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A Potential Biomaterial and Scaffold for Tissue Engineering- Amniotic Membrane

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Abstract

In a variety of clinical applications human amniotic membrane (AM) has been employed as a graft biomaterial. There is tenacious issue associated with preparation, storage, and sterilization. To decode these issues, hyperdry AM (HD-AM) using far-infrared rays, vacuum air, and microwaves are developed and then sterilized by g-ray irradiation. Amniotic membrane is a thin membrane that completely covers the embryo on the inner side of the fetal placenta. The membrane contains various growth factors, proteins and stem cell reserves that may enhance wound healing with tissue regeneration. The amniotic membrane has other biological properties important for Tissue engineering, including anti-inflammatory, anti-microbial, anti-fibrosis, anti-scarring, as well as low immunogenicity.

Keywords: amniotic membrane, periodontal regeneration, scaffold, tissue engineering, stem cells

Introduction

Periodontitis is a chronic, inflammatory condition that results in the loss of the supporting structures of the tooth such as alveolar bone, cementum and periodontal ligaments which leads to tooth loss.

Periodontal regeneration has long been the optimum goal in periodontal therapy. However, treating and restoring the original structure, properties, and function of the disease periodontium presents a significant challenge. By definition, periodontal regeneration implies the regeneration of the cementum, periodontal ligament, alveolar bone in a specific sequence which i.e., cementum, periodontal ligament which is based on a number of essential factors. (Figure 1)

Tissue engineering (TE) is described as the creation of biological substitutes for the purpose of restoring, preserving or improving tissue function and it necessitate the use of engineering and life sciences principles and procedures. As a result, designing and selecting the biomaterials used for scaffolding is an important step in tissue engineering. During tissue engineering, cell seeding onto scaffolds is the first step in establishing a three-dimensional culture, which plays a crucial role in determining the progression of the tissue formation. Amniotic membrane is an excellent candidate to use as a native scaffold for tissue engineering as it can be easily obtained, processed and transported.

The use of amnion as a medication originates from traditional Chinese medicine. However, the first written scientific report, concerning its application as a skin graft substitute, is dated 1910. More advanced studies focusing on possible clinical applications of amniotic membrane began in the second half of the 20th century. Amniotic membrane was one of the first biomaterials to be employed in tissue engineering, as a scaffold for cell proliferation and differentiation. Some attempts have also been made to use the amniotic membrane as a conduit for peripheral nerve regeneration.

A tissue-engineering approach is a fundamental concept for regenerating the hierarchical structures of the periodontium, in which periodontal tissues are generated in the laboratory under controlled conditions and subsequently surgically implanted. The use of advanced scaffolds is recommended due to the intricacy of the periodontal tissue architecture and the need for finely coordinated wound healing response, innovative scaffold designs that can manage the spatiotemporal needs for periodontal regeneration have the potential to improve treatment outcomes dramatically. The use of such scaffolds could be complementary to current clinical procedures such as Guided Tissue Regeneration and the use of bioactive molecules, and have the ability to be combined with cell-based approaches.

