clock. **c** 6 months postoperatively with complete integration of AM and no scarring. **d** 1 year postoperatively. The ocular surface is smooth and without signs of irritation. **e** recurrence of OSSN temporally 3.5 years after treatment. The tumor covers the limbus from II to VI o'clock and is growing over the limbus up to 2 mm. **f** Re-excision of temporal conjunctiva and grafting of AM, first day postoperatively. **g** 3 Weeks after the second procedure with good reepithelization of the bulbar surface

gained accreditation by the Public Agency of the Republic of Slovenia for Medicinal Products and Medical Devices.

After identification of a possible donor, her consent is obtained and blood drawn to test for the presence of several viral markers as demanded by local regulations: lues, hepatitis B surface antigen (HBsAg), antibodies to the hepatitis B core antigen (anti-HBc) and hepatitis B surface antigen (anti-HBs), hepatitis C antibody (anti-HCV), combined testing for human immunodeficiency virus antibody and p24 antigen (anti-HIV 1/2/0, HIVp24Ag), hepatitis B virus DNA (HBV DNA), hepatitis C virus RNA (HCV RNA) and HIV1 RNA. Due to the increased possibility of bacterial contamination of AM after vaginal delivery, placentas are collected after elective Cesarean section (Madhavan et al. 2002; Adds et al. 2001a). AM is removed from the placenta, repeatedly rinsed with 2 % gentamicin in saline solution, layered onto nitrocellulose paper with the epithelium side up and soaked in the media containing MEM with gentamicin. AM is then transferred to the laboratory, cut into smaller pieces (4 × 4 cm) and stored in the freezing medium (50 % glycerol—50 % MEM—25 μg/ml gentamicin). The samples are taken (in duplicates) to test for the sterility of the medium and AM pieces itself as required by

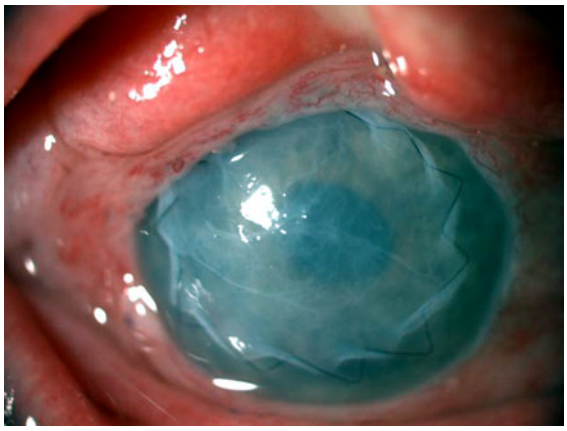

Fig. 4 AM as a graft in bullous keratopathy

European Pharmacopoeia for ophthalmologic preparations. AM is quarantined at −80 °C until the results of the tests are available. If the donor blood sample is negative for viral markers and the sterility of the end product is confirmed, AM is released for use. The 6-month quarantine to cover the window period and additional HIV testing is not required due to use of nucleic acid amplification test (NAT) that narrows the window period significantly (Weusten et al. 2011; Busch et al. 2005). If any test gives positive results, the duplicate samples are tested and if the results are positive again, all the AM pieces, prepared from the same starting material, are discarded. When needed, a required number of AM samples are thawed just before their use. AM is stored at −80 °C for no longer than 2 years. All the reagents used for the preparation and storage of AM have a European Conformity (CE) certificate. AM processing is performed in a laboratory that meets good practice requirements.

Since 2000, 19 of harvestings of AM were performed, which resulted in 332 grafts for treatment of ophthalmic patients. 250 patients have been treated by grafting of AM at the Eye Hospital, University Medical Centre Ljubljana, Slovenia. The most common indica-

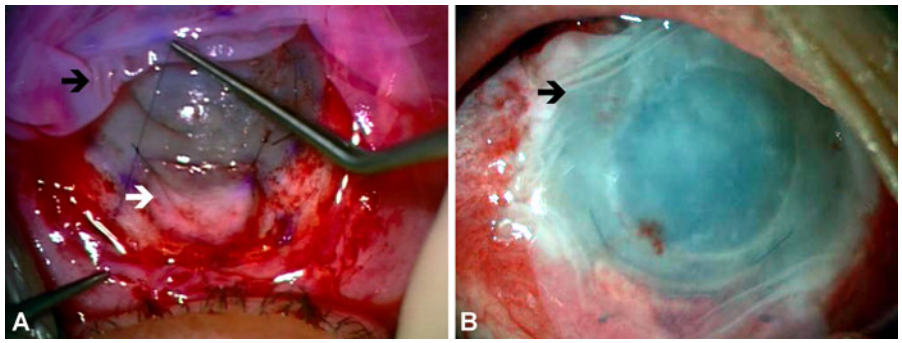

Fig. 5 **a**, **b** AM as a patch (*black arrows*) in total limbal stem cell deficiency, combined with autologous limbal stem cell transplantation (*white arrow*)

