ROLE OF AMNIOTIC MEMBRANE TRANSPLANTATION IN OCULAR SURFACE RECONSTRUCTION

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INTRODUCTION
The normal ocular surface is covered by corneal, limbal and conjunctival epithelial cells. These cells along with the stable tear film are responsible for good visual acuity and integrity of the globe. Any damage to these cells due to any systemic or ocular pathology can cause breakdown of ocular surface which results in ocular pathologies, which mostly are refractory to medical treatment and need surgical intervention like lamellar keratoplasty or therapeutic penetrating keratoplasty which further depends on the availability of the donor cornea, but now the replacement of this can be done with amniotic membrane (AM) in various ocular surface procedures.

HISTORICAL PERSPECTIVE
The first therapeutic use of AM was successfully achieved by Davis in 1910 for skin transplantation. In 1940, De Roth used a fresh fetal membrane as a graft for conjunctival surface reconstruction. Sorsby et al in 1946 and 1947 reported the successful use of AM as a patch graft in the treatment of acute ocular burns. Kim and Tseng successfully reintroduced it, in 1995 by using noncryopreserved human amniotic membrane (HAM) as a xenograft in rabbit eyes. The success of this procedure is attributed to the cryopreservation of the amniotic membrane in recent era, which renders the amniotic epithelial cells nonviable and thus nonimmunogenic.

STRUCTURE OF AMNION:
The AM is the inner avascular layer of the three-layered foetal membrane. It consists of five layers from within outward as described by Bourne.

1. A single layer of highly metabolically active, columnar to cuboidal epithelium
2. A thin basement membrane
3. A compact layer of reticular fibres virtually devoid of cells
4. A loose network of reticulum containing fibroblasts, called fibroblast layer
5. A spongy layer of wavy bundles of reticulum bathing in mucin which forms interface with the chorion

AM is 0.02-0.5mm with 3 layers broadly as epithelial monolayer, thick basement membrane and avascular hypocellular stroma. It contains no blood vessels and has no direct blood supply.

Properties of amniotic membrane: AM stimulates re-epithelialization as it acts like a basement membrane and facilitates the migration of epithelial cells. It produces growth factors like hepatocyte and transforming growth factor which modulates proliferation and differentiation of stromal fibroblasts. The basement membrane of the AM, cornea and conjunctiva contain collagen types IV, V and VII, fibronectin and laminin, which are very effective in facilitating corneal epithelial cell adhesion and anchorage. Its anti-cicatricial activity reduces the tendency for scar tissue proliferation. It act against fibroblast multiplication by creating obstacle to growth factors. The stromal matrix of the membrane is rich in fetal hyaluronic acid which suppresses proliferation and myofibroblastic differentiation of normal corneal and limbal fibroblasts as well as normal conjunctival and pterygium fibroblasts which reduces scars during conjunctival surface reconstruction. By antiprotease activity it prevents neovascularization. It has anti-inflammatory quality due to suppression of certain inflammatory cytokines that originate from the ocular surface epithelia, including IL-2, IL-8, interferon γ, tumor necrosis factor-β, basic fibroblast growth factor and platelet derived growth factor.
The AM attracts and sequesters inflammatory cells infiltrating the ocular surface and contains various forms of protease inhibitors. No immunological rejection is associated as it does not express HLA-A, -B or -DR antigens so immunosuppressants are not required postoperatively. The basement-membrane has high tensile strength and is one of the thickest membranes found in human tissue so it is utilized to cover larger ocular surface area, especially in cases of fornix reconstruction. The structural integrity, transparency and elasticity and resistant to cryopreservation of the amniotic basement membrane makes it currently the most widely accepted tissue replacement for ocular surface reconstruction. It maintains a normal epithelial phenotype, allows the surrounding epithelial cells to migrate over it and differentiates these epithelial cells into conjunctival and corneal epithelial cells. In tissue cultures AM supports epithelial cells grown from explant cultures and maintains their normal morphology and differentiation. The resultant cultured epithelium can be transplanted with the AM to reconstruct damaged corneas. As it takes nourishment by simple diffusion from the surrounding host tissue so it get accepted by ocular surface. It is used in corneal ulcers as it creates resistance for infection. As it contains large amount of nerve growth factor it facilitates nerve re-growth so used effectively in corneal surface reconstruction for regaining sensitivity. It acts as a biological bandage and a carrier for EX VIVO expansion of corneal epithelial cells and serve as stem cell niche.

