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VIABILITAS FIBROBLAST FREEZE – DRIED AMNIOTIC AND FRESH AMNIOTIC IN BHK21 CELL

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ABSTRACT

Amniotic membrane is the inner most lining of the human placenta that is normally discanded after parturition. The membrane has numerous growth factors, proteins and stem cell reserves that help in accelerating wound healing with regeneration of the lost tissues. The preserved human amniotic membrane is a novel tissue engineerrined biomaterial that is recently trial in field of medicine dentistry to regenerate the lost tissues and accelerate repair. This review paper unfolds the inherent structure, properties, mechanisms and the application of this neglected tissue that makes it a potential for regeneration especially in the field of oral and periodontal surgeries. This research has been conducted to measure the difference of viabilitas fibroblast concentration between fresh amniotic and freeze – died amniotic membranes. Experimental three group test design was exployed with amniotic membrane. Amniotic membrane was divided into two parts. The first parts was without preservation amniotic, the test with BHK21 cell for control group, the second part was with reservation freeze –dried amniotic and the last experiment was with preservation fresh amniotic membrane. The result experiment are viabilitas fibroblast freeze – dried amniotic more than fresh amniotic membrane.

KEYWORDS *Growth Factor, Regeneration, Freeze – Dried Membrane, Fresh Amniotic Membrane*

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INTRODUCTION

Periodontal disease is an inflammatory disease caused by infection with periodontopathogenic bacteria, namely P Gingivalis and Aggregatebacter Actinomycetemcomitansi, which can cause destructive, progressive damage to the tooth support tissue and can cause alveolar bone destruction and damage to the periodontal ligament. (Newman et al. 2006)

Treatment in the field of periodontia should ideally result in new tissue regeneration (Fleckenstein 2006). Damage to periodontal tissue is treated in various ways, both surgically and non-surgically. Flap surgery using bone graft combined with GTR has been widely performed in the hope of regenerating periodontal tissue, but so far there is still no affordable material for surgical treatment in the field of periodontia.

Fresh amniotic membrane has clinical, biological and logistical limitations, namely not durable, inefficient and requires time in serological testing (the examination needs to be repeated 6 months later for the possibility of a window period). The healing effect on fresh amniotic membrane is thought to be greater than preserved amniotic membrane because the preservation process of amniotic membrane can reduce active cells including growth factors. Several processes involved in the preservation of amniotic membrane include freezing, lyophilization and radiation reported to affect the quality of amniotic membrane. (Hennerbichler et al 2007)

RESEARCH METHOD

This type of research is a laboratory experiment that uses special techniques to evaluate the viability of BHK21 fibroblast cells with Freeze-Dried and Fresh Amnion membranes. The independent variable in this study was the type of amniotic membrane, while the dependent variable was the viability of BHK21 fibroblast cells. Some controlled variables include the use of Eagle's medium and 10% bovine serum, the length of incubation, as well as the procedure for planting and separating fibroblast cells. This study was conducted at PUSVETMA Surabaya for 24 hours using tools such as standard and CO2 incubators, Roux bottles, petri dishes, multi-channel pipettes, microplates, shakers, and Elisa Reader. Materials used included BHK21 fibroblast cell line, Eagle media and 10% bovine serum, PBS and versene trypsin, amniotic membrane (Freeze-Dried and Fresh), MTT, and DMSO. The research procedure consisted of planting fibroblast cells, incubation, cell washing, adding growth media, planting amniotic membrane, incubation again, and measuring absorbance using an Elisa Reader. Preparations were carefully made to ensure the validity and consistency of the data.

RESULT AND DISCUSSION

Fibroblasts are found in the periodontal ligament, and are the stem cells that produce: reticulum, elastin, glycosaminogen and glycoproteins from amorphous intercellular substance. Fibroblasts are a major element in the wound healing process. In tissue damage, stimulation of fibrocytes occurs, resulting in fibroblast mitosis. During the wound healing process, fibroblast cell proliferation is required and fibroblasts differentiate into myofibroblasts (Hinz 2012).