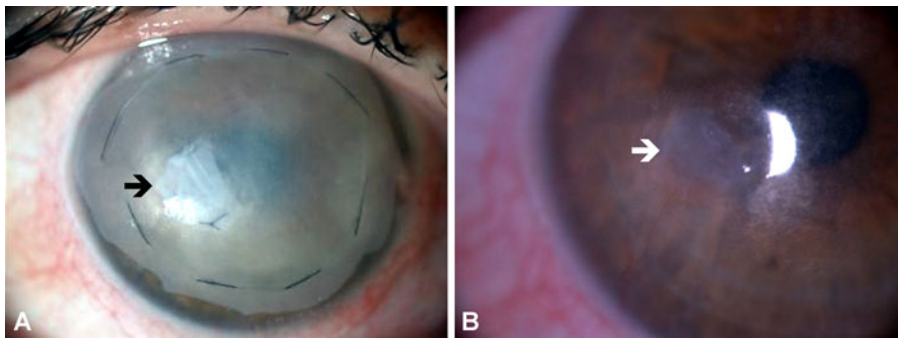

Fig. 6 **a** Combined technique: AM graft (*black arrow*) covered by AM patch in neurotrophic ulcer. **b** The same patient several months later with integrated AM (*white arrow*)

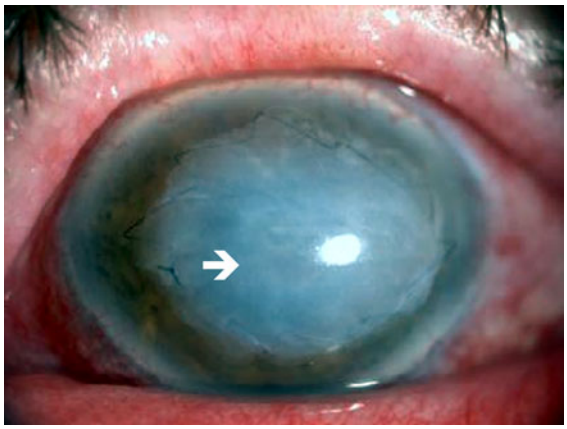

Fig. 7 Fill-in technique in perforated corneal ulcer. Note multilayered AM in the centre of the cornea (*white arrow*) filling the perforation, covered by AM patch

tions were: corneal disease (persistent corneal epithelial defects, non-healing corneal ulcers and corneal perforations), chemical and thermal burns and reconstruction of the conjunctiva after excision of malignant tissue from the ocular surface (own data, unpublished), and less common: bullous keratopathy, Stevens-Johnson syndrome, ocular cicatricial pemphygoid, revision of filter blebs after glaucoma surgery, limbal stem cell deficiency.

The AM was used in either of three manners: (1) graft, (2) patch, (3) combined or (4) fill-in technique. In the first case, AM is transplanted with the epithelial side up. We attached the AM to the cornea using 10–0 Nylon, and to the conjunctiva with 7–0 and 8–0 Vicryl. The fixated AM served as a scaffold for epithelial cells, which migrate to and then proliferate on the AM. The AM remained incorporated into the tissue, or in the case of the cornea, AM partially reabsorbed in a certain period of time. Examples of AM used as grafts are shown in Figs. 2, 3 and 4. In the second case, AM was used as a patch to protect the damaged ocular surface, to promote epithelization, to reduce scarring and inflammatory response of the tissue underneath (Fig. 5). Both methods can be combined (Fig. 6). Occasionally, AM was used in the 'fill-in' technique. Several small cuts of AM were used to fill a deep stromal defect due to corneal ulcer or small corneal perforation (Fig. 7). These techniques are small variations to previously described techniques from different authors (Dua et al. 2004; Rahman et al. 2009). An overview of the indications and used techniques is given in Table 1.

Table 1 Indications for the AM use in ophthalmic surgery

Tissue	Indication	Technique
Conjunctiva	Stevens-Johnson syndrome	Graft, patch
	Ocular cicatritial pemphygoid	Graft
	Chemical/thermal burns	Graft, patch
	Reconstruction of the conjunctiva	Graft
Cornea	Limbal stem cell deficiency	Graft, patch
	Bullous keratopathy	Graft
	Persistent corneal epithelial defects	Graft, patch, combined
	Corneal ulcer, corneal perforation	Graft, patch, combined, fill-in technique

Conclusions

Relatively inherent biological qualities of AM prompted its widespread and successful use for a plethora of ophthalmic indications. However, one of the main disadvantages of using AM is that the process is still not standardized. Several differences in AM harvesting, processing, patient selection, surgical indications and surgical techniques make prospective randomised trials almost impossible and with it the comparison of these different protocols. The biochemical composition and histological appearance of AM change through the pregnancy. Thickness and transparency of AM differ at different sites of the membrane, as well as protein content (Hopkinson et al. 2006a). The thickness could affect the integration of AM with ocular surfaces, while transparency affects vision. There might also be racial variations within the donor age and gestational age influence the levels of different growth factors in AM (López-Valladares et al. 2010). Usually none of these factors are considered when the outcomes of the AM use are evaluated. Also, there are differences between use of fresh or frozen AM and—in the case of the preservation—the type and length of it. Further investigation of the molecular mechanism whereby AM exerts its therapeutic actions should therefore help us unravel the most effective protocol for use in ocular surface reconstructions.

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