**HARVESTING OF AMNIOTIC MEMBRANE**

It is obtained from prospective donors undergoing elective caesarean section, who are negative for diseases including human immunodeficiency virus, hepatitis A and C, cytomegalovirus, syphilis and tuberculosis. After taking placenta from the maternal source, it should be transferred to laboratory under all aseptic conditions and blood clots should be removed from it under laminar flow followed by cleaning with sterile saline solution containing antibiotics and antifungal (50 mg/ml penicillin, 50 µg/ml streptomycin, 100 mg/ml of neomycin as well as 2.5 mg/ml of amphotericin B) as proposed by Kim et al. The amnion is separated from the chorion by blunt dissection. The separated membranes are cut in different sizes placed on nitrocellulose paper strips with the epithelial side up. Dulbecco Modified Eagles Medium/glycerol (1:1) is used for cryopreservation and the tissues are frozen at -80 degrees until further use. The technique of cryopreservation and thawing renders amniotic epithelial cells nonviable and tissue nonimmunogenic. Amnion stored in 50-85% glycerol is effective for over a year and has antibacterial properties. HAM deprived of amniotic epithelial cells by incubation with EDTA when freeze dried, vacuum packed and sterilized with gamma-irradiation at 25kGy retained most of the physical, biological and morphologic characteristics of cryopreserved AM.

**Fresh/ Preserved amniotic membrane:** Both fresh and preserved AM have been found to function equally well when transplanted onto the ocular surface. Ideally, serologic tests on the maternal donor must be done both at the time of procurement and six months later to observe the "window period" of infections. This dual testing eliminates the slightest risk of disease transmission which is not possible with fresh one. Patients have to be brought to the hospital at a short notice unlike with preserved membrane, which allows more flexibility in scheduling surgery. Wastage of tissue is not there with frozen one where up to 30 grafts can be prepared from one placenta. The epithelial cells are nonviable in both and the viability may be associated with low-grade inflammatory response. The preserved membrane needs-70° temperature refrigeration, which restrains its use in many institutions.

**Placement of amniotic membrane:** Epithelial side is smooth and should be always kept facing outward, and stromal side which is rough spongy layer is kept downward i.e towards the surface to be treated. The stromal side can be identified by presence of strands just like vitreous strands which can be seen with bud, sponge or forceps. When used as biological bandage, it is sutured with epithelial side down. The stromal side traps inflammatory cells and induces apoptosis reducing inflammation. Sutures used for AM transplant are 10-0 nylon, 8 to 10-0 vicryl and are placed as interrupted, running or mattress type which are placed tangential to limbus. AM can be used in a number of indications, either as a 'substrate' to replace the damaged ocular tissue or as a 'patch' (biological dressing) or combination of both.

**OCULAR SURFACE RECONSTRUCTION**

When used for ocular surface reconstruction, there are three basic principles that govern the manner in which the membrane is applied to the eye.
1. **Graft or inlay technique.** The AM is intended to act as a substrate or scaffold for epithelial cells to grow and is therefore incorporated into the host tissue (cornea or conjunctiva). This is usually placed basement membrane side up. Epithelialization occurs on the membrane “substrate transplant”.

2. **Patch or overlay technique.** Here the AM functions essentially as a cover or a biological bandage contact lens, protecting the underlying healing epithelial surface. Epithelialization occurs beneath the membrane in this technique.

3. **Multilayered or fill in technique.** Here AM is used in multiple small pieces to fill the entire depth of a corneal ulcer or crater. Fresh AM can be folded in a 3-layered sandwich pattern to achieve complete filling of the corneal defective depth with stromal side on the corneal surface and epithelial side up, and then trimmed to the size of the measured corneal defect. Fibrin glue can also be applied under the membrane layers to obtain stability and adherence to the corneal defect. The final layer is slightly larger than the others and sutured with 10-0 nylon suture. No bare sclera or corneal epithelial defect should be there at the end of surgery. Intraoperatively subconjunctival triamcinolone acetonide may be given to reduce subconjunctival fibrosis along the edges of excised conjunctiva.