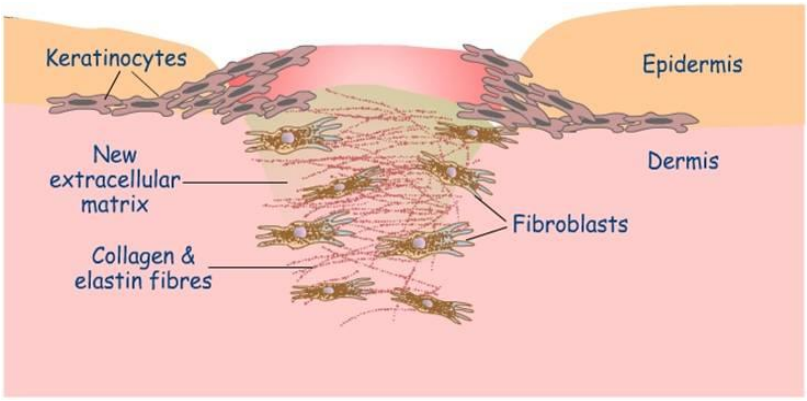
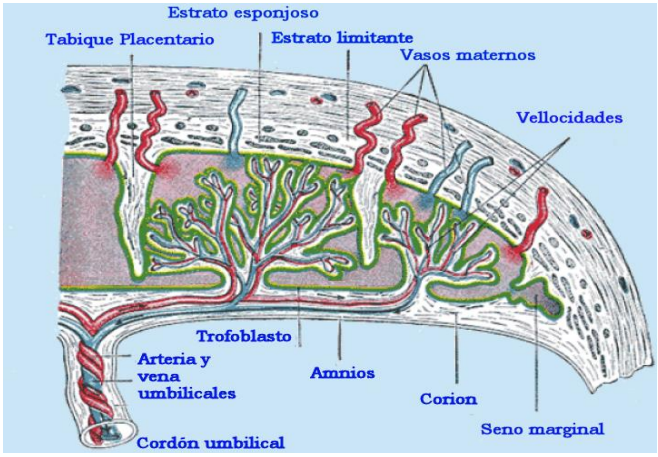


Figure 1. Fibroblast (source: https://dentosca.wordpress.com/proses-penyembuhan-luka/)

The amnion or amniotic membrane is the innermost layer of the placenta. Microscopically, the amniotic membrane is a thin, transparent, strong membrane and is attached to the placenta. The normal thickness of the amniotic membrane is 0.05- 2.02 mm, surface area 1600 cm. (Porolini et al 2008).

The human amniotic membrane is the deepest layer of the placenta and histopathologically consists of three layers, namely:

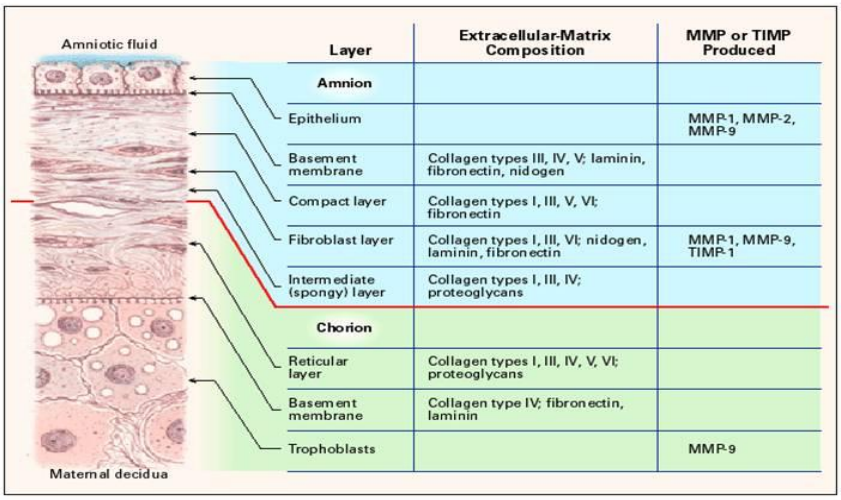
1. Epithelium
2. Membrane Basalis basement membrane
3. Avasclar hypocellular stromal matrix



