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DIFFERENTIAL CONCENTRATIONS OF GROWTH FACTORS IN FREEZE DRY VERSUS CRYOPRESERVED PRESERVED HUMAN AMNIOTIC MEMBRANE AND DETERMINATION OF THE REGENERATIVE POTENTIALS.

[1]Alekhya ayalapuram [1]Post graduate student
[2]Shiva charan yadav [2]Post graduate student
[3]Tejaswini Annam [3]Post graduate student

[1,2,3] Department of periodontics,[1]Army college of Dental sciences,Secunderabad, Telengana; [2] College of Dental sciences Davangeree, Karnataka; [3]PMNM Dental college, Bagalkot, Karnataka.

ABSTRACT: Placenta is normally discarded after birth as medical waste, its procurement as a cell source is easy and raises no Ethical controversy. Progress in our understanding of the biology and properties of placenta-derived cells has encouraged researchers to investigate their effects in animal models of different diseases, with the ultimate aim of developing clinical applications based on the use of these cells. The placenta is a temporary feto-maternal organ that maintains feto-maternal tolerance and also harbor stem/progenitor cells with properties which make them attractive candidates for application in regeneration. The Amniotic Membrane(AM) represents the innermost layer of the placenta and is composed of a single epithelial layer, a thick basement membrane and an avascular stroma. Recent reports indicate that human Amniotic Membrane express stem cell markers and have the ability to differentiate toward all three germ layers. These properties, the ease of isolation of the cells, and the availability of placenta as a discard tissue, make the amnion a potentially useful and noncontroversial source of cells for transplantation and regenerative medicine. However, it has been demonstrated that different processing, storage and sterilization methods do affect Human Amniotic Membrane (HAM) properties. The aim of this review is to enlighten with differential regenerative potentials of freeze dry and cryopreserved HAM.

KEYWORDS: Placenta, Amniotic Membrane, cryo preserved

INTRODUCTION

Cells derived from perinatal sources, such as the placenta, placental membranes, umbilical cord and amniotic fluid have attracted attention from researchers and clinicians as a potential source of cells for regenerative medicine[1,2.] The reason for this interest is that these cell types all possess some degree of plasticity and immunomodulatory capability[3], properties that are fundamental to their potential therapeutic applications.

Human amniotic membrane (HAM) has been used in a variety of surgical procedures. First employed in skin transplantation by (Davis 1910), HAM was subsequently found to be useful as a biological wound dressing for burns (Ramakrishnan and Jayaraman 1997; Branski et al. 2008), acute (Tekin et al. 2008) and chronic wounds (Gajiwala and Lobo 2003; Insausti et al. 2010), and in the reconstruction of the dura mater (Tomita et al. 2012; De Weerd et al. 2013),oral cavity (Lawson 1985), vaginal vault (Ashworth et al. 1986), tendons (Ozbölu¨k et al. 2010) and nerves (O'Neill et al. 2009). HAM has also long been used in ophthalmic surgery, the earliest reported application being in 1940 when De Rötth used fetal membranes to correct symblepharon.

Structure of HAM:

Extra- embryonic tissue of foetus gives rise to HAM. The amniochorionic membrane forms the outer limits of the sac that encloses the foetus, while the innermost layer of the sac is the AM. The innermost layer, nearest to the foetus, is called the amniotic epithelium and consists of a single layer of cells uniformly arranged on the basement membrane.

The basement membrane is one of the thickest membranes found in all human tissue. The support provided to the foetus by the basement membrane throughout gestation stands testimony to the structural integrity of this remarkable membrane.

The compact layer of stromal matrix adjacent to the basement membrane forms the main fibrous skeleton of the AM.The collagens of the compact layer are secreted by mesenchymal cells situated in the fibroblast layer. Interstitial collagens (types I and III) predominate and form parallel bundles that maintain the mechanical integrity of AM. Collagens type V and VI form filamentous

connections between interstitial collagens and the epithelial basement membrane.

The intermediate layer (spongy layer or *zona spongiosa*) of the stromal matrix sits adjacent to the chorionic membrane. Its abundant content of proteoglycans and glycoproteins produces a spongy appearance in histologic preparations, and it contains a nonfibrillar meshwork of mostly type III collagen.