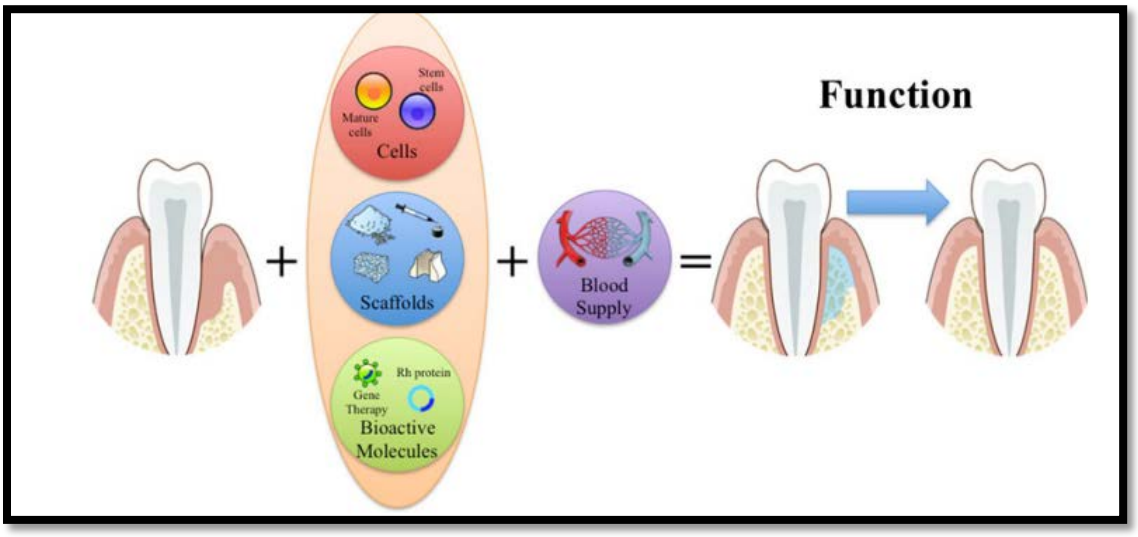

Figure 1: Schematic illustration of principles and requirements for periodontal engineering

Basic structure of amniotic membrane and their function

Amniotic membranes develop from extra-embryonic tissue and consist of a foetal component (the chorionic plate) and a maternal component (the deciduas). Foetal and maternal component are held together by the chorionic villi and connect the cytotrophoblast shell of the chorionic sac to the decidua basalis. The amnio chorionic membrane forms the outer limits of the sac that encloses the foetus, while the innermost layer of the sac is the amniotic membrane. The component of amniotic membrane are epithelial monolayer, a thick basement membrane, and an avascular stroma. (Figure 2) The amniotic membrane contains no blood vessels or nerves, instead, the nutrients are supplied directly by diffusion of the amniotic fluid.

Amniotic membrane has a variety of metabolic functions including transport of water and soluble materials and the production vasoactive peptides, growth factors and cytokines. The basic functions of the amniotic membrane are to provide the protection towards the environment of suspension, where an embryo can grow which is free from pressures of the structures that surround its body. This is due to tensile strength of amniotic membrane that is especially associated

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with the condensed layer of interstitial collagen type I, II and elastin. Amniotic membrane plays an important role during birth, due to the substances produced by the epithelium of amniotic membrane allow the initiation of uterine contractility. Prostaglandins, especially prostaglandin E_2 and enzymes included into the synthesis of prostaglandins, and a number of molecules which might be produced in the amniotic epithelium and that have a fundamental position in the physiology of contraction.