Amniotic layer (Sangwan et al 2007)

Sangwan et al, 2007 in his book explains that, the membrane layer consists of :

1. The amniotic epithelium consists of a single layer of cuboidal cells with a large number of microfili on the apex surface.
2. Membrane basement of the amniotic membrane consists of collagen types IV, V and VII, coupled with fibronectin and laminin. Laminin is very effective as a facilitator of epithelial cell adhesion with type V collagen helping epithelial cells to approach the stroma (Modesti et al 1984; Sangwan et al 2007).
3. Membrane amnion produces basic Fibroblast Growth Factor (FGF). These growth factors act as stimulators of epithelialization, regulating proliferation and differentiation of stromal fibroblasts (Sangwan et al 2007).



Layer	Extracellular-Matrix Composition	MMP or TIMP Produced
Amnion		
Epithelium		MMP-1, MMP-2, MMP-9
Basement membrane	Collagen types III, IV, V; laminin, fibronectin, nidogen	
Compact layer	Collagen types I, III, V, VI; fibronectin	
Fibroblast layer	Collagen types I, III, VI; nidogen, laminin, fibronectin	MMP-1, MMP-9, TIMP-1
Intermediate (spongy) layer	Collagen types I, III, IV; proteoglycans	
Chorion		
Reticular layer	Collagen types I, III, IV, V, VI; proteoglycans	
Basement membrane	Collagen type IV; fibronectin, laminin	
Trophoblasts		MMP-9

Histology of amniotic membrane (Seyffarth et al 2004)

Various important components contained by the amniotic membrane provide unique characteristics obtained from the use of amnion. Membrane amnion is immunologically non-immunogenic (inert), does not show the presence of major histocompatability antigens (HLA-A, HLA-B, HLA-C and antigens). With the use of amnion as a GTR does not cause immunological rejection reactions. Amnion membrane is flexible, strong semi-transparent and has high tensile strength. Clinically important properties of amniotic membrane include bacteriostatic properties, anti-angiogenesis properties, anti-inflammatory, anti-cicatric properties, reepithelialization effects and can be a substrate for cell growth of epithelial cells both invivo and invitro (Gomes et al 2005).

Amniotic membrane specifically has several beneficial properties including anti-adhesion, bacteriostatic, anti-angiogenesis, anti-inflammatory, anti-cicatric, very high tensile strength, protects wounds, reduces pain and has a reepithelializing effect. (Hennerbichler et al 2007; Parolini et al 2008; Niknejad et al 2008).

The unique and favorable characteristics of the amniotic membrane are inseparable from its extracellular matrix components. Amniotic membrane is known to contain cologen type I, III, IV, V, VI, fibronectin, nidogen, proteoglycans, hyaluronan and laminin. In addition, the amniotic membrane also contains a variety of growth factors... Some of the growth factors found in the amniotic membrane are Epidermal Growth Factor (EGF) Transforming Growth Factor (TGF). Fresh amniotic membrane taken directly from the placenta after the mother gives birth. Amniotic membranes to be used as biomaterials require special treatment before use. This is related to its safety risk as occurs in other organ/tissue transplatation

Several methods of amniotic membrane preparation and preservation have been developed and applied in various tissue banks in the world. Based on the preservation process and storage method, amniotic membrane is divided into two, namely without preservation (fresh) and with preservation (fresh frozen and freeze dried). In clinical use, ideally the membrane should be sterile, easy to obtain, easy to distribute and can be stored for a long time without change. One form of amniotic membrane preservation that is considered capable of overcoming these obstacles is freeze-dried amniotic membrane (Yan-Hong and Hong-Guang 2007).

Fresh amniotic membrane has clinical, biological and logistical limitations, namely that it is not durable, inefficient and requires time in serological testing (the examination needs to be repeated 6 months later for a possible window period). While freeze dried

amnion is not re-examined because it has been lyophilized and sterilized with gamma rays. To find out the difference between the two membrane materials as a tissue material that can have an effect on tissue repair, a study is needed to determine the extent of changes in the effectiveness of amniotic membrane after freeze-dried preservation and fresh amnion in the application of BHK21 cells to see fibroblast viability in freeze dried amnion and fresh amnion in the process of tissue healing.

