"PROCESSING OF HYDRATED AMNION CHORION MEMBRANE FROM PLACENTA AND COMPARING WITH DEHYDRATED AMNION CHORION MEMBRANE FOR THE PROLIFERATION OF PERIODONTAL LIGAMENT FIBROBLAST ACTIVITY– AN IN-VITRO STUDY."

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ABBREVATIONS

SR. NO.	ABBREVATION	FULL FORM
1.	ACM	Amnion chorion membrane
2.	HACM	Hydrated Amnion chorion membrane
3.	dHACM	Dehydrated Amnion chorion membrane
4.	PDL	Periodontal ligament
5.	KGF	Keratinocyte growth factor
6.	PDGF	Platelet derived growth factor
7.	TGF – α	Transforming growth factor α
8.	TGF – β	Transforming growth factor β
9.	NGF	Nerve growth factor
10.	TNF- α	Tumour necrosis factor α
11.	EGF	Epidermal growth factor
12.	bFGF	Basic fibroblast growth factor
13.	CTGF	Connective tissue growth factor
14.	IL-1β	Interleukin -1β
15.	MTT	3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide
16.	EDTA	Ethylenediamine tetra acetic acid
17.	FBS	Fetal bovine serum
18.	DMEM	Dulbecco's modified eagle medium

19.	DMSO	Dimethyl sulfoxide
20.	CO_2	Carbon dioxide
21.	BP Handle	Bard Parker handle
22.	Units of measurement	
	ml	Millilitre
	nm	Nano-metre
	µL	Micro-litre
	Gy	Gray
23.	γ- radiation	Gamma radiation
24.	PBS	Phosphate buffer saline
25.	OD	Optical density
26.	Hr	Hours
27.	p- Value	Probability value
28.	HGF	Hepatocyte growth factor
29.	CTGF	Connective tissue growth factor
30.	ELISA	Enzyme linked immunoassay
31.	HLA-G	Human leukocyte antigen
32.	rhFGF - 2	recombinant human fibroblast growth factor-2
33.	PGE_2	Prostaglandin E_2

ABSTRACT:

Background:

Periodontitis is a multifactorial chronic inflammatory disease of periodontium leading to periodontal destruction eventually leading to bone loss. The primary goal of periodontal therapy is to restore the supporting tissues and prevent further attachment loss. Factors such as appropriate cells, signals, scaffolds are required for the successful outcome of periodontal regeneration. Different methods have been used in the past along with incorporation of biomaterials like derivatives and placental membranes for periodontal regeneration that will provide favourable wound healing.

Aim:

To process and assess the efficacy of Hydrated amnion chorion membrane (HACM) and Dehydrated amnion chorion membrane (dHACM) on proliferation of Periodontal ligament fibroblast cells.

Materials and methods:

A total of 18 systemically healthy patients meeting the eligibility criteria were included in this study. The amnion chorion membrane from placenta was obtained from the Department of Obstetrics and Gynaecology and were processed as hydrated and dehydrated based on different processing methods. Periodontal ligament fibroblast cells were cultured from the freshly extracted premolars, for orthodontic reasons in Basic science research centre (BSRC). The cultured PDL fibroblast cells were exposed to Hydrated and Dehydrated amnion chorion membrane. The MTT assay was performed to assess the proliferation of PDL fibroblast cells. The proliferative activity of the PDL fibroblast cells were assessed after 24 hours and 48

hours using ELISA reader. The statistical analysis was performed using SPSS software. Intra and Inter-group comparison of hydrated and dehydrated amnion chorion membrane at 24 and 48 hours was done using Mann Whitney U test. The p value <0.05 was considered as statistically significant.

Results:

Both Intra and Inter-group comparison of the hydrated and dehydrated amnion chorion membrane at 24 and 48 hours showed statistically significant (<0.05) proliferation of periodontal ligament fibroblast cells.

Conclusion:

In the current study properties of both hydrated and dehydrated amnion chorion membrane showed proliferation of periodontal ligament fibroblast cells. However, dehydrated ACM showed significant proliferation of PDL fibroblasts.

Keywords: Periodontitis, Amnion chorion membrane, Hydrated amnion chorion membrane, Dehydrated amnion chorion membrane, Periodontal ligament fibroblasts, Wound healing

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INTRODUCTION

Periodontitis is a globally prevalent inflammatory disease characterized by periodontal tissue destruction. The main purpose of periodontal treatment is to prevent further attachment loss and predictably restore the periodontal supporting structures that were lost because of disease.[1] This occurs by sequential entry of macrophages that elaborate growth factors which further regulate fibroblasts, smooth muscle cells and endothelial cells which will accelerate wound healing.

Wound healing is a complex biological process requiring interactions among distinct resident cell types, inflammatory cells, platelets, and stem cells. Growth of new blood vessels into the wound through angiogenesis is a critical aspect of this process, to promote the adequate delivery of nutrients and regulatory factors required for tissue remodeling and regeneration.[2] The destruction of growth factors and their receptors is due to a drop in the mitogenic activity and recruitment of additional cells to the wound bed. Fibroblasts, the building blocks of dermal tissue, is affected by the chronic wound environment.[3]

Fibroblasts are the dominant cell types of the connective tissue in the body. They help in production of PGE_2 and collagen that help in exacerbation of inflammatory disease state. The PDL fibroblast produces and maintains the connective tissue attachment which firmly anchors the tooth to alveolus. Collagen is the major structural component and is responsible for rapid and extensible remodeling of attachment fibers.[4]

Placental membranes have a long-documented history for being used to treat both chronic and acute wounds.[5] The fetal membranes possess unique inherent biologic properties that enhance wound healing and may propagate regeneration.[6] The first documented use of amniotic membranes as a surgical dressing in skin transplantation was reported by Davis in 1910. It has been used in conjunctival reconstruction, glaucoma, burn, pterygium and bullous keratopathy. It has also been successfully applied in different clinical conditions such as vaginal reconstruction, abdominal surgery with an enterocutaneous fistula, gastroschisis and omphalocele in infants.[7] Several medical reports have described the amnion tissue as a biological dressing in treatment of skin loss in Stevens-Johnson's diseases, replacement of normal nasal mucosa in Rendu-Osler-Weber diseases, periodontal surgery, chronic varicose ulcers, decubitus ulcers and open infected wounds with good outcome.[8] Excellent results have been obtained in treatment of second and third degree burns and open infected wounds.[9]

Placental membranes have their origin from extraembryonic tissue. This tissue is composed of a fetal component (the chorionic plate) and a maternal component (the decidua). The fetal component includes the amnion and chorion membranes which separate the fetus from the endometrium.[10]

Amnion, the inner layer of fetal membranes shows a thickness of 20–50µm and lines the amniotic cavity.[11] It consists of a monolayer of simple epithelium attached to a thick basement membrane and an underlying avascular stromal region that is further subdivided into compact, fibroblast and spongy layers.[12] The epithelium, the layer closest to the developing fetus, consists of a single layer of epithelial cells uniformly arranged on the basement membrane. The basement

membrane is a thin layer composed of collagens III and IV and non-collagenous glycoproteins laminin, nidogen, and fibronectin. The compact layer is a dense layer almost entirely devoid of cells and forms the main fibrous structure of the amnion. The fibroblast layer is the thickest layer of the amnion and consists of fibroblasts embedded in a loose collagen network with islands of non-collagenous glycoproteins. The outermost spongy layer forms the interface between the amnion and chorion, composed of a nonfibrillar meshwork of collagen III and an abundant content of proteoglycans and glycoproteins.[13] It is known to be non-immunogenic, to reduce inflammation, pain and scarring and provide a matrix for cell colonization as well as a natural biological barrier.[14]

It contains an array of growth factors, including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF), transforming growth factor α (TGFα) and transforming growth factor β (TGF β) , nerve growth factor (NGF) and hepatocyte growth factor (HGF), known to play critical roles in the physiological processes leading to normal wound healing and tissue regeneration.[11] It also contains anti-fibrotic, anti-inflammatory, anti-angiogenic, anti-microbial properties and immunomodulatory effect.[15]

Chorion is the outermost layer and is in contact with amnion on its inner aspect and the maternal decidua on its outer aspect. It is three to four times thicker than amnion. Chorion is composed of a reticular layer, basement membrane, and trophoblast layer, which is adhered to the maternal decidua. The reticular layer contacts the spongy layer of the amnion and forms a majority of chorion's thickness.

The reticular network is composed of collagens I, III, IV, V, and VI. The basement membrane anchors the trophoblasts to the reticular layer with collagen IV, fibronectin, and laminin. The trophoblast layer is the deepest layer, consisting of 2–10 layers of trophoblasts which contact with the decidua.[16]

There are various methods of processing hydrated and dehydrated amnion chorion membranes. According to literature both have variations on healing after application.[17] In the present study we are evaluating the effects of hydrated and dehydrated amnion chorion membrane on healing by using the periodontal ligament fibroblast cells as they secrete a variety of growth factors such as platelet-derived growth factor (PDGF), interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), connective tissue growth factor (CTGF), transforming growth factor (TGF-β) and fibroblast growth factor (FGF) which promote collagen synthesis and modulate the functions of immune cells, keratinocytes, endothelial cells and mast cells which help in wound healing.[18]

Hence, this study aims to process Hydrated and Dehydrated amnion chorion membrane and evaluate their effect on proliferation of Periodontal ligament fibroblast cells *in vitro*.

AIMS AND OBJECTIVES

AIM OF THE STUDY: -

To process and assess the efficacy of Hydrated amnion chorion membrane (HACM) and Dehydrated amnion chorion membrane (dHACM) on proliferation of Periodontal ligament (PDL) fibroblast cells.

OBJECTIVES OF THE STUDY: -

- To process and assess the efficacy of proliferation of Periodontal ligament fibroblast with Hydrated amnion chorion membrane.
- To process and assess the efficacy of proliferation of Periodontal ligament fibroblast with Dehydrated amnion chorion membrane.
- To compare the efficacy of proliferation of Periodontal ligament fibroblast treated with processed Hydrated and Dehydrated Amnion chorion membrane

REVIEW OF LITERATURE

Periodontal disease is a complex, multifactorial, chronic inflammatory disease, affecting the periodontal tissue due to the presence of local factors leading to further destruction. The main goal of periodontal treatment is to prevent further attachment loss and predictably restore the periodontal supporting structures that were lost because of disease. This occurs by sequential entry of macrophages that elaborate growth factors which regulate fibroblasts, smooth muscle cells and endothelial cells which will accelerate wound healing. Literature reviews the use of placental membranes to treat both chronic and acute wounds due to its inherent biologic properties as it increases the bioavailability of factors that enhance wound healing and propagate regeneration.

Various studies have reviewed the use of Amnion chorion membrane and its effect on different cell lines i.e., mesenchymal stem cells, human gingival fibroblasts and PDL fibroblasts. Under the guidance of certain biologic clues, the resident cells of the periodontium mainly fibroblasts actively participate in tissue repair and remodeling thus promoting tissue regeneration.

AMNION CHORION MEMBRANE

History:

The placenta is a simple source of biological membrane without major restrictions as it is considered a biological waste. With an estimated birth rate of 18.3 worldwide in 2019, this is a readily available biomaterial. The use of placental tissue for the treatment of wound started more than 100 years ago when Davis in 1910 first

used them as skin substitutes for treating open wounds. Sabella and Stern in 1913 described its use for burnt and ulcerated skin surfaces and reported the accelerative effect of the membrane on epithelialization and reduction in pain.

The human placenta is composed of two membranes: the amniotic membrane, which is in contact with the fetus and the outer chorionic membrane. Amnion is the inner layer with a thickness of 20–50µm and lines the amniotic cavity. It is composed of five layers, containing no vasculature. The amniotic epithelium rests on a basement membrane, followed by two layers of connective tissue – the compact, fibroblast and the spongy layer. The fibroblast layer is the thickest layer which consists of a loose fibroblast network with Hoffbauer cells which are the macrophages. Chorion is the outermost layer and is three to four times thicker than amnion. It is composed of a reticular layer, basement membrane, and trophoblast layer, which is adhered to the maternal decidua. The reticular layer is composed of collagens I, III, IV, V, and VI and the basement membrane anchors the trophoblasts to the reticular layer with collagen IV, fibronectin, and laminin.

Amnion chorion membrane was selected because it is easy to procure and is widely available. It has low cost of harvesting with good clinical outcomes and can be used as a scaffold for tissue engineering. It contains immunoregulatory factors such as HLA-G and Fas ligand, which have been linked to its low immunogenicity. It is also known to display anti-inflammatory, anti-microbial, anti-scarring, antifibrotic effect and enhances wound healing.

Properties:

Anti-microbial activity:

N. Kjaergaard et al (2001)[19] described the effects of amnion and chorion on bacterial growth in vitro. Five strains of Hemolytic streptococci group B, one strain of Hemolytic streptococcus group A, Staphylococcus aureus, Staphylococcus saprophyticus, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Acinetobacter calcoaceticus and Lactobacillus species were tested in blood-agar medium, transparent agar medium and nutrient broth medium. All strains of bacteria were inhibited, but the most pronounced inhibition was obtained for streptococcus group A, S. aureus and S. saprophyticus by both chorion and amnion membrane.

M Zare Bidaki et al (2017)[20] compared the anti-bacterial efficacy of amnion and chorion membrane on 8 bacterial strains using disk diffusion method on bacteriologic media and concluded growth inhibitory effects on 8 bacterial strains (E. coli, B cereus, K pneumonia, S pyogenes, P aeruginosa, S aureus, S flexneri, L plantarum) and the number of bacterial growth inhibition zone was more around chorion membrane than the amnion membrane.

H Ashraf et al (2019)[21] evaluated wound biomodification by assessing antimicrobial properties present within a human-derived composite amnion-chorion membrane with porcine-derived collagen membrane Bio-Gide®. The same number of colony-forming units per milliliter for each bacterial species (Aggregatibacter actinomycetemcomitans, Streptococcus mutans, and Streptococcus oralis) was inoculated on each of the paper discs with and without tetracycline. And found ACM

inhibited growth of all bacterial strains, similar to the negative control tetracycline discs.

Anti-inflammatory effects:

S Kothiwale et al (2018)[22] evaluated anti-inflammatory effect of chorion as a barrier membrane in periodontal pocket therapy by assessing interleukin-11 (IL-11) level in gingival crevicular fluid (GCF). The patients were randomly divided into two groups, flap surgery and flap surgery with chorion membrane placement. After Phase 1 therapy, clinical and biochemical parameters were recorded at baseline and at 4 weeks. Plaque index (PI), sulcus bleeding index (SBI), probing pocket depth (PPD), clinical attachment level and IL-11 in GCF were assessed using ELISA kit and concluded adjunctive use of chorion membrane in flap surgery provides an additive anti-inflammatory effect along with improvement in clinical outcomes enhancing the long-term prognosis.

Y Hao et al (2000)[23] conducted a study to identify the potential antiangiogenic and anti-inflammatory proteins expressed in human amniotic membrane tissue in which human amniotic epithelial and mesenchymal cells were isolated from human amniotic membranes by sequential trypsin and collagenase digestion. Antiangiogenic and anti-inflammatory proteins were detected by the reverse transcriptase-polymerase chain reaction (RT-PCR) technique and were confirmed by DNA sequencing of PCR amplified transcripts and concluded human amniotic membrane epithelial and mesenchymal cells express various antiangiogenic and anti-inflammatory proteins.

Wound healing:

McQuilling et al (2019)[5] examined the effects of dehydrated amnion and chorion (dACM) graft and investigated how factors released from this graft interact with cells important to the wound micro environment using in vitro models. Adult human fibroblasts were cultured in presence of assay media conditioned with dACM for 5 days at 4°C at a concentration of 1 cm^2 dACM membrane per milliliter (ml) of assay media, under standard conditions, and concluded the factors released from dehydrated amnion/chorion membranes (dACM) stimulated cell proliferation of fibroblast which are important for wound repair in vitro.

Koob et al (2014)[12] analyzed the Dehydrated amnion chorion membrane (dHACM) for extracellular matrix (ECM) composition through histological staining and growth factors content via ELISA. Histology of dHACM demonstrated preservation of native amnion and chorion layers with intact, nonviable cells, collagen, proteoglycans, and elastic fibers in individual layers. An array of 36 cytokines known to be involved in inflammation and wound healing were identified along with upregulation of basic fibroblast growth factor (bFGF), granulocyte colony-stimulating factor (GCSF), and placental growth factor (PLGF).

Koob et al (2014)[13] described PURION® Processed dehydrated human amnion/chorion membrane tissue allografts to support wound angiogenesis. It demonstrated that dHACM grafts contain angiogenic growth factors retaining biological activity; promote amplification of angiogenic cues by inducing endothelial cell proliferation and by upregulating production of endogenous growth factors and support the formation of blood vessels in vivo, and have potential to promote revascularization and tissue healing within poorly vascularized, non-healing wounds.

Processing and preservation:

For clinical use, amniotic membrane can be prepared and stored in following forms: Fresh storage, air-dried, cryopreserved and freeze-dried membrane. Fresh storage of the amniotic membrane uses 85% glycerol solution and are refrigerated at 4°C. Air-dried method uses electric controlled temperature oven at 60°C and laminar airflow for preservation followed by γ-radiation for sterilization. In cryopreservation, the membrane is stored in -80°C with glycerol and Dulbecco's modified eagle medium in a ratio of 1:1. Whereas, freeze-dried is a preservation method that removes water from tissue by sublimation and are sterilized with γ-radiation.

Tae Gi Kim et al (2018)[24] conducted a study to compare the effects of drying and fresh-freezing on human amniotic membrane (HAM) and amnion/chorion membrane (HACM) in terms of histological and structural characteristics and cytokine levels. HAM and HACM were dried, electron beam-irradiated or fresh-frozen. The ultrastructural characteristics of HAM and HACM were evaluated using light and transmission electron microscopes. The authors concluded that dehydration process maintained the histological structure of HAM/HACM and a variety of growth factors and cytokines were identified. HAM, processed with dehydration method, had a higher EGF level than that processed with the fresh-freezing method.

McQuilling et al (2017)[3] compared the growth factor and cytokine content of amnion and chorion layers and determined the effects of dehydration on them by proteomic analysis. Signaling molecules from tissue samples were evaluated using quantitative multiplex proteomics microarrays, and data were analyzed based on a per cm^2 basis and also on pg/mg of extracted protein for potency. Both dehydrated samples saw a significant drop in total growth factor and cytokine content. In conclusion, processing

of placental membranes can have a dramatic effect on total growth factor and cytokine.

Hamid et al (2014)[25] studied the effects of gamma radiation on human amniotic membrane following different preservation methods where morphological structure of human amniotic membrane was studied under scanning electron microscopy (SEM). The amniotic membrane was preserved by either air drying or submerged in glycerol before gamma irradiated at 15, 25 and 35 kGy. The surface morphology of glycerol preserved amnion was found comparable to the fresh amniotic membrane which were beautifully arranged, homogonous in size and tended to round up. The cell structure in air-dried preserved amnion seemed to be flattened and dehydrated. SEM revealed changes of cell morphology of glycerol preserved amnion were visible at 35 kGy while radiation doses lower than 25 kGy for sterilization did not affect the appearance of the preserved amnion.

PERIODONTAL LIGAMENT FIBROBLAST

Fibroblasts are mesenchymal cells that deposit collagen and elastic fibres of the ECM in connective tissue. The periodontal ligament (PDL) is a key contributor to the process of regeneration of the periodontium. Endothelial cells create a rich vascular network and are found throughout the PDL tissue along with fibroblasts, macrophages, undifferentiated adult/mesenchymal stem cells, and neural element. PDL fibroblasts are the main cellular component of the periodontium and represent a heterogeneous population of cells that express a rich repertoire of proteins. When compared to gingival fibroblasts, genes encoding transmembrane proteins and cytoskeleton-related proteins tend to be up-regulated in PDL fibroblasts.

Fibroblasts are present in healthy healing wound, from late inflammatory phase until full epithelialization has occurred. They are summoned to migrate to the wound area, proliferate and carry out a number of key activities under the tight regulation of injury mediated factors and progressively changing environment of the healing wound, which is critical to end state of the wound.

Patricio C. Smith et al (2019)[26] in their review article described the role of periodontal fibroblasts during wound healing and its function in restoring the structure of periodontal tissues. Wound closure is regulated by epithelial cells and connective tissue cells, and stated the role of gingival and periodontal fibroblasts in healing and repair of tissues is crucial and begins by organizing and synthesizing the collagen fibers of connective tissue promoting attachment. Thus, cell populations are needed for regeneration and actively functional fibers will restore the periodontium.

Wenjun Zhu et al (2015)[27] demonstrated the culture and identification of PDLSCs (PDL stem cells) in their review article and stated PDLSCs possess characteristics of MSCs. The culture media affects the biologic features of PDLSCs and those cultured in α- MEM (α minimum essential medium) had higher proliferation and osteogenic potential than those cultured in DMEM (Dulbecco's modified eagle's medium). Cumulative data suggests the sequential use of different types of growth factors seems to be effective for the differentiation of PDLSCs for periodontal regeneration.

Sarita Dabra et al (2011)[28] reviewed the role of growth factors, mode of action and molecular signaling pathways in periodontal pathologies. Growth factors participate in tissue repair by regulating proliferation, differentiation and mitotic activity of cells and extracellular matrix synthesis. The differentiation of PDL fibroblasts in specific tissue cell type is enhanced in presence of signaling molecules. The availability of

growth factors greatly improves the predictability of tissue engineering for regeneration, and the use of GFs will populate PDL space, root and bone surfaces with desired cell population required for formation of new attachment apparatus.

Giuseppe Polimeni et al (2006)[29] described factors influencing wound healing post periodontal surgery. The review compiled current scientific evidence and stated the biologic and clinical factors mainly the cells from PDL, primary wound closure, space maintenance and wound stability together contribute to successful periodontal regeneration. The periodontal wound healing and regeneration of lost periodontium is influenced by biomaterial used and the technique used to achieve periodontal regeneration.

MATERIALS AND METHODS

SOURCE OF DATA:

The study was conducted in the Department of Periodontics, KAHER'S KLE V.K Institute of Dental Sciences, Belagavi. An ethical clearance (Annexure I) was obtained before conducting the study from the Ethical Committee, KAHER'S KLE V.K Institute of Dental Sciences, Belagavi.

The amnion chorion membrane was obtained from the Department of Obstetrics and Gynaecology, KLE'S Dr. Prabhakar Kore hospital, Belagavi, based on the following inclusion and exclusion criteria for the study:

INCLUSION CRITERIA:

- Systemically healthy patients
- Patients with caesarean/normal delivery
- Age- 20 -35 years

EXCLUSION CRITERIA:

- Patients with any complications during delivery.
- Patients with premature delivery

STUDY DESIGN:

The following study was an In-vitro study.

The laboratory procedures were undertaken at KLE'S Dr. Prabhakar Kore's Basic Science Research Centre (BSRC), Belagavi. Primary cultures of human PDL fibroblast cells were obtained from freshly extracted premolar teeth. After which, sufficient numbers of cells were obtained by subsequent propagation.

The PDL fibroblasts cells were then exposed to two different groups for the in-vitro experiment:

- **HYDRATED (n=18):** PDL fibroblasts exposed to Hydrated amnion chorion membrane
- **DEHYDRATED (n=18):** PDL fibroblast exposed to Dehydrated amnion chorion membrane

PROCESSING OF HYDRATED AND DEHYDRATED AMNION CHORION MEMBRANE:

- A pilot study was done to process and store Hydrated and Dehydrated amnion chorion membrane in the Skin Bank, KLE's Dr Prabhakar Kore Hospital and KLE Society's Belgaum Cancer Hospital, Belagavi.

ARMAMENTARIUM USED IN STUDY

FOR PROCESSING HYDRATED AMNION CHORION MEMBRANE:

- Vials
- Plastic container
- Gown
- Head cap

- Surgical gloves
- BP Handle
- Surgical blade No. 11
- Plastic sheet
- 70% Isopropyl alcohol
- Pad's packet
- Steel scale
- Meshing board
- Non-toothed forceps
- Glycerol
- Normal saline
- Antibiotics- Penicillin G, Streptomycin, Neomycin, Amphotericin B
- Biosafety Cabinet/Laminar airflow
- Shaking Incubator
- Dulbecco's modified eagle medium (DMEM)

FOR PROCESSING DEHYDRATED AMNION CHORION MEMBRANE:

- Vials
- Plastic container
- Gown
- Head cap
- Surgical gloves
- BP Handle
- Surgical blade No. 11
- Plastic sheet

- 70% Isopropyl alcohol
- Pad's packet
- Steel scale
- Meshing board
- Non-toothed forceps
- Glycerol
- Normal saline
- Antibiotics- Penicillin G, Streptomycin, Neomycin, Amphotericin B
- Biosafety Cabinet/Laminar airflow
- Shaking Incubator
- Dulbecco's modified eagle medium (DMEM)
- Hot air oven
- Nitrocellulose paper
- Zip lock pouches
- Linear accelerator machine (X-ray radiation)

PROCEDURE FOR OBTAINING AMNION CHORION MEMBRANE:

Amnion chorion membrane was obtained from placenta of systemically healthy patients under sterile conditions immediately after caesarean/normal delivery from the Department of Obstetrics and Gynaecology, KLE's Dr. Prabhakar Kore hospital, Belagavi. The membrane was freed from umbilical cord and was sequentially washed free of blood clots under running tap water until it was cleaned properly. It was stored in saline solution inside a plastic container at 4°C in the Skin Bank, KLE's Dr Prabhakar kore hospital.

The ACM was then treated with 15 ml of balanced saline solution containing antibiotics (50µg/ml of Penicillin, 50µg/ml of Streptomycin, 100µg/ml of Neomycin and 2.5µg/ml of Amphotericin B) for 30 minutes in a plastic container in shaking incubator.

The membranes were then placed in the meshing board under laminar airflow where, they were divided into two equal parts using BP blade 11. One half of the membrane was used for preparation of Hydrated amnion chorion membrane and the other half was used for processing Dehydrated amnion chorion membrane.

PROCEDURE FOR PROCESSING HYDRATED AMNION CHORION MEMBRANE:

After the membranes, were divided into two equal halves under laminar airflow, one half of the ACM was placed using a non-toothed forceps in a sterile vial containing Dulbecco's modified eagle medium (DMEM) and glycerol (86%) in a ratio of 1:1 and was preserved at -80°C in KLE's Dr Prabhakar Kore Basic Science Research Centre (BSRC), Belagavi.

After storage, samples were sent to KLE's Dr Prabhakar Kore Basic Science Research Centre (BSRC), Belagavi for sterility test. It showed negative presence of microorganisms and was further stored.

PROCEDURE FOR PROCESSING DEHYDRATED AMNION CHORION MEMBRANE:

The other half of the ACM was spread in nitrocellulose paper using a tweezer. It was then allowed to dried in a hot air oven at 60°C for 40 minutes till the moisture

content of the membrane was reduced. The dried-membrane was also sent for sterility test to evaluate the bioburden level for colony forming units of bacteria. After the sterility test showed negative results, the membranes were sent for sterilization using X-ray radiation at 25 Gy for 10 minutes in KLE's Belgaum cancer hospital. And finally stored in sterilized pouches at room temperature.

PDL-FIBROBLASTS ISOLATION AND CELL-CULTURE:

Human PDL-fibroblast cells were cultured from the premolars extracted for orthodontic reasons. After extraction, tooth was placed in DMEM supplemented with 0.5 μL/ml of amphotericin, 200 μL/ml penicillin G, 200 μL/ml streptomycin and washed with Phosphate buffer saline (PBS). Under the laminar air-flow the tooth was removed from the bottle and was transferred to a sterile glass petri dish. The tooth was first washed with PBS in the petri dish and transferred to another petri dish to which 2ml of DMEM was added. The tissue over the mid-root surface was scrapped with a BP blade no. 22 attached to its handle and macerated into smaller portions.

The obtained tissue was seeded into a 24 well plate to which Fetal bovine serum (FBS) and DMEM supplemented with 0.5μL/ml of amphotericin B, 200μL/ml penicillin G, 200gL/ml streptomycin were added. These plates after the addition of the scraped tissue with the above mentioned was placed in the CO_2 incubator at 37^oC in a humidified atmosphere containing 5% CO_2 and allowed to incubate. The media was changed every 2 to 3 days and the cells were sub-cultured on acquiring sufficient confluency.

Once the cell growth was 60-70% confluent by about 4-6 weeks, it was subjected to the process of trypsinisation. The medium was removed from the wells completely and the cell layer was washed with PBS. To allow the cells to detach from the plate, 0.05% trypsin in Ethylenediamine tetra acetic acid (EDTA) buffer was added to the wells which was allowed to stand for 2-3 minutes. Then reaction was stopped by addition of complete media and the cell count was determined by using Neubauer's cell chamber, dilutions were prepared to get the required cell density. Cells were seeded in 96 well plates and were allowed to attach to the plate overnight.

PDL fibroblasts were subjected to Hydrated and Dehydrated amnion chorion membrane to perform the proliferation.

CELL PROLIFERATION:

- PDL fibroblast cell proliferation using hydrated and dehydrated amnion chorion membrane was determined by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

PRINCIPLE OF MTT ASSAY:

- MTT assay is a colorimetric assay which determines cell proliferation. The reduction of yellow MTT by mitochondrial succinate dehydrogenase is evaluated in this assay. MTT that enters the cells is taken up by the mitochondria and is reduced to an insoluble, colored (dark purple) formazan product. The crystals formed are solubilized with an organic solvent (i.e., DMSO). The solubilized formazan is measured spectrophotometrically at 570 nm. The amount of color

produced is directly proportional to the number of viable cells. Reduction of MTT takes place only in metabolically active cells, the level of activity is a measure of the viability of the cells, the amount of color produced is directly proportional to the number of viable cells.

PROCEDURE:

The cells were seeded at a concentration of 1 x 10^5 cell/well in 24 well plates and were allowed to attach overnight. The cells were treated with Hydrated amnion chorion membrane appropriately diluted with DMEM media and the other group was treated with Dehydrated amnion chorion membrane diluted with DMEM. The plate was kept for incubation for 24 and 48 hours in CO_2 incubator at 37^{o}C in a humidified atmosphere containing 5%CO_2. 20µl of 5 mg/ml MTT reagent was added to wells after 24 hours and 48 hours and the plate was kept for 4hr incubation in dark place at room temperature. (Aluminum foil was used as MTT reagent is photosensitive). The supernatant was carefully removed without disturbing the precipitated Formazan crystals and 100µl of DMSO was added to dissolve the crystals formed. The optical density was measured using spectrophotometer at a wavelength of 570 nm. The proliferation effect of Hydrated and Dehydrated amnion chorion membrane was assessed by spectrophotometric determination of color change due to conversion of MTT into "Formazan blue" by living cells.

The proliferation activity of PDL fibroblasts was assessed in the following groups:

Group 1 (Hydrated): PDL fibroblasts treated with Hydrated amnion chorion membrane

Group 2 (Dehydrated): PDL fibroblasts treated with Dehydrated amnion chorion membrane

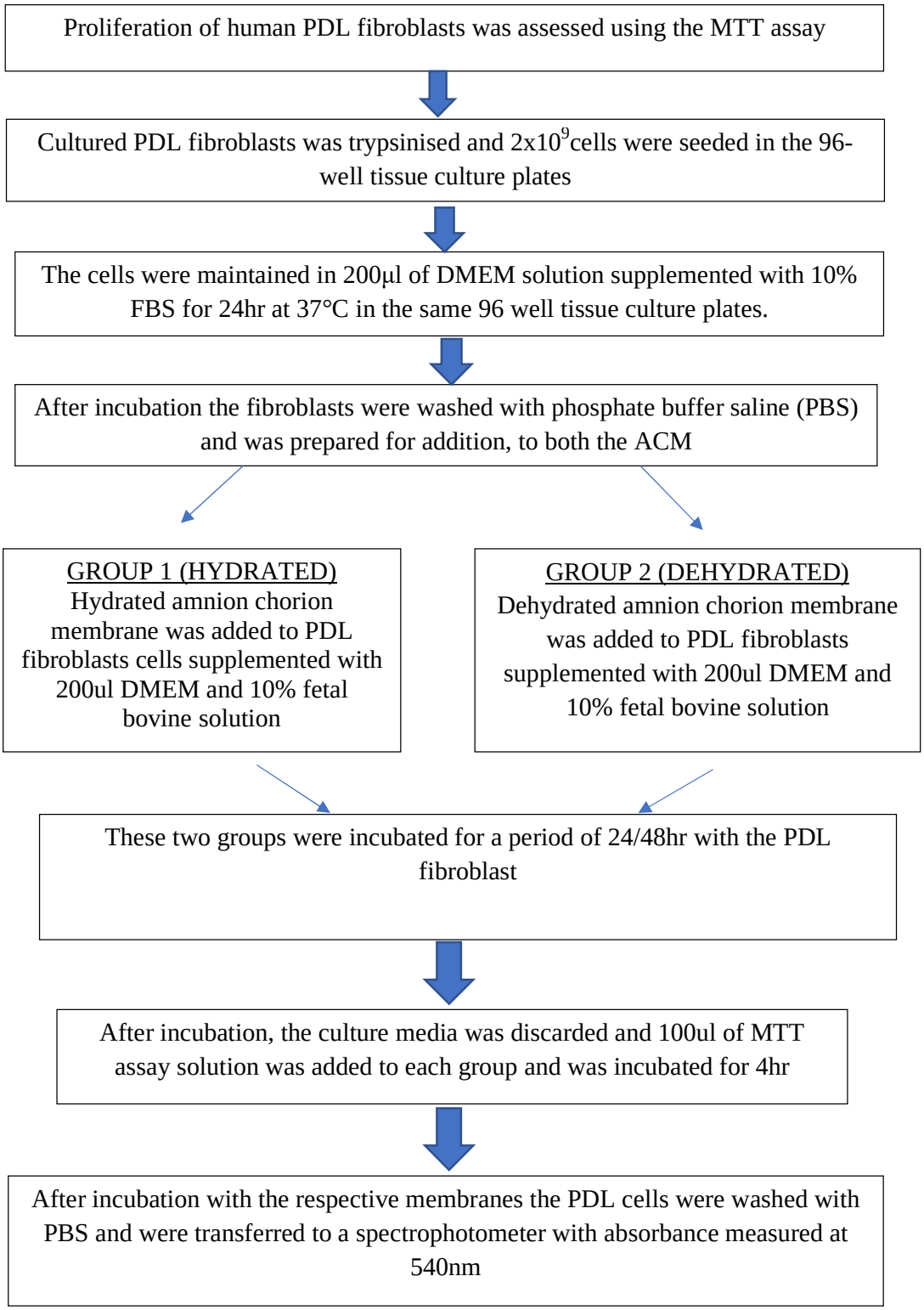
Negative control: PDL Fibroblasts treated with only DMEM.

The proliferation activity of PDL fibroblasts was compared to a standard group i.e., DMEM media only which served as a negative control and the effects of Group 1 (Hydrated) and Group 2 (Dehydrated) were compared with standard group.

The relative proliferation activity of PDL fibroblasts in Group 1 (Hydrated) and Group 2 (Dehydrated) were calculated as percentage of control. The % cell viability of negative group was taken as 100%. The results were calculated as follows: [Graph 1]

$$\frac{\text{(OD of groups)}}{\text{(OD of negative control)}} \times 100$$

PROLIFERATION ASSAY:



SATISTICAL ANALYSIS

The mean and standard deviation was calculated for the proliferation of PDL fibroblast for both Hydrated and Dehydrated groups at 24 and 48 hours.

Intra group comparison for Hydrated and Dehydrated groups at 24 and 48 hours was performed using Mann Whitney U Test, while the Inter group comparison for Hydrated and Dehydrated groups at 24 and 48 hours was performed using Mann Whitney U Test.

Probability value (p-value) of <0.05 was considered to be statistically significant.

The data was processed for statistical analysis using Statistical analysis software SPSS 20.0.

PHOTOGRAPHS

FOR PROCESSING OF HYDRATED AND DEHYDRATED AMNION CHORION MEMBRANE:

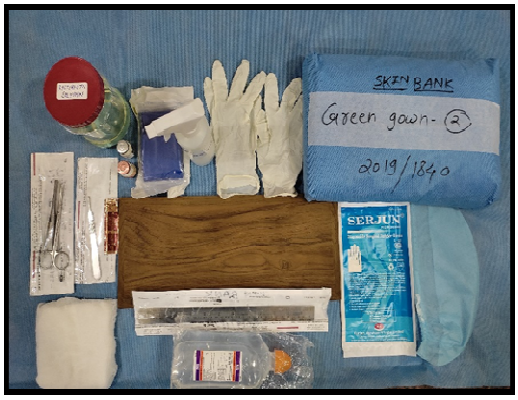


Figure 1: Armamentarium

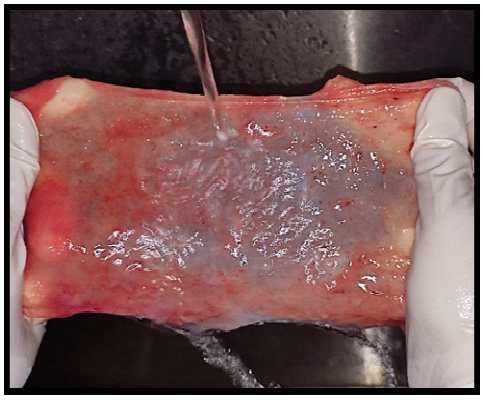


Figure 2: Washing of the amnion chorion membrane in running tap water

Figure 3: Shaking Incubator

Figure 4: Hot air oven

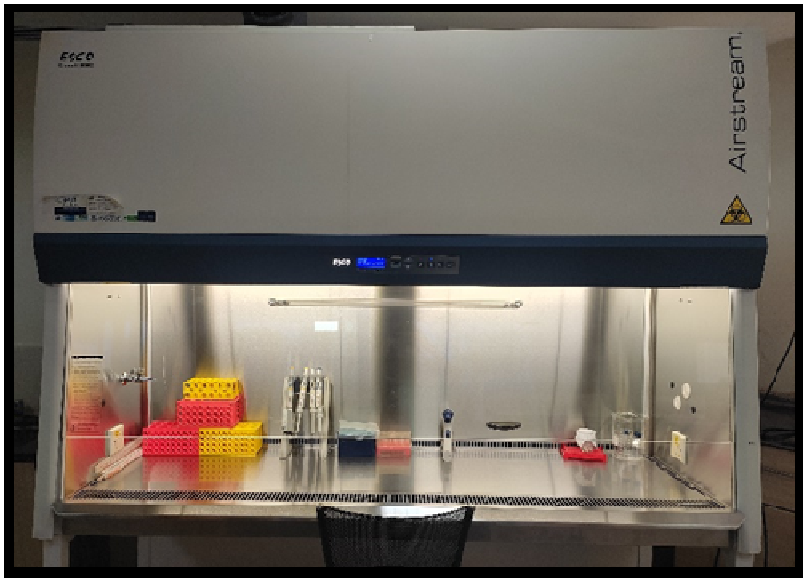


Figure 5: Laminar airflow/Biosafety cabinet

Figure 6: Linear accelerator machine for X-ray radiation

Figure 7: Processed Hydrated and Dehydrated amnion chorion membrane

FOR CULTURING OF PDL FIBROBLAST CELLS:

Figure 8: Collection of teeth

Figure 9: Cultured PDL fibroblasts under light microscope

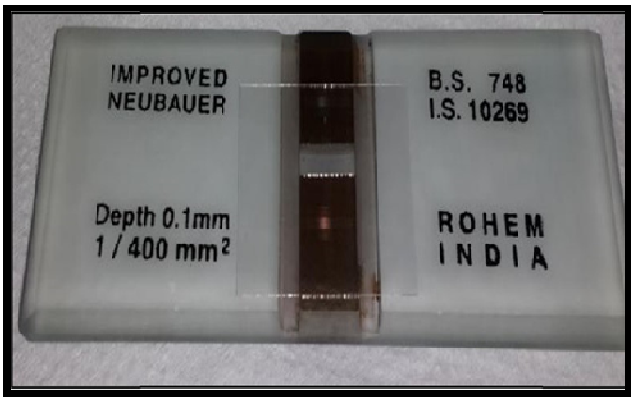
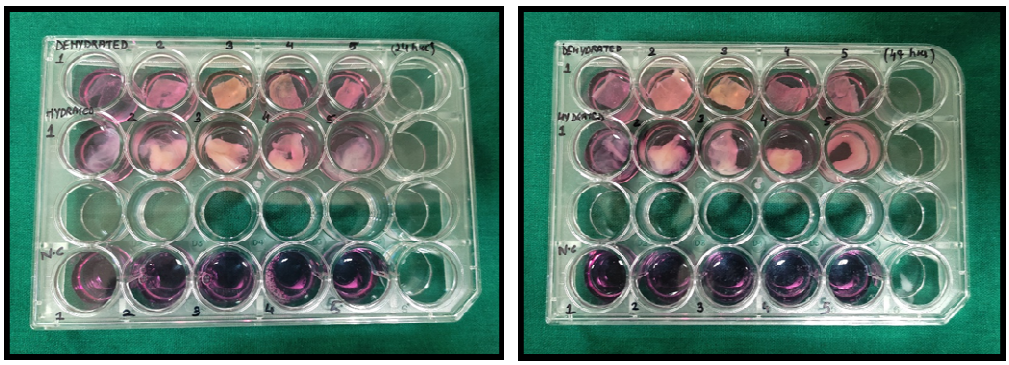


Figure 10: Neubauer's chamber used for cell counting



For 24hours **For 48 hours**

Figure 11: Incubation of the hydrated and dehydrated amnion chorion membranes

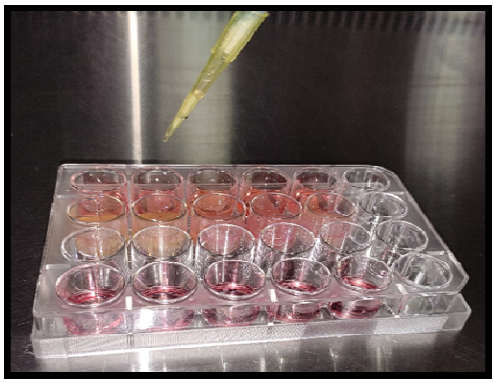


Figure 12: MTT reagent added after 24 and 48 hours

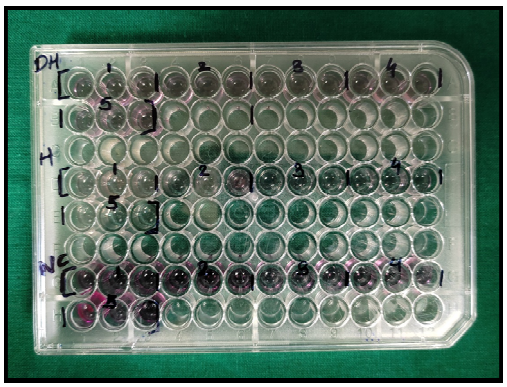


Figure 13:96 well plate to assess the proliferation of PDL fibroblast cells with ELISA reader

RESULTS

1. PROLIFERATION (MTT ASSAY)

TABLE 1: Mean and Standard Deviation of optical density of hydrated and dehydrated ACM at 24 hours (Graph 1)

GROUPS	MEAN	STANDARD DEVIATION (SD)
GROUP 1 (HYDRATED)	0.3438	0.36921
GROUP 2 (DEHYDRATED)	0.4032	0.23825
NEGATIVE CONTROL	0.368	0.076

Observations:

The mean value of optical density observed at the end of 24 hours for proliferation of PDL fibroblast in Group1, Group 2 and negative control group were 0.343±0.36, 0.403±0.23 and 0.368±0.07 respectively

TABLE 2: Comparison of optical density at 24 hours by Mann Whitney U test Between (Inter) groups:

HOUR	Groups	N	Mean	Std. Deviation	Std. Error Mean	Z value	p-value
24H **(INTER GROUP)**	HYDRATED	18	0.3438	0.36921	0.08702	-2.468	**0.014***
	DEHYDRATED	18	0.4032	0.23825	0.05616		

Observations:

*p value < 0.05 is statistically significant.

‡ Test applied – Mann Whitney U test for inter-group comparison

Z test in between Group 1 and Group 2 for intergroup comparison of Proliferation of PDL fibroblast at 24 hours; Z =-2.468; p – values < 0.05

The mean value of optical density observed at the end of 24 hours for proliferation of PDL fibroblast in Group1 was 0.343±0.36 and Group 2 was 0.403±0.23.

Inter-group comparison between Group 1 and Group 2 by Z test at 24 hours showed statistically significant improvement in the Group 2 ($p<0.05$) than that of Group 1.

TABLE 3: Mean and Standard Deviation of optical density at 48 hours (Graph 2)

GROUPS	MEAN	STANDARD DEVIATION (SD)
GROUP 1 (HYDRATED)	0.3404	0.11479
GROUP 2 (DEHYDRATED)	0.4022	0.20517
NEGATIVE CONTROL (NC)	0.368	0.076

Observations:

The mean values of optical density observed at the end of 48 hours for proliferation of PDL fibroblast in the Group1, Group 2 and NC were 0.340±0.11, 0.402±0.20 and 0.368±0.076 respectively.

TABLE 4: Comparison of optical density at 48 hours by Mann Whitney U test Between (Inter) groups:

HOUR	GROUPS	N	Mean	Std. Deviation	Std. Error Mean	Z value	p-value
48H (INTER GROUPS)	HYDRATED	18	0.3404	0.11479	0.02706	-2.341	**0.019***
	DEHYDRATED	18	0.4022	0.20517	0.04836		

Observations:

*p value < 0.05 is statistically significant.

[‡] Test applied –Mann Whitney U test for inter-group comparison

Z test in between Group 1 and Group 2 for intergroup comparison of Proliferation of PDL fibroblast at 48 hours; Z = -2.341; p – values < 0.05

The mean value of optical density observed at the end of 48 hours for proliferation of PDL fibroblast in Group1 was 0.340±0.114 and Group 2 was 0.402±0.205.

Inter-group comparison between Group 1 and Group 2 by Z test at 48 hours showed statistically significant improvement in the Group 2 ($p<0.05$) than that of Group 1.

TABLE 5: Comparison of optical density within (Intra) groups at 24 and 48 hours by Mann Whitney U test:

	HOUR	N	Mean	Std. Deviation	Std. Error Mean	Z Value	p-value
HYDRATED	24	18	0.3438	0.36921	0.08702	-2.025	**0.043***
	48	18	0.3404	0.11479	0.02706		
DEHYDRATED	24	18	0.4032	0.23825	0.05616	-1.962	**0.050***
	48	18	0.4022	0.20517	0.04836		

Observations:

*p value < 0.05 is statistically significant.

‡ Test applied –Mann Whitney U test for intra-group comparison

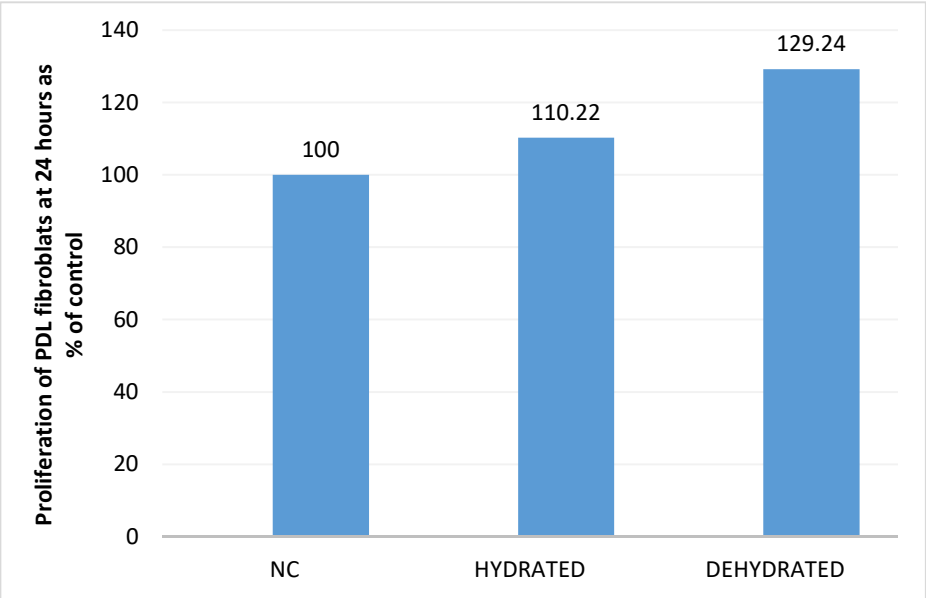
Z test within Group 1 for intragroup comparison of Proliferation of PDL fibroblast at 24 and 48 hours; Z = -2.025; p – values < 0.05. Whereas, Z test within Group 2 for intragroup comparison of Proliferation of PDL fibroblast at 24 and 48 hours; Z = -1.962; p – values = 0.05.

The mean value of optical density observed at the end of 24 and 48 hours for proliferation of PDL fibroblast in Group1 was 0.343±0.36 and 0.340±0.114.

The mean value of optical density observed at the end of 24 and 48 hours for proliferation of PDL fibroblast in Group 2 was 0.403±0.23 and 0.402±0.205.

Intra-group comparison at the end of 24 and 48 hours showed statistically significant improvement in the Group 1 and Group 2 ($p<0.05$).

GRAPH 1: Proliferation of PDL fibroblasts at 24 hours:



Observations:

*p=<0.05

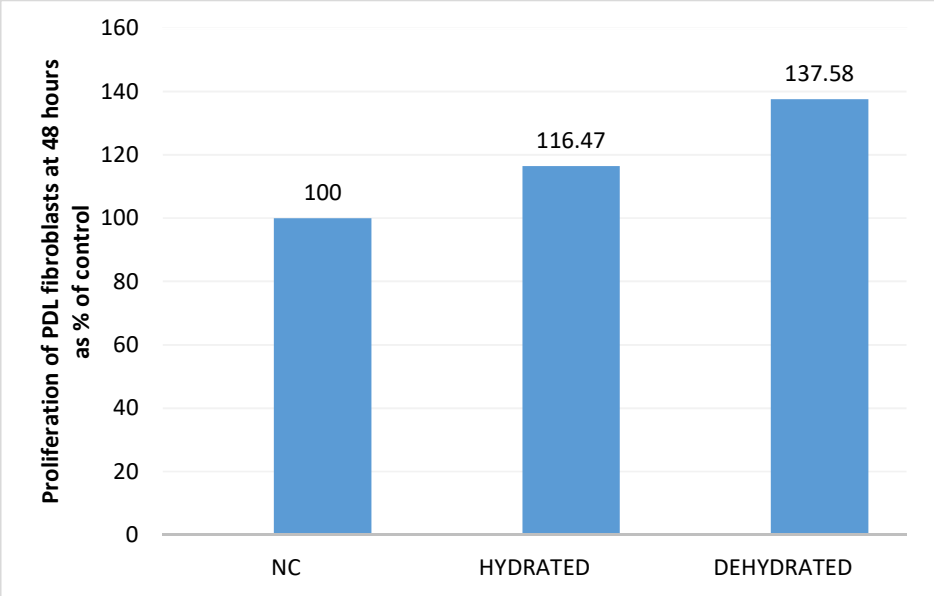
*Denotes statistically significant differences when compared to NC.

Graph 1 depicts the difference in proliferation rates seen after 24 hours in Hydrated and Dehydrated ACM in comparison to the negative control (NC) whose value was set at 100.

The rate of proliferation in PDL fibroblasts increased by 10% for hydrated ACM after 24 hours and 30% for dehydrated ACM when compared with Negative control group. At the end of 24 hours, dehydrated ACM showed 20% more proliferation in comparison to hydrated ACM.

Dehydrated ACM shows statistically significant difference from Negative Control after a time period of 24 hours.

GRAPH 2: Proliferation of PDL fibroblasts at 48 hours



Observations:

*p=<0.05

* Denotes statistically significant differences when compared to Negative Control.

Graph 2 depicts the difference seen after 48hours in hydrated and dehydrated ACM when compared to negative control (NC) which was set at 100.

The rate of proliferation in PDL fibroblasts increased by 16% for hydrated ACM after 48 hours and 38% for dehydrated ACM when compared with Negative control group. At the end of 48 hours, dehydrated ACM showed 22% more proliferation in comparison to hydrated ACM.

Dehydrated ACM showed statistically significant difference from Negative Control after a time period of 48 hours.

DISCUSSION

Periodontal disease is a chronic inflammatory condition which lead to destruction of the periodontal tissues. The ultimate goal of the periodontal therapy is to prevent further attachment loss and to restore the supporting tissue lost as a result of inflammatory periodontal disease.[30] A successful outcome of periodontal regeneration requires the following essential factors which include appropriate cells, signals, scaffolds, blood supply, mechanical loading, and pathogen control. The use of biologics such as recombinant human fibroblast growth factor (rhFGF)-2 produces clinically favorable wound healing.[31]

Wound healing is a dynamic process involving several types of cells, extracellular matrix, cytokines, and growth factors. Cytokines and growth factors secreted by macrophages induce the proliferation and migration of fibroblasts and endothelial cells into the wound site.[31] Fibroblast is the dominant cell of the connective tissue in the body producing PGE_2 and collagen which help in exacerbation of inflammatory disease state. The PDL fibroblast produces and maintains the connective tissue attachment which firmly anchors the tooth to alveolus.[3,4] The development and use of biomaterials such as amnion chorion membrane, enamel matrix derivative, collagen has supported the paradigm shift focusing on actual periodontal tissue regeneration.

Therefore, the present study was aimed to process and assess the efficacy of hydrated amnion chorion membrane and dehydrated amnion chorion membrane for proliferation of periodontal ligament fibroblast cells.

Amnion chorion membrane was selected based on its inherent properties of anti-inflammatory, anti-fibrotic, anti-microbial, anti-angiogenic, stimulation of cell proliferation and migration into the wound environment and increased production of extracellular matrix (ECM) allowing the wound to heal in a faster and an efficient way. Growth factors such as basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF), transforming growth factor β1 (TGF-β1) and various cytokines like IL-6 and chemokines, identified in amnion chorion membrane have been shown to be chemotactic for fibroblasts.[30]

Periodontal ligament fibroblast cells were selected because periodontal ligament is a key contributor to the process of periodontal regeneration and PDL fibroblasts are the main cellular components of periodontium representing a heterogenous population of cells which express signaling of proteins for wound healing.[32]

Studies have been conducted on amnion chorion membrane to evaluate its effect on cell behavior of fibroblasts. These studies have shown that amnion chorion membrane positively impacts the cellular activity and enhances cellular proliferation, migration, differentiation of human gingival fibroblasts, PDL fibroblasts and osteoblasts.[33]

To our knowledge, until now, there have been no studies conducted using comparison of hydrated and dehydrated amnion chorion membrane after processing on proliferation of PDL fibroblasts.

In this in-vitro study, hydrated and dehydrated amnion chorion membrane was processed and evaluated for proliferation of PDL fibroblast cells.

The study included a total of 18 systemically healthy patients with age between 20-35 years with normal/caesarean delivery who reported to the Department of obstetrics and gynecology, KLE'S Dr. Prabhakar Kore hospital, Belagavi. Systemically healthy patients with normal/caesarean delivery were selected fulfilling the eligibility criteria mainly to avoid any risk of transmissible infections such as human immunodeficiency virus, hepatitis virus types B and C, and syphilis due to inadequate aseptic preparation.[34] Reasons for mean age less than 35 years could be due to patient's systemic problems including diabetes, hypertension which may create complications such as pre-mature delivery of the fetus.

The present study compared the proliferative activity of PDL fibroblasts to a standard group i.e., DMEM media which served as a negative control (NC). The effects observed in Group 1 (Hydrated) and Group 2 (Dehydrated) were compared to the standard group. Proliferation of PDL fibroblasts was observed using MTT assay. The activity of the PDL fibroblasts were assessed after 24 hours and 48 hours respectively.

In our study, the proliferative activity of PDL fibroblast cells was checked after 24 hours and 48 hours because after 24 hours there is a cell attachment and stabilization and an increase in cell viability leading to a 2-fold increase in number of cells within 48 hours.[35]

At 24 hours, Group 1 (hydrated) showed statistically significant ($p= 0.043$) difference in the proliferation of periodontal fibroblasts than the Negative control as the hydrated amnion chorion membrane contains a number of growth factors and cytokines which aid in proliferation of PDL fibroblasts.

Whereas, at 24 hours, statistical difference was noted between Group 2 and the Negative control (p=0.050), and between Group 1 and Group 2 (p=0.014) as dehydrated group has greater retention of growth factors such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factor 2 (FGF-2), transforming growth factor α (TGF α) , platelet derived growth factors (PDGF), placental growth factor (PIGF), granulocyte-colony stimulating factor (GCSF) and various cytokines and chemokines which occur naturally in the native tissue. (Table 3,4,5)

After 48 hours, the difference between Group 1 and Negative control (p=0.043) and between Group 2 and Negative control (p=0.050), was statistically significant. However, at 48 hours, statistical difference between Group 1 and Group 2 was (p=0.019). (Table 3,4,5)

When the proliferation rate of fibroblasts was compared to the Negative control whose value was set at 100, proliferation noted in cells of Group 1 increased by 10% and those of Group 2 by 30% at the end of 24 hours. At 24 hours, Group 2 showed an increase in proliferation by 20% than Group 1 (Graph 1).

At 48 hours, Group 1 showed an increase by 16% and Group 2 by 38% when compared to the negative control. When noted at 48 hours, Group 2 showed an increase in proliferation by 22 % compared to Group 1. However, the results showed a statistical difference between the study groups 1 and 2. (p= 0.014 at 24 hours, p= 0.019 at 48 hours). [Table 4]

In this study, proliferation of PDL fibroblast was seen in hydrated ACM showing statistically significant proliferation rates ($p<0.05$) at 24 and 48 hours. This

was due to preserved viable cells such as growth factor and Extra cellular matrix (ECM). Duan-Arnold et al in 2015[36] reported in their study where-in tissue resident cells, ECM and growth factors were preserved and promoted fibroblast proliferation in hydrated/cryopreserved ACM.

The proliferation of PDL fibroblast cells in dehydrated ACM was also statistically significant ($p < 0.05$) at 24 and 48 hours. This could be attributed to the presence of growth factors, cytokines and chemokines in dehydrated ACM in light of the results observed by Koob et al in in-vitro conditions [2,13] where they found quantifiable levels of the growth factors such as platelet-derived growth factor-AA (PDGF-AA), PDGF-BB, TGFα, TGFβ1, bFGF, EGF, PLGF and granulocyte colony stimulating factor (GCS) along with presence of ILs 4, 6, 8 and 10, TIMPs 1, 2 and 4; which have known roles in regulating wound healing processes and have been chemotactic for dermal fibroblasts.

Similarly in another study by McQuilling et al in 2017[5] which confirmed the presence of variety of growth factors including bFGF, TGF-β1, PDGF, KGF, cytokines including IL-6 and extra cellular matrix proteins, chemotactic to dermal fibroblasts in dehydrated amnion chorion membrane.

Hilmy et al in 1997[37] reported precise processing techniques can have dramatic effects on the bioburden levels of the tissue being prepared for sterilization by irradiation. In the present study, the processing was carried out in aseptic environment, where bioburden test of the samples was negative. The dehydrated ACM was sterilized by X-ray radiation exposed to a dose of 25Gy using linear accelerator machine. After sterilization of the dehydrated membrane, it was also assessed for sterility.

Dhall et al in 2018[38] compared the structural, molecular and functional properties of viable lyopreserved amnion membrane (VLAM) with viable cryopreserved amnion membrane (VCAM) using in-vitro and in-vivo wound models and concluded that both VLAM and VCAM retain anti-inflammatory and pro-angiogenic properties that was corelated with faster closure of wound in diabetic mice. In-vitro experiments stated that both VLAM and VCAM have same clinical benefits in management of acute and chronic wounds. Whereas, Kim et al in 2019[24] studied the histological, structural and the level of cytokines in dehydrated ACM and hydrated/cryopreserved ACM discovered a wide array of growth factors and cytokines present in dehydrated ACM.

Within the limitations of the study, the properties of both hydrated and dehydrated amnion chorion membrane proved beneficial in proliferation of PDL fibroblast, however dehydrated ACM showed significant proliferation of PDL fibroblasts. Further, various assays can be incorporated in similar studies for evaluating the proliferation of PDL fibroblast in both the groups.

SUMMARY AND CONCLUSION

The present study was conducted with the aim to process and assess the efficacy of Hydrated amnion chorion membrane and Dehydrated amnion chorion membrane on proliferation of Periodontal ligament fibroblast cells. The study was conducted in Department of periodontics, KAHER's KLE V K Institute of Dental Sciences, Belagavi.

A total of 18 systemically healthy patients meeting the eligibility criteria were included in this study. The amnion chorion membrane from placenta was obtained from the Department of Obstetrics and Gynecology and were processed as hydrated and dehydrated based on different processing methods. Periodontal ligament fibroblast cells were cultured from the non-diseased premolars, extracted for orthodontic reasons in Basic science research center (BSRC). The cultured PDL fibroblast cells were exposed to Hydrated and Dehydrated amnion chorion membrane. The MTT assay was performed to assess the proliferation of PDL fibroblast cells. The proliferative activity of the PDL fibroblast cells were assessed after 24 hours and 48 hours using ELISA reader.

Intra-group comparison at 24 hours and 48 hours showed statistically significant proliferation of PDL fibroblasts in both hydrated and dehydrated amnion chorion membrane. Whereas, Inter-group comparison between hydrated and dehydrated amnion chorion membrane showed statistically significant proliferation of PDL fibroblasts at both 24 hours and 48 hours.

Within the limitations of the study, the following conclusions were made:

1. On Intra-group comparison at 24 hours and 48 hours, hydrated group showed statistically significant proliferation of PDL fibroblast cells. ($p<0.05$)
2. Intra-group comparison of the dehydrated group showed statistically significant proliferation of PDL fibroblasts at 24 hours and 48 hours. ($p<0.05$)
3. Inter-group comparison between hydrated and dehydrated group presented statistically significant proliferation of PDL fibroblasts at both 24 hours and 48 hours. ($p<0.05$)

Therefore, it can be concluded that hydrated and dehydrated amnion chorion membrane helps in proliferation of PDL fibroblasts. This was ascribed due to presence of growth factors such as basic fibroblast growth factor (bFGF), transforming growth factor β1 (TGF-β1), and platelet derived growth factor (PDGF) in amnion chorion membrane have shown to be chemotactic for fibroblast proliferation which helps in wound healing.

Within the limitations of the study, the properties of both hydrated and dehydrated amnion chorion membrane proved beneficial in proliferation of PDL fibroblast, however dehydrated ACM showed significant proliferation of PDL fibroblasts. Further, studies on large samples and various assays incorporating in similar studies are essential to evaluate the proliferation of PDL fibroblast in both the groups.

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ANNEXURES:

ETHICAL CLEARANCE CERTIFICATE

Research and Ethics Committee
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Sl. No. : 1487

CERTIFICATE

This is to Certify that the synopsis titled

Processing of Hydrated Amnion Chorion Membrane from Placenta and compare with Dehydrated Amnion Chorion membrane for the proliferation of Periodontal ligament fibroblast Activity – An Invitro Study *submitted by*

Dr. **REG. NO. IK0220002** *P. G. Student /*

Staff, Guided by *rom Department of*

Periodontics. *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date : 5/5/21

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

BIOSTATISTIC CLEARANCE CERTIFICATE

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Biostatistics Clearance Certificate

This is to certify that the Biostatistics aspect of the Dissertation / Research work of REG. NO. IK0220002 ıst Graduate Student, under the guidance of Professor, Department of Periodontics entitled **"Processing of hydrated amnion chorion membrane from placenta and comparing with dehydrated amnion chorion membrane for the proliferation of periodontal ligament fibroblast activity– an invitro study."** has been done under my guidance and considered satisfactory.

Place: Belagavi
Date: 13/12/22

Dr. T. B Prasad
Name & Signature of Biostatistician

PLAGIARISM CHECK REPORT

Scientific Correspondence and Review Committee

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Date : 26/12/2022 **Serial No.:** 141

PLAGIARISM CHECK REPORT

REG. NO. IK0220002

Name of the Applicant :
UG / PG / Ph.D / Staff : Post graduate student
Batch & Year : 2020 – 2023
Department : Periodontics

The soft copy of Research Work / Manuscript by **REG. NO. IK0220002** ..... entitled

"Processing of hydrated amion chorion membrane from placenta and comparing with dehydrated amnion chorion membrane for the proliferation of Periodontal ligament fibroblast activity – An invitro study"

under the guidance of .. ..has been submitted for Anti-Plagiarism check to the Scientific Correspondence & Review Committee of KLE VK Institute of Dental Sciences using "Turn-it-in" software.

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