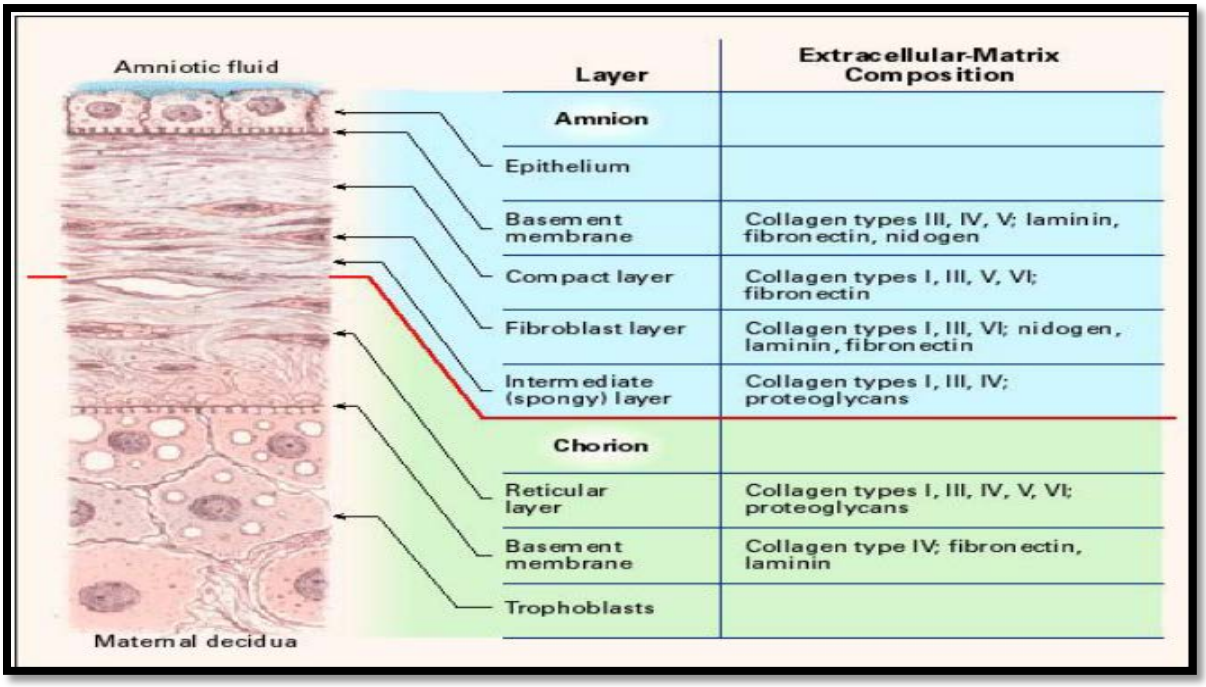

Figure 2: Schematic presentation of the structure of the foetal membrane at term. The Extracellular matrix components of each layer are shown. Adapted from Parry and Strauss (1998); with some modifications.

Histology Of Human Amniotic Membrane

Histologically amniotic membrane is a thin, strong, transparent, avascular membrane made up of three basic layers: epithelial layer which is single, a thickened basement membrane, and a mesenchymal layer which is avascular.

At ultrastructure level mesenchymal layer can be further divided in to compact, fibroblast and spongy layer. The extracellular matrix consists of collagen (types I, III, IV, V, VI), laminins, reticular fibres, proteoglycans, glycoproteins, cell signalling proteins (such as cytokines), and growth factors that are essential to the healing process. There are no nerves, muscles, or lymphatics in the amniotic membrane instead, the nutrients it requires are supplied directly by diffusion out of the amniotic fluid and/or from the underlining decidua.

The basic structure of Human amniotic membrane consists of a monolayer epithelium, a thick basement membrane and an avascular stroma. The shape of epithelium cells are flat, cuboidal and columnar which is present as a single layer in the amniotic fluid. The stroma has three sub layers; a compact layer, a fibroblast layer and a spongy layer. The epithelial layer is characterised by: collagen type III and IV, non-collagenous glycoprotein, fibronectin, vitronectin, tumour necrosis factor–alpha, nerve growth factor. In the stromal there are various proteins, such as anti-angiogenic factors, anti-inflammatory proteins, natural inhibitors to protease and several growth factors. These properties along with their mechanism are summarised in Table 1.

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Table 1: Intact Amniotic Membrane Biological Characteristics

Amniotic Membrane Features	Contributing Factors
Biological Properties	
Anti-Inflammatory Effect	Trapping inflammatory cells and driving apoptosis through its pro-apoptotic agents; production of anti-Inflammatory factors by its epithelial cells; suppression the pro-inflammatory cytokines such as IL- 1α and 1β; production of MMP's inhibitors; Expression of migration inhibitory factor (MIF); Expression of anti-inflammatory cytokines such as Interleukin 1 Receptor Antagonist; secretion of anti-inflammatory factors such as prostaglandin E_2, Tumor growth factor- β and Tumor necrosis factor α from mesenchymal and Epithelial cells of amniotic membrane.
Antibacterial And Antiviral Effect	Expression of natural antibacterial molecules such as β-defensins, elastin, and cystatin E; adhesion to wound surfaces and to act as a barrier against bacterial infiltration.
Low Antigenicity	Lack of human leukocyte antigens A, B, B_2, C and DR antigens. Macroglobulin on the surface of Amniotic membrane epithelial cells; absence of blood vessels, lymphatic drainage and nerves in its structure.
Anti-Scarring and Anti-Adhesive Effect in Wound Healing	Reduction of proteases activity due to the inhibitors of metalloproteinase enzyme; the fibroblastic activity is decrease because of the downregulation of Tumor necrosis factor β and presence of hyaluronic acid in amniotic membrane.
Angiogenesis And Anti - Angiogenesis Properties	There is a secretion of pro apoptotic agents; secretion of Interleukin-1,2,10 Receptor antagonist, and endostatin which all inhibit the secretion of tumour growth factors such as EGF, KGF and HGF
An Anticancer Agent with Low Tumorigenicity Promotion of Epithelization	Its hyaluronic acid content and proteins such as fibronectin, laminin, collagens and proteoglycan act as a ligan for integrin receptor
Support Cell Adhesion and Growth Support	Amniotic membranes act as a substrate, on which epithelial Cells grow on by facilitating the following i.e. Epithelial cell migration, Basal epithelial cell adhesion, Epithelial cell differentiation and Prevention of apoptosis.
Promotion of Epithelization	Presence of anti- microbial factors: Bactericidin, Beta-lysin Lysozyme and Transferrin 7S immunoglobulin. It acts as a physical barrier as human, Membranes closely adhere to underlying structure
Anti-microbial	There is no immune rejection from human amniotic membranes because as it lacks histocompatibility antigens such as HLA-A, HLA-B or HLA-DR