Amnion also contains high levels of transferrin which can reduce the possibility of immunologic reactions from the recipient. Thus Amnion does not have a resistant effect so it is clinically safe to use as a wound cover (Akle et al.1981). The application of the use of Freeze - Dried amnion membrane causes the occurrence of Fibroblast viability in BHK21 Fibroblast cell line greater than fresh Amnion in BHK21 cell line. Amnion contains various growth factors (PDGF, VEGF, KGF, EGF, FGF, and TGF) that can stimulate epithelialization, inhibit the inflammatory process, inhibit scar tissue formation, inhibit Angio Genesis and also as an anti-microbial agent. Amnion's special properties that are very beneficial are that it can stimulate epithelialization, inhibit the inflammatory process and scar tissue formation (anti-cicatric), inhibit angiogenesis and as an antimicrobial agent. (Koizumi et al, 2000).

From this study, it was found that fibroblast viability was higher in freeze-dried amnion membrane compared to Fresh Amnion, this is because freeze-dried amnion membrane that has undergone lyophilization and preservation did not undergo changes in structure and physiological function so that at the time of the study it still had the same function as before lyophilization and preservation (Ahn et al 2006).

Amnion contains 8 growth factors (EGF, TGF α, KGF, HGF, β FGF, TGF β1, β2, and β3) and 2 growth factor receptors (KGFR, and HGFR) (Koizumi et al, 2000). Growth factors play an important role in the revitalization of EGF, KGF, and HGF. EGF plays a role in the process of proliferation and differentiation of epithelial cells. EGF stimulates the proliferation of epithelial cells, indicating that EGF plays an important role in wound healing. The growth factor content in amnion can stimulate cell growth and potentially accelerate wound healing through :

1. Angiogenic i.e. stimulating blood vessels
2. Chemotactic i.e. attracting different cell types to the wound area
3. Mitogenic i.e. stimulation of cell proliferation
4. Regulation i.e. synthesis and degradation of extracellular matrix (ECM) components
5. Effects of cytokine and growth factor synthesis by neighboring cells.

Growth factors contained in amniotic membrane that have a role in fibroblast proliferation are TGF β (Transforming Growth Factor β), FGF (Fibroblast Growth Factor), KGF (Keratinocyte Growth Factor), and PDGF (Platelet Derived Growth Factor). These unique and favorable characteristics in amniotic membrane make it more potential as a biomaterial, so it is widely used as a clinical application material.

In this experiment, fibroblast viability in Fresh Amnion is lower than freeze-dried amnion because Fresh Amnion cannot be stored for too long in a fresh state because it will quickly decay. This is because Fresh Amnion that does not go through the stages of preservation and lyophilization of the structures contained therein, namely proteins and amino acids, is still in a wet (fresh) state, so it is highly favored by microbes (Virender 2007).

From the results of this study, it was found that fibroblast viability in fresh amnion was lower than freeze-dried amnion. As for the application of freeze-dried amnion membrane which has decreased its function as a growth factor in this study, fresh amnion membrane can still function as a scaffold.

CONCLUSION

This study concluded that the use of Freeze-Dried amnion membrane causes fibroblast viability in BHK21 cell line higher than the use of Fresh Amnion. Amnion contains various growth factors (PDGF, VEGF, KGF, EGF, FGF, and TGF) that can stimulate epithelialization, inhibit inflammatory processes, scar tissue formation, angiogenesis, and function as antimicrobial agents. The higher viability of fibroblasts in Freeze-Dried Amnion membrane is due to the lyophilization and preservation process that does not change their physiological structure and function. Amnion contains eight growth factors and two growth factor receptors that play an important role in wound revitalization and healing. Growth factors in amnion stimulate fibroblast proliferation, making it a potential biomaterial for clinical applications. The lower fibroblast viability in Fresh Amnion is due to the inability to store in a fresh state leading to spoilage, while the Freeze-Dried Amnion membrane shows minimal decrease in function as a Growth Factor.

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