The spongy layer is loosely connected to the chorionic membrane; hence, the AM is easily separated from the chorion by means of blunt dissection.[4,5]

Different techniques in isolation and cultivation of HAM :

Special processing and sterilization is recommended to ensure consistent quality and preservation of the properties of AM. Various methods have been tried to preserve the AM include:

- HYPOTHERMIC STORAGE AT *4°C,*
- *FREEZE DRYING THROUGH LIQUID NITROGEN AT -196°F, Γ-STERILIZATION,*
- *GLYCEROL PRESERVATION AND CRYOPRESERVATION*.

Infrared-frared rays and microwaves

The media and storage temperature used for the preservation process affects the viability of cells and growth factors in the AM.

Sterilization with γ-rays has no significant effect on growth factor content in the human AM. While storage of AM in glycerol at 4°C will result in immediate cell death, cooling will preserves the membrane for an indefinite time and make it bacteriologically pure and immunologically inert[37].

Cryopreservation with dimethylsulphoxide (DMSO) at -80°C is an important modality for preservation of these tissues as it keeps the viable for a longer period of time but causes loss of some angiogenic factors and cell rupture[37,38].

To overcome these problems with cryopreservation, freeze dried - irradiated (Lyophilized) is the one of the most commonly used preservation technique that preserves the original size and shape with minimum cell rupture [39]. The membrane is first freeze dried at -60°C under vacuum (atmospheric pressure 102 mm of Hg) for 48 hours and then irradiated with 2.5 mega Rads (25 K Gray) in a batch type cobalt-60 irradiator [40,41]. The freeze dried membrane can be processed for use by soaking in normal saline for 1 minute. It returns to a layered structure similar to that of fresh amnion when it absorbs water unlike the hyperdry AM. However upon hydration, the freeze dried amnions did not recover their thickness, and their histologic appearance was different from a fresh AM[42].

Far-infrared rays and microwaves

The *FAR-INFRARED RAYS AND MICROWAVES* are also used for sterilization of amniotic membrane which is known as the Hyper-dry-amnion. During the drying process, the temperature inside the hyperdrying device should not exceed 35°C as high temperatures on the surface that can reach 60°C can decrease the degradation of tissue-protein.

Compared to cryopreserved amnion, which can be preserved for less than 3 months at 80°C, “Hyper-dry amnion” can be preserved at room temperature indefinitely until the packet is cut open. It is easily cut to the desired size and shape just before application.

Gluteraldehyde fixation

GLUTERALDEHYDE FIXATION is recently introduced method to fix the AM that provide better stability and properties. This requires neither antibiotics nor the use of special storage techniques and renders the amnion sterile and non-immunogenic. Gluteraldehyde treated amnion (SAM) is employed successfully as a microvascular interpositional graft in many experimental animals and is the area of further research [43].

Properties of HAM

IMMUNE SYSTEM

I. Suppression of inflammation

- The exact mechanism of the anti-inflammatory properties of amniotic membrane is not clear.
- It is hypothesized that it decreases influx of inflammatory cells to the wound area and consequently reduces inflammatory mediators by serving as a barrier.
- It entraps T lymphocytes when it is applied as a patch in vivo.[7]
- Matrix metalloproteinases released by infiltrating neutrophils and macrophages are taken care of by inhibitors of matrix metalloproteinases found in the amniotic membrane. Presence of various tissue inhibitors of metalloproteinases 1, 2, 3, and 4, interleukin-10, and interleukin-1 receptor antagonists and endostatin which inhibit endothelial cell proliferation, angiogenesis, and tumor growth has also been observed in amniotic membrane [6].
- The presence of proteinase inhibitors may facilitate wound healing.
- Two very potent pro-inflammatory mediators, interleukin-1 and interleukin-1□, are suppressed by matrix of stroma of amniotic membrane.

- Shimmura et al. in 2001 reported that amniotic membrane reduces inflammation through entrapment of inflammatory cells[9].

II. Antibacterial factors and antiviral factors [5-6.]

α-Defensins:

- Microorganisms upon their entry into the body are eliminated by our immune system through an adaptive immune response, -defensins, a major group of antimicrobial peptides and an integralpart of the innate immune system, which are expressed at surfaces of mucosa by epithelial cells and leukocytes.
- Amniotic membranes also have the ability to produce α–defensins[12] with the predominant type present in amniotic epithelium being α3-defensin.[13]
- Kjaergaard et al. in 2001 have also shown in vitro antimicrobial effects of the amnion and chorion against certain microorganisms.[6]
- It has been shown that AM, through adhesion to the wound surface, can act as an antibacterial barrier and reduce bacterial infiltration.[6] Thus, it has been speculated that AM, because of its antibacterial properties, could decrease the risk of infection.

Cystatin E

- Its antiviral properties are exhibited by presence of cystatin E, the analogue of cysteine proteinase inhibitor.[9] There is still further need for studies to verify these properties of the amniotic membrane.[12]

Barrier

- Amniotic membrane may prevent infiltration and adhesion of microorganisms to wound surfaces by acting as a barrier.[9]

Hemostatic Property Of Collagen Fibers

- The collagen fibers of amniotic basement membrane prevents hematoma formation in clean surgical wounds. This reduces bacterial load and risk of infection by preventing accumulation of microbes.[13]

Adhesion

- This attachment prevents formation of dead space and accumulation of serous discharge.[9]

Fibrin filaments in healing wound

- Bacterial entrapment and stimulation of migration of phagocytes also occur by fibrin filaments formed during wound healing. These filaments cause adhesion of the wound bed to amniotic membrane collagens.[9]

There is a report that bacterial proliferation is reduced even in contaminated wounds by amniotic membrane.[14]

III. Induction Of Apoptosis Of Inflammatory Cells.

EPITHELIAL TISSUE

1. Stimulation of growth of epithelial cells **[15-19]**

- Amniotic membrane facilitates migration of epithelial cells,[15]
- Reinforces basal cell adhesion,[16]
- Promotes epithelial differentiation,[17]
- Prevents epithelial apoptosis,[18] and
- Promotes epithelialization in healing of wounds.[19]
- Its basement membrane serves as a safe and suitable bed for the growth of epithelial cells.
- Sufficient oxygenation for epithelial cells is provided by its good permeability in contrast to other synthetic materials.

Thus, amniotic membrane is an ideal tissue,which facilitates the growth of epithelial cells, helping in their migration and differentiation.[20,21]

2. Epidermal growth factor and keratinocyte growth factor.[22,23]

- Various growth factors produced by amniotic membrane can stimulate epithelialization.[36]
- AM contains growth factors that hasten formation of granulation tissue by stimulating the growth of fibroblasts.[7]
- It can also promote expansion and maintenance of epithelial progenitor cells in vivo and can produce endothelin-1 and parathyroid hormone related protein.[22]
- Brain natriuretic peptide and corticotrophin releasing hormone are also produced by membrane epithelial cells which play roles in increasing cellular proliferation and calcium metabolism.[23]
- Expression of mRNA for epidermal growth factor,hepatocyte growth factor receptor, and keratocyte growth factor receptor was demonstrated by Koizumi et al. in 2000 in cryopreserved amniotic membrane.[36]
- It can be claimed that the presence of various growth factors in AM, such as platelet-derived growth factors alpha and beta and transforming growth factor beta, is likely to induce faster sealing of the defects and limited loss of the grafting material in the test group.[36]

MESENCHYMAL TISSUE:

1. TGF-b-suppression of myofibroblastic differentiation [24,25]
2. Reduction of scarring- *Antifibrosis Property*. [24,25]
   - Fibroblasts are naturally responsible for scar formation during wound healing and are activated by transforming growth factor .

- Amniotic membrane reduces the risk of fibrosis by down-regulation of transforming growth factor and its receptor expression by fibroblasts.
- Therefore, scaffold of an amniotic membrane modulates wound healing by promoting reconstruction of tissues rather than promoting formation of scar tissue.

3. Axonal regeneration [26]
4. Neural growth factor[27]

BIOMECHANICAL PROPERTIES:

1. Avascular,[28]devoid of cells, no immune response [29-31]
2. Durable and puncture resistant[32]
3. Elastic and translucent [33]
4. Serving as a physical barrier [32]

- The significant less REC observed in the test group might be a result of the thinness of the AM, which resulted in better adaptation of the membrane over the bony defect and consequently better coverage of gingiva over the membrane. Although in this study a double layer of AM was used, no postoperative membrane exposure or uneventful healing was observed. AM is suggested for use in areas with limited thickness and height of gingiva, as full coverage of membrane is more easily accomplished.
- In the meantime, AM vascularizes healthy granulation tissue and stimulates neovascularization in the neighboring tissues.[33]
- Also, AM provides a protein-enriched bioactive matrix that facilitates cell migration.[34] Hence, it can be speculated that the use of AM as a membrane for GTR could stimulate vascularization of the granulation tissue in the defects and promote cell migration and wound healing.
- The presence of *laminin-5* in high concentrations throughout AM, with its high affinity for gingival epithelial cells, could accelerate healing and integration of the membrane with gingival tissue.[33,35] In other words, it has been claimed that AM has the ability to form an early physiologic “seal” with the host tissue. This precludes bacterial contamination.[35] This quality of good integration of AM with the overlying gingiva may account for the smaller amount of REC observed in the test group. For confirmation of this hypothesis, further studies with histologic examination at different points during the healing period are necessary.[36]

Advantages of HAM in regeneration:

1. Pluripotency of amnion-derived cells,
2. Anti-inflammatory and low immunogenic characteristics of amniotic membrane /amnion-derived cells,
3. Non-tumorigenicity,
4. Little ethical problems with usage.

In fact, it remains unclear whether stripping the epithelial layer is beneficial or not prior to preservation of AM for clinical use. The fact that most cytokines and growth factors are present at higher concentrations in the epithelium, however, would support the maintenance of this layer[36]. Furthermore, destruction of the amniotic epithelium with vacuolic degeneration and dissolution of the connective tissue layers into single fibre bundles in HAM preserved by different methods and sterilized by irradiation has been reported (**Von Versen-Höynck et al. 2004**).

Biomechanical characterization of different AM preparations, including cryopreserved and denuded dehydrated samples, showed that frozen preparations have more elasticity and require greater force to be broken (**Chuck et al. 2004**). These differences may affect surgical preferences and uses because of ease of handling and resistance.

The method employed to preserve HAM until surgery must guarantee that such tissue properties are preserved. The ideal method would be one that facilitates transport and prolonged storage without deterioration. Currently, cryopreservation is widely considered the only preservation method to guarantee the maintenance of such properties. This method requires a deep-freezing facility (to − 80 °C), which is expensive and frequently unavailable, especially in underdeveloped countries. Moreover, maintaining the cold chain makes transportation difficult.

Lyophilization is a preservation method that consists of removing water from a tissue by sublimation. This results in the inhibition of deleterious chemical reactions that lead to tissue alteration. Lyophilized tissue can then be stored at room temperature for long periods and its transportation is easy, thus resolving the two main disadvantages of cryopreservation.

The immunogenicity of cryopreserved tissue is generally thought to be less than that of fresh tissue. However, ≥50% of Amiotic Epithelial cells, cryopreserved for 2 months, remained viable and able to grow in culture and Akle et al. reported that low-grade inflammatory responses were observed when viable amniotic epithelial cells were present, suggesting that live amniotic membrane is immunogenic.[29]

High levels of EGF, KGF, HGF and bFGF in AM with amniotic epithelium as compared to AM without amniotic epithelium suggest an epithelial origin for these growth factors.[36]

six growth factors have been associated with HAM, they are bFGF, EGF, HGF, KGF, NGF and TGF-β1. Basic FGF is a potent angiogenic factor and an endothelial cell mitogen, and has been described as a multipotent cytokine regulating cell growth and differentiation, matrix

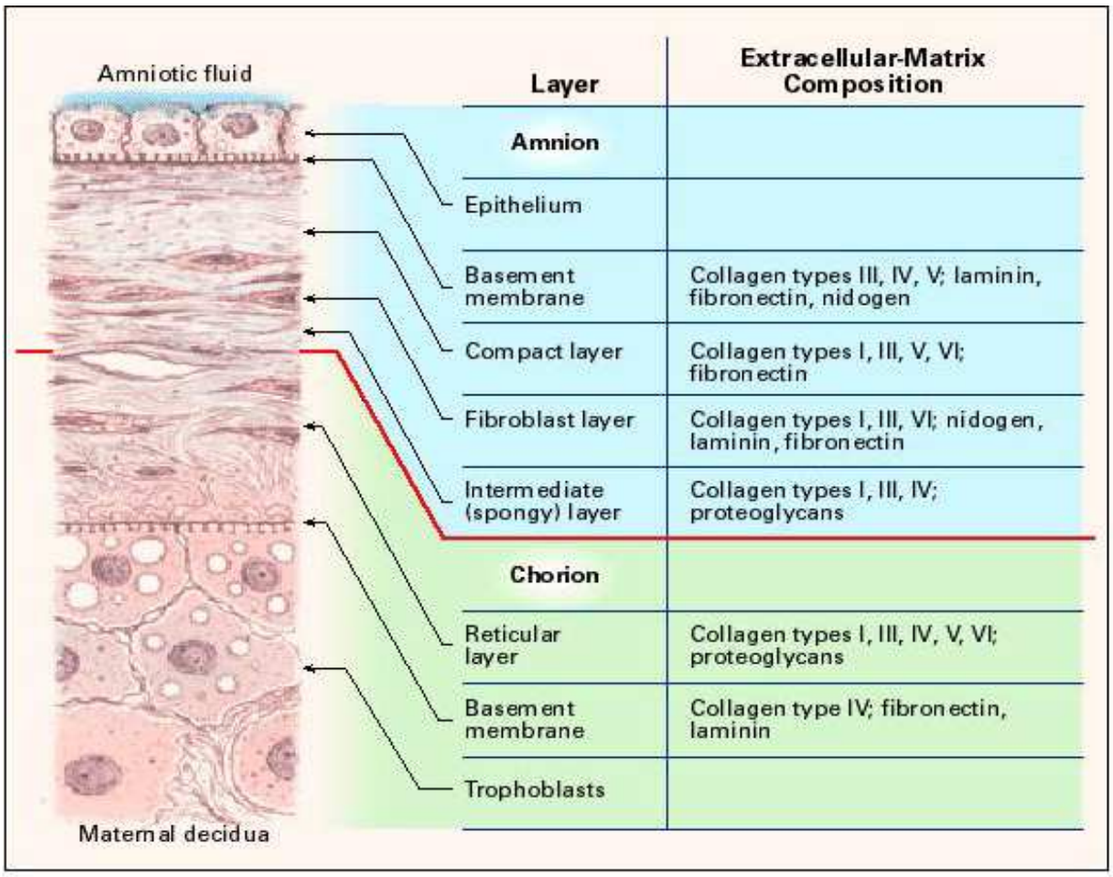

Fig.1. Schematic presentation of the structure of the foetal membrane at term. The Extracellular matrix components of each layer are shown. Adapted from Parry and Strauss (1998); with some modifications.

composition, chemotaxis, cell adhesion and migration in a variety of cell types (Makino et al. 2010). bFGF is known to stimulate proliferation of cultured fibroblasts.

Members of the PDGF family are mitogenic factors for cells of mesenchymal origin. PDGF-BB modulates endothelial proliferation and angiogenesis (Battegay et al. 1994).

EGF is a potent mitogen for epithelial cell growth, promoting wound healing following transplantation [36]

Studies on C-HAM demonstrated the expression and significant levels of all of them, in both intact and denuded HAM.[36,25] Moreover, these growth factors are particularly involved in the wound-healing. [36]

The facilitation of wound healing is one of the most important properties of AM and determines most of its clinical indications. [8,24,26]

EGF, HGF, FGF, and TGF-b 1 content in a tissue-suspension obtained from frozen, freeze-dried, powdered and 46-irradiated HAM, reporting that the freeze-drying process causes a reduction in total protein compared to freezing alone, while powdering causes a significantly increased release of EGF (Russo et al. 2012).[47]

Lim et al. compared decellularized and dehydrated human amniotic membrane with cryopreserved human amniotic membrane, and reported significant differences in the composition and ultrastructure of dehydrated HAM as shown by histological and immunohistochemical examination (Lim et al. 2010). Nakamura et al. reported no statistically significant differences in the physical strength of cryopreserved HAM or freeze-dried HAM treated with γ–irradiation.[46]

Ultrastructural analysis provided additional evidence of the damage caused by γ-rays, in contrast to the absence of any severe damage evidenced in fresh-frozen

and freeze-dried samples. γ-irradiation induced major damage to the epithelium, basement membrane and lamina densa, which was more severe after exposure to 20 and 30 kGy γ –irradiation but the amniotic structure-key features responsible for the favorable clinical outcomes obtained with HAM were well preserved only in fresh-frozen and freeze-dried HAM samples.(Adolfo Paolin 2016).[44]

In 2014, Hamid et al. demonstrated changes to the cell morphology of glycerol-preserved amnion exposed to 35 kGy, while air-dried HAM underwent changes at 25 kGy. and concluded, that cell structure preservation of glycerol-preserved amnion after radiation is probably due to the radio-protectant properties of glycerol, which removes water and limits the formation of free radicals (Ab Hamid et al. 2014).[45]

CONCLUSION

conclusion, processing the amniotic membrane under sterile conditions to guarantee safety at every step as an alternative to final sterilization with c irradiation is strongly recommended in order to avoid alteration of the biological characteristics of the amniotic membrane.

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Corresponding Author

Alekhya ayalapuram
Postgraduate student
Department of periodontics,
Army college of Dental sciences,
Secunderabad, Telengana
Email: - ayalapuramalekhya13@gmail.com
Phone No;8019989867