Amniotic Membrane Transplantation

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The past decade has witnessed the revival of amniotic membrane transplantation (AMT) in ophthalmology. The importance of amniotic membrane lies in its ability to reduce inflammation and scarring, enhance epithelialization and wound healing, and in its antimicrobial properties. Amniotic membrane has recently been used as a substrate for culturing limbal stem cells for transplantation. It has also been used extensively in corneal conditions such as neurotrophic ulcers, persistent epithelial defects, shield ulcers, microbial keratitis, band keratopathy, bullous keratopathy, and following photorefractive keratectomy and chemical injuries. Other indications for AMT include ocular surface reconstruction surgery for conjunctival pathologies such as squamous neoplasia, pterygium, and symblepharon. In this review we describe the basic structure and properties of amniotic membrane, its preparation process and its applications in ophthalmology.

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INTRODUCTION

In 1910, Davis was the first to report the use of fetal membranes for skin transplantation. In 1913 Stern and Sabella separately began using amniotic membrane for management of skin burns and superficial wounds. Thereafter, amniotic membrane was used as a surgical dressing for burns and as an adjunctive tissue in surgical reconstruction of the oral cavity, bladder and vagina and also for tympanoplasty, arthroplasty, repair of omphaloceles, prevention of adhesions in pelvic and abdominal surgery.[1] In 1940, De Roth first reported use of fetal membranes in the ocular surface. This investigator used fresh fetal membranes including amnion and chorion as a biological dressing for management of conjunctival defects.[2] Sorsby in 1946 and Simmons in 1947 used chemically processed and dried amniotic membrane for ocular chemical burns.[3]

Despite several reports on the beneficial role of amniotic membrane in treating a variety of ocular disorders, its use was abandoned for five decades up to 1995 when Kim and Tseng[4] reintroduced it to ophthalmology by applying non-cryopreserved amniotic membrane as a xenograft in experimental eye disease in rabbits. Their early attempts were focused on cellular biological ways to preserve amniotic membrane and at the same time maintain its biologic properties. Their first experimental models involved preservation in 100% glycerol at 4°C for one week. Thereafter, the cryopreservation method was developed in which amniotic membrane was kept in 50% glycerol at -80°C.

The poor clinical outcomes in the early 20th century might have been due to inefficient tissue processing leading to loss of the biologic properties of amniotic membrane; the present

success is in part due to cryopreservation of amniotic membrane resulting in maintenance of its physiologic properties and loss of epithelial cells which results in lack of immunogenicity. Several studies have demonstrated the benefits of amniotic membrane transplantation (AMT) in the management of ocular surface disorders. The preservation method by Kim and Tseng[5] is still the best way for maximal maintenance of its biologic properties.

In this article we review the characteristics and preparation methods of amniotic membrane as an ideal material for treatment of a variety of ocular surface conditions and also discuss indications, surgical techniques and outcomes of AMT in ophthalmology.

EMBRYOLOGY

Following implantation of the blastocyst in the endometrium, its wall becomes the chorion, the outer layer of membranes surrounding the fetus. The chorion has many villi on its outer surface which are more pronounced at the site of the placenta and attenuated outside this area resulting in formation of chorion frondosum (villous chorion) and chorion laeve (nonvillous chorion), respectively. In the same early stages, cleavage occurs within the cellular mass of the embryo and the trophoblasts. The innermost trophoblastic cells, the so called amniogenic cells, form the amniotic membrane (amnion) which is visible by day 10 post-conception (Fig. 1).[6]

By the end of the third month of gestation, the exocoelomic or chorionic cavity separates the chorion laeve from the amnion. The amniotic sac fills with fluid, expands gradually and adheres to the inner surface of the chorion; the chorionic cavity disappears but the two layers do not fuse histologically and remain separable (Fig. 2). The amnion is a thick and hard but flexible sheet of tissue at full term.[6]

HISTOLOGY AND PHYSIOLOGY

In 1962 Bourne described several layers in the amniotic membrane. The innermost layer, adjacent to the amniotic fluid, is a single homogenous layer of cuboidal epithelial cells which is presumably derived from embryonic ectoderm. This epithelium is firmly fixed to a basement membrane which is in turn attached to a condensed acellular layer composed of collagen types I, II, and V.[6]

Amniotic epithelial cells have many microvilli at the apical surface and some processes at the basal aspect which extend toward the basement membrane. These processes have a hemidesmosome type of attachment with multiple filaments. These cells have a large irregular nucleus with a large homogenous nucleolus as well as many intracytoplasmic organelles and pinocytic vesicles.[6] The ultrastructure of amniotic epithelial cells suggests that it has an active secretory function as well as intra- and transcellular transport functions.[7] The distribution of collagen type IV subchains in the basement membrane of the amnion resembles that of the conjunctiva but not the cornea.[8]

External to the amniotic membrane epithelium is a layer of mesenchymal fibroblast-like cells which are probably derived from the mesodermal embryonic plate. These cells are scattered in a full term membrane. The outermost layer of the amnion is the zona spongiosa which is an almost acellular layer adjacent to the laeve chorion. Human amniotic membrane is totally free of smooth muscle cells, nerve fibers and lymphatics or blood vessels[6] (Fig. 3).