TGF-B= transforming growth factor-beta, IL= interleukin, HLA= human leukocyte antigen

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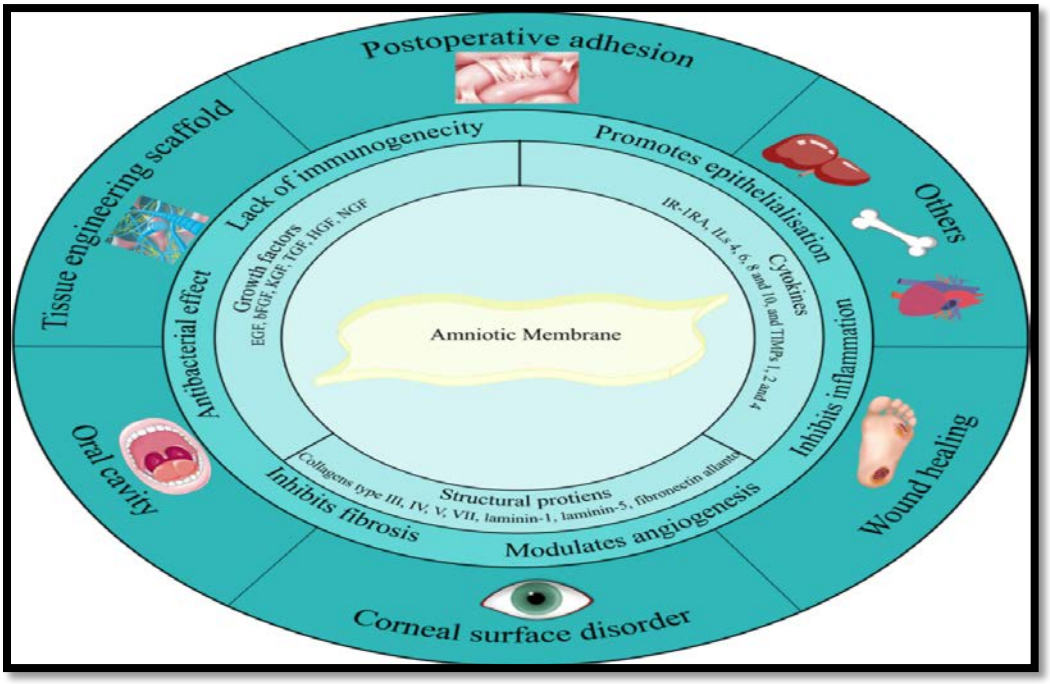

Figure 3: Amniotic membrane components, characteristics, and applications. Amniotic membrane is rich in growth factors, cytokines, and structural proteins. These biological factors have given Amniotic Membrane its unique features such as antibacterial effect, anti-inflammatory activity, anti-scarring, and anti-fibrosis potential. Additionally, it has been used as a natural scaffold in tissue engineering.

Forms of Amniotic Membrane

Frozen membrane

Amniotic membrane is frozen at -196° F by passing it through liquid nitrogen. The membrane is preserved for an unlimited time period by cooling, which produces bacteriologically natural and immunologically inert material. Cryopreservation with dimethyl sulphoxide (DMSO) at -80°C permit retention cells in the AM at approximately 50% for several months. The various angiogenic growth factors and cytokines are eliminated during cryopreservation of the AM. The viability of AEC's is diminished if the AM is cryopreserved in 50% glycerol. It has been referred to that storage of the AM in glycerol at 4°C leads to immediate cell death.[12]

Freeze dried irradiated (lyophilized)

After being extracted from the placenta the membrane is freeze dried at -60°C under vacuum (atmospheric pressure 102) for 48 hours. In a batch type cobalt-60 irradiator, it is then irradiated with 2.5 mega rads (25 K Gray). The liquid moisture of the membrane is sublimated to gaseous state using the freeze-drying process without going through the intermediate solid stage. This approach aids in the preservation of membrane natural size and form while minimizing cell rupture. By soaking the freeze-dried membrane in normal saline for 1 minute, it can be prepared for usage.[13]

Decellularized and then-lyophilized human amniotic membrane had the slowest resorption rate as compared to fresh, cryopreserved, and lyophilized human amniotic membrane.

Cryopreservation often increases human amniotic membrane thickness, whereas lyophilization decreases it. Recent studies have compared fresh, cryopreserved, lyophilized, and decellularized-then-lyophilized human amniotic membrane.

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In vivo, fresh human amniotic membrane and decellularized-then-lyophilized human amniotic membrane were significantly stronger than cryopreserved human amniotic membrane and lyophilized human amniotic membrane. Thus, the decellularization process increased the physical and mechanical properties of human amniotic membrane. It made human amniotic membrane significantly more stretchable than fresh human amniotic membrane, significantly enhancing the tearing strength and significantly decreasing the human amniotic membrane rate of resorption.

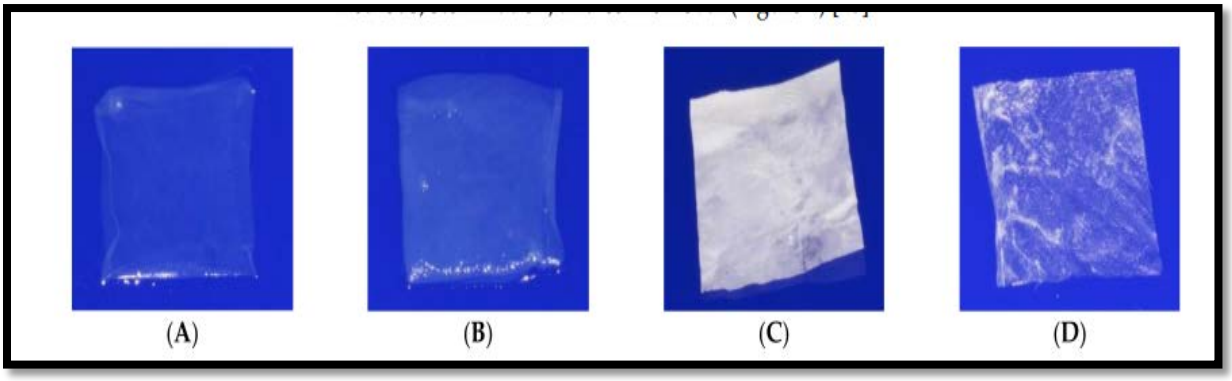

Figure 4: Human Amniotic Membrane Formats A) Fresh b) Cryopreserved c) Lyophilized d) Decellularized and Lyophilized

Preparation of Amniotic Membrane

Amniotic membranes were obtained from women undergoing caesarean section surgeries. The membranes were washed under aseptic conditions with PBS containing 5 ml of 0.5% levofloxacin and stored at 80°C in DMEM and glycerol until required. After thawing, the overlying amniotic epithelial cells were removed by gentle scraping with a cell scraper after incubation with 0.02% EDTA at 37°C for 2 h.

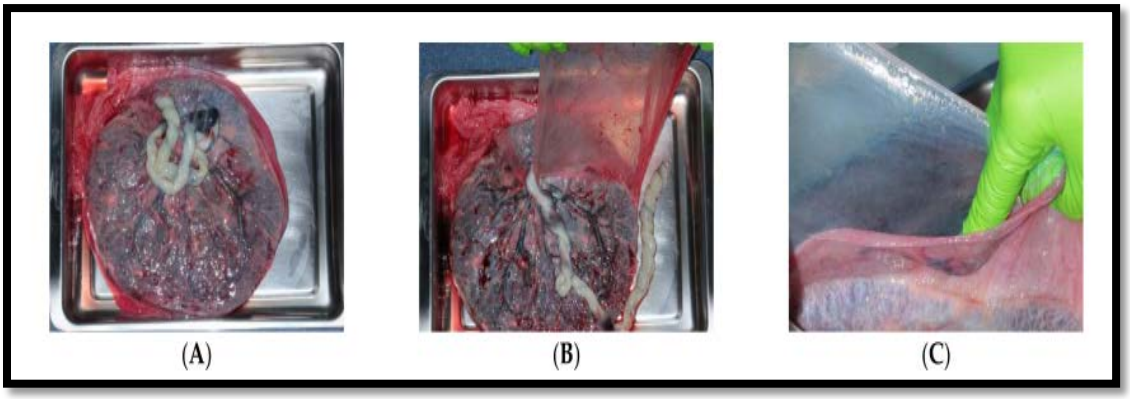

Figure 5: Human Amniotic Membrane Collection a) Placenta b) Amnion Chorion c) Amniotic Membrane from the Chorion

Mechanism of Amniotic Membrane

The mechanisms concerned in accelerated wound healing through amnion membrane may be divided as follows:

- Immunomodulative and Immune privilege
- Anti-microbial (broad spectrum impact against bacteria, fungi, protozoa and viruses)
- Reduction of pain

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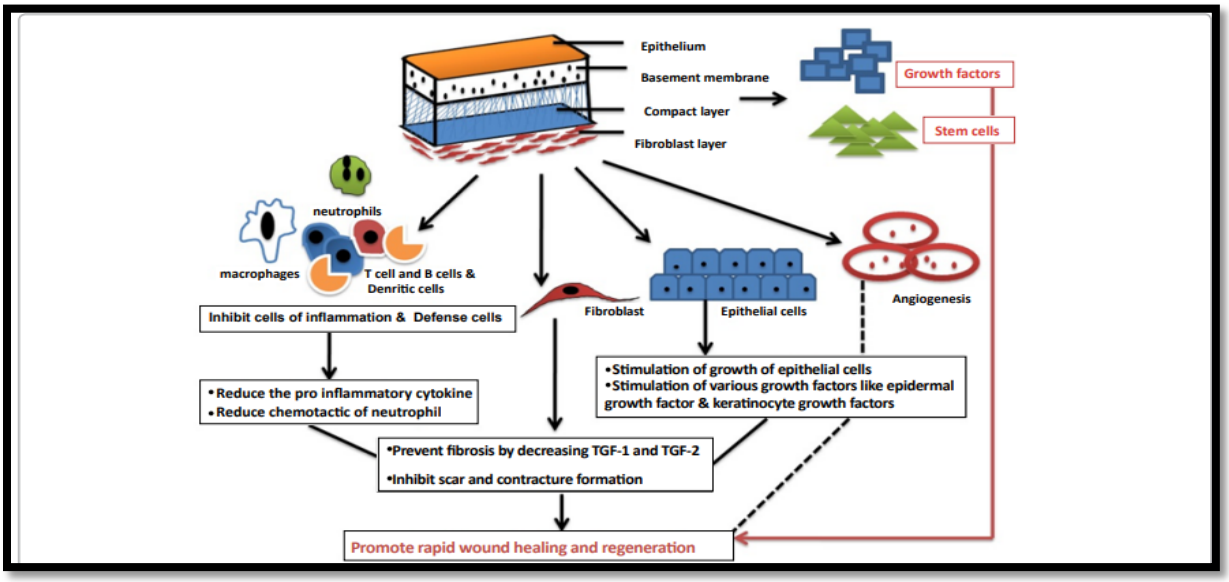
- Anti-scarring and anti-Inflammatory
- Tissue reparative activities with advanced bone remodelling, osteogenesis and chondrogenesis
- Speed fibrogenesis and angiogenesis
- Increased extracellular matrix deposition
- Potent source of mesenchymal stem cells



Application in Periodontics

Amniotic membrane provides good results in terms of root coverage procedure, increased the width of attached gingiva, excellent in cosmetic procedure which may result as barrier membrane for intrabody defects and furcation involvement in addition to intraoral soft and hard tissue restoration.

Velze et al. evaluated cryopreserved amniotic membrane for tissue healing in terms of lesion size, epithelialization, pain, infection, inflammation, and scarring during dental implant surgery. Their trial showed that cryopreserved amniotic membrane was effective in helping cicatrisation, wound healing, supported the growth of the epithelium, reinforced adhesion, and decreased the pain of subjects.[12]

Adachi et al. reported an analysis of Periodontal ligament cells obtained from maxillary third molar, cultivated on amniotic membrane to determine the distribution of factors responsible for maintaining the characteristics of periodontal ligaments. The immunofluorescent and electron microscopic findings suggested a improved proliferation of periodontal ligament cells with lateral conjugation and adhesion to amniotic membrane as well as maintenance of their inherent properties with strong cell-cell adhesion structures (desmosomes and tight junctions) to adjacent cells.[14]

Samandari et al. investigated the effects of amniotic membrane (AM) in bone induction and wound healing after vestibuloplasty surgery on animal samples. Amniotic membrane has also used as a barrier membrane for the promising regenerative management of class III furcation involvement in combination with composite allograft. A recent retrospective observational report documented the use of amniotic membrane for combination GTR treatment of periodontal intrabony defects. The postoperative observations showed improved density of radiographical bone fill as

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compared to base line level. There are some inherent benefits of placental barrier (Amniotic Membrane) over conventional GTR membrane. Evidences are also present regarding the use of amniotic membrane for the purpose of gingival recession. Placental barrier (AM) may provide an effective alternative to autograft tissue treatment for root coverage procedures. A histological study demonstrated that amniotic membrane transplantation for gingival wound healing may induce rapid epithelialization and both granulation tissue and collagen formation but suppress inflammation.[1]

Tsuno et al. have successfully treated intraoral alveolar wounds with bone exposure during vestibuloplasty of the reconstructed mandible with the use of hyper dry amniotic.[15]

Figure 6: A human amniotic membrane, preserved by glycerol, was soaked in normal saline before being used.

The amniotic membrane as a scaffold for tissue engineering

A most important prerequisite for selecting a scaffold is its biocompatibility. Biocompatibility is the property of being biologically well suited because of not generating a toxic, injurious, carcinogenic, or immunological reactions in living tissue. Scaffolds must not be destroyed by inflammation but it should be able to react with host response. Their mechanical properties should include permeability, stability, elasticity, flexibility, plasticity at a rate congruent with tissue replacement. Scaffolds should also allow cell adhesion and the potential for delivery of bio modulatory agents such as growth factors and genetic materials.

The Amniotic membrane is a scaffold with a template of the ECM. AECs secrete collagen type III and IV and non-collagenous glycoproteins (laminins, nidogen, and fibronectin) that form the basement membrane of the amniotic membrane.

Amniotic membrane successfully completed the prerequisite for choosing as a scaffold for tissue engineering. The attachment of a cell to a scaffold is largely affected by the components of the scaffold's extracellular matrix (ECM). When each epithelial and mesenchymal cells are seeded on a cellular scaffold that are comprised of the amniotic

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membrane, the cells have been interconnected and are able to penetrating the porous shape of the amnion scaffold. Cultivation of endothelial cells on an amniotic membrane scaffold has also been reported as a potential approach for vascular tissue engineering.

The Amniotic membrane can be used either with amniotic epithelium or without it. To remove the amniotic epithelium, the amniotic membrane is incubated in EDTA at 37°C and the cells are gently scraped with a cell scraper under a microscope. Although complete removal of cellular components from amniotic membrane is important for the denudation in which the structural components of the remaining scaffold must be retained.

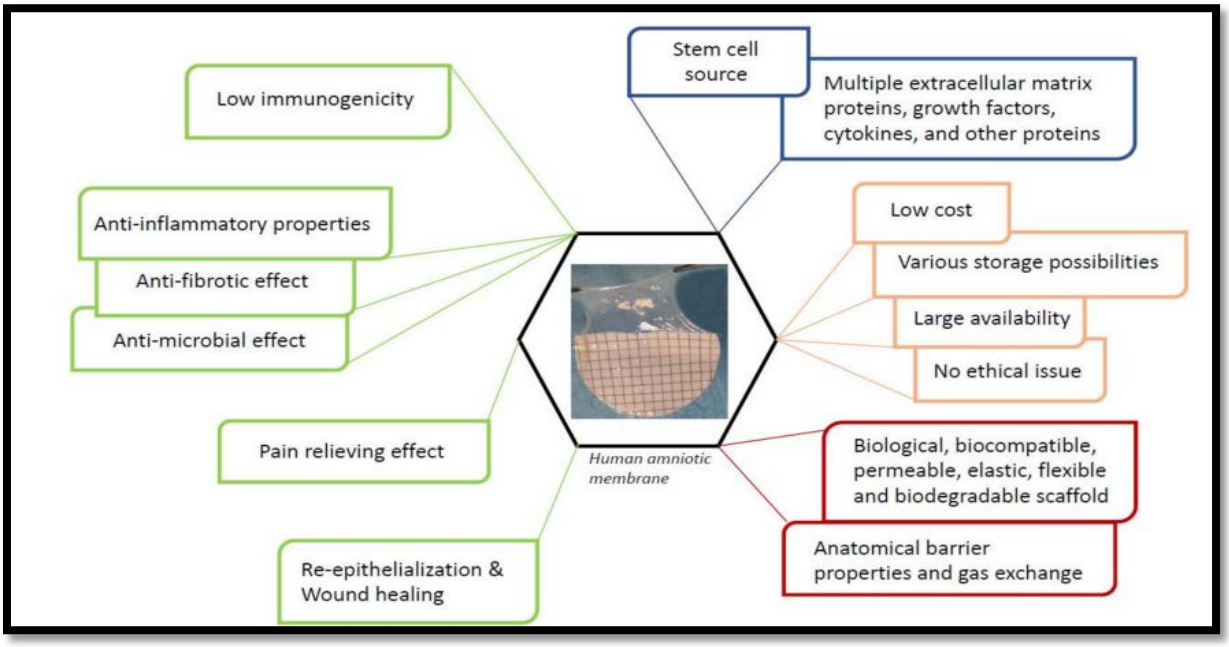

Figure 7: Human Amniotic Membrane Properties as An Ideal Scaffold for Tissue Engineering

Table 2: Studies associated with Amniotic Membrane as a Scaffold for Tissue Engineering

Authors	Tissue Engineering Application	Amniotic Membrane Formats	Modalities of Amniotic Membrane Usage	Sides of Cells Seeding	Assessment
Amemiya et al., 2008	Periodontal	Cryopreserved + Deepithelialized	Single membrane/Cover	Basement membrane	In vivo
Iwasaki et al., 2013	Periodontal	Decellularized + Cryopreserved	Single membrane/Cover	NS = Not Specified;	In vitro/Ex vivo + In vivo
Amemiya et al., 2014	Periodontal	Deepithelialized	Single membrane/Cover	NS = Not Specified;	In vitro/Ex vivo + In vivo
Wu et al., 2015	Periodontal	Deepithelialized	Single membrane/Cover	Basement membrane	In vitro/Ex vivo + In vivo
Honjo et al., 2015	Periodontal	Cryopreserved + Deepithelialized	Amnion placed on a cell culture insert	Basement membrane	In vitro/Ex vivo

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Amniotic epithelium as a source of stem cells for Tissue Engineering

Amniotic epithelial cells (AECs) have numerous traits that lead them a rich supply of stem cells for Tissue engineering. The amniotic epithelium originates from the epiblast before gastrulation. Recent studies aimed at defining the characteristics of AECs stems cells have determined that these cells express the surface markers associated with embryonic stem cell, e.g., SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81.

Another important advantage of AECs over HESCs is related to their cultivation method. AECs create their own feeder layer with number of cells spreading out at the lowest layer of the culture dish. The basal layer of AECs that attaches to the culture dish may play an important role of an autologous feeder layer which serve as a substrate for attachment which provide secreted factors that help in maintain undifferentiated AEC. The other advantage of AECs is that an average yield is more than 100 million AECs per amnion collected. The robust proliferation of these cells in the presence of certain growth factors such as EGF.

Table 3: Studies associated with Amniotic membranes as substrate/scaffolds, for periodontal progenitor/stem cells

Type of Study	Scaffold	Cell	Outcome
In-vitro	Amniotic membrane	Human periodontal Ligament cell sheets	Cells were able to differentiate, proliferate and express the essential protein for cell- substrate adhesion
In-vitro	Amniotic membrane	Periosteal-derived Cell sheet	Amniotic membranes showed good adhesion with periosteal derived cell sheet
In-vitro	Amniotic membrane	Human dental pulp derived cells	Amniotic membranes showed good adhesion to dental pulp periosteal derived cell sheet
Pre-clinical	Amniotic membrane	Periodontal ligament stem cells	Periodontal regeneration was enhanced and showed a monolayer of the cells on the amnion surface cells
In-vivo	Amniotic membrane	Adipose-derived stem cells	Periodontal regeneration was enhanced in the surgically created bone defect

Conclusion

The Amniotic membrane has numerous traits, which make it potentially appropriate for use in tissue engineering. The epithelial layer of the amniotic membrane includes cells that have similar characteristics to stem cells. In addition, there are numerous other benefits that recommend AECs are an excellent source of cells for tissue engineering. The amniotic membrane can act as a scaffold for tissue engineering. The salient of the amniotic membrane include anti-inflammation, anti-fibrosis, anti-scaring, anti-microbial, low immunogenicity and affordable mechanical property, which can be essential to be used in tissue engineering. Amniotic membranes are being utilized in a huge variety of procedures and disciplines in medicine, dentistry and stem cell research, owing to its pleiotropic properties and having a benefit of being a biological membrane. Amniotic membranes have shown great potential as suitable scaffolds, and therefore, can serve as an

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alternative material in periodontal tissue regeneration and engineering. Further clinical research is needed to establish its function and efficiousness.

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