EVALUATION OF HUMAN AMNIOTIC MEMBRANE AS A SCAFFOLD FOR PERIODONTAL TISSUE ENGINEERING: AN *IN VITRO* STUDY

by

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LIST OF ABBREVIATIONS

α-MEM	Alpha modified eagle's medium
µl	Microlitre
Adj. Sig.	Adjusted significance
ADSCs	Adipose derived stem cells
APCs	Alveolar periosteal cells
APCs	Apical papilla cells
BMMSCs	Bone marrow mesenchymal stromal cells
BMP	Bone morphogenic protein
CAL	Clinical attachment loss
CO_2	Carbon dioxide
DAPI	6-diamidino-2-phenylindole
DFDBA	Demineralized freeze dried bone allograft
DMSO	Dimethyl sulfoxide
DPSCs	Dental pulp stem cells
ECM	Extracellular matrix
ECs	Endothelial cells
EMD	Enamel matrix derivatives
FBS	Fetal bovine serum
GTR	Guided tissue regeneration
HA	Hydroxyapatite
hADSC	Human adipose tissue-derived stromal cells
HAM	Human amniotic membrane

HAM-DE	de-epithelialized HAM
HAM-E	HAM with epithelial layer attached
HMDS	Hexamethyldisilizane
HPDLFs	Human periodontal ligament fibroblasts
IGF	Insulin like growth factor
IL	Interleukin
LASERS	Light Amplification by Stimulated Emission of Radiation
LEC	Limbal epithelial cell
ml	Millilitre
MMPs	Matrix metalloproteinases
MSCs	Mesenchymal stem cells
NADPH	Nicotinamide adenine dinucleotide phosphate
PBS	Phosphate buffered saline
PDGF	Periodontal derived growth factor
PDL	Periodontal ligament
PDLSCs	Periodontal stem cells
Pen Strep	Penicillin streptomycin
PGA	Polyglycolic acid
PLA	Polylactic acid
PLGA	Poly (lactic-co-glycolic acid)
PMNs	Polymorphonuclear neutrophils
PPD	Probing pocket depth
PRP	Platelet-rich plasma
RFU	Relative fluorescence unit

ROI	Region of interest
SCAPs	Stem cells from apical papilla
SEM	Scanning electron microscopy
Sig.	Significance
Std. Error	Standard error
TCP	Tricalcium phosphate
TGF	Transforming growth factor
TNF-α	Tumor Necrosis Factor Alpha
UK	United Kingdom
USA	United States of America
USM	Universiti Sains Malaysia
UV	Ultraviolet

PENILAIAN MEMBRAN AMNION MANUSIA SEBAGAI PERANCAH UNTUK KEJURUTERAAN TISU PERIODONTAL: KAJIAN IN-VITRO

ABSTRAK

Membran amnion manusia (HAM) mempunyai sifat-sifat biologi bersesuaian untuk penjanaan semula tisu periodontal seperti tindak balas imunogenik rendah, anti-fibrosis, anti-radang dan kaya dengan komponen matriks luar sel. Kajian ini bertujuan untuk menilai keupayaan membran ini sebagai perancah untuk pertumbuhan sel utama dalam tisu periodontal, iaitu sel fibroblas ligamen periodontal manusia (HPDLFs).

HPDLFs yang diperolehi secara komersial (Lonza, USA) telah dikulturkan di atas HAM yang diawet gliserol (USM Tissue Bank, Malaysia). Pelekatan dan morfologi permukaan HPDLFs telah dilihat melalui analisa histologi dan mikroskop imbasan elektron (SEM) masing-masing. Manakala kadar pembiakan sel pada hari 1, 3, 7, 14 dan 21 dinilai dengan menggunakan ujian alamarBlue® dan pelabelan nuklear DNA menggunakan 6-diamidino-2-phenylindole (DAPI).

Hasil penelitian histologi, menunjukkan HPDLFs membiak dari satu lapisan sel kepada beberapa lapisan sel pada membran amnion dari hari pertama hingga ke-7. Pada hari 14 dan 21, lapisan sel HPDLFs telah dikurangkan kepada lapisan sel tunggal dengan penampilan yang lebih leper dan sel-sel berbentuk gelendong yang lebih panjang. Analisa SEM menunjukkan pelekatan HPDLFs telah berlaku dengan baik pada membran amnion dari hari pertama hingga ke-3 dan seterusnya berlaku pertindihan pada hari ke-7. Dalam tempoh tersebut sel mengekalkan bentuk leper mereka. Namun, pada hari ke-14 dan 21, sel-sel telah menunjukkan perubahan pada morfologi dan kemudian bertukar menjadi bentuk bulat. Berdasarkan analisa statistik (*Friedman's Two-Way Analysis of Variance*

oleh *Ranks* diikuti dengan perbandingan cara berpasangan) menggunakan SPSS 22.0, ujian pembiakan menunjukkan kebolehan sel membiak pada membran amnion telah meningkat dengan ketara dari hari pertama hingga ke-7 (p=0.002). Tetapi, kadar pembiakan sel menunjukkan pengurangan yang ketara pada hari 14 (p=0.002) dan hari 21 (p=0.005). Pewarnaan DAPI pada nuklear DNA telah menunjukkan kehadiran HPDLFs hingga hari ke-7 sahaja.

Keputusan kajian ini menunjukkan bahawa membran amnion mampu berfungsi dengan baik sebagai perancah untuk HPDLFs dalam tempoh 7 hari. Kerencatan pertumbuhan sel kemudiannya mungkin disebabkan oleh beberapa perkara seperti penyekatan persandaran ketumpatan pertumbuhan atau pelepasan *matrix metalloproteinase* oleh HPDLFs yang mungkin telah mendegradasikan membran.

Kesimpulannya, penemuan ini mencadangkan bahawa membran amnion manusia mempunyai potensi sebagai perancah untuk penjanaan semula tisu periodontal. Walau bagaimanapun, perilaku sel yang berhubungkait dengan membran dalam kultur untuk tempoh yang lebih lama memerlukan penelitian lanjut.

EVALUATION OF HUMAN AMNIOTIC MEMBRANE AS A SCAFFOLD FOR PERIODONTAL TISSUE ENGINEERING: AN *IN VITRO* STUDY

ABSTRACT

Human amniotic membrane (HAM) has many biological properties suitable for periodontal tissue regeneration such as low immunogenicity, anti-fibrosis, anti-inflammation and rich in extracellular matrix component. This study aimed to evaluate the ability of this membrane as a scaffold for the growth of the predominant cells in periodontal tissues, human periodontal ligament fibroblasts (HPDLFs).

Commercially available HPDLFs (Lonza, USA) were seeded on glycerol preserved HAM (USM Tissue Bank, Malaysia). HPDLFs attachment and surface morphology were observed through histological analysis and scanning electron microscopy (SEM) respectively. While the cell proliferation was assessed using alamarBlue® proliferation assay and nuclear labeling of DNA using 6-diamidino-2-phenylindole (DAPI) at day 1, 3, 7, 14 and 21.

Histologically, HPDLFs showed mono layer to multilayers attachment on HAM from day 1 to day 7. On day 14 and 21, HPDLFs cell layers were reduced to single cell layer with more flattened appearance and longer spindle shaped cells. SEM analysis demonstrated that HPDLFs had attached appropriately on HAM surface at day 1 to day 3 and became overlapping at day 7, while maintaining their flat shape. However, by day 14 and 21 the cells demonstrated alteration in their morphology and later became rounded in shape. Based on statistical analysis (Friedman's Two-Way Analysis of Variance by Ranks

followed by pairwise comparison) using SPSS 22.0 proliferation assay showed that HPDLFs viability on HAM had increased significantly from day 1 to day 7 (p=0.012). However, the proliferation of cells showed significant reduction at day 14 (p=0.002) and day 21 (p=0.005). DAPI staining of nuclear DNA showed the presence of HPDLFs up to day 7 only.

This study showed that HAM is able to function well as a scaffold for HPDLFs within 7 days. Retardation of cellular growth after 7 days could be due to possible reasons such as density dependent inhibition of growth or the release of matrix metalloproteinases by the HPDLFs that might have degraded the membrane.

In conclusion, the findings suggest that HAM could be a promising scaffold for periodontal regeneration. However, cells' behaviour in relation to the membrane over longer culture duration requires further investigations.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Periodontitis is the 6th most prevalent health condition in the world (Kassebaum *et al*., 2014). It is a microbial-induced inflammatory condition of the periodontium resulting in the loss of the structures supporting and surrounding the tooth. These structures include alveolar bone, cementum, periodontal ligament and gingiva. If the condition is left untreated eventually, it will lead to tooth loss (Han *et al.*, 2014). To halt the on-going disease process, nonsurgical periodontal therapy such as scaling and root planing are carried out to disrupt bacterial biofilm and limit the disease progression. Although, these conventional therapies may avert the disease process, they are unlikely able to restore or rebuild the affected complex tri-phasic interphase of cementum, bone and periodontal ligament.

The main goal of periodontal therapy has always been to regenerate the loss of the diseased periodontal structures, to maintain its form and function (Nakahara, 2006). To achieve this goal, regenerative approaches including guided tissue regeneration (GTR) with membrane and/ bone graft placement, use of growth factors, and enamel matrix derivatives (EMD) (Esposito *et al.*, 2009) have been introduced. However, the results of these approaches are frequently associated with uncertain or poor clinical predictability (Esposito *et al.*, 2009; Chen *et al.*, 2010). Therefore, new strategies are needed to overcome the challenges.

In 1993, Langer and Vacanti had proposed tissue engineering as a promising alternative for restoring or reconstructing lost tissue (Langer and Vacanti, 1993). Tissue engineering aims to restore, regenerate or improve the defective or damaged tissues due to various disease conditions. It uses the principle of *de novo* 'engineering' of tissue constructs either *ex vivo* (extra corporeally) or *in situ* (intra corporeally). The engineering process utilizes the regenerative capacity of stem cells and their proliferation/ differentiation within a three-dimensional (3D) framework (scaffold) (Semenov, 2011). Since then, the concepts of tissue engineering, stem cell research, gene therapy and therapeutic cloning have vastly revolutionized the modern therapeutics (Koh and Atala, 2004; Rodriguez-Vazquez *et al.*, 2015). Presently, these modern concepts and principles are applied in regenerative dentistry to regenerate the periodontal structures.

Tissue engineering involves the use of desired regenerative cells that are cultured in laboratories over a substrate as two-dimensional (2D) cell sheet or three-dimensional (3D) structure and then transferred to the defect site to enhance the regenerative process. Apart from cellular strategies in periodontal regeneration, the use of bioactive scaffolds (Shimauchi *et al.*, 2013) alone for mechanical support or as carrier for regenerative cells with or without growth factors are also being used. The recent advancements in the scaffold design and biomaterials have enhanced the present capabilities of scaffolds to recapitulate the natural extracellular matrix (ECM) function both temporally and spatially. Many natural, synthetic, semi synthetic and hybrid scaffolds have been constructed and applied for tissue regeneration (Causa *et al.*, 2007).

Amniotic membrane is a well reputed natural biomaterial and has been in use for various medical procedures for over a century (Niknejad *et al.*, 2008). It is the inner most

layer of the fetal membrane and completely enfolds the embryo, demarcating the amniotic cavity. The reasons for its diverse applications apart from being easily available and cheap is, its exceptional biological properties. These include anti-inflammatory, anti-scarring, anti-microbial (Chopra & Thomas, 2013), anti-angiogenic, promoter of epithelialization (Mamede *et al*., 2012), and anti-carcinogenic properties (Niknejad *et al*., 2014). Over the past few decades, with the advancements in tissue engineering, biomaterial sciences and scaffold designs, this 'miracle' membrane was introduced into periodontal tissue engineering (Litwiniuk and Grzela, 2014).

Adachi *et al.* (2014a; 2014b) demonstrated that periodontal cells cultured on human amniotic membrane (HAM) substrate express essential proteins for cell-substrate adhesion and maintaining tissue integrity. HAM also showed good adhesion with periosteal-derived cell sheet (Amemiya *et al.*, 2014) and dental pulp-derived cell sheet (Honjo *et al.*, 2014). Iwasaki *et al.* (2014) used rat periodontal model (by surgically creating periodontal defects into first maxillary molars of rats) and transplanted periodontal ligament stem cells (PDLSC)-amnion into these sites showing enhanced periodontal regeneration.

Despite the advances in tissue engineering, the ideal scaffold that is cheaper, easily available and has all the biophysical and biochemical properties necessary for periodontal reconstruction is yet to be established. With the best of our knowledge, lack of data available on the efficacy of HAM as a scaffold for periodontal ligament fibroblast culture. Therefore, in this study we have evaluated the efficacy of this membrane as a scaffold for human periodontal ligament fibroblasts (HPDLFs) for future use in periodontal tissue engineering.

1.2 Justification of study

In order to regenerate periodontium we need a good biocompatible scaffold that would integrate and support the growth of regenerative cells while providing mechanical strength. Stem cell based-periodontal tissue regeneration is becoming extensively studied domain due to its remarkable results in pre-clinical studies (Iwasaki *et al.*, 2014; Wu *et al.*, 2016). This technique can be expensive and time consuming as it requires laboratory procedures and expensive scaffolds.

In the past few years much advancement has been made to search for the ideal scaffold which, among many benefits, should be cost effective and easily available. On this advantage, amniotic membrane is being investigated. Its ability to harness the cells of regenerative capacity has been established in a study by Adachi *et al*. (2014). In another study, its regenerative capability as a scaffold in rat periodontal models has also been demonstrated (Iwasaki *et al*., 2014). However, none have investigated the ability of HAM to attach and proliferate the periodontal fibroblasts, which are the predominant cells of periodontal tissue and play key role in its repair and regeneration.

This research will help us get one step closer to our goal of clinical transition by providing an alternate source of scaffolds which will be locally procured and processed, and introduce amniotic membrane in periodontal clinical practices. With the best of our knowledge, there are very limited data available on the use of HAM as scaffolds for HPDLFs.

1.3 Objectives of the study

1.3.1 General objective

To evaluate the efficacy of human amniotic membrane as a scaffold for human periodontal ligament fibroblasts cell growth.

1.3.2 Specific objectives

i. To evaluate the attachment of cultured human periodontal ligament fibroblasts on human amniotic membrane histologically by using hematoxylin and eosin staining.
ii. To determine the proliferation rate of the cultured human periodontal ligament fibroblasts on human amniotic membrane using alamarBlue® proliferation assay.
iii. To evaluate the surface morphology of the cultured human periodontal ligament fibroblasts on human amniotic membrane using scanning electron microscopy.

1.4 Research question

1. Is there any difference in the attachment and proliferation level of HPDLFs seeded on HAM on different observation days?

1.5 Null hypothesis

1. There is no difference in the attachment and proliferation level of HPDLFs seeded on HAM on different observation days.

CHAPTER 2

LITERATURE REVIEW

2.1 Development of periodontium

Through the understanding of the development and formation of periodontal tissues we can formulate mechanisms for repair and regeneration by retracing the footsteps of development (Bartold *et al.*, 2000).

During the first three weeks of embryonic development cells undergo rapid proliferation and migration. By about 8 days, cells are differentiated into two distinct layers: endoderm and ectoderm. By the third week, another layer is formed between the endoderm and ectoderm, that is, the mesoderm. In the next few weeks the ectoderm thickens to form raised margins called neural folds which later fuse to form the neural tube. The cells at the lateral borders or crest of this neuroectoderm dissociate from their neighbours to form the neural crest cells. Most of the dental structures originate from cells derived from these neural crest cells. These structures include dentine and cementum and the supporting structures of the tooth (periodontal ligament and the alveolar bone). On the other hand, the enamel is formed from the cells which originate from the ectoderm.

The tooth development begins by the sixth week of embryogenesis starting from the thickening of the oral epithelium lining the future sites of dental arches to form dental lamina. Morphologically, the tooth development is divided into three stages, namely, bud, cap and bell stage (Figure 2.1). In the bud stage, the epithelial cell of dental lamina begins to invaginate into the underlying connective tissue. Continuous proliferation of epithelium

forms a parabolic or cap like structure. At this stage, enamel organ is at its earliest stage of development. Underneath the epithelial cap, the mesenchymal cells proliferate to form the dental papilla. The dental papilla will later on form the dentine and the dental pulp. The dental papilla continues to encapsulate the enamel organ to form the dental follicle from which the future root cementum, periodontal ligament and alveolar bone will develop. During the bell stage, the enamel organ continues to grow and form bell like structure with four types of cells. The outer enamel epithelium forms the outer portion of enamel organ. The inner enamel epithelium lines the inner portion of enamel organ, which will eventually differentiate into ameloblasts and form enamel. Stratum intermedium lines the inner enamel epithelium, and stellate reticulum lies between stratrum intermedium and outer enamel epithelium.

Once the bell stage is complete, crown formation begins. Odontoblasts deposit dentine and lay the foundation of matrix for the ameloblasts to differentiate into secretory cells and produce enamel matrix. After the enamel and dentine formation is nearly completed at future cementoenamel junction, the root formation begins.

After crown development is complete, the outer and inner enamel epithelium form bilayer cell structure called Hertwig's epithelial root sheath which separates the dental papilla from dental follicle. The cells of the inner enamel epithelium induce the adjacent cells of dental papilla to form odontoblasts which deposit root dentine. The Hertwig's root sheath disintegrates into fragments, but never completely disappears and leaves behind remnants called epithelial cell rests of Malassez. After the disintegration of Hertwig's root sheath, the dental follicle cells attach and align onto the matrix coating dentine surface. These dental follicle cells differentiate into cementoblasts and form the root cementum.

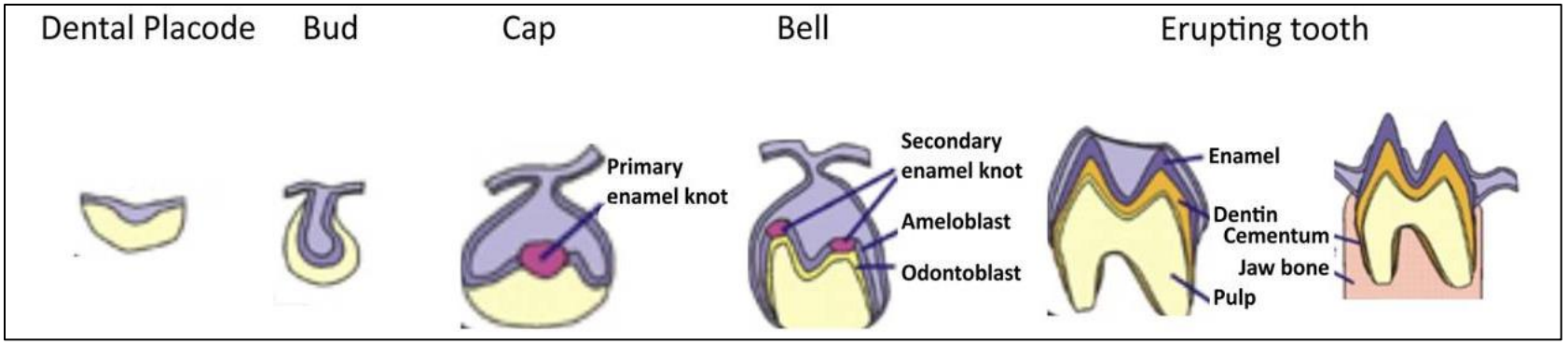

Figure 2.1 Stages of tooth development, adopted from Bei (2009)

The cementum deposition occurs during tooth development and also after eruption during normal functions. Cementoblasts, cementocytes and fibroblasts deposit cementum, while cementoblasts are the primary source (Schroeder, 1992; Bosshardt and Schroeder, 1996). These cells are located within the periodontal ligament and have their origin from dental follicle cells. Moreover, the mesenchymal cells in the periodontal ligament have the potential to differentiate into periodontal ligament fibroblasts and cementoblasts.

The periodontal ligament is mainly produced by the fibroblasts. These periodontal ligament fibroblasts have their origin from dental follicle cells and differentiate during root development. As the root is being formed, the cementoblasts deposit cementum on the root surface, and the fibres of periodontal ligament fibroblasts (PDLFs) appear. The fibres are inserted into the cementum and the alveolar bone, and become mature as the tooth erupt.

The alveolar bone (bone lining the tooth socket) is formed during root formation and is derived from cells called osteoblasts which have their origin in dental follicle. The gingival tissue is formed from both ectodermal origin, forming the superficial epithelium, and mesodermal origin, forming the underlying connective tissue. The non-keratinized junctional epithelium of gingiva has origins in enamel organ. Whereas, both the non-keratinized sulcular epithelium and the keratinized gingival epithelium has oral mucosal origin (Bartold and Narayanan, 1998).

2.2 Periodontal tissue and its biology

Periodontium is the supporting structure of the tooth comprising gingiva and the attachment apparatus. The attachment apparatus is composed of alveolar bone, periodontal

ligament (PDL) and cementum (Newman, 2011) (Figure 2.2). It forms a biological and functional tissue which corresponds to age or environmental changes.

The outer surface of periodontium is formed by the gingiva. It covers the alveolar bone and the tooth root just coronal to cementoenamel junction and acts as a barrier to mechanical and microbial damage. Anatomically, it is divided into marginal or unattached, attached and interdental papilla (Newman, 2011).

A gingival sulcus is present in the form of shallow crevice or space surrounding the tooth on one side and the epithelial lining of the marginal gingiva on the other. Clinically this space can be measured using a metallic instrument called periodontal probe. In clinically normal gingiva, this probing depth is 1 to 3 mm (Highfield, 2009). This is one of the most important clinical parameters used to evaluate the periodontal status.

Histologically, gingiva consists of gingival epithelium and the gingival connective tissue. The gingival epithelium is formed by continuous lining of stratified squamous epithelium. Morphologically and functionally, gingival epithelium is divided into three areas. The oral or outer epithelium, sulcular epithelium and the junctional epithelium. Apart from its function as a barrier, the gingival epithelium, also helps in selective interchange with oral environment.

The gingival connective tissue is formed of collagen fibers (88.4%), fibroblasts (8.4%), blood and lymph vessels (7.3%), edema (4.9%) and residual tissues (unidentified cells and nerves) (2.7%) (Daniel and Dupont, 1980). The connective tissue has cellular and extracellular components composed of fibers and ground substance. While the ground substance is mainly composed of water, it also has proteoglycans (mainly hyaluronic acid chondroitin sulphate) (Bartold *et al.*, 1983) and glycoproteins (mainly fibronectin)

(Romanos *et al.*, 1993). Fibronectin helps fibroblasts to bind to the fibers (Klebe, 1974; Yamada *et al.*, 1976; Yamada and Olden, 1978; Grinnell and Feld, 1979) and laminin, another glycoprotein, helps it to bind to epithelial cells (Terranova and Lyall, 1986; Pakkala *et al.*, 2002). Laminin receptor on gingival epithelial cell surface also indicates its role in maintaining cemento-epithelial junction by interactions of cementum laminin and its receptor on gingival epithelium (Sengupta *et al.*, 1991).

Underneath the gingiva, the teeth are located within the alveolar bone (bony socket) of the alveolar processes of maxilla and mandible. The alveolar process consists of thin lamella of bone lining the tooth socket wall and containing inserting Sharpey's fibers (Johnson, 1987) and the thicker outer layer of bone, lining the labial and lingual aspects of the alveolar process. The thick layer constitutes of cortical plates and spongy bone.

The periodontal ligament (PDL) is present across the space between the root surface and the alveolar bone. It is a fibrous connective tissue and is produced mainly by fibroblasts (Lekic and McCulloch, 1996). It is highly cellular and vascular. It has increased collagen turnover, due to which it has high remodelling rate (Mensing *et al*., 2011). Presence of multipotent post-natal periodontal stem cells (PDLSCs) in PDL have also been demonstrated (Ivanovski *et al*., 2006). Cementum is a hard tissue covering the root surface (Denton, 1939). It provides anchorage to the periodontal ligament by insertion of Sharpey's fibers and also protects the pulp by covering the porous dentine on the root surface (Short and Johnson, 1990).

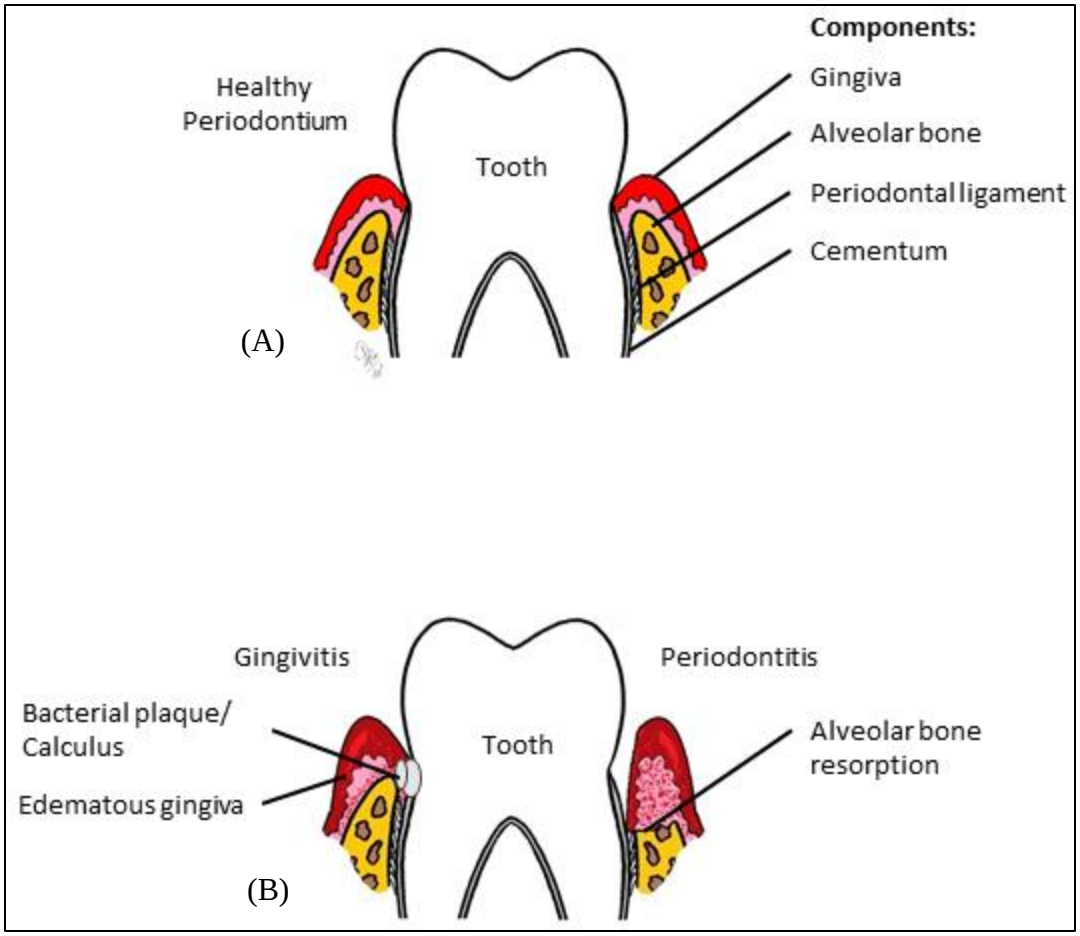

Figure 2.2 (A) Healthy periodontium. (B) Diseased periodontium (gingivitis and periodontitis).

2.2.1 Human periodontal ligament fibroblast cells

HPDLFs are the predominant cells in the periodontal tissues and are of mesenchymal origin. They are large spindle shaped or stellate cells with an oval nucleus (Nanci, 2013). They have an extensive cytoplasm with an abundance of organelles associated with the synthesis and secretion of protein. HPDLFs possess well- developed cytoskeleton with adherens and gap junctions (Nanci and Bosshardt, 2006). HPDLFs from young donors ages between 15-19 years were observed to be small, whereas HPDLFs from older donors ages 20-80 years showed diverse and large-spread out shapes (Sawa *et al.*, 2000).

HPDLFs have a finite population doubling (PD) number of 7.1±2.9 (Sawa *et al.*, 2000). Whereas, for human fibroblasts, the proliferative life span *in vitro* ranges from 30-60 population doublings and it decreases with increase in donor age (Dimri *et al.*, 1995). The periodontal cells in tissue proliferate at a daily rate of 0.5-2 percent, however, an increase in the proliferation of the fibroblast cells is seen during orthodontic forces and injury (Gould *et al.*, 1980).

HPDLFs play an important role in the development, function and regeneration of periodontal tissues (McCulloch, 1995). Fibroblasts synthesize collagen (Kumada and Zhang, 2010; Limeback *et al.*, 2016), elastic fibers (Tsuruga *et al.*, 2002; Sawada *et al.*, 2006), glycoproteins and glycosaminoglycans (Bartold and Page, 1985; Baglole *et al.*, 2013). Fibroblasts also produce cytokines by which they mediate tissue destruction and stimulate bone resorption by osteoclastic stimulation (Genco, 1992). Most extra cellular matrix components are mediated by the production of matrix metalloprotenases (MMPs)

and tissue inhibitor of matrix metalloproteinases (TIMPs) by these fibroblasts (Sorsa *et al.*, 2004; Verstappen and Hoff, 2016). Therefore, by the balance of these enzymes, the periodontal fibroblasts maintain the periodontal structures during physiological turn over.

HPDLFs have cementoblast-like and/ or osteoblast-like properties and can form mineralized nodules *in vitro*. They also express bone-associated markers, form cementum-like tissues and modulate osteoclastogenesis *in vivo* (Nohutcu *et al.*, 1997; Flores *et al.*, 2008). Primary periodontal cell cultures at early passages maintain the rich phenotypic and functional heterogeneity of fibroblasts characteristic of the original tissue when compared to late passages (Goseki *et al.*, 1996; Itaya *et al.*, 2009).

2.3 Classification of periodontal disease

Periodontitis is the inflammatory condition of the periodontium induced by bacteria. As the disease progresses, it causes degenerative changes in the periodontium, resulting in the loss of periodontal ligament and alveolar bone. As a result, the gingival sulcus deepens and forms periodontal pocket (Chen *et al*., 2012).

The 1999 classification of periodontal diseases enlisted, apart from the gingival diseases, seven major categories of destructive periodontal diseases (Armitage, 1999):

- Chronic periodontitis
- Aggressive periodontitis
- Periodontitis as a manifestation of systemic diseases
- Necrotizing periodontal disease
- Abscesses of the periodontium
- Combined periodontic-endodontic lesions

- Developmental or acquired deformities and conditions

2.3.1 Pathogenesis of periodontal disease

The dental biofilm/ bacterial plaque accumulation is considered to be the primary initiating agent in the aetiology of periodontal diseases. During the disease process, the periodontal tissues undergo several changes. During the initial 2-4 days of plaque accumulation, histologically low grade inflammation can be seen which is characterized by dilation of vascular network and increase in vascular permeability, this is known as initial lesion. However, there are no signs of clinical inflammation during this stage. Neutrophils and monocytes then migrate from the gingival vasculature to the site of the chemotactic stimuli, that is, the connective tissue near the gingival sulcus. Gingival crevicular fluid (GCF) also increases due to increases hydrostatic pressure in the local microcirculation. After one week of plaque accumulation the gingiva becomes slightly swollen due to edema and also erythematous due to capillary proliferation and vasodilation. This corresponds to the early clinical sign of gingivitis and hence called early lesion. The flow of gingival crevice fluid is increased along with notable collagen destruction. Polymorphonuclear cells (PMNs) migrate towards gingival sulcus and phagocytose bacteria (Miller *et al.*, 1984; Scott and Krauss, 2012). Fibroblasts begin to show cytotoxic effects with decreased capacity for collagen formation.

If the condition persists it becomes as established lesion which clinically correlates to moderate to severe chronic gingivitis. A predominance of plasma cells and B lymphocytes can be seen with an increase in immunoglobulin G1 (IgG1) and G3 (IgG3) subclasses (Okada *et al.,* 1983). Blood flow is impaired, and the collagenolytic activity is increased. Neutrophils accumulate in the tissue and release their lysosomal contents to kill

bacteria which are still not phagocytose, causing further destruction. Neutrophils migrate towards the sulcus and release matrix metalloproteinases (MMP-8 and MMP-9), making the junctional and sulcular epithelium ulcerated. Hence, giving the common feature of chronic gingivitis of bleeding on probing.

The final stage is demarcated by the transition from gingivitis to periodontitis known as the advanced lesion or phase of periodontal breakdown. The bacterial plaque products differentiate bone progenitor cells into osteoclasts or act directly on osteoblasts and inhibit their actions and reduce their numbers. The host inflammatory cells release factors that play a role in periodontal destruction such as prostaglandins, including interlukin-1a (IL-1a) and IL-b, and tumor necrosis factor alpha (TNFa) (Page, 1991; Chiang *et al.*, 1999). The junctional epithelium migrates apically and the pocket deepens. All these events leading up to the irreversible attachment loss and bone loss which can be observed histologically and clinically.

2.3.2 Risk factors

Any attribute, exposure or characteristic that can predispose an individual to disease or injury can be referred to as a risk factor (World Health Organization, 2014). Although the bacterial plaque is essential for the periodontal disease initiation, there are various factors which determine the severity of periodontal disease, its progression and response to therapy.

The plaque-induced chronic periodontitis is a slowly progressing disease, however, when coupled with a systematic condition the rate of periodontal destruction is increased due to altered host responses. Diabetes and periodontal disease have two-way relationship,

in which diabetes increasing the risk for periodontitis and periodontal inflammation negatively affecting the glycaemic control (Nishimura *et al.*, 2003; Preshaw *et al.*, 2012). Some environmental and behaviour factors also affect the severity and extend of periodontal disease. While dietary habits, alcohol consumption, socioeconomical conditions and stress levels influence periodontal status, smoking is a major risk factor for bone loss, furcation involvement and deeper pockets (Albandar *et al.*, 2000; Newman, 2011). Several genetic risk factors for periodontitis have also been identified, such as IL-1, TNF-α, Fcgamma receptor, CD14, TLR-2 and TLR-4 gene polymorphisms, which suggests genetic predisposition to periodontitis. (Loos *et al.*, 2005; van Dyke and Dave, 2005).

2.4 Management of periodontal diseases

2.4.1 Initial phase

The initial periodontal therapy, also known as phase 1 periodontal therapy or non-surgical periodontal therapy, aims to control the bacterial infection by completely removing plaque and calculus, which harbors the bacteria, correcting defective restorations, treating carious lesions, managing orthodontic tooth movements, treatment of food impaction areas and extracting hopeless teeth. Motivating the patient to maintain good oral hygiene practices by adhering to effective daily plaque removal at home is one specific aim of the initial therapy (Newman, 2011). In order to reduce plaque, mechanical and/ or chemical plaque reduction techniques can be adopted. Scaling and root debridement are done to mechanically remove the calculus deposits. The bacterial load can also be reduced chemotherapeutically using systemic antimicrobials (oral antibiotic) such as clindamycin,

amoxicillin/ clavulanate and metronidazole (Slots, 2002) or local antimicrobials which may be introduced directly into the periodontal pockets (Kinane, 2000; Nair and Anoop, 2012). Kinane (2000) demonstrated the effectiveness of slow release devices: Actisite (tetracycline fiber), Dentomycin (minocycline) and Elyzol (metronidazole dental gel) adjunctively with root planing as compared to root planing alone. Although all three locally applied antimicrobial systems were effective, Actisite placement gave the greatest probing pocket depth reduction during six months after treatment.

2.4.2 Corrective or surgical phase

After the initial periodontal therapy phase, if the periodontal pocket or defect persists, more advanced therapy may be required to treat the condition. One such periodontal surgical therapy which is limited to the gingival tissues only without involving the osseous structures is called gingivectomy (Newman, 2011). It involves removing the pocket wall surgically and cleaning the area. Apart from the conventional gingivectomy using knifes and blades, now gingivectomy using LASERS (Light Amplification by Stimulated Emission of Radiation) are also being used (Shankar *et al.*, 2013; Kumar *et al.*, 2015b). Moreover, periodontal flap surgeries may be undertaken to allow better visibility and access to bone and root surface. Flaps can also be displaced to treat various mucogingival related defects or for access to pocket depths and their reduction/ elimination (Newman, 2011).

Moreover, depending on defect size and/ or tooth root exposure, bone graft materials are being used. These can be autografts (from same individual), allografts (from different individual from the same species), xenografts (from different species) or

alloplastic (non-biological material). Autografts can be from intraoral site or from extraoral sites such as iliac crest autografts. Allografts are usually available from tissue banks as undecalcified freeze-dried bone allograft or demineralized freeze-dried bone allograft. Alloplastic materials include hydroxyapatite-HA and tricalcium phosphate-TCP (Bansal *et al.*, 2014; Gupta, 2014; Jain *et al.*, 2014; Matsuura *et al.*, 2015).

2.5 Periodontal regeneration and tissue engineering

The American Academy of Periodontology (1992) defines periodontal regeneration histologically as regeneration of the tooth's supporting tissues, including alveolar bone, periodontal ligament and cementum over a diseased root surface. This has to be assessed not only through clinical parameters but also re-entry evaluations and histological analysis in animal models.

Bartold et al. (2000) mentioned that there are at least four criteria, based on the natural morphology of the dento-gingival complex, that determine periodontal regeneration: (a) A functional epithelial seal must be re-established, no more than 2 mm in length, at the most coronal portion of the tissues, (b) Both the periodontal ligament and the dentogingival fiber complex must be reproduced by the insertion of new connective tissue fibers (Sharpey's fibers) into the previously exposed root surface, (c) New acellular, extrinsic fiber cementum must be reformed on the previously exposed root surface and (d) Alveolar bone height must be restored to within 2 mm of the cemento-enamel junction. To obtain such a degree of regeneration all the processes involved in periodontal reconstruction have to be strongly rooted in the biological stages of development. Therefore, a model of regeneration is adopted in tissue engineering, in which careful

selection of regenerative cells intermixed with a suitable extracellular matrix has to be constructed.

Tissue engineering is a relatively new but rapidly progressing field which aims to develop procedures and biomaterials to support and facilitate the development of new tissues to replace lost/ damaged tissues, with great potential to fulfil the functional needs. Tissue engineering can be classified into passive and active tissue engineering based on the role of cells played in the regeneration of the structures. Passive tissue engineering consists of procedures like guided tissue regeneration (GTR) in which regenerative cells are not actively introduced into the defect rather a barrier membrane is placed to passively induce the innate regenerative cells to construct the structures. Whereas, in active tissue engineering growth factors and cell therapy plays an active role. Growth factors, such as enamel matrix derivatives (EMD) (Venezia *et al.*, 2004; Esposito *et al.*, 2009), platelet-rich plasma (PRP) (Rosello-Camps *et al.*, 2015) or specific growth/ differentiation factors such as periodontal growth factor (PDGF), insulin like growth factor 1 (IGF-1), bone morphogenetic protein 2 (BMP-2) and 7 (BMP-7) are used to stimulate periodontal regeneration (Lynch *et al.*, 1989; Kaigler *et al.*, 2011; Nevins *et al.*, 2013; Rao *et al.*, 2013).

2.5.1 Guided tissue regeneration

Over the past few decades, guided tissue regeneration (GTR) has been established as an effective treatment modality for periodontal reconstruction (Needleman *et al.*, 2002). The principle behind this technique is to prevent the epithelial migration along the cemental wall of pocket, which forms long junctional epithelium, by placing a barrier (membrane) to cover the bone and periodontal ligament. Initially nonresorbable membranes were used

but they required a second surgical procedure to remove them. Therefore, biodegradable membranes were introduced into this procedure. They were procured from different species such as bovine, porcine or cargile (from the peritoneum of the ox). Other membranes were derived from polylactic acid, Vicryl (polyglactin 910) and freeze dried dura mater. However, the results of these procedures were usually associated with poor clinical predictability and less effective in producing periodontal regeneration (Chen *et al.*, 2010).

2.5.2 Cell-based periodontal regeneration

The recent advances in stem cell research has led to cell-based tissue engineering strategies for periodontal regeneration. These strategies include the use of regenerative cells integrated in a 3D scaffold or cultured on a substrate to form cell sheets and then transferred into the defect.

Cells that have been used for periodontal regeneration include: dental pulp stem cells (DPSCs) (Gronthos *et al.*, 2000; Aimetti *et al.*, 2014), periodontal ligament stem cells (PDLSCs) (Ding *et al.*, 2010; Zhu and Liang, 2015), bone marrow mesenchymal stromal cells (BMMSCs) (Yang *et al.*, 2010), alveolar periosteal cells (APCs) (Jiang *et al.*, 2010), stem cells from apical papilla (SCAPs) (Sonoyama *et al.*, 2008), and alveolar bone mesenchymal stem cells (MSCs) (Li *et al.*, 2008; Lin *et al.*, 2009).

The use of cell based approaches have been shown to enhance periodontal regeneration in various *in-vitro* and *in-vivo* settings (Rios *et al.*, 2011; Abbayya *et al.*, 2015; Yan *et al.*, 2015). However, it is still indistinct whether the transplantation of autologous cells differentiate into osteoblasts, cementoblasts, and fibroblasts to form bone, cementum,

and periodontal ligament, respectively or whether they engage the surrounding host cells to support and facilitate the regenerative process.

In particular, PDL tissue contains multipotent stem cell populations and contributes to the regeneration of complex periodontal structures (Seo *et al.*, 2004; Liu *et al.*, 2008; Pejcic *et al.*, 2013; Zhu and Liang, 2015). BMMSCs have been reported to differentiate into PDL, and the transplantation of BMMSCs has facilitated periodontal regeneration (Kawaguchi *et al.*, 2004; Hasegawa *et al.*, 2006; Yang *et al.*, 2010). APCs have also been reported to improve periodontal regeneration (Yamamiya *et al.*, 2008; Kawase *et al.*, 2009; Jiang *et al.*, 2010). Moreover, PDLSCs hold the potential to form bone, cementum and periodontal ligament-like structures and to enhance overall periodontal regeneration (Ding *et al.*, 2010; Hynes *et al.*, 2012). These cells are being used in combination with various natural and synthetic biomaterial scaffolds along with required growth factors to regenerate periodontal tissues. But the ideal combination of cells, scaffolds and growth factors is yet to be established.

2.6 Challenges of periodontal regeneration and the pivotal role of scaffolds

Scaffolds are three-dimensional (3D) porous solid biomaterials that mimic the natural extracellular matrix (ECM) (Dhandayuthapani *et al.*, 2011). They are designed to perform some or all of the following functions (Langer and Tirrell, 2004): (a) To promote cell-biomaterial interactions and cell adhesion, (b) To promote ECM deposition, (c) To permit sufficient transport of essential gases, nutrients, and regulatory factors for cell survival, proliferation, and differentiation and (d) To provoke a minimal degree of inflammation or toxicity in vivo.

While the “gold standard” for periodontal regeneration and healing is the autograft or allograft (Reynolds *et al.*, 2003; O'Brien, 2011), these approaches are integrally limited by the amount of available donor tissue and demands a second surgical site, resulting in additional trauma to the patient and associated risks such as pain, risks of rejection by the patient's immune system, infection, and donor-site morbidity (Chen and Jin, 2010; Hughes *et al.*, 2010). Perhaps one of the greatest challenges faced in periodontal regeneration, regardless of tissue type, is promoting healing three-dimensionally (Abou Neel *et al.*, 2014).

The principle of tissue engineering embodies the creation of a highly porous scaffold structure that has the appropriate physical, chemical, and mechanical properties to enable cell penetration and tissue reconstruction in three dimensions. In addition to defining the 3D geometry for the tissue to be engineered, the scaffold provides the microenvironment ‘niche’ for regenerative cells, supporting cell attachment, proliferation, differentiation, and neo-tissue formation (Yang *et al.*, 2001; Dhandayuthapani *et al.*, 2011).

The contribution of scaffolds in periodontal tissue regeneration is crucial as they serve as carriers to facilitate delivery of regenerative stem cells and/ or growth factors at a local defect site. Moreover, scaffolds should have certain properties conductive for regeneration while fulfilling its primary purpose of delivering cells or drugs to the target sites, such as follows (Garg *et al.*, 2012):

1) Biocompatibility: not causing any immune or inflammatory reaction.
2) Biodegradability at a controllable rate in approximation with the rate of tissue regeneration.

3) Mechanical properties: maintaining volume (Chen *et al.*, 2009), shape and mechanical strength to protect cells.

4) Architecture: Porous structure to allow cells to be well integrated into the scaffold and show tissue-ingrowth.

5) Physical and chemical structure supporting cell adhesion, proliferation and migration.

2.6.1 Types of scaffolds

Scaffolds can be broadly classified into synthetic (artificial) and biological (natural) (Table 2.1). Biological scaffolds are derived from human and animal tissues, and synthetic scaffolds from polymers. Although synthetic scaffolds are available and tailored to the needs of the structures to be reconstructed, natural sources of scaffolds are an endless substitute for synthetic products and more readily available. Natural scaffolds also have the advantage of providing specific cell interactions, biocompatible and are a cheap alternative. Among such scaffolds, amniotic membrane holds a unique interest due to its already established biological properties such as immunomodulatory action and tissue-like characteristics which makes it highly acceptable by the natural host tissues (Akle *et al.*, 1981; Wang *et al.*, 2006). A deeper understanding of the structure and composition of this membrane will help understand its present role and possible future applications in this vast arena of tissue engineering.