**INDICATIONS OF CORNEAL SURFACE RECONSTRUCTION**
- Persistent epithelial defects
- Non-healing stromal ulcers
- Partial limbal stem cell deficiency
- Total limbal stem cell deficiency
- Bullous keratopathy
- Band keratopathy
- Mooren's ulcer (Fig 3a, b)

**INDICATIONS OF CONJUNCTIVAL SURFACE RECONSTRUCTION**
- Pterygium surgery
- Chemical burns
- Cicatrizizing conjunctivitis
- Ocular surface squamous neoplasia (OSSN)
- Leaking blebs
- Filtering surgery
- Symblepharon release (Fig 3c)

Fornix formation
- Socket reconstruction
- Entropion correction
- Other indications are scleral melt and substrate for ex vivo expansion of limbal stem cells

**AMNIOTIC MEMBRANE AS STEM CELL NICHE:**
It has been shown that intact AM epithelium contains higher levels of growth factors compared with epithelially denuded amniotic membrane. However, Koizumi indicated that denuded membrane promotes better corneal epithelial cell colonization than intact and limbal corneal epithelial cell colonize more readily than central. Migrating limbal stem cells on denuded membrane have a smooth, uniform leading edge. The basal cells grown on bare amniotic membrane are nicely columnar, and the more superficial cells seem fairly well differentiated into wing cells and surface cells. Expanded epithelium from limbal explants on intact membrane adopts a limbal epithelial phenotype whereas that expanded on epithelially denuded amniotic membrane reveals a corneal epithelial phenotype (Grueterich et al).

**Cultivation of limbal stem cells:** Epithelial stem cell differentiation ex vivo helps to develop an equivalent of human cornea using tissue culture techniques. These cultivated limbal cells provides the most successful alternative for ocular surface reconstruction for various conditions. AM epithelium favours limbal stem cell growth and provides the limbal epithelium with an adapted microenvironment and a solid basal layer.

**Cultures of explanted tissue:** Corneal limbal explants have been used to cultivate corneal epithelial stem cells since these are easy to prepare and there is no danger of damaging the donor corneal epithelium through enzymatic treatment. After informed consent is obtained from the patients or guardians, limbal biopsy is performed on the healthy contralateral eye or a healthy area of the ipsilateral eye. The procedure includes careful dissection of a 1 × 2 mm² piece of limbal epithelium with 1 mm into clear corneal stromal tissue at the limbus 100um in depth including the palisades of Vogt. The harvested tissue should exclude the tenon’s capsule and conjunctiva as conjunctival epithelial cells interfere with corneal epithelial proliferation. The biopsy should be taken
from superior limbus to ensure the inclusion of immature cells in it. The obtained tissue is placed with Ham’s F_{12} medium containing 50μg/ml gentamicin and 1.25μg/ml amphotericin B until it is processed. Limbal tissue is exposed for 5 min to Dispase II(1.2u/ml in Mg^{2+} and Ca^{2+} free Hank’s balanced salt solution HBBS) at 37 degree C under humidified 5% CO_{2}. The explants are then cultured in DMEM (Dulbecco Modified Eagle’s Medium) which is 1:1 mixture of DMEM and Ham’s F_{12} medium containing 0.01mg/l epidermal growth factor, 0.25mg/l insulin, 0.1mg/l cholera toxin and hydrocortisone, 5% autologous serum at 37 degree C under humidified 5% CO_{2}. The medium is renewed every 2-3 days. The amniotic membrane is thawed at 37 °C for 30 min before use and its epithelium is removed by digestion with 0.1% trypsin-EDTA at 37 °C, followed by scraping. The de-epithelized membranes with basement membrane side up are then spread on a glass slide (used as culture inserts) in a Petri plate tucking the edges for a uniform surface.21,22 Limbal tissue is shredded into 4-6 fragments, and placed on the de-epithelized membranes onto the basement membrane side in the center, separately and allowed to settle down by overnight incubation at 37 °C with 5% CO_{2} and 95% air. The medium is changed on alternate days for 10–14 days with daily monitoring of cell growth till the epithelial cell covers an area 2-3cm. Every week bacteriological testing is performed to assess contamination. The mycoplasma content test and Gram’s test are performed 24 hrs before transplantation.

After1–2 weeks, the growth of monolayer is terminated by replacing the medium with 10% buffered formalin. The membrane with the cultured limbal tissue and the cultured cells is fixed in formalin and processed for routine histopathology with paraffin embedding. The sections are cut at 4–5 cm and after deparaffinization, stained with hematoxylin and eosin and periodic acid-Schiff’s stains. In contrast to the cuboidal epithelium of the normal amniotic membrane the cultured cells form an epithelium of 1–2 layered cells over the amniotic membrane. Immunostaining on the formalin-fixed, paraffin-embedded sections can be done using prediluted antibodies to cytokeratin 3 (K3) and cytokeratin 19 (K19) from DAKO (Copenhagen, Denmark) to confirm the corneal phenotype of the cultured cells. 23

SURGICAL TECHNIQUE OF AMNIOTIC MEMBRANE TRANSPLANTATION

AM is thawed at room temperature just before its use and is rinsed in balanced salt solution. It is gently separated from the nitrocellulose paper with blunt forceps as shown in Fig1(a) and can be sutured to the ocular surface with its epithelium-basement membrane side up and the stromal side in contact with the eye (preferred technique) or stromal side up, away from the eye. The stromal side of the membrane is sticky, similar to vitreous and the epithelial basement membrane side is shiny and non-sticky.

The membrane is then gently spread on to the ocular surface and trimmed to the appropriate shape and size Fig 1(b). In cases of corneal pathologies like persistent epithelial defect as in Fig 2(a) the membrane is secured in place using 10-0 nylon interrupted sutures to the cornea as shown in Fig 2(b). In corneal/limbal diseases (e.g. chemical injury) a membrane much larger than the affected area is needed. Multilayered approach is preferred in deep corneal ulcers, descemetoceles or perforation. In reconstruction of the conjunctival fornices a spacer (e.g retinal band) is used to maintain the fornices until epithelialization has occurred. A therapeutic contact lens is routinely used to protect the membrane in place and enhancement of healing. Tarsorrhaphy may also offer additional protection. The sutures and contact lens are often removed after 2 to 4
weeks. Recommended post-operative topical treatment consists of preservative free antibiotic and corticosteroid drops.

The cultured stem cell with membrane is transplanted 10–14 days after the limbal biopsy. Following strict aseptic precautions, a drop of 1:1000 epinephrine is instilled into the conjunctival cul de sac to ensure hemostasis. A plane of dissection is noted and tissue is excised. The AM with its monolayer of cultured limbal epithelial cells, is then transferred to the ocular surface and anchored in place at the limbus with interrupted 10–0 monofilament nylon sutures placed circumferentially. The knots are trimmed and buried. The peripheral membrane is anchored to the conjunctiva with 8–0 vicryl sutures. Subconjunctival gentamycin and dexamethasone injection is given at the end of surgery.

**POST-OPERATIVE COMPLICATIONS**

Post-operative infection, dislocation of membrane as a result of loose, broken sutures, hemorrhage under the membrane and early disintegration of the membrane have also been observed. Lack of its beneficial effect may also occur possibly due to problems with processing.

Tseng et al. have devised Prokera Fig. 6(a,b) which comprises AM attached to a soft contact lens-sized conformer for easy insertion. Further non-surgical innovations such as AMX and Prokera have made access to amnion easier than ever before. AMX is a topical application of amniotic membrane extracts, currently available for use in Europe.
CONCLUSIONS
The amniotic membrane transplantation on cornea and conjunctiva contributes as a basement membrane and a source of biological factors. The spectrum of clinical indications continues to expand and encompass a varying range of ocular surface pathology. The relative ease of the procedure, repeatability and freedom from intraocular intervention makes it an attractive surgical option. The low rate of intraoperative, postoperative complications and the avoidance of immunosuppression are the advantageous features of this procedure which have made it acceptable in surgical armamentarium of ocular surface surgeon.

The future of AM transplantation looks promising in the management of ocular surface disorders. With continued technological advancements in tissue processing, newer preserved forms such as the low-heat dehydrated amniotic membrane are commercially available. Sutureless applications with fibrin glue have been aimed at making the procedure easier and more comfortable for the patient.

The AM devoid of epithelial cells provides an excellent substrate for culturing limbal stem cells and conjunctival epithelial cells. It acts as “cell transporter”. Thus patients with partial or total limbal stem cells deficiency may benefit from transplantation of AM alone or limbal stem cells cultured on AM. The results of long term studies on this are still awaited. In nutshell AM due to its properties is a boon to the field of ocular surface reconstruction.

REFERENCES:


