MECHANISM OF NF-κB SIGNALING IN BMP-2 - INDUCED DENTAL STEM CELL ODONTOGENEIC DIFFERENTIATION ON HUMAN AMNIOTIC MEMBRANE SCAFFOLD

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by

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TABLE OF CONTENTS

LIST OF APPENDICES

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celcius
α	Alpha
β	Beta
γ	Gamma
κ	Kappa
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar
™	Trademark
3D	Three-dimensional
bp	Base pair
cm	Centimetre
C_T	Cycle threshold
g	Gram
L	Litre
mg	Milligram
min	Minutes
mm	Millimetre
mM	Milimolar
ml	Millilitre
ng	Nanogram
s	Second

U	Unit
V	Voltage
wt	Weight
Akt	Protein Kinase B
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
AQPs	Aquaporins
BMMSCs	Bone marrow-mesenchymal stem cells
BMPs	Bone morphogenetic proteins
BMSCs	Bone mesenchymal stem cells
BSA	Bovine serum albumin
BSP	Bone sialoprotein
CBFA1	Core-binding factor subunit alpha-1
cDNA	Complementary DNA
CD29	Integrin beta-1
CO_2	Carbon dioxide
COL-1	Collagen type 1
COX-2	Cyclooxygenase-2
ddH_2O	Double-distilled water
DEPC	Diethyl pyrocarbonate
DFSCs	Dental follicle stem cells
DMP1	Dentin matrix protein 1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPCs	Dental pulp cells

DPP	Dentin phosphoprotein
DPSCs	Dental pulp stem cells
DSP	Dentin sialoprotein
DSPP	Dentin sioalophosphoprotein
ECM	Extracellular matrix
EG	Embryonic germ
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESCs	Embryonic stem cells
et al.	and others
FBS	Fetal bovine serum
GSH	Glutathione
HA/TCP	Hydroxyapatite/tricalcium phosphate
HAM	Human amniotic membrane
HDAC	Histone deacetylases
hDPCs	Human dental pulp cells
H_2O_2	Hydrogen peroxide
IFN- γ	Interferon gamma
IFRD1	Interferon-related developmental regulator-1
IGF	Insulin-like growth factor
IKK	Iκβ -kinase
IL	Interleukin
IL-1β	Interleukin-1 beta
iNOS	Inducible nitric oxide synthases
kGy	Kilogray

LB	Lithium borate
LPS	Lipopolysaccharide
MEM	Minimum essential medium
MEPE	Matrix extracellular phosphoglycoprotein
MG 132	Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated b cells
NFM	Neurofilament M
NO	Nitric oxide
NOSs	Nitric oxide synthases
NTC	Non-template control
OCN	Osteocalcin
OCT4	Octamer-binding transcription factor 4
OD	Optical density
ODT	Odontoblast
OPG	Osteoprotegerin
OPN	Osteopontin
OSX	Osterix
p65	Transcription factor p65
PBS	Phosphate buffered saline
PDLSCs	Periodontal ligament stem cells
PI3K	Phosphoinositide-3-kinase
PDGF	Platelet-derived growth factor
PDTC	Ammonium pyrrolidinedithiocarbamate

QR	Relative quantity
R^2	Correlation coefficient
RANKL	Receptor activator of nuclear factor kappa-B ligand
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcriptase - polymerase chain reaction
Runx2	Runt-related transcription factor 2
S	SHED
SA	SHED + HAM
SAB	SHED + HAM + BMP-2
SABM	SHED + HAM + BMP-2 + MG 132 inhibitor
SABP	SHED + HAM + BMP-2 + PDTC inhibitor
SCAP	Stem cells from the apical papilla
SEM	Standard error mean
SHED	Stem cells from human exfoliated deciduous teeth
SOX2	Sex determining region Y
TE	Tissue engineering
TCP	Tricalcium phosphate
TGFs	Transforming growth factors
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
WNT	Wingless/Integrated
TCP	Tricalcium phosphate

MEKANISME PENGISYARATAN NF-κB YANG DIARUH OLEH BMP-2 DALAM PEMBEZAAN ODONTOGENIK SEL TUNJANG GIGI DI ATAS PERANCAH MEMBRAN AMNIOTIK MANUSIA

ABSTRAK

Pengisyaratan tapak jalan NF-κB terlibat dalam organogenesis dan pengeruptan gigi manakala, penyingkiran tapak jalan NF-κB mengakibatkan penyekatan dalam perkembangan gigi. Walaubagaimanapun, pengaruh tapak jalan NF-κB dalam perkembangan gigi dan kajian odontogenesis masih tidak jelas sehingga kini. Oleh itu, kajian ini dijalankan untuk mengkaji mekanisme tapak jalan isyarat NF-κB dalam pembezaan sel-sel tunjang dari gigi susu muanusia yang terlupas (SHED) kepada sel-sel menyerupai odontoblas. Analisis pengisyaratan NF-κB terbahagi kepada dua fasa. Fasa pertama dijalankan bertujuan untuk mengenalpasti kepekatan optimum perencat *carbobenzoxy-L-leucyl-L-leucyl-L-leucina*l (MG 132) dan *ammonium pyrrolidinedithiocarbamate* (PDTC). SHED dikultur di atas membran amniotik manusia (HAM) dan dirawat dengan protein morfogenetik tulang-2 (BMP-2). Kumpulan eksperimen dibahagikan kepada empat seperti berikut: SHED sahaja (S), SHED dikultur di atas membran amniotik (SA), SHED dikultur di atas membran amniotik dirawat dengan BMP-2 (SAB), SHED dikultur di atas membran amniotik dirawat dengan BMP-2 dan perencat MG 132 (SABM), dan SHED dikultur di atas membran amniotik dirawat dengan BMP-2 dan perencat PDTC (SABP). Berdasarkan kepada keputusan penghasilan protein NF-κB, 0.1 μM daripada MG 132 manakala 25 μM daripada PDTC dipilih sebagai kepekatan optimum untuk menghalang pengisyaratan NF-κB. Kemudiannya, fasa kedua dijalankan untuk mengkaji

pengekspresan gen bagi penanda sel tunjang, odontogenik, dan NF-κB . Pasca rawatan, SHED dituai pada hari 1, 7, 10, dan 14. Analisis selanjutnya dianalisa menggunakan tindak balas rantai polimerase transkriptase berbalik masa nyata (real time RT-PCR). Sepanjang eksperimen, ekspresi penanda gen sel tunjang, *Nestin*, *Nanog*, dan *CD29* adalah perbezaan nyata dalam kesemua kumpulan. Selain itu, keputusan kajian ini menunjukkan bahawa SHED yang dirawat dengan BMP-2 dan dikultur di atas HAM menunjukkan peningkatan dalam ekspresi penanda odontogenik iaitu *dentine sialophosphoprotein* (*DSPP*) (1.80 ± 0.06; 2.41 ± 0.01) dan *alkaline phosphatase* (*ALP*) (2.01 ± 0.01; 4.60 ± 0.21) pada hari 1 dan 7. Ini menunjukkan bahawa SHED berjaya dibezakan kepada sel-sel menyerupai odontoblas. Sementara itu, rawatan dengan PDTC atau MG 132 menunjukkan penurunan ekspresi *ALP* dan *DSPP*, menandakan bahawa isyarat NF-κB terlibat secara langsung dalam pembezaan dan pemineralan SHED. Sebaliknya, interaksi di antara HAM, BMP-2, dan SHED meningkatkan ekspresi *Aquaporin 5* (*AQP5*) pada hari ke-7 dan 10 (1.15 ± 0.01; 2.43 ± 0.24) dan *Interleukin-8* (*IL-8*) pada hari ke-14 (0.88 ± 0.36). Walau bagaimanapun, rawatan dengan perencat menurunkan ekspresi gen tersebut pada hari ke-14 bagi *AQP5* dan *IL-8* untuk keseluruhan eksperimen. HAM membantu menigkatkan ekspresi *Interleukin 1 beta* (*IL-1β*) tetapi mengurangkan ekspresinya dengan penambahan BMP-2. Penambahan perencat turut menyebabkan pengurangan pada gen tersebut. Selain itu, ekspresi t*umour necrosis factor* (*TNF-α*) menurun bagi semua kumpulan apabila ditambah dengan BMP-2. Corak ekspresi yang sama turut ditunjukkan oleh *RANKL* bagi semua kumpulan, mencadangkan bahawa *RANKL* kurang dipengaruhi oleh HAM dan BMP-2. Hasil kajian juga menunjukkan bahawa HAM dan BMP-2 meningkatkan ekspresi *Osteoprotegerin* (*OPG*) pada peringkat awal pembezaan iaitu pada hari 1 (1.96 ± 0.05). Walau bagaimanapun, PDTC dan MG 132

mengurangkan ekspresi *OPG* selepas hari ke-7. Semua analisis statistik dilakukan pada tahap signifikan iaitu $p<0.05$. Kesimpulannya, berdasarkan analisis pengekspresan gen, kajian ini mencadangkan bahawa perencatan NF-κB secara langsung terlibat dalam pembezaan odontogenik SHED apabila dikultur di atas HAM dan dirawat dengan BMP-2.

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ABSTRACT

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway is involved in the tooth organogenesis and eruption process. With the elimination of NF-κB pathway could lead to a developmental detention of teeth. However, the influence of NF-κB signalling in tooth development as well as odontogenesis study remains unclear. Hence, this study was conducted to investigate the mechanism of NF-κB signalling in the differentiation of stem cells derived from human exfoliated deciduous teeth (SHED) into odontoblast-like cells. Analysis of NF-κB signalling was divided into two phases. The first phase was aimed to identify the optimal concentration of carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG 132) and ammonium pyrrolidinedithiocarbamate (PDTC). SHED were cultured on human amniotic membrane (HAM) and treated with bone morphogenetic protein-2 (BMP-2). Experimental groups were assigned into four as follows: SHED only (S), SHED cultured on HAM (SA), SHED cultured on HAM treated with BMP-2 (SAB), SHED cultured on HAM treated with BMP-2 and MG 132 inhibitor (SABM), and SHED cultured on HAM treated with BMP-2 and PDTC inhibitor (SABP). Based on the NF-κB protein expression, 0.1 µM and 25 µM of MG 132 and PDTC, respectively, were selected as an optimal concentration to inhibit NF-κB signalling. Thereafter, the second phase of the study was aimed to investigate the expression of stem cell, odontogeneic, and NF-κB gene markers. Following treatment, SHED were harvested on day 1, 7, 10 and 14. Further analyses were carried out using real time reverse transcription polymerase chain reaction (real time RT-PCR). Results showed that the

expression levels of stem cell gene markers, *Nestin*, *Nanog*, and *CD29* were fluctuated in all groups. Besides that, the results of the present study showed that SHED treated with BMP-2 and cultured on HAM showed an increased at day 1 and 7 in the expression of odontogeneic markers, namely, *dentine sialophosphoprotein* (*DSPP*) (1.80 ± 0.06; 2.41 ± 0.01) and *alkaline phosphatase* (*ALP*) (2.01 ± 0.01; 4.60 ± 0.21). This indicated that SHED had successfully differentiated into odontoblast-like-cells. Meanwhile, treatment with PDTC or MG 132 showed a decreased expression of *ALP* and *DSPP* indicating that NF-κB signalling is directly involves in SHED differentiation and mineralisation. On the other hand, the interaction of HAM and BMP-2 with SHED increased the *Aquaporin 5* (*AQP5*) expression at day 7 and 10 (1.15 ± 0.01; 2.43 ± 0.24) and *Interleukin-8* (*IL-8*) expression at day 14 (0.88 ± 0.36). While, the treatment with inhibitors decreased the expressions of both genes especially at day 14 for *AQP5* and for *IL-8* was throughout the experiment. As for *Interleukin 1 beta* (*IL-1β*), HAM induced its expression while, the addition of BMP-2 decreased its expression. Addition of the inhibitors also down-regulated its expression. Besides, *Tumour necrosis factor* (*TNF-α*) expression was down-regulated in all the groups when BMP-2 was added. A similar pattern of *Receptor activator of nuclear factor kappa-B ligand* (*RANKL*) expression were demonstrated for all the treatment groups suggesting that *RANKL* was minimally affected by HAM and BMP-2. Results also demonstrated that HAM and BMP-2 increased *Osteoprotegerin* (*OPG*) at day 1(1.96 ± 0.05). However, PDTC and MG 132 reduced the expression of *OPG* after day 7. All statistical analyses were performed at the significance level of $p<0.05$. In conclusion, based on the gene expression analysis, this study suggested that inhibition of NF-κB directly involves in odontogeneic differentiation of SHED when cultured on HAM with the treatment of BMP-2.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Dental caries is the most prevalent infectious disease among children and adults. This oral problem affects the quality of life of approximately 90% of the world's population (López and Baelum, 2007). Dental caries is characterised by infected and necrotic dental pulp tissue (Cooper *et al.*, 2010). The dental pulp tissue provides vitality and sensitivity to the tooth. The pulp tissue is highly vascularised, innervated and also serves as a source of stem cells. These characteristics enable the pulp to play a significant role in homeostasis and formation of reparative dentin (Galler *et al.*, 2011).

One of the clinical treatments for dental caries is root canal therapy. This involves cleaning and replacement of the infected and necrotic pulp tissue with a mineral trioxide compound (Goldberg, 2016). As a result of replacing a living tissue with a trioxide compound, the tooth loses its vitality and sensitivity. Sometimes, the tooth is also exposed to infections and the complications associated with it (Cordeiro *et al.*, 2008). In adolescents, root canal treatment poses an even greater problem of root maturation (McTigue *et al.*, 2013).

These drawbacks in conventional therapy can be overcome by the use of tissue engineering (TE) strategies to regenerate the dental pulp. The three core factors that ensure the success of TE are the types of cells, scaffold and growth factors used (Toda

et al., 2007). Stem cell research has become a new era and is regarded as one of the important fields for the understanding of tissue regeneration and its implementation in regenerative medicine. Stem cells exist in an undifferentiated state, capable of proliferating over extended periods of time through self-renewing divisions and later differentiating into a variety of cells that contribute to organ formation and function (Chagastelles and Nardi, 2011). Since the discovery and characterisation of multi-potent mesenchymal stem cells (MSCs) from bone marrow, it remains an exciting prospective cell source for regenerative medicine applications because of their strong proliferative potential and multi-lineage differentiation capability. Moreover, the identification of stem cells from several dental tissues has made pulp tissue regeneration a realistic clinical possibility.

Despite the many types of dental stem cells available such as dental pulp stem cells (DPSCs) (Gronthos *et al.*, 2000), periodontal ligament stem cells (PDLSCs) (Gould *et al.*, 1977; Gronthos *et al.*, 2006), stem cells from the apical papilla (SCAP) (Sonoyama *et al.*, 2006), and stem cells from human exfoliated deciduous teeth (SHED) (Miura *et al.*, 2003). This study was carried out on the SHED since the focus of this research is odontogenesis. Stem cells from dental pulp are categorised under adult stem cells and derived from ectoderm (Ulmer *et al.*, 2010). It exhibits the MSC properties which has a self-renewal ability and able to transdifferentiate into another type of cell (Huang *et al.*, 2009a). Therefore, markers that have been used for identifying MSCs are *Nestin*, *Nanog*, and, *CD29* (Huang *et al.*, 2009). SHED were selected in this study since there are less issues involved in isolation and can be obtained naturally from exfoliated deciduous teeth (Wang *et al.*, 2010a).

The success in tissue regeneration also depends on the use of a suitable scaffold to carry specialised cells *ex vivo* or to orchestrate and differentiate the homing of endogenous cells *in vivo* (Alshehadat *et al.*, 2016). With regard to that, human amniotic membrane (HAM) was selected due to its ability to promote cell growth and differentiation of stem cells (Díaz-Prado *et al.*, 2010). It is found in the innermost layer of the placenta, consisting of prominent basement membrane and subjacent avascular stromal (Solomon *et al.*, 2001). HAM is natural in origin which raises no ethical issue, abundantly available and easy to acquire (Alviano *et al.*, 2007). Unlike the synthetic scaffold, no modification of structure is needed in HAM to allow micro channel formation (Willerth and Sakiyama-Elbert, 2008). Thus, it can serve as an excellent scaffold for tissue regeneration, especially due to the presence of extracellular matrix (ECM) components that help in cell proliferation and differentiation (Niknejad *et al.*, 2008). In this study, glycerol-preserved HAM was used as it mimics the structural integrity of fresh HAM (Lo and Pope, 2009). It has been shown that when epithelial and mesenchymal cells are seeded on a cellular scaffold created from the amniotic membrane, the cells were highly interconnected and capable of penetrating the porous structure of the amnion scaffold (Niknejad *et al.*, 2008).

Another essential component that is needed to promote odontogeneic differentiation is the growth factor. Bone morphogenetic proteins (BMPs) have a prominent role in tooth development and formation (Chen *et al.*, 2004), and play a major role in skeletal development, bone formation, and MSC differentiation (Chen *et al.*, 2012a). Among the osteogeneic BMPs, BMP-2 has been proven to enhance MSC differentiation into odontoblast-like cells (Saito *et al.*, 2004).

There are many specific markers to determine odontoblastic differentiation. In this study, odontoblastic differentiation markers such as dentine sialophosphoprotein (*DSPP*) and alkaline phosphatase (*ALP*) (Wei *et al.*, 2007) which play important roles in stimulating tertiary dentine formation in human dental pulp were selected (Casagrande *et al.*, 2010). Tertiary dentine is a third type of dentine secreted as a response to external pathological stimuli such as caries. Besides that, Aquaporin 5 (*AQP5*), a membrane water channel involved in osteo/odontoblastic differentiation (Yi *et al.*, 2012) was also selected. The protein is observed in dental lamina, inner enamel epithelium, stratum intermedium, stellate reticulum and the outer enamel epithelium of a tooth (Felszeghy *et al.*, 2004).

This complex biological event of odontogenesis involves nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway. The pathway is known to play a role in tooth organogenesis and eruption process (Ohazama and Sharpe, 2004). The receptor activator of nuclear factor kappa-B ligand (RANKL)/ osteoprotegerin (OPG) system is considered as an important signal transduction pathway in the formation, differentiation, and activation processes of osteoclast (Boyce and Xing, 2007). The elimination of NF-κB is an effective approach to inhibit osteoclast formation and bone resorptive activity (Abu-Amer, 2013). This may result in a developmental detention of teeth (Courtney *et al.*, 2005). NF-κB protein complex interacts with other signalling pathways such as Notch signalling and phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K)/ protein kinase B (Akt) pathway during the tooth development and inflammation (Cai *et al.*, 2011; Kuan *et al.*, 2012). It regulates a variety of pro-inflammatory mediators, including cytokines tumor necrosis factor (TNF-α) and Interleukin 1 beta (IL-1β) and chemokines, Interleukin 8

(IL-8) (Chang *et al.*, 2005). However, the current knowledge of the role of this pathway on tooth development as well as odontogenesis is yet to be explored.

1.2 Research justification

Numerous study have been carried out exhibiting the capacity of mesenchymal stem cells (MSCs) to differentiate into odontoblasts like cells with different results and success rate. However, to the best of our knowledge, there is no study have addressed on the dental pulp regeneration focusing on NF-κB signalling. The inhibition of NF-κB signalling pathway was carried out to promote odontogeneic differentiation of SHED seeded on HAM due to the fact that the inhibition of NF-κB also inhibits osteoclastogenesis. Hence, we presume that inhibiting osteoclastogenesis would indirectly promote odontogenesis. This study used HAM for three-dimensional (3D) construct consisting of SHED and BMP-2. The use of this construct is based on the consideration of the abilities of SHED, HAM, and BMP-2 to induce odontogenesis *in vitro*.

1.3 Research objectives

1.3.1 General objective

The study was conducted to investigate the mechanism of NF-κB signalling in bone morphogenetic protein-2 (BMP-2) – induced dental stem cell odontogeneic differentiation on human amniotic membrane scaffold.

1.3.2 Specific objectives

1. To determine the optimal inhibitor concentration of NF-κB inhibitors namely, ammonium carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG 132) and pyrrolidinedithiocarbamate (PDTC), in stem cells from human exfoliated deciduous teeth (SHED) induced with BMP-2 in the presence and absence of human amniotic membrane (HAM) using ELISA.

2. To assess the morphological changes of SHED seeded on HAM in the presence and absence of NF-κB inhibitors using inverted microscope.

3. To determine the gene expression levels of stem cell markers namely, *Nestin*, *NANOG*, and *CD29*, in BMP-2 induced SHED, seeded on HAM, in the presence and absence of NF-κB inhibitors.

4. To determine the gene expression levels of odontoblast specific markers namely, *dentine sialophosphoprotein* (*DSPP*) and *alkaline phosphatase (ALP*), in BMP-2 induced SHED, seeded on HAM, in the presence and absence of NF-κB inhibitors.

5. To determine the gene expression levels of NF-κB inducers namely, *Interleukin-8* (*IL-8*), *interleukin 1 beta* (*IL-1β*), and *tumor necrosis factor* (*TNF-α*), in BMP-2 induced SHED, seeded on HAM, in the presence and absence of NF-κB inhibitors.

6. To determine the gene expression levels of NF-κB downstream effectors associated with osteo-odontoblastic regulators namely, *receptor activator of nuclear factor*

kappa-B ligand (RANKL) and *osteoprotegerin (OPG),* in BMP-2 induced SHED, seeded on HAM, in the presence and absence of NF-κB inhibitors.

7. To determine the gene expression levels of *Aquaporin 5* (*AQP5*), in BMP-2 induced SHED, seeded on HAM, in the presence and absence of NF-κB inhibitors.

1.4 Research questions

Does NF-κB signalling plays a role in regulating odontogeneic differentiation of SHED induced by BMP-2 cultured on HAM?

1.5 Research hypothesis

NF-κB signalling plays a role in regulating odontogeneic differentiation of SHED induced by BMP-2 cultured on HAM.

CHAPTER 2

LITERATURE REVIEW

2.1 Dental pulp and tooth loss

2.1.1 Dental pulp

Dental pulp has essential functions to sustain teeth by supplying nutrient and oxygen, innervation, reactionary/reparative dentin formation and immune response (Nakashima *et al.*, 2009). The pulp is small in scale but has a complex structure that requires challenging therapeutic strategies for repair and regeneration (Janjić *et al.*, 2016). An evidence of pulp-like tissue regeneration *de novo* showed that dental pulp stem cells have risen into odontoblast-like cells (Huang *et al.*, 2009b).

2.1.2 Dental pulp structure

Dental pulp is derived from neural crest cell and is a specialised loose connective tissue located in the centre of the tooth (Sonoyama *et al.*, 2008). The dental pulp is encased with a rigid, non-compliant shell and its survival depends on the blood vessels accessing the interior of the tooth through apical foramina and accessory canal (Ramazanzadeh *et al.*, 2009). The structure contains cellular, fibrillary, neurovascular, and ground substance elements (Trowbridge and Kim, 1994). The cellular elements include mesenchymal stem cells (MSCs), odontoblasts, fibroblasts, and defensive cells such as lymphocytes, mast cells, basophils, macrophages, eosinophils, neutrophils, and

plasma cells (Yu and Abbott, 2007). Proliferation and condensation of these cells forms the dental papilla from which the mature pulp is derived (Tziafas and Kodonas, 2010). The primary function of pulp is to produce primary dentin during early tooth development, secondary dentin throughout the entire life span of the tooth, and tertiary dentin under pathogenic stimuli (Zhang and Yelick, 2010).

2.1.3 Odontoblast

Odontoblast, a layer of cells which lines at the periphery of the pulp in the inner dentin surface, is the specialised cell type capable of synthesising dentin (Zhang and Yelick, 2010) (Figure 2.1). Interactions between epithelial and dental papilla cells promote tooth morphogenesis by stimulating a sub-population of mesenchymal cells to differentiate into odontoblasts (Yen and Sharpe, 2008). Odontoblasts are thought to arise from the proliferation and differentiation of a precursor population, residing somewhere within the pulp tissue (Gronthos *et al.*, 2000). In addition, the dental pulp has an inherent capability to produce reparative dentine when the local environment is favourable (Kitagawa *et al.*, 2007). Morphologically, odontoblasts are columnar polarised cells with eccentric nuclei and lengthy cellular processes aligned at the outer edges of dentin (Gronthos *et al.*, 2000) (Figure 2.2). Unlike osteocytes, odontoblasts are not incorporated in the matrix, except for their processes that are embedded in the tubules. This is why dentine is not considered as an individual tissue but rather as the dentine-pulp complex (Simon *et al.*, 2011).

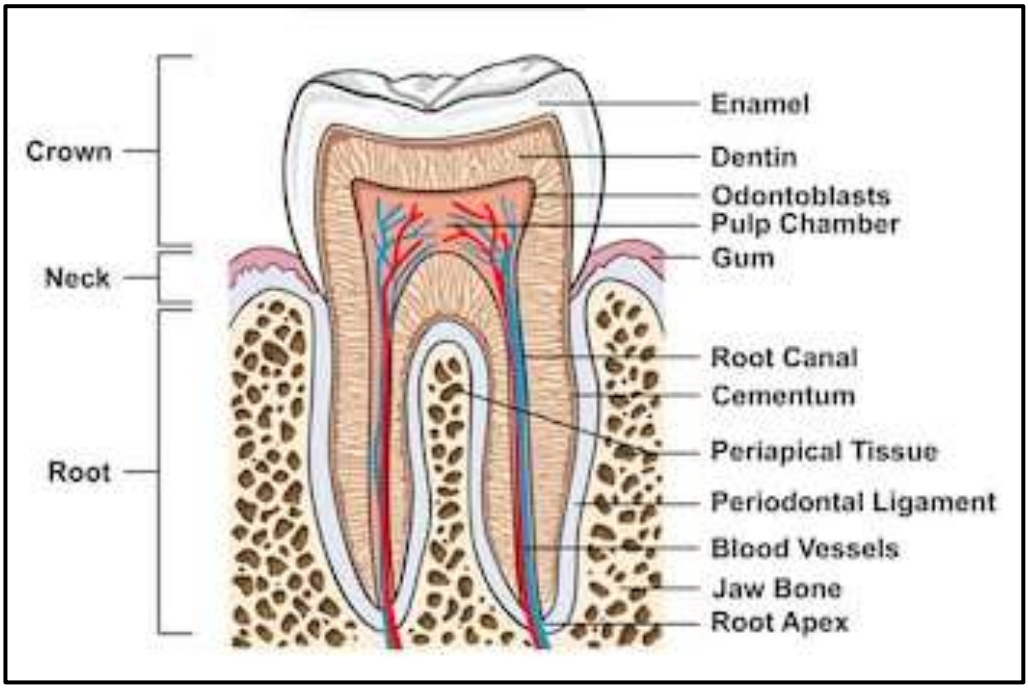

Figure 2.1: Cross-sectional image of tooth structure with exposed dentine. A tooth is made up of several components including the dentin and enamel. (Adapted from: https://www.shutterstock.com/search/pulp+cavity).

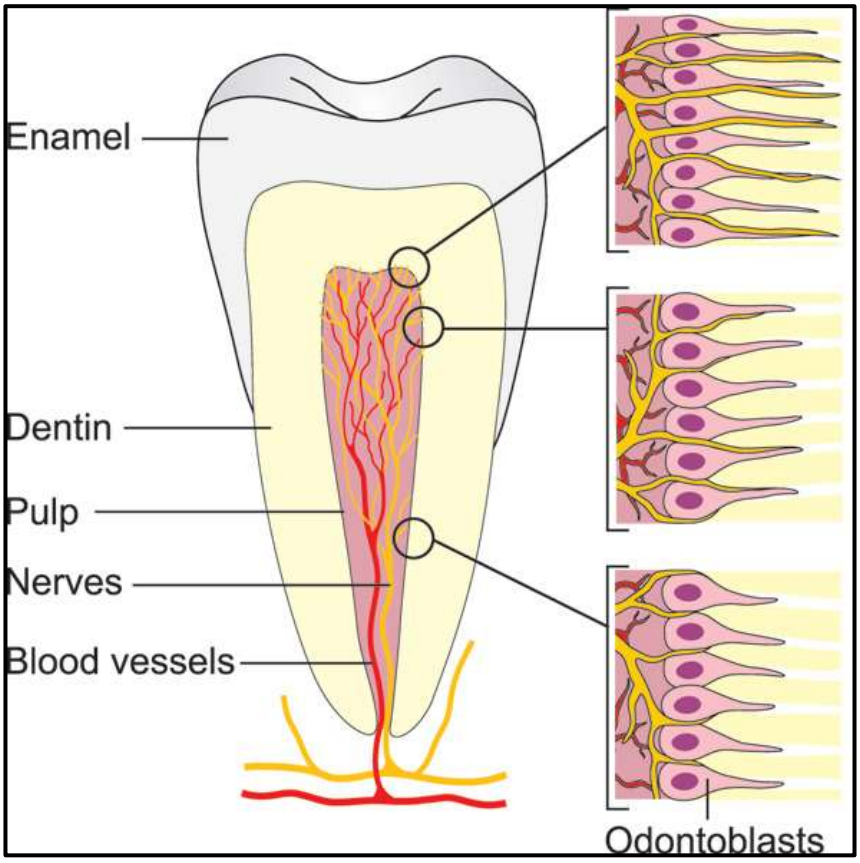

Figure 2.2: Cross sectional schematic diagram of odontoblast and dentine tubules of tooth. Dentinal tubules form around each odontoblast process. (Adapted from: http://www.tannlegetidende.no/i/2016/1/d2e198).

2.1.4 Odontogenesis

Odontogenesis or tooth formation is a process that occurs within developing embryos via sequential and reciprocal interactions between mesenchymal and epithelial cells (Volponi *et al.*, 2010). The principles of early odontogenesis are being used to devise methods to generate teeth in humans (Modino and Sharpe, 2005).

Odontogenesis starts with the condensation of the neural-crest derived ectomesenchymal cells around the epithelial bud (Volponi *et al.*, 2010). The formation is commonly divided into the following stages: initiation stage, the bud stage, the cap stage, the bell stage, and the maturation. The staging of odontogenesis is an attempt to categorise changes that take place along a continuum; frequently deemed difficult to determine the current stage of a particular developing tooth (Krivanek *et al.*, 2017).

2.1.4(a) Odontoblast specific gene markers

Odontoblast phenotype is widely used in previous studies to determine the odontogeneic differentiation of stem cells (Shi *et al.*, 2005; Bakopoulou *et al.*, 2011). In this study, two cell surface markers expressed by odontoblast were analysed, which are dentine sialophosphoprotein (DSPP) and alkaline phosphatase (ALP). These proteins are essential for suitable development and mineralisation of hard tissues such as bone and dentine (Suzuki *et al.*, 2012). Other available proteins involved in odontogenesis are dentin matrix protein 1 (DMP1) (Lu *et al.*, 2007), osteocalcin (OCN) (Yu *et al.*, 2007), osteopontin (OPN) (Rathinam *et al.*, 2015), collagen-1 (COL-1) (Paduano *et al.*, 2016), runt-related transcription factor 2 (Runx2) (Hu *et al.*, 2011),

osterix (OSX) (Zhang *et al.*, 2011), and matrix extracellular phosphoglycoprotein (MEPE) (Staines *et al.*, 2012).

DSPP is an ECM protein, typically found in dentin- and bone-specific gene, which plays an important role in dentin mineralisation and tooth development (Wu *et al.*, 2008). The organic components of dentin consist of 90% type I collagen and 10% non-collagenous proteins namely, dentin sialoprotein (DSP) and dentin phosphoprotein (Fratzl *et al.*, 2004). It was reported that, *DSPP* up-regulates osteoblast marker genes in primary human adult MSCs, mouse osteoblastic and fibroblastic cell lines (Jadlowiec *et al.*, 2004). Besides bone and dentin, *DSPP* also plays an important role in growth factor function and cellular signalling transduction in human tissues (Qin *et al.*, 2002; Alvares *et al.*, 2006).

ALP is an endogenous enzyme present in many organs such as ovary and thymus (McComb *et al.*, 2013). The enzyme activity is most often involved at the beginning of osteo/odontoblast differentiation (Min *et al.*, 2010). As an enzyme marker of both osteoblasts and odontoblasts, ALP plays a vital role in calcified tissue formation and extracellular matrix metabolism (Lee *et al.*, 2006). According to Beck (2000), this enzyme is bound to the membrane of osteoblasts and functions to enhance osteogenesis by degrading pyrophosphate. It works by inhibiting crystallisation at the calcification site and degrades organic phosphate esters, to increase the inorganic phosphate concentration (Beck *et al.*, 2000). In addition, it has also been reported that the activity of *ALP* in odontoblast-like cells is higher than in undifferentiated cells (Pang *et al.*, 2006). Therefore, *ALP* is believed to stimulate the formation of mineralised nodule

and calcium deposition in most cells that have differentiated into odontoblasts, as it is considered as an early marker of odontoblastic differentiation (Wu *et al.*, 2008).

2.1.4(b) Aquaporin5 and its role in odontogenesis

Aquaporins (AQPs) are the type of integral membrane proteins that facilitate water movement into the cell by forming pores in the membrane of biological cells (Borgnia *et al.*, 1999). AQP5 is localised in many types of cells such as salivary acinar, alveolar, and cornea cells. Moreover, it is one of the 13 membrane proteins from the family of aquaporins that control the movement of water (Yakata *et al.*, 2011). Among these, AQP1, AQP2, AQP4, AQP5, AQP6, or AQP8 are exclusively selective for water, while AQP3, AQP7, AQP9, or AQP10 are proposed as aqua glyceroporins which can transport water and small neutral solutes such as glycerol (Nico and Ribatti, 2011). AQP5 controls cell homeostasis as it is down-regulated in atrophic salivary acinar cells (Azlina *et al.*, 2010). Felszeghy (2004) demonstrated that AQP5 is expressed in various human and mouse dental structures during odontogenesis but there was no *AQP5* expression detected in dental pulp cells when dental hard tissues were present. However, after pre-dentin formation, an intense signal was evident for these water channel throughout the entire cytoplasm of young odontoblasts (Felszeghy *et al.*, 2004). Meanwhile, previous study reported that *AQP5* is involved in osteogeneic stem cell differentiation (Yi *et al.*, 2012) and associates with NF-κB signalling (Yao *et al.*, 2010). It was reported that *AQP5* is down-regulated during osteogeneic stem cell differentiation (Yi *et al.*, 2012), and the down-regulation of *AQP5* is associated with NF-κB signalling (Yao *et al.*, 2010). These findings relentlessly supported the negative role of *AQP5* in osteogeneic stem cell differentiation. Other than that, some findings

also suggested AQP5 is associated with cancer cell proliferation and migration (Jung *et al.*, 2011; Wang *et al.*, 2015).

2.1.5 Dental pulp impairment

When the teeth suffer from impairment caused by chemical, mechanical, thermal or microbial causes, they lead to inflammation and disease affecting local tissue, lymph as well as vascularity (Demarco *et al.*, 2011) (Figure 2.3). Dental caries, one of the common health problems, frequently occurs among children (Werle *et al.*, 2016). The carious infection is caused by cariogenic bacteria such as *Streptococci* and *Lactobacilli*. The interaction between the two strains of bacteria resulting in acidic environment and thus deepened the lesion (Farges *et al.*, 2015). The infection at the pulp area is difficult to manage since it has minimal blood supply affecting the inflammatory regulation to combat infections (Demarco *et al.*, 2011). Injured dental pulp has limited potential for self-recovery. If the infected pulp area has a severe inflammation, it leads to irreversible pulpitis (Farges *et al.*, 2015). Irreversible pulpitis indicates that vascularity function can no longer be achieved in that infected area (Wang *et al.*, 2010c).

On the other hand, if the inflammation is mild, involves moderate attrition, erosion, or superficial fracture, the odontoblasts can usually survive and continue to produce the dentin barrier beneath the injury, allowing the underlying soft pulp tissue to retain its function (Zhang and Yelick, 2010). The essential strategy to preserve dental pulp when it is infected is, to protect the remaining odontoblasts (Ward, 2002). However, when the infections are strong and rapidly progressing, deep dentin caries, severe abrasion,

or fracture, the primary odontoblasts will be destroyed (Zhang and Yelick, 2010). In such cases, the post-mitotic terminally differentiated odontoblasts lack the ability to proliferate to replace the injured odontoblasts or to produce new dentin (Ricucci *et al.*, 2014). However, the MSCs within the dental pulp have the ability to differentiate into odontoblasts and to secrete reparative dentin (Obeid *et al.*, 2013).

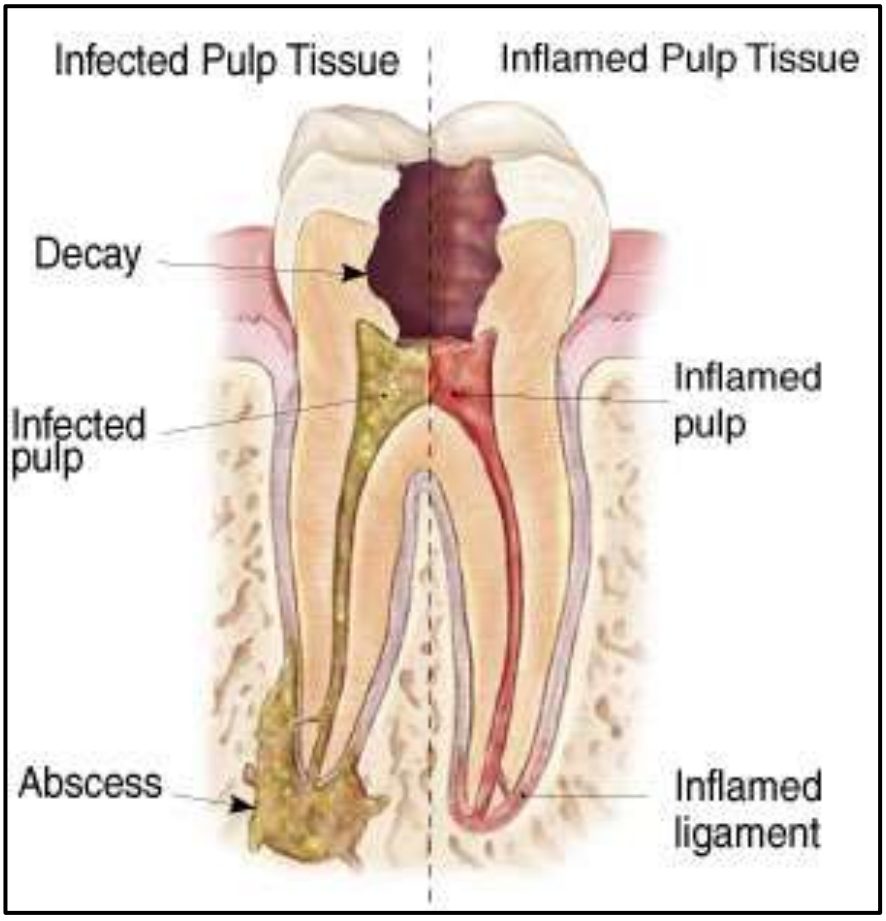

Figure 2.3: Cross sectional schematic diagram of tooth exposing damaged pulp. Bacteria and other decayed debris that goes into the pulp chamber results into an infected or abscessed tooth. (Adapted from: http://dentalwestwood.com/root-canals/).

2.1.6 Advancement in dental pulp treatment

Traditional treatment in dental pulp impairment is regenerative endodontics. It is based on the concept of tissue engineering (TE). Regenerative endodontics can be defined as biologically based procedures designed to replace damaged structures, including dentine and root structures, as well as cells of the pulp-dentine complex (Murray *et al.*, 2007). Clearly, it is a field of active research that is dynamically growing in both basic research and clinical applications. It has been reported that the scope of regenerative endodontics may be increased to include the replacement of periapical tissues, periodontal ligaments, gingiva and even the whole tooth. This would give patients a clear alternative to the artificial tooth implants that are currently available (Garcia-Godoy and Murray, 2006).

Since stem cells could be differentiated into other cell lineages, dental pulp regeneration has caught the interest of many researches (Huang *et al.*, 2009a). The possible approach taken to regenerate dental pulp is through transplantation of stem cells into the pulp area for the differentiation into functional tissue including vascularisation (Trope, 2010; Huang, 2011). Pulp vasculature is essential for nutrient and oxygen transportation and inflammatory regulation (Nakashima *et al.*, 2009).

2.2 Understanding tissue engineering concept for dental pulp regeneration

Regeneration of pulp is an unmet need in endodontic therapy (Nakashima *et al.*, 2009). TE paves the way for researches in the new field of regenerative endodontics to

overcome the limits of conservative treatment (Janjić *et al.*, 2016). TE based approaches have been considered as an attractive strategy for dental pulp regeneration (Nakashima and Reddi, 2003; Nör, 2006; Rosa *et al.*, 2013). TE evolved from the field of biomaterials development and perhaps is best defined as the involvement of cells, scaffolds, and signalling factors, alone or in combination,that aims to improve or replace biological tissues. TE was also known as an interdisciplinary field that applies the principle of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ (Lanza *et al.*, 2011). In addition, TE also aims to stimulate the body either to regenerate tissue on its own or to grow tissue outside the body which can then be implanted as natural tissue (Nadig, 2009). TE with the triad of; i) an appropriate cell source ii) a supportive matrix (scaffold) and iii) inductive biological factors or signals create better regenerative therapies (Murray *et al.*, 2007). Therefore, choice of scaffolds, growth factors, and cells are the key considerations in developing a strategy for TE.

2.2.1 Scaffold

Scaffold is developed to support the host cells during TE, promoting their differentiation and proliferation throughout their formation into a new tissue (Niknejad *et al.*, 2008). Therefore, the design and selection of the biomaterials used for scaffolding is a critical step in TE (Mano *et al.*, 2007). Scaffolds can be artificial or natural. Natural scaffolds are usually more biocompatible when compared with synthetic scaffolds (Liu *et al.*, 2007). During TE, cell seeded onto scaffolds is the first step in establishing a 3D culture, and plays a crucial role in determining the progression of the tissue formation (Song *et al.*, 2008). Successful cell seeding of the

scaffold depends on the type and source of the living cells as well as ECM components of the scaffold (Benders *et al.*, 2013) to support 3D tissue formation in the therapeutic application (Badylak *et al.*, 2009). Since the construction of a scaffold must mimic the ECM, therefore the natural source scaffold that can be easily obtained which is human amniotic membrane (HAM) were chosen. ECM components of the HAM such as collagen, elastin, laminin, and fibronectin have been shown to be an excellent candidate of a scaffold for TE application (Chen *et al.*, 2012b).

2.2.1(a) Human amniotic membrane (HAM)

HAM thickness varies from 0.02 mm to 0.5 mm and consists of three main histological layers: the epithelial layer, the thick basement membrane and the avascular mesenchymal tissue (Mamede *et al.*, 2012a). The basement side comprised of epithelium and basement membrane while the stromal side has three distinct layers; which are compact, fibroblast and spongy layers (Hashim *et al.*, 2016). The basement membrane contains large amounts of proteoglycans that are rich in heparan sulphate and that serves as a permeable barrier to amniotic macromolecules and several molecules with a structural function enabling the maintenance of membrane integrity (Toda *et al.*, 2007). Those molecules are actin, α- actinin, spectrin, ezrin, several cytokeratins, vimentin, desmoplakin and laminin (Mamede *et al.*, 2012a). The most investigated molecules is laminin because it contributes to cell survival, differentiation, shape, movement and is involved in the maintenance of tissue phenotypes (Takashima *et al.*, 2004; Toda *et al.*, 2007).

Besides that, the outer layer of HAM is composed of mesenchymal fibroblast-like cells that are probably derived from the mesodermal embryonic plate scattered in a full-term membrane (Díaz-Prado *et al.*, 2011) (Figure 2.4). The content of collagen-rich mesenchymal layer increases its tensile strength. The outermost layer of the amnion as zona spongiosa, because its abundant content of proteoglycans and glycoproteins produces a spongy appearance in histological preparations (Rocha and Baptista, 2015). This layer lying adjacent to the chorion laeve is an almost acellular structure and contains a non-fibrillar meshwork mostly of type III collagen (Benirschke *et al.*, 2012).

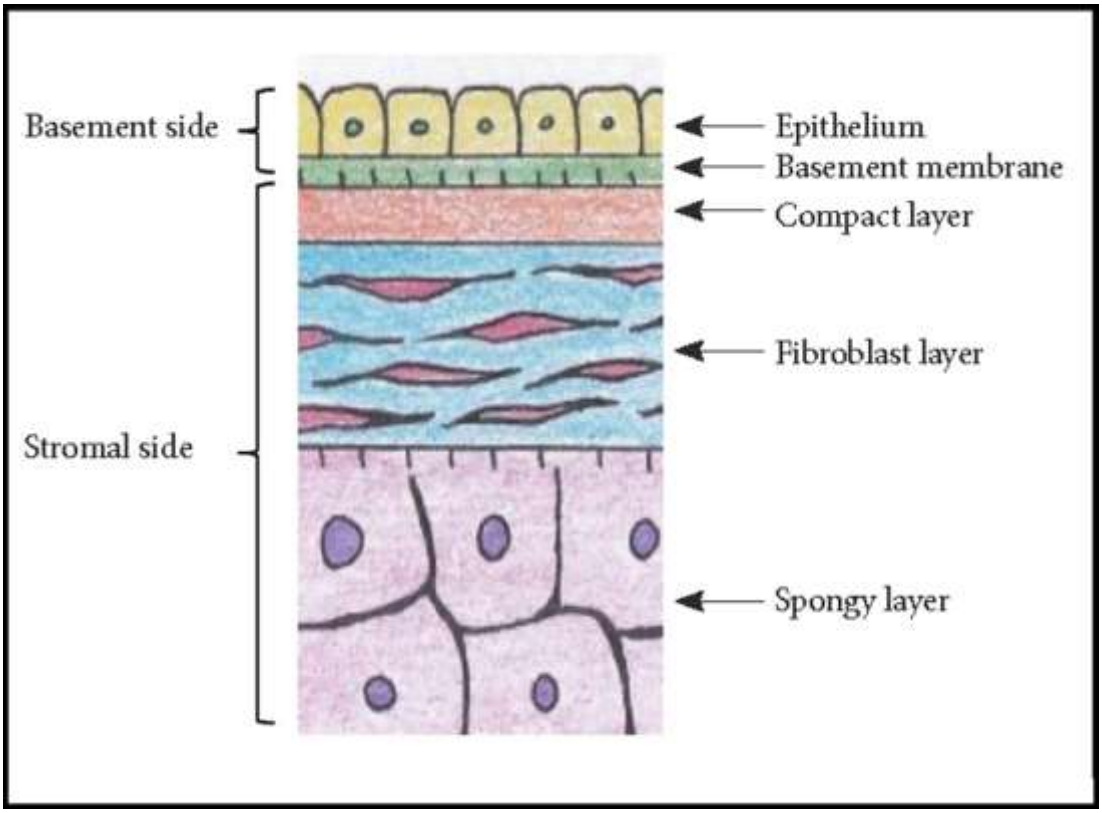

Figure 2.4: Schematic diagram of human amniotic membrane (HAM). Basement side comprised of epithelium and basement membrane while stromal side has 3 distinct layers; which are compact, fibroblast and spongy layers. (Adapted from Hashim *et al*., 2016).

2.2.1(b) Applications and potentials of HAM in tissue engineering

Possessing many favourable properties, such as in-expensiveness and its availability, makes HAM a potential biomaterial scaffold for TE applications, especially for soft TE. In addition, this material is made up of many ECM proteins, cytokines, and growth factors that could enhance cell proliferation and function (Gholipourmalekabadi *et al.*, 2015). When epithelial and mesenchymal cells are seeded on a cellular scaffold created from the amniotic membrane, the cells were highly interconnected and capable of penetrating the porous structure of the amnion scaffold (Niknejad *et al.*, 2008).

In early 19th century, HAM was initially designed for skin transplantation and, later, HAM was also used for management of skin burns and superficial wounds (Mamede *et al.*, 2012a). Studies have indicated that the use of this membrane is mainly associated with the early induction of the repair process, as well as the promotion of pain relief and hemostasis (Rinastiti *et al.*, 2006). Interestingly, the antibacterial properties of this membrane have also been reported when it is applied to infected wounds. Their findings demonstrated that the high concentration of lysozyme is a powerful bactericidal enzyme that against many gram-negative microorganisms (Werber and Martin, 2013).

The special properties and availability of HAM made it an ideal candidate for many other purposes in research including periodontal regeneration. HAM is known to be suitable substrate for culturing periodontal ligament cells as these cells are capable of proliferating, maintaining their original properties and provide strong cell adhesion on the basement side of cryopreserved HAM (Adachi *et al.*, 2014).