The durability of fetal membranes is mostly due to the amniotic membrane. The tractional resistance of the amniotic membrane is related mainly to the condensed layer of interstitial collagens type I and II. Collagens type V and VI are less important in this respect. Collagen type I is the main interstitial collagen in tissues with high tractional resistance such as bone and tendon, but in other tissues, collagen III is known as the main factor of integrity and firmness.[6] Elastin exists in small amounts in the amniotic membrane and its elasticity is mainly due to collagen type III. Owing to the presence of interstitial collagens, one of the important properties of amniotic membrane is its resistance to proteolytic factors.[9]

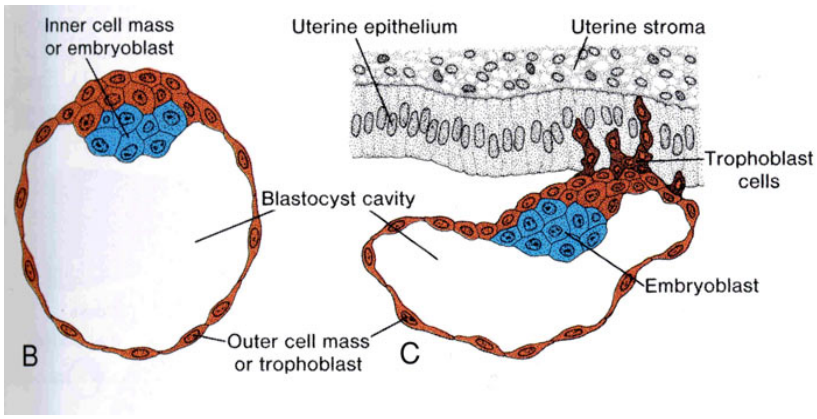

Figure 1 Embryonic membranes and placenta are derived from the outer cell mass and the fetus is derived from the inner cell mass of the blastocyst.

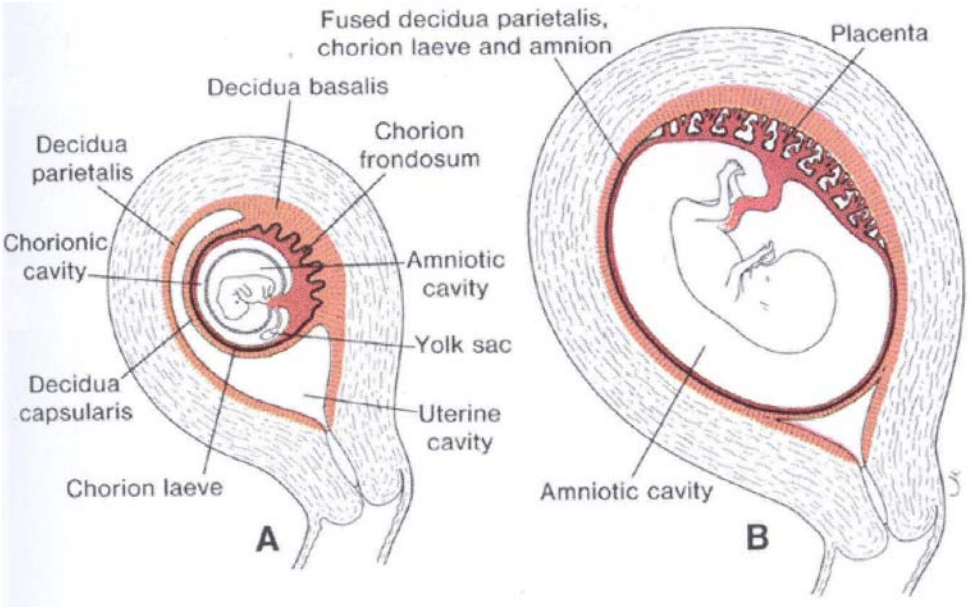

Figure 2 Amniotic membrane is the innermost membrane lining the amniotic cavity (A). Amnion and chorion join to form the fetal membrane by the end of the first trimester of pregnancy (B).

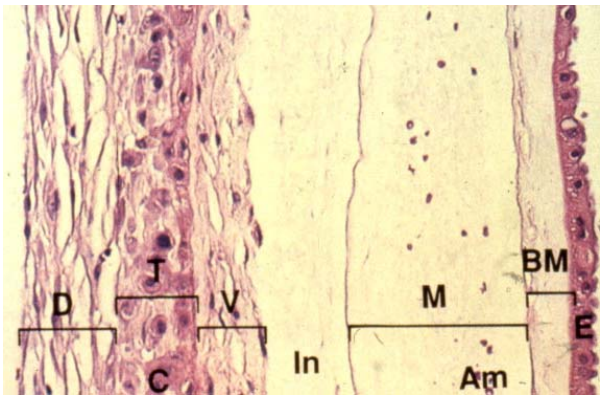

Figure 3 The amniotic membrane is composed of a cuboidal epithelial layer (E) attached to basement membrane (BM) which separates it from the underlying avascular stroma (M).

Interstitial collagens of the amniotic membrane are produced by various cells: collagens type I and III are mainly produced by mesenchymal cells, basal membrane proteins of the amnion such as collagen type IV, fibronectin and laminin are mainly produced by amniotic epithelial cells. The amniotic membrane is not just a simple avascular membrane, it has multiple metabolic functions such as transport of water and soluble materials and production of bioactive factors including vasoactive peptides, growth factors, and cytokines.[9]

PROPERTIES

Certain characteristics of amniotic membrane make it a suitable substrate for ocular surgery:

1) Promotion of Epithelialization

Amniotic membrane serves as a basement membrane which facilitates epithelial cell migration,[10] reinforces adhesion of basal epithelial cells,[11] promotes epithelial differentiation[12] and prevents epithelial apoptosis.[13] It also improves corneal sensation and tear stability by an unknown mechanism.[14] It produces various growth factors which can stimulate epithelialization.[15] It is believed that amniotic membrane can promote expansion and maintenance of progenitor epithelial cells in vivo.[16] It can also produce endothelin-1 and parathyroid hormone related protein. The epithelial cells of the membrane produce brain natriuretic peptide and corticotrophin releasing hormone which have roles in increasing cellular proliferation and calcium metabolism.[9] In the year 2000, Koizumi et al[16] demonstrated that cryopreserved amniotic membrane can express mRNA for epidermal growth factor (EGF) as well as two growth factor receptors including hepatocyte growth factor (HGF) and keratocyte growth factor (KGF) in addition to producing the related growth factors.

Amniotic membrane can accelerate epithelial healing through several mechanisms of action mentioned above. Its basement membrane serves as a safe and suitable bed for the growth of epithelial cells. Laminin isoforms, present in the basement membrane, facilitate adhesion and expansion of corneal epithelial cells. The ability of the basement membrane of the amnion to support expansion of progenitor cells can explain application of AMT for treatment of partial limbal stem cell deficiency.[17,18]

It has also been reported that amniotic membrane can act as a bandage contact lens allowing epithelialization to occur under its cover.[19] Moistened by tear, amniotic membrane can provide a wet media for ocular surface re-epithelialization. It also has good permeability providing sufficient oxygenation for epithelial cells which is in contrast to many synthetic materials.

2) Inhibition of Fibrosis

Fibroblasts are naturally responsible for scar formation during wound healing and are activated by transforming growth factor β (TGF-β). Amniotic membrane inhibits expression of TGF-β receptors in fibroblasts resulting in less fibrosis.[20] It has been shown that amniotic membrane suppresses TGF-β signaling of fibroblasts in the cornea and limbus[21] and in the conjunctiva and pterygia.[22] Chui and Tseng[20] applied dispase-treated amniotic membrane with and without corneal epithelial cells onto the corneal stroma of rabbit eyes and found that amniotic membrane inhibits keratocyte differentiation to myofibroblast and helps maintain corneal transparency.

3) Inhibition of Inflammation and Angiogenesis

AMT has been reported to be effective for treating severe neurotrophic corneal ulcers.[23] The exact mechanism of the anti-inflammatory properties of amniotic membrane is not clear. It is postulated to serve as a barrier, decreasing influx of inflammatory cells to the affected area and consequently reducing inflammatory mediators. When applied as a patch in vivo, amniotic membrane entraps T lymphocytes.[24] In an experimental study on rabbit eyes, the

stromal matrix of the amniotic membrane attracted inflammatory cells.[25] Additionally, other anti-inflammatory factors such as tissue inhibitors of metaloproteinase (TIMPs-1,2,3,4), interleukin-10 (IL-10), and IL-1 receptor antagonists as well as endostatin which inhibits endothelial cell proliferation, angiogenesis, and tumor growth have been isolated in human amniotic membrane.[26] The presence of proteinase inhibitors may facilitate wound healing.[27] Thrombospondin-1, an antiangiogenic factor, is secreted by the epithelium of amniotic membrane. IL-1α and IL-1β, two very potent pro-inflammatory mediators, are suppressed by the stromal matrix of the amniotic membrane.[28]

In 2001 Shimmura et al[24] reported that amniotic membrane reduces inflammation through entrapment of inflammatory cells. They applied cryopreserved amniotic membrane onto injured ocular surface for one week and performed immunohistochemical studies afterwards. Cells underwent staining for CD_4, CD_8, CD_{14}, and CD_{20} and TUNEL staining for apoptosis (TUNEL: Terminal deoxynucletidyl transferase-mediated dUTP Nick End Labeling). The results revealed that CD_4, CD_8, and CD_{14} positive cells, the main inflammatory cells, existed in large numbers in the amniotic membrane and that most of the entrapped cells were involved in apoptosis. It has also been shown that cultivating corneal epithelial cells on amniotic membrane matrix results in suppression of IL-1α and IL-1β.[27] Secretion of certain antiangiogenic factors, such as thrombospondin-1 and TIMP 1-4 may also explain anti-inflammatory properties of the amniotic membrane.[26] It has also been reported that corneal neovascularization is significantly suppressed by amniotic membrane.[29,30]

4) Lack of Immunogenicity

It was initially believed that amniotic epithelial cells do not express HLA-A, B, or DR antigens,[31] however subsequent studies showed that both amniotic epithelial and mesenchymal cells and fibroblasts express all HLA class I molecules including class Ia (HLA-A, B, C, DR) and class Ib (HLA-G, E) antigens.[32] However, HLA class II antigens are not expressed by amnion epithelial cells.[31,33]

INFγ and other immunologic factors have been recognized in the amniotic membrane. It seems that in the presence of viable epithelial cells, amniotic membrane may induce immunologic reactions. One study revealed that transplantation of fresh amniotic membrane is associated with a mild inflammatory reaction probably due to expression of HLA-I antigens by viable epithelial cells.[34] However cryopreserved amniotic membrane does not lead to immunologic rejection. The main reason is loss of epithelial cells due to cryopreservation.[31,33] FDA-approved methods of amniotic membrane processing include Delbeco modified eagle medium (DMEM) or cryopreservation in glycerol 50%, both of which result in the death of all amniotic epithelial cells leading to non-immunogenicity.[35] Human amniotic membrane has the ability to suppress T lymphocytes in allografted limbus cells, this implies immunosuppressive properties which can increase the success rate of grafting.[36]

5) Antimicrobial and Antiviral Properties

Amniotic membrane may decrease the risk of infection due to antimicrobial properties.[37] Amniotic membrane has cystatin E, the analogue of cysteine proteinase inhibitor, which exhibits antiviral properties.[38] Kjaergaard et al[39] have also shown in vitro antimicrobial effects of the amnion and chorion against certain microorganisms. Further studies are needed to verify the antimicrobial and antiviral characteristics of the amniotic membrane.

Amniotic membrane may function as a barrier against bacterial infiltration by adhesion to the wound surface, reducing bacterial load. In clean surgical wounds, the hemostatic property of collagen fibers in amniotic basement membrane prevents hematoma formation reducing microbial accumulation and thereby the risk of infection. Adhesion to the wound surface prevents dead space formation and serous discharge accumulation, which is another mecha-

nism of action against infection. Furthermore, formation of fibrin filaments during wound healing results in adhesion of the wound bed to amniotic membrane collagens leading to bacterial entrapment and stimulation of phagocyte migration. There is a report that amniotic membrane may decrease bacterial proliferation even in contaminated wounds.[40]

6) High Hydraulic Conductivity

This property of the amniotic membrane makes it attractive for revision of leaking blebs after glaucoma filtering surgeries.[41]

7) Other Properties

Amniotic membrane acts as a biologic dressing resulting in significant pain relief in burns owing to adhesion to the wound surface and coverage of dermal nerve endings. It also prevents wound surface drying, which accelerates wound healing.[42]

AMNIOTIC MEMBRANE PREPARATION

Amniotic membrane may be contaminated by normal vaginal flora during normal vaginal delivery, therefore amniotic membrane is obtained under sterile conditions through elective cesarean section after a full-term pregnancy. The donor is initially evaluated for the possibility of blood borne infectious by taking a careful history of high risk sexual behavior, intravenous drug abuse, blood transfusion or malignant disease and by performing physical examination specifically for tattoos and needle marks. Following identification of a potential donor, she is informed about the method of obtaining the tissue and its usage. After obtaining written consent, serum samples of the potential donor are tested for anti-HIV-1 and 2 antibodies, hepatitis B surface antigen, hepatitis B core antigen, anti-hepatitis C virus antibody, and rapid plasma reagin test for syphilis. The serologic tests are repeated 6 months later in seronegative subjects to recognize infections in the window period of the previous tests. Until confirmation of the seronegative state of the donor, the amniotic membrane is preserved at -80ºC.[43] This screening, does not, however, exclude the possibility of infections with pathogens for which no test is available such as Creutzfeldt-Jakob disease. Contamination of the tissue may also occur during preparation and processing procedures, therefore absolute aseptic techniques should be applied at all stages.[43]

Donor material including placenta and fetal membranes are placed in a sterile organ bag composed of bilayered polyethylene containing RPMI-1640 solution within it in a sterile container and transferred to the preparation unit at 4ºC temperature. Based on FDA-approved standards, good tissue practice principles, and standards of the American Association of Tissue Banks, tissue processing should be performed in clean room condition with neatness class of 100 according to the 209E classification. Tissue processing may also be performed under a lamellar flow hood in a clean room with neatness class of less than 100.[18] There are several methods of tissue preparation and preservation which are described hereunder.

1) Heat-dried Amniotic Membrane

In this method, after preparing the tissue under a lamellar flow hood in a clean room with a neatness class of 100, the tissue in dried overnight in an oven at 40±2°C. It is then sterilized using 25 KGY gamma irradiation. In this method, the membrane loses many of its biologic properties due to the high temperature employed and serves as a biologic dressing which is often used for management of burns.[40]

2) Air-dried Amniotic Membrane

After separating and washing, the amniotic membrane is flattened under a lamellar flow hood and exposed to the air overnight to get dried. Packing and sterilization using gamma irradiation is then performed. Although high temperature is not applied in this method,

some properties of the amnion are lost or altered remarkably due to dehydration. This type of prepared amniotic membrane is also often used for wound dressing.[44]

3) Lyophilized (Freeze-dried) Amniotic Membrane

In this method, placental membrane is cut into pieces and rapidly frozen at -50 to -80ºC. Thereafter it is dried under high vacuum using a freeze drier device. Tissue water is extracted through sublimation reaching a final water content of 5-10%. At the end, packing and sterilization using gamma irradiation is performed. This type of preparation induces minimal changes in amniotic membrane properties and the product can be stored at room temperature. The technique is complex and more expensive than the previous two methods. This preparation is mainly used for management of wounds.[45]

4) Preservation in Cold Glycerol

Glycerol has been used as a cryoprotective agent for a long time. Due to its high osmotic pressure it extracts interstitial water from the amniotic membrane. In this method, 80% glycerol is used for drying the amniotic membrane which can thereafter be preserved at 4ºC for a long time, although it loses some of its biologic properties. This type of preserved amnion is used for dressing burn wounds. This low-cost and simple method may be suitable for developing countries.[46]

5) Cryopreserved Amniotic Membrane

Under a lamellar flow hood in a clean room with neatness class of 100 and under sterile conditions, the placenta is washed free of blood clots with isotonic solutions. Next, the amniotic membrane is separated from the chorion and washed again with isotonic solutions containing antibiotics. The amniotic membrane is then flattened onto a nitrocellulose paper (Silifone) with the stromal surface (the adhesive side) in contact with the paper. The membrane and the paper are cut into pieces of desired size (usually 2×2, 3×3, 5×5, or 10×10 cm) and placed in a solution containing glycerol or DMSD (digital media sustainable development) as a cryoprotective agent. Due to the toxicity of DMSD, glycerol may be preferable.[46]

The cryopreservation method of amniotic membrane introduced by Tseng et al is as follows. The placenta is first washed free of blood clots with balanced salt solution containing penicillin 50 μg/ml, streptomycin 50 μg/ml, neomycin 100 μg/ml, and amphotericin B 2.5 μg/ml. The inner amniotic membrane is separated from the chorion by blunt dissection. Thereafter, the membrane is flattened onto nitrocellulose paper with the epithelium/ basement membrane surface up. The membrane and the paper are cut into 4×4 cm pieces and placed in a sterile vial containing DMEM and glycerol at a volume ratio of 1:1 and frozen at -80ºC. The membrane is defrosted immediately before use by warming the container to room temperature for 10 minutes.[4,5,43] With this method, all amniotic epithelial cells are destroyed and consequently the tissue loses its immunogenicity. Presence of laminin 5, an adhesive glycoprotein, preserves the ability of the membrane to stimulate migration and adhesion of corneal epithelium.[47]

In an alternative method, amniotic membrane is first placed at 4ºC inside a solution containing glycerol and DMEM in order to absorb the preservative agents. The tissue can next be preserved by two methods: (1) The vial containing the amniotic membrane and the solution is frozen at -80ºC. (2) The tissue is packed within two layers of polycarbonate and aluminum foil and then frozen at -80ºC. This type of preserved amniotic membrane maintains maximum biologic properties compared to other methods and according to extensive studies is the best method of tissue processing for use in ocular surface disorders.[48]

Adds et al[49] compared the viability of amniotic epithelial cells in fresh vs frozen amniotic membrane grafts and found that the majority of epithelial cells are lost after placing fresh am-

nion at 4ºC for a while. Kruse et al[36] investigated the effect of cold on the viability of amniotic epithelial cells using trypan blue staining and cell culture. The study revealed that the majority of epithelial and stromal cells lose their viability in cryopreserved amniotic membrane, but most of the cells were alive in fresh membranes. However, Alizarin red staining showed that cellular border undergoes little change in cryopreserved amnion compared to fresh preparations. They concluded that the morphology of epithelial and stromal cells remains unchanged in cryopreserved amnion but they undergo severe functional damage and lose their ability to grow and divide.

It seems that the viability of amniotic epithelial cells has little effect on the biologic properties of the membrane for ocular disorders; these properties are correlated on the integrity of its matrix. However, damaged epithelial cells may serve as a nutrient layer for migration and growth of conjunctival and corneal epithelial cells (such as 3T3 cell line).[36]

Both types of the above-mentioned products are currently produced by the Iranian Tissue Bank (Research and Preparation Center).

6) Antibiotic Impregnated Amniotic Membrane (AIAM)

After separation, the amniotic membrane is placed in an antibiotic solution composed of 5 types of wide-spectrum antibiotics and an antifungal agent overnight and then frozen at -80°C. The resultant amniotic membrane is suitable for management of infected wounds by providing an appropriate concentration of antibiotics to the wound surface.[39]

QUALITY CONTROL

Amniotic membrane is a biologic transplant and must be collected, processed, preserved, and distributed in a standard manner in all stages. The European Tissue Bank, the American Tissue Bank and the FDA have set standards with a common principal of maintaining biologic properties of the tissue and minimizing the risk of transmission of infectious agents. For this purpose, in addition to application of strict criteria for selecting seronegative donors, several microbiological tests are performed at each step. Samples for aerobic, anaerobic and fungal cultures are obtained from the transferring solution and the solution left from irrigation of the membrane as well as from pieces of membrane before placing in antibiotic solutions and before packing.[48]

All tissues must be traceable from the donor to the recipient and vice versa. For this reason, all prepared tissues are labeled with a link to registered information about the donor, tissue processing and the recipient.

TISSUE ALLOCATION

After registering the name of the patient in the recipient waiting list, an appropriate piece of membrane is selected based on the clinical condition and lesion size and type. The frozen piece of amniotic membrane is packed in a foam containing dry ice and supplied for use. In case of immediate use, it is placed at room temperature for defrosting, however it can be stored at -20°C (household freezer) for three months or at -80°C for six months. The total storage time is 12-18 months at -80°C.

SURGICAL TECHNIQUE

Amniotic membrane can be used as a graft (inlay), patch (overlay) or in multiple layers. If stored at -80ºC it should first be warmed up to room temperature. The membrane is always sutured to the ocular surface with the epithelial side up and the mesenchymal (stromal) side in contact with the eye to facilitate adherence.[18] It is important to distinguish the two surfaces which is easiest when the membrane is fresh. During preparation the membrane is always mounted on nitrocellulose paper with the epithelial side up. It is also possible to determine the mesenchymal or stromal side of the membrane at the time of application using fine blunt forceps or a surgical sponge. A fine strand of vitreous-like substance can be drawn up from

the stromal side but not from the epithelial side with forceps. The stromal side also has a tendency to adhere to the surgical sponge.

After removing necrotic tissue and loose epithelium from the corneal defect, the amniotic membrane is spread onto the eye taking care to avoid any blood or liquid underneath. It is then sutured to the cornea with 10.0 nylon sutures or to the conjunctiva with 8.0 or 9.0 Vicryl or 10.0 nylon sutures.[18]

Inlay Graft

The amniotic membrane is spread onto the ocular surface with the epithelial side up and cut to appropriate size, keeping the final piece slightly larger than the size of the defect. The membrane acts as a basement membrane allowing migration and growth of corneal epithelium.[39]

Overlay or Patch

Amniotic membrane covers the whole corneal surface including the limbus and is sutured to the limbus or corneal midperiphery with running sutures using 10-0 Nylon.[50] The amniotic membrane acts as a bandage contact lens and provides a barrier against inflammatory cells or tear proteins. The above techniques may be combined so that one layer of amniotic membrane is first applied as an inlay graft and the second layer is added as an overlay.

Multilayer Technique

More than one layer of amniotic membrane may be used to fill a deep defect. The arrangement of layer surfaces is not important except for the uppermost layer which should be placed with the epithelial side up to allow coverage by corneal epithelial cell.[51]

Postoperative Care

Subconjunctival triamcinolone acetate (10-12 mg) may be injected adjacent to the incised edges in cases with pre-existing inflammation such as pterygium, Stevens-Johnson syndrome (SJS), and ocular cicatricial pemphigoid (OCP).[52] Postoperatively, a large bandage hydrophilic contact lens may be put in place and topical steroids and antibiotics are used accordingly. Transparency of the amniotic membrane allows visualization and monitoring of the underlying epithelial defect.

In the presence of severe inflammation, the membrane disintegrates faster and the procedure may need to be repeated.[50] Processing with glutaraldehyde may increase the membrane's resistance to breakdown.[53]

INDICATIONS FOR AMT IN OPHTHALMOLOGY

Persistent Epithelial Defects and Neurotrophic Ulcers

Treatment for a persistent epithelial defect (PED) of the cornea includes correcting the underlying condition, suppressing inflammation and conservative ocular surface management. Surgical interventions are used when medical treatment fails.

Properties of amniotic membrane such as stimulation of epithelialization and reduction of inflammation, make it an attractive alternative for PEDs,[43,50,51,54,55] deep ulcers,[43,51,54,56,57] neurotrophic ulcers,[23,43,51] and perforations[54,56,57] of the cornea with a reported success rate of 50-90%. A multilayer AMT can restore stromal thickness in eyes with non-infected deep corneal ulcers or perforations and may provide a substrate for collagen synthesis. It also provides growth factors for re-epithelialization.[54]

Limitation to AMT include continuous tissue destruction under the graft and need for sufficient limbal stem cells, presence of normal keratocytes in adjacent tissues and intact corneal sensory innervation for healing.[54] Amniotic membrane combined with fibrin glue is FDA approved for management of corneal perforation $\leq$2 mm in diameter. This combination provides better adherence to adjacent epithelium and prevents detachment of the glue.[58]

Shield Ulcers

Visual loss in vernal keratoconjunctivitis with shield ulcers may be due to secondary microbial keratitis, scarring or vascularization, which can lead to amblyopia, sterile ulcer, or perforation.[59] If medical treatment fails, surgical intervention including mechanical debridement, superficial keratectomy and phototherapeutic keratectomy (PTK) with or without contact lenses becomes necessary.[60] Incomplete removal of a grade III plaque may result in relapse whereas complete removal can result in stromal thinning. AMT has been used in 7 eyes of four patients with grade II and III shield ulcers unresponsive to conventional treatment resulting in healing of all eyes within 7-14 days.[61] Randomized clinical trials are needed to verify the role of and compare AMT and surgical debridement in shield ulcers unresponsive to medical treatment.

Infectious Keratitis

AMT has been used in bacterial, fungal and parasitic keratitis.[62] Decision about the time of AMT for this indication is difficult. The effect of AMT on acanthamoeba and fungal keratitis is a matter of doubt since fungal hyphae and acanthamoeba cysts persist in the corneal stroma for a long time. This problem is compounded by poor penetration and the fungostatic effect of antifungal agents. Monitoring the course of the condition through the membrane may also become difficult in these cases.

Bullous Keratopathy

AMT has been used for bullous keratopathy with poor visual potential; this has resulted in relief of pain despite persistence of the underlying pathology. In cases with long-term follow up (45 months) no relapse of bulla or pain was seen.[63-65] The mechanism of this effect is not known but has also been observed in severe skin burns.[40]

AMT has also been used as an alternative to Gunderson conjunctival flap because of better cosmetic results and avoiding damage to limbal stem cells or inducing lid retraction or ptosis.[65] Randomized clinical trials are needed to compare these methods; however based on our experience, AMT does not decrease corneal edema and the condition recurs within a few weeks in most patients.

Band Keratopathy

Ocular pain in this condition is due to surface instability and epithelial irregularity. In one study, 16 eyes underwent superficial keratectomy with or without ethylenediaminetetraacetic acid (EDTA) application followed by AMT which resulted in ocular surface stabilization and relief of pain in 15 eyes (93.75%).[66] It is unclear whether this effect is due to replacement of damaged corneal basement membrane with amniotic basement membrane or acceleration in repopulation of corneal basal epithelial cells.

Chemical Burns

The acute phase of chemical injuries is associated with severe ocular surface inflammation and epithelial interruption leading to tissue melting. The goals of treatment in the acute phase include reduction of inflammation, stimulation of epithelialization and prevention of tissue necrosis. Achieving these goals may decrease scar formation and visual loss in the chronic phase. AMT can facilitate healing of the corneal and conjunctival surfaces in mild and moderate chemical injuries and prevent symblepharon formation in severe cases. In severe chemical burns, diffuse inflammation in the ocular surface, deep stromal ischemia and near total destruction of limbal stem cells occur. AMT in the acute phase may decrease inflammation, prevent further damage to the stem cells and reduce symblepharon formation.[67,68] These results have been obtained from small groups of patients, therefore definite conclusions cannot be made.

Joseph et al[69] have reported complete failure of AMT in 4 cases with acute grade IV

ocular burn with total loss of vision. They stated that the Roper-Hall classification system[70] for chemical injuries may not be adequate; recently modifications have been applied in classification of ocular injuries in terms of the extent of limbal ischemia and conjunctival involvement.[71]

Photorefractive and Phototherapeutic Keratectomy

In rabbits, AMT following photorefractive keratectomy inhibited inflammation, apoptosis and proliferation of keratocytes; it also reduced subconjunctival fibroblast hyperplasia and the resultant corneal irregularity but had no effect on epithelial healing.[72] These factors may lead to corneal haze reduction. We found only one report of AMT in human eyes after PRK and laser assisted subepithelial keratomileusis: three eyes developed severe corneal opacity and regression following photorefractive surgery and underwent AMT after epithelial debridement and PTK in order to reduce subepithelial fibrosis. Visual acuity improved from <20/100-20/40 with some reduction of corneal opacity.[73] Longer follow up and more cases are required to draw a conclusion.

Conjunctival Surface Reconstruction

Conjunctival autografts and mucous membrane grafts may be used for ocular surface reconstruction. Disadvantages include poor cosmesis, risk of infection, scar formation and limited sources.[71,74-77] In 1944 de Rotth[2] first introduced the use of fetal membranes for conjunctival reconstruction, but the outcomes were discouraging. Thereafter, several studies reported the successful use of fetal membranes in a variety of ocular surface disorders such as ocular surface reconstruction in OCP,[78,79] SJS,[78,80] and after excision of conjunctival dysplasia, tumors,[10,81,82] and removal of cicatricial tissue.[10,52,83] It has also been used in two cases of acute toxic epidermal necrolysis (TEN) for covering the whole ocular surface and eyelid skin. After 33-36 months, there was no symblepharon, the ocular surface was moist and vision was preserved.[84] AMT has also been reported for treatment of conjunctivochalasis.[85,86]

Since the distribution of the alpha subchain of collagen type IV in amniotic membrane resembles that of the conjunctiva but not the cornea,[8] it seems to be more suitable as a substrate for replacement of conjunctiva. Amniotic membrane decreases scar formation and inflammation and stimulates epithelialization which are beneficial for ocular surface reconstruction. It has been shown that conjunctival epithelial cells cultivated on amniotic membrane acquire the normal phenotype of conjunctival epithelium without goblet cells.[87,88] Impression cytology of the reconstructed conjunctiva revealed normal conjunctival epithelial cell phenotype with goblet cells.[89]

Advantages of AMT in ocular surface reconstruction include better cosmesis, visibility for monitoring tumor recurrence,[52] providing a thinner layer compared to oral mucous membrane,[90] preservation of eyelashes in cases of TEN,[84] and reversing entropion due to release of cicatricial tissue.[80] Factors that may adversely affect AMT success include dry eye,[91] previous treatment with mitomycin-C or beta irradiation,[43] and total destruction of conjunctival stem cells.[52]

Pterygium

Amniotic membrane can suppress fibroblast proliferation in normal conjunctiva as well as in pterygia[22] and has been used in pterygium surgery. The recurrence rate after AMT was 10.9% for primary and 37.5% for secondary pterygia, which is much higher than recurrence rates of conjunctival autografts.[92] These values were reduced to 3% and 9.5% respectively, after modifying the surgical technique,[93] which are comparable with the results of conjunctival autograft[94] and much better than that of the bare sclera technique.[95] AMT has also been used in combination with conjunctival or limbal autografts[11] and intraoperative mitomycin-C.[92]

AMT has been suggested as first line treatment for pterygium, especially in double-headed cases and in eyes with large conjunctival defects and also in order to retain the superior bulbar conjunctiva for future glaucoma surgery.[96]

Partial Limbal Stem Cell Deficiency (LSCD)

Cases of unilateral partial LSCD do not always need limbal stem cell transplantation and can be managed by close observation and repeated epithelial debridement or sequential sector conjunctival epitheliectomy[97] or AMT.[98,99] In cases of partial LSCD, within the range of 90-330º, AMT was effective in terms of relieving symptoms, recovery of ocular surface stability and visual improvement in the majority of cases after 12-34 months of follow up.[100]

Stem Cell Cultivation

Ocular surface reconstruction using keratoepithelioplasty[101,102] and limbal transplantation[103] are relatively successful, however there are drawbacks to these techniques. In cases with unilateral LSCD in which the autograft is harvested from the fellow eye, the donor eye is at risk of iatrogenic LSCD. In bilateral LSCD, limbal allografts require long term immunosuppression.

Pellegrini et al[104] harvested 1 mm^2 of limbal tissue from healthy eyes, cultivated the limbal cells in vitro and transferred the expanded cells to the recipient bed using a bandage hydrophilic contact lens. Koizumi et al[105] used amniotic membrane as a substrate for cultivating limbal cells for autologous transplantation in rabbits. Amniotic membrane provides a suitable bed for culturing limbal epithelial cells by facilitating adherence, proliferation and differentiation of cultivated cells and maintaining the colonogenicity of epithelial progenitor cells.[106]

Ex vivo cultivated limbal stem cells on amniotic membrane differ from cells cultivated on fibroblast nutrient layer 3T3, in that they proliferate slowly and lack keratin-3 (K_3) or connexin-43.[107] Subsequent studies revealed that denuded amniotic membrane provides a better miniature media for proliferation of human limbal epithelial cells and differentiation to corneal transient amplifying cells together with more expression of connexin-43.[108] Rabbit limbal and corneal epithelial cells also showed faster growth on denuded vs intact amniotic membrane[109] indicating that denuded amniotic membrane provides a better medium for culturing epithelial cells.

Amniotic membrane culture system supports limbal stem cells by expressing trk A[110] which is also expressed by human corneal basal epithelial cells.[111] trk A stimulates a signaling system which stimulates the growth of nerve fibers, suppresses apoptosis in limbal stem cells and prolongs their life span.[110] Amniotic membrane also produces a variety of growth factors including EGFb, FGF, keratocyte growth factor, TGF-α and TGF-β which stimulate epithelial proliferation. During the first 96 hr of culturing limbal stem cells on amniotic membrane, TGF-β and TGF-β_2 release is decreased which results in regulation of cell proliferation.[112] Nerve growth factor (NGF) is also produced by amniotic membrane and has shown a mitogenic effect on rabbit limbal and corneal epithelial cells.[113]

Amniotic membrane has also been used for culturing conjunctival epithelial cells which results in a mainly non-goblet cell phenotype possessing microvilli, intercellular connections with increased desmosomes and hemidesmosomes.[92] Amniotic membrane maintains conjunctival epithelial progenitor cells for producing both goblet and non-goblet cells. Differentiation to goblet cells needs a more specific medium and may depend on the presence of fibroblasts.[114] Further studies are needed to verify this mechanism of action.

In all recent studies, an explant of limbus was used for cultivating epithelial stem cells. Preparing the explant is easy without any risk to the donor corneal epithelium due to enzymatic treatment. The cell suspension culture technique provides more stem cells by incubation of the limbal ring with 1.2 IU dispase for an hour before transferring onto amniotic

membrane.[115] It is believed that the resultant epithelium has better mechanical strength and structural integrity due to increased number of desmosomes and decreased interstitial spaces between basal epithelial cells. Air lifting culture system keeps the cellular plate wet which is similar to in vivo conditions and results in a stratified epithelial layer with better barrier function. The resultant epithelium has more desmosomes, less intercellular junctions and the same non-permeability of apical cells compared to in vivo condition.[116]

Transplantation of rabbit limbal epithelial cells cultivated on rabbit amniotic membrane has shown that using an additional human amniotic membrane as a patch for two weeks results in increased cell life span. Eyes with severe lid abnormality had a poor outcome.[117] Since ocular surface inflammation has an adverse effect on stem cell viability,[118] AMT in combination with or following limbal transplantation may increase stem cell survival. The results of conjunctivolimbal autograft with and without human AMT in rabbit eyes revealed no difference between the two methods in terms of corneal opacity and epithelium phenotype but the outcomes of both groups were better than untreated cases.[119] If rabbit amniotic membrane had been transplanted, the cornea could have been clearer, since xenograft reaction may have confounded the results.

Penetrating Keratoplasty following Stem Cell Transplantation

One eye underwent penetrating keratoplasty (PK) 5.5 months after transplantation of human limbal epithelial cells cultivated on amniotic membrane. Histopathologic examination and immunofluorescent staining of the corneal button revealed that ex vivo expanded limbal epithelium retained the phenotype of limbal epithelial progenitor cells including presence of integrins $\alpha_3\beta_1$ and $\alpha_6\beta_4$ and lack of K_3 or connexin-43 in basal epithelium.[120] Phenotypic study of the corneal button after PK in another case who had previously received keratolimbal allograft with amniotic membrane revealed a corneal phenotype including stratified epithelium on an eosinophilic basement membrane. K_3 was seen throughout the whole thickness of the epithelium and connexin-43 was found in basal epithelium, but MUCSAC was not found. Tests for Laminin-5 and integrins $\alpha_6\beta_1$ were also positive in basal epithelial cells.[121] These findings indicate that in keratolimbal allograft with amniotic membrane, the resulting cells acquire corneal phenotype whereas after transplantation of cultivated limbal epithelial cells, they retained the phenotype of limbal progenitor cells.

Regrafting of Limbal Epithelial Cells Cultivated on Amniotic Membrane

Three eyes with previously failed limbal stem cell transplantation due to epithelial rejection underwent ocular surface reconstruction by regrafting of allolimbal stem cells cultivated on amniotic membrane. The procedure was successful for more than one year.[122]

Cultured Conjunctival and Limbal Epithelium for Bilateral Ocular Surface Reconstruction

In a case of bilateral ocular surface injury, 4 clock hours of limbal tissue was harvested from the eye with less severe damage. Conjunctival tissue was also harvested from the area adjacent to the site of limbal harvesting. The specimens were cultivated on two separate amniotic membranes with the limbal tissue at the center and conjunctival tissue at the periphery of each membrane for three weeks. The expanded epithelium was then transferred to the ocular surface. After one year, the ocular surface was stable and visual acuity was improved.[123]

Filtering Blebs

Amniotic membrane has been used as an alternative to antifibrotic agents in filtering surgery to prevent scar formation under the scleral flap.[124] In a rabbit model, fibroblast growth was

much less in amniotic membrane roofed blebs compared to conventional blebs. However with long-term follow up there was no difference between the two groups in terms of IOP reduction but bleb survival was greater in the amniotic membrane group.[125] A randomized clinical trial demonstrated that the results of AMT for repair of leaking filtering blebs were not as good as conjunctival advancement. In this study, the existing bleb was excised and replaced with amniotic membrane or conjunctival advancement. After two years, bleb survival was 46% in the amniotic membrane group vs 100% in the conjunctival advancement group.[126] Since a bleb covered with amniotic membrane resembles avascular blebs following antimetabolite application, they seem to be at risk of delayed leakage.

Recently, AMT has been successfully used in two patients for treatment of delayed bleb leakage after trabeculectomy with mitomycin-C. The technique of surgery was different from the previous study; amniotic membrane covered the existing bleb, providing coverage of the leaking area and maintaining bleb function and also reducing the risk of postoperative infection due to the antimicrobial properties of amniotic membrane.[127]

Cicatricial Entropion

Cicatricial entropion is caused by a variety of ocular or systemic disorders such as chronic blepharoconjunctivitis, trachoma, thermal or chemical burns, SJS, or OCP. AMT combined with the split lid procedure has been used in 18 patients with a total success rate of 96%. This procedure resulted in accelerated epithelialization of the bare tarsus but did not prevent keratinization of the lid margin or tarsal shrinkage.[128]

CONCLUSION

Amniotic membrane transplantation has been successfully used in a variety of ocular surface conditions. This membrane has unique properties such as promotion of normal epithelialization and suppression of inflammation and scar formation. These properties are beneficial for treatment of various conjunctival and corneal disorders such as PED, neurotrophic ulcers, shield ulcers, chemical injuries, pterygium surgery and conjunctival surface reconstruction. Since most published studies are case reports or case series, randomized controlled trials are needed to establish the role of AMT in ocular surface reconstruction.

Based on current evidence, denuded amniotic membrane provides a better substrate for culturing limbal stem cells and conjunctival epithelial cells which is probably due to production of several growth factors and the ability to maintain the colonogenicity of epithelial progenitor cells. Patients with total or partial LSCD may benefit from AMT alone or in combination with transplantation of limbal stem cells cultivated on amniotic membrane. The role of AMT in band keratopathy, bullous keratopathy, glaucoma filtration surgery, and entropion needs further evaluation.

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