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Technique for Processing and Preservation of Human Amniotic Membrane for Ocular Surface Reconstruction

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***Abstract*—**Human amniotic membrane (HAM) is a useful biological material for the reconstruction of damaged ocular surface. The processing and preservation of HAM is critical to prevent the patients undergoing amniotic membrane transplant (AMT) from cross infections. For HAM preparation human placenta is obtained after an elective cesarean delivery. Before collection, the donor is screened for seronegativity of HCV, Hbs Ag, HIV and Syphilis. After collection, placenta is washed in balanced salt solution (BSS) in sterile environment. Amniotic membrane is then separated from the placenta as well as chorion while keeping the preparation in BSS. Scrapping of HAM is then carried out manually until all the debris is removed and clear transparent membrane is acquired. Nitrocellulose membrane filters are then placed on the stromal side of HAM, cut around the edges with little membrane folded towards other side making it easy to separate during surgery. HAM is finally stored in solution of glycerine and Dulbecco's Modified Eagle Medium (DMEM) in 1:1 ratio containing antibiotics. The capped borosil vials containing HAM are kept at -80°C until use. This vial is thawed to room temperature and opened under sterile operation theatre conditions at the time of surgery.

***Keywords*—**HAM, AMT, ocular transplant

I. Introduction

HUMAN amniotic membrane (HAM) is the innermost semi-transparent layer of the fetal membranes that has been gaining popularity because of favorable results produced when used in ocular reconstruction. It has some unique properties including the facilitation of migration of epithelial cells, the reinforcement of basal cell adhesion and the induction of epithelial differentiation. Moreover, it has anti-inflammatory and anti-bacterial activities along with the ability to modulate stromal scarring, these characteristics has led to the use of HAM in the treatment of ocular surface pathology [1].

HAM possesses anti-adhesive and bacteriostatic properties. It also protects wound and reduces pain. Moreover, HAM epithelium produces various growth factors as Interleukin 6 (IL-6) and 8 (IL-8) which are the predominant cytokines associated with amniotic cells.

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Higher levels of various growth factors are found in HAM with epithelium than without epithelium indicating an epithelial origin for these growth factors [2].

Studies on human amniotic membranes preserved at -80°C for one month revealed the presence of EGF, TGF α, KGF, HGF, bFGF, TGF β1 and β2. The basement membrane facilitates migration of epithelial cells, reinforces adhesion of basal epithelial cells and may promote epithelial differentiation [2].

Another unusual characteristic of amniotic membrane is the lack of immunogenicity. Human amnion cells do not express HLA-A, B, C or DR antigens of β2 microglobulin on their surfaces [3]. It has been demonstrated that mRNA for both anti-angioganic and anti-inflammatory factors is present in the epithelial cell layer of amniotic membrane; it should therefore be applied epithelial cell surface down in order to deliver a high concentration of these factors to the damaged ocular surface [4]. Amniotic membrane has been used *in vivo* as substrate for epithelial growth in the management of persistent epithelial defects following infection in neourotrophic cornea and chemical injuries and for recurrent erosions syndromes and persistent epithelial defects associated with cicatricial conditions [5].

Collagen type I, III, IV, V, and VII, laminin and fibronectin have been identified in amniotic basement membrane and stroma [6]. The presence of a rich extracellular matrix and collagen provides anti-inflammatory properties to the stroma that arise from entrapment of inflammatory cells, presence of various growth factors, inhibition of proteinase activity and decreased lipid peroxidation [7].

Several studies have demonstrated that amniotic membrane has antimicrobial properties. Amniotic membrane is equal to isograft and superior to allograft at decreasing bacterial levels. Antibacterial effect of amnion has been demonstrated against a wide range of bacterial infections including the hemolytic streptococcus group A, *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* [2].

Since 1910, HAM has been used sporadically in clinical practice to encourage epithelialization in burns, as graft over skin ulcers and in intra-abdominal and reconstructive surgery [8]. HAM as a graft was first used for conjunctival reconstruction in 1940 [9]. With standardization of the technique and further understanding of pathobiology, HAM has been routinely used in the ocular surface reconstructive surgery since 1995 [10]. Amniotic membrane has been used as a surgical material because of the properties shown by clinical

and experimental data that amniotic membrane facilitates the proliferation and differentiation of epithelial cells, maintains the original epithelial phenotype, promotes goblet cell differentiation and reduces scarring, vascularization, and inflammation [11,12,13]. In addition, rapid epithelialization has also been noted after with Amniotic membrane transplant (AMT) [14]. The current study was carried out to evaluate the HAM processing technique applied at Al-Shifa trust eye hospital, Rawalpindi, Pakistan.

II. MATERIALS AND METHODS

A. Processing of Amniotic Membrane for Ocular Transplant

Amniotic membrane is relatively cheap and freely available tissue especially in developing countries as the donor consent is readily available with less legislative considerations [15]. Membranes used for ocular pathologies are obtained from cases that have under gone elective caesarian section, after consent and are seronegative for hepatitis B and C, syphilis and human immunodeficiency virus. An elective caesarian delivery helps in the right choice of a consenting donor and planned collection of HAM because placenta collected after natural vaginal delivery may have structural defects linked with stretching of the membrane during labour and delivery and may be infected by normal vaginal flora, herpes, chlamydia or other contaminant bacteria. Due the risk of infection with HIV and hepatitis C, tissue transplantation laws in different countries require different protocols for preservation, testing and storage [16]. Protection against transmission of viruses is effected by donor selection and testing for serological markers of presently known transmissible viruses at the time of donation and again 3-4 months later. This time window omits any chances of infection transfer that may be diagnosed later on.

The AM can be preserved in glycerol, by irradiation, cryopreservation or lyophilization techniques. Preservation at -80°C is done either with glycerol or dimethyl sulfoxide (DMSO). Glycerol has antiviral and antibacterial properties that are dependent on concentration, time and temperature [17].

Processing and preparation of the amniotic membrane is carried out under sterile conditions. An antibiotic cocktail to cover Gram-negative and Gram-positive bacteria and fungi is used in washing and storage solution.

For preservation, amniotic membrane is washed in balanced salt solution containing ampicillin, streptomycin and amphotericin B. It is scrapped manually to remove all the debris. Once the amnion is clear and transparent, it is spread uniformly without folds or tears on individually sterilized 0.22µm nitrocellulose membranes of the required size (47mm or 25 mm, commercially available-Millipore or Sartorius) with the epithelial/basement layer surface up. The HAM around the nitrocellulose membrane is cut and allowed to adhere to the cellulose membrane. It is then preserved at -80ºC in the solution containing Modified Eagle's Medium (MEM) and glycerol in 1: 1 ratio.

The filter membrane along with the adherent HAM is placed carefully in the preservative medium in 50ml wide mouthed screw capped irradiated transparent plastic bottles. The preservative medium used is 1:1 (vol/vol) ratio of sterile glycerol (sterilized by autoclave) and MEM with 3.3 % L-glutamine, 25 ug/ml gentamicin, 50 units/ml penicillin, 100ug/ml ciprofloxacin and 0.5mg/ ml Amphotericin B. The bottles are labeled with the appropriate size and date of preparation. The HAM is stored at –80°C to facilitate the devitalization of the epithelial cells.

A random bottle from the batch is left on the work bench at room temperature for about an hour and about 5ml of the same is inoculated into 100ml of brain heart infusion medium and 100ml of thioglycolate broth medium to check the bacterial and fungal sterility. These media are incubated for 21days and if no growth of bacterium or fungus is observed, the batch should be considered as free of cultivable microbial agents.

Upon confirmation of the HIV negative status of donor by repeat serology done 3-4 months after the collection of HAM, the membranes are released. The membrane may be used up to 6 months after preparation. The use of HAM after 6 months is recommended as the cellular viability is found to be reduced to 50% in two months [18]. In addition damage of HAM due to cryopreservation results in decrease levels of associated growth factors [19].

III. RESULTS

We, at Al-Shifa Trust Eye Hospital, Rawalpindi, have so far processed 19 batches of HAM in the time span of 20 months. One placenta provided 25-30 membrane pieces of 45mm size. If the size was reduced to 25mm then we had more than 40 pieces in a batch. The size of piece depended upon the ocular complication of patients undergoing AMT. The patients that had full corneal epithelial removal required larger piece whereas the one that was under going a patch graft on little portion of cornea needed smaller piece.

Of all, only one of the processed batches had positive growth for Gram positive bacteria. The full sterility was maintained in the preparation for 95% membranes (Fig.1).

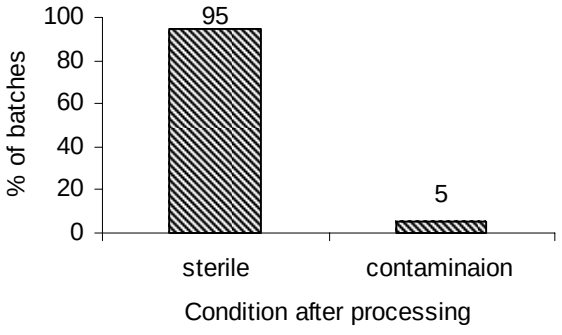

Fig. 1 Percentage of HAM showing sterility and contamination

IV. DISCUSSION

In our hands, the above mentioned protocol was found to be safe and amniotic membranes harvested following this protocol are expected to have long shelf life. However, HAM preparation in a laboratory requires expertise in preparation of media and solutions and good laboratory practice of sterilization and preservation of biological materials.

Most probably the one that showed contamination was due to some handling artifact during handling. Perhaps, the gloves or the media caused such infection to be reported. This can be predicted because the culture sterility tests for the preparation room, glycerol and DMEM were negative prior to the preparation.

Kruse *et al.* [20] showed that cryopreservation considerably damages the viability and proliferative capacity of amniotic membrane. They concluded that amniotic membrane grafts after cryopreservation function primarily as a matrix and not by acting as transplanted functional cells. Kubo and his co workers [21] have shown that after two months of freezing at least 50% of amniotic cells are viable and capable of proliferation; after 18 months of cryopreservation they were not able to demonstrate a significant amount of cell survival.

Adds *et al.* [22] examined fresh membranes obtained by elective caesarean section and normal vaginal deliveries. They found bacterial contamination in all membranes, notably; they recovered a greater number of different bacterial species from membranes obtained by vaginal deliveries.

A. Indications for Amniotic Membrane Transplant

The role of AMT in ocular disorders has been recently re-evaluated. Severe ocular injuries due to trauma or disease can compromise ocular surfaces and deplete the stem cell population that repairs the damaged corneal epithelium ultimately leading to pain, scarring, vascularization, and loss of sight. In addition, there are a number of conditions affecting the conjunctiva and the eyelids that can cause pain and corneal injury, as well as interfere with the normal appearance and functioning of the eye. The choice of ocular surface reconstruction is dependent upon the extent of involvement of the cornea (i.e., epithelium, basement membrane or stroma), extent of limbal ischemia, conjunctival necrosis etc [23].

B. Complications Associated with Amniotic Membrane Transplantation

The complications associated with the ocular AMT can be suture granuloma, persistant inflammation, hematoma, dehiscence, shrinkage of the graft, failure to achieve the intended effect with AM, infection, dislodged or loose AM, hemorrhage and early disintegration have also been reported. It is not a good substance for use as a graft due to the lack of mechanical strength and resultant wound leakage [21].

The prepared membrane should be handled properly avoiding microbial contamination during transportation to the operating room and should be done in the ice. The color of the storage medium after thawing should be light pink. A change to yellow is taken as an indication of microbial contamination; any membrane having such appearance should be discarded. When the HAM is detached from nitrocellulose membrane in the bottle and surgeon is unsure of the epithelial side of HAM, it is identified by its stickiness to the tip of the cotton swab [24].

One must not drop view of the potential danger of spread of viruses and bacteria. As membrane from a single donor can be used in a number of patients, the danger of single donor to multiple recipients cannot be over ruled. However, sufficient donor screening to cover the window period of 3-4 months, appropriate handling and storage and repeated microbiological tests can minimize the risk.

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