CELL GROWTH EFFECT OF PERIVITELLINE FLUID FROM HORSESHOE CRAB ON STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH

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by

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LIST OF ABBREVIATIONS

α-MEM	Alpha Minimum Essential Medium
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar
ºC	Degree Celsius
%	Percentage
2-DGE	Two-dimensional gel electrophoresis
3D	Three-dimensional
ADV	Average Density Values
ASCs	Adult stem cells
BCL2L11	Bcl-2-like protein 11
b-FGF	Basic fibroblast growth factor
BLAST	Basic Local Alignment Search Tool
BMMSCs	Bone marrow-derived mesenchymal stem cells
BMP	Bone morphogenetic protein
bp	Base pair
CA	Chromosome aberration
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
cDNA	Complementary DNA
CO_2	Carbon dioxide
DMSCs	Mesenchymal stem cells derived from dental tissues
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleoside
dNTP	Deoxyribonucleotide triphosphate
DPSCs	Dental pulp stem cells
DTT	Dithiothreitol
ECM	extracellular matrix
EthD-1	Ethidium homodimer-1
ESCs	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSI	Gonado-somatic index
HPLC	High-performance liquid chromatography
iPSCs	Induced pluripotent stem cells
LAL	Limulus Amoebocyte Lysate
LB	Lithium borate
MDCK	Madin Darby Canine Kidney
MDM2	Mouse double minute 2 homolog
mg	Milligram
mM	Millimolar
MMLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase
MSCs	Mesenchymal stem cells
NAD	Nicotinamide adenine dinucleotide
NCBI	National Centre for Biotechnology Information
NFM	Neurofilament M

NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
PI 3	Phosphoinositide- 3 kinase signalling pathway
PtdIns(3,4,5)*P*3	Phosphatidylinositol 3,4,5-trisphosphate
PTEN	Phosphatase and tensin homolog
PVF	Perivitelline fluid
RNA	Ribonucleic acid
Rpm	Rotation per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHED	Stem cells from human exfoliated deciduous teeth
TGFβ	transforming growth factorβ
T. gigas	*Tachypleus gigas*
TNF	Tumour necrosis factor
TP53	Tumor protein p53
UMT	Universiti Malaysia Terengganu
VEGF	Vascular endothelial growth factor

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KESAN CECAIR PERIVITELLINE DARIPADA BELANGKAS TERHADAP PERTUMBUHAN SEL TUNJANG DARI GIGI SUSU MANUSIA YANG TERKELUPAS

ABSTRAK

Cecair perivitelline (PVF) daripada telur belangkas yang telah disenyawakan dilaporkan mampu untuk membantu proses embriogenesis, serta dapat memberi impak positif dalam pertumbuhan dan pembezaan sel dimana ia sekaligus mampu mendorong kepada regenerasi sesetengah organ di dalam organisma terpilih. Kesan PVF (0.019 mg/ml) terhadap sel tunjang dari gigi susu manusia yang terkelupas (SHED) dikaji dari segi kebolehhidupan sel dengan menggunakan kit HIDUP/MATI kebolehhidupan/sitotoksisiti untuk sel-sel mamalia. Kebolehhidupan sel di dalam kumpulan SHED dengan rawatan PVF serta kumpulan kawalan (SHED tanpa rawatan PVF) dikaji dengan melihat dan menganalisa sel hidup dan mati menggunakan mikroskop pendaflour. Peratusan sel hidup dan mati untuk kedua-dua kumpulan tersebut juga dikenalkpasti dengan menggunakan pembaca mikroplat pendarfluor. Kedua-dua analisis ini telah dijalankan selama 3 hari berturut-turut, hari 1, 2 dan 3. Dapatan kajian melaporkan bahawa PVF adalah efektif dalam meningkatkan tahap kebolehhidupan SHED di mana peratusan sel hidup yang lebih tinggi direkod untuk kumpulan SHED dengan rawatan PVF. Peratusan sel hidup untuk kumpulan SHED dengan rawatan PVF kekal tinggi daripada 90% untuk tiga hari manakala di dalam kumpulan kawalan, peratusan sel hidup susut secara perlahan-lahan kepada 76.09% pada hari yang ketiga. Disamping itu, imej daripada mikroskop pendaflour juga menunjukkan lebih banyak sel yang hidup dan sihat untuk kumpulan SHED dengan

rawatan PVF sehingga hari ketiga. Manakala kumpulan kawalan menunjukkan lebih banyak kewujudan sel mati (merah). Kemudian, ekspresi gen yang terlibat dalam peraturan kitaran sel telah dikaji dan dibanding diantara kumpulan rawatan dan kawalan SHED menggunakan tindak balas rantai polimerase transkripsi terbalik (RT-PCR) pada hari 1, 3, 7, 14 dan 21. Ujian Mann-Whitney telah digunakan untuk menentukan perbezaan signifikan bagi ekspresi gen untuk kedua-dua kumpulan SHED. Gen pengawal kitaran sel, *CDKN2A, PTEN* and *TP53* menunjukkan ekspresi tinggi yang signifikan dalam kumpulan rawatan PVF berbanding kumpulan kawalan ($p \leq 0.05$) pada hari ke 7 hingga hari ke 21 sekaligus mencadangkan kemampuan PVF dalam membantu pertumbuhan dan proliferasi SHED. Manakala, ekspresi onkogen, *MDM2* kekal rendah di dalam kumpulan rawatan PVF sepanjang experimen sekaligus menunjukkan bahawa PVF tidak menyebabkan pembantutan kitaran sel ke atas SHED. Ekspresi yang rendah dan malap dilihat untuk gen pengaktif apoptotik, *BCL2L11* di dalam kumpulan rawatan PVF daripada hari 1 sehingga hari 14, dengan kenaikkan mendadak pada hari ke-21. Dapatan kajian merumuskan bahawa kesesakan sel SHED di dalam bekas kultur yang sempit itu telah mengaktifkan ekspresi *BCL2L11* sekaligus mengaktifkan laluan isyarat kematian sel pada hari ke-21. Secara keseluruhan, kajian semasa menyimpulkan bahawa PVF bertanggungjawab dalam peraturan kitaran sel, proliferasi dan pertumbuhan serta meningkatkan kebolehhidupan SHED.

CELL GROWTH EFFECT OF PERIVITELLINE FLUID FROM HORSESHOE CRAB ON STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH

ABSTRACT

Perivitelline fluid (PVF) from the fertilized eggs of a horseshoe crab has been reported to support embryogenesis, enhance cell growth and differentiation as well as promote organ regeneration in certain organisms. The effect of PVF (0.019 mg/ml) on stem cells from human exfoliated deciduous teeth (SHED) were investigated with regard to cell viability using LIVE/DEAD viability/cytotoxicity kit for mammalian cells. The cell viability in PVF treated SHED and control group (SHED without PVF treatment) were assessed by observing the live and dead cells using fluorescence microscope and the percentage of live and dead cells using fluorescence microplate reader for 3 consecutive day(s), 1, 2 and 3. The results showed that the viability of SHED was as demonstrated by the higher live cell percentage in the PVF treated group compared to control. The percentage of live cells in PVF treated group remained higher than 90% for 3 days while in the control group, the live cell percentage dropped to 76.09% on the third day of test. Moreover, the fluorescence microscopy images also demonstrated more live and viable SHED in the PVF treated group until day 3 compared to the control which showed the presence of dead (red) cells. The expression of selected cell cycle regulatory genes in control and PVF treated SHED were compared using reverse transcriptase polymerase chain reaction (RT-PCR) on day(s) 1, 3, 7, 14 and 21. Mann-Whitney test was used to determine the significant difference in the gene expression between both groups of SHED. Cell cycle regulatory genes,

CDKN2A, PTEN and *TP53* expressed significantly higher in the PVF treated group compared to control ($p \leq 0.05$) on day 7 until day 21 which proposes that PVF treatment enhances SHED growth and proliferation. On the contrary, the expression of *MDM2*, an oncogene, remained at low levels in the treated group throughout the whole experiment indicating that PVF did not result in cell cycle arrest in SHED. Faint and low expression of apoptotic activator gene, *BCL2L11* was observed in the PVF treated group from day 1 until 14 with a sudden peak on day 21, demonstrating that overcrowding of SHED in the confined culture flask could have induced the expression of *BCL2L11* which activates cell death signalling pathways towards the 21st day of incubation. Hence, it can be concluded that PVF plays a role in cell cycle regulation, proliferation and growth and increases the viability of SHED.

CHAPTER 1

INTRODUCTION

1.1 Background of study

The increasingly high demand of stem cells to support tissue engineering and regenerative medicine has opened new windows for exploration in a wide variety of stem cells such as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and somatic stem cells. However, immunorejection, tumorigenesis as well as ethical morals involving ESCs and iPSCs have made it less practicable for its application in the clinical level (Lo and Parham, 2009). Attention has been brought to the most common type of somatic stem cells, namely, mesenchymal stem cells (MSCs), which could be isolated from various tissues including bone marrow, adipose tissue, skin, umbilical cord, and placenta (Kern *et al.*, 2006). Ever since the discovery of bone marrow in the 1960's, it has remained to be the most common source for obtaining MSCs (Charbord, 2010). Nevertheless, the aspiration process of obtaining the bone marrow is not only an invasive and a painful procedure but could also cause some amount of trauma to the donor. Therefore, researchers have identified and characterized alternative sources of MSCs which are obtained from the human teeth.

The most common alternative sources of MSCs are dental pulp stem cells (DPSCs) which are extracted from the pulp of permanent teeth and stem cells from human exfoliated deciduous teeth (SHED). Both of them are claimed to be originated from

the cranial neural crest and express early markers for both mesenchymal and neuroectodermal stem cells (Gronthos *et al.*, 2000; Miura *et al.*, 2003). DPSCs and SHED express trophic factors that could enhance the neuronal survival, proliferation, differentiation, and migration (Nosrat *et al.*, 2001; Nosrat *et al.*, 2004; Arthur *et al.*, 2009). Both these stem cells have been identified as a novel population of stem cells that have the capacity of self-renewal and multilineage differentiation (Gronthos *et al.*, 2000; Gronthos *et al.*, 2002; Miura *et al.*, 2003; d'Aquino *et al.*, 2008), mimicking the trait of MSCs.

However, in the current study, SHED was chosen as it possesses higher level of proliferation as compared to DPSCs (Nakamura *et al.*, 2009). This is due to the higher expression of genes related to cell proliferation and extracellular matrix, including several cytokines such as fibroblast growth factor and tumour growth factor beta detected in SHED. SHED are known for their high plasticity since they could differentiate into wide range of cells, namely, adipocytes, neural cells and odontoblasts (Miura *et al.,* 2003). Researches have also confirmed that the proliferation rate of SHED is much higher than in DPSCs and bone marrow-derived mesenchymal stem cells (BMMSCs) (Miura *et al.*, 2003; Nakamura *et al.*, 2009; Pivoriūnas *et al.*, 2009), thus making SHED an excellent candidate to be explored for its potential in regenerative medicine, especially, in dental tissue regeneration. Most importantly, studies have shown that SHED were capable of promoting the growth of bone and pulp/dentin complexes *in vivo* (Rodríguez-Lozano *et al.*, 2012), which in turn could contribute in the clinical application of regenerative medicine. Besides, SHED have been reported to have the potential to be used as cellular resource for neuroregeneration therapies (Sakai *et al.*, 2012). The main benefits of SHED are that

they are economically friendly, as SHED could easily be obtained non-invasively from deciduous teeth that are routinely extracted among children and then discarded as medical waste. However, due to the physiological absorption of roots in deciduous teeth, only a small amount of dental pulp remains in exfoliated deciduous teeth. Hence, one of the main constraints of SHED is its limited supply and challenges in maintaining its “stemness” throughout the multiple passaging during artificial cell cultures (Yu *et al.*, 2014).

The advancement of tissue engineering needs is not an easy task as their degree of stemness would usually decline after a number of passages. In the present study, perivitelline fluid (PVF) was selected to be tested on SHED. A number of studies have been done previously on PVF which is extracted from the fertilized eggs of horseshoe crab. It is reported that PVF has depicted its relevance as a supplement material in enhancing biological processes such as proliferation of cells and in promoting the generation of certain organs in certain organisms due to its richness in proteins and important amino acids (Ghaskadbi *et al.*, 2008; Dolashka and Voelter, 2013; Srijaya *et al.*, 2013; Musa *et al.*, 2015). Horseshoe crab has been one of the most versatile marine species that offers valuable biological sources that contributes to biomedical researches and testing. PVF from horseshoe crab has been studied in many areas reflecting its capability to promote many cell biological processes such as cell proliferation (Musa *et al.*, 2015), angiogenesis (Ghaskadbi *et al.*, 2008), and gonadal development (Srijaya *et al.*, 2013). PVF is rich in molecules such as haemagglutinins, haemocyanins and lectins which could stimulate growth and differentiation of cells. It was also further confirmed that the compound called lectins present in PVF has essential roles in stimulating embryogenesis at early stages by interacting with

endogenous glycoproteins or *N*-acetylhexosamines (Nagai *et al.*, 1999). PVF has also been demonstrated to be non-toxic and non-mutagenic to DPSCs which reflect the potential of using PVF as a supplement in enhancing the proliferation and cell viability of SHED as well (Musa *et al.*, 2015).

In order to understand the PVF's potential on SHED, this study analysed the effect of PVF on SHED by taking into account the expression of selected genes which are known to be involved in cell-cycle regulation and proliferation which are Cyclin-Dependent Kinase Inhibitor 2A (*CDKN2A),* Mouse double minute 2 homolog (*MDM2),* Tumor Protein p53 (*TP53)* and Phosphatase and tensin homolog *(PTEN)*. Besides that, this study also aimed to analyse the expression *BCL2L11*, an apoptotic activator gene to find out whether PVF promotes or inhibits the apoptotic process in SHED. Cell viability test using Live/Dead Cytotoxicity Kit was also done to assess the cell viability of SHED after treatment with PVF.

1.2 Problem statement

A number of studies have been conducted to assess the effect of PVF in many types of cells. The outcome of these researches has given light on the potential of PVF in sustaining and enhancing as well as inducing various cell biological processes (Ghaskadbi *et al.*, 2008; Srijaya *et al.*, 2013; Musa *et al.*, 2015). SHED are one of the best sources of obtaining MSCs as it has the capability to self-renew as well as differentiate into multiple cell lineages. However, the extent of practicability of using SHED in many medical applications has not been fully applied which could be due to insufficient knowledge in terms of the mechanisms in sustaining and enhancing its

growth and stemness. Besides, even though SHED have the characteristics that mimic MSCs, it is still a challenge to translate SHED into the real clinical application as it has much lower cell yield as compared to the bone marrow derived MSCs. Therefore, to explore the potential of PVF as a supplement in supporting stem cell growth, this study is warranted to assess its effect on SHED.

1.3 Justification of the study

With the huge potential that SHED could offer to support today's regenerative medicine application, it has now become one of the most discussed tools to substitute bone marrow derived MSCs. PVF obtained from fertilized eggs of horseshoe crab has brought attention regarding its potential in supporting cell biological processes as it contains high amount of proteins and amino acid content that has been widely documented in many papers to be effective in promoting cell growth and differentiation in various types of cells (Ghaskadbi *et al.*, 2008; Srijaya *et al.*, 2013; Musa *et al.*, 2015). Therefore, this study would pave way to explore and look into the potential of PVF in supplementing the cell growth and regulation process in SHED before further application step could be implemented.

1.4 Objectives

1.4.1 General objectives

The general objective is to study the effects of perivitelline fluid from fertilized eggs of horseshoe crab on the cell growth regulation of stem cells from human exfoliated deciduous teeth.

1.4.2 Specific objectives

1. To compare the gene expression of *CDKN2A, MDM2, TP53, PTEN* and *BCL2L11* in stem cells from human exfoliated deciduous teeth treated with and without perivitelline fluid.
2. To determine the cell viability in terms of cell morphology and live/dead cell percentage of stem cells from human exfoliated deciduous teeth treated with and without perivitelline fluid.

1.5 Research hypothesis

PVF increases cell viability and enhances the cell cycle regulatory gene expression in stem cells from human exfoliated deciduous teeth.

CHAPTER 2

LITERATURE REVIEW

2.1 Regenerative medicine and stem cell therapy

The field of regenerative medicine is now being actively explored due to the abundance of research and development in stem cell therapy aiming at treating various types of diseases. Regenerative medicine is defined as a translational research in tissue engineering and molecular biology that revolves around the process of substituting, engineering or regenerating human cells, tissues or organs for normal function restoration and establishment (Shankar, 2011). A successful translation of stem cell therapy relies on four crucial factors that work together; choice of stem cells, environment/scaffold, necessary biomolecules such as cytokines and growth factors, and physical and mechanical forces to support the development of the cells. Stem cell therapy is deemed promising in the new era of treating diseases such as cardiac (Orlic *et al.*, 2001) and bone defects (Peng *et al.*, 2002) which has been supported by many preclinical studies on animals.

There are few parameters which are crucial for its successful translation in the clinical world. First, the mechanism of action needs to be carefully characterized and identified as the repair response upon receiving the treatment does not solely depend on the transplanted cells, but rather is a dynamic and correlated signalling network between the transplanted cells and host cells (Mahla, 2016). Hence, this relationship also involves the secretion of paracrine factors that are stimulated by the injured host

(Caplan, 2007). Regenerative medicine using stem cells is an interesting branch to be explored as stem cells have the potential of differentiate into different types of cells when cultured in different types of environment. Studies have shown that human neuronal stem cells could be differentiated into muscle cells when transplanted into skeletal muscle (Galli *et al.*, 2000), while bone marrow cells could be transformed into neuronal cells when introduced into a neural environment (Zhao *et al.*, 2002; Mezey *et al.*, 2003).

2.2 Embryonic stem cells

Embryonic stem cells (ESCs) possess pluripotency trait and originate from the inner cell mass of embryo during blastocyst stage. Due to its pluripotency, ESCs are made of undifferentiated cells that are deemed to have the full potential in giving rise to all types of tissues of the three embryological germ layers; ectoderm, mesoderm and endoderm. Since its first isolation in 1998, it was reported that the main pluripotency markers such as OCT4, SOX2, and NANOG were highly present in ESCs thus explaining its pluripotent trait (Thomson *et al.*, 1998). This has given a new perspective in the advancement of regenerative medicine as ESCs are highly anticipated to cure all kinds of diseases (Thomson *et al.*, 1998).

A number of studies have been conducted to support this theory. ESCs were shown to be able to generate hepatic cells and the generated hepatic cells were reported to be successfully used for the treatment of liver injuries as well as in high throughput drug screening (Avior *et al.*, 2015; Tolosa *et al.*, 2015; Carpentier *et al.*, 2016). Besides that, ESCs were also able to produce insulin by generating β-cells with the help of

progenitors ($CD24^+$, $CD49^+$ & $CD133^+$) as reported in a previous study (Salguero-Aranda *et al.*, 2016). Furthermore, ESCs have been shown to be able to produce chondrocytes, the cartilage forming cells embedded in fibrin gel. Positive results were observed as the new chondrocytes that were transplanted effectively healed defective cartilage in the knee joints of mice in 3 months without any adverse effects (Cheng *et al.*, 2014). ESCs have also been shown to have the potential of being an alternative treatment in cardiovascular diseases as ESCs could be differentiated into electro physiologically active cells of the heart; sinoatrial node pacemaker cells that help in promoting natural regulation of heart pace (Vedantham, 2015). However, due to ethical concerns, the application and translation of ESCs into the clinical realm has been restricted. Hence, this has opened exploration of new alternatives of stem cell sources such as adult stem cells (ASCs).

2.3 Adult stem cells

Adult stem cells (ASCs) have been studied quite extensively as it turns out to be a more acceptable alternative against the controversial ECSs. ASCs have a different potential of differentiation as compared to ESCs by its more limited multipotent differentiation capability. It is suggested that ASCs could give rise to only a group of specialized cell types that originate from a specific embryological germ layer, either from ectoderm, mesoderm or endoderm (Caplan, 2007). ASCs also produce progenitor or precursor cells that can eventually differentiate into specific types of cell lineage. Another characteristic of ASCs is that they can replicate and make identical daughter cells repetitively, indicating that they have the capability to proliferate and self-renew. Another highly debated characteristic of ASCs is the possibility of plasticity or in other

terms; unorthodox differentiation or trans differentiation to take place in stem cells derived from adult tissues (Sykova and Forostyak, 2013). The term plasticity is used to refer to the condition whereby ASCs could generate or differentiate into specialized cells which originate from another tissue origin besides from where they initially originated (Wu *et al.*, 2002). Previous studies have demonstrated that BMMSCs from mesoderm were able to differentiate into neural tissues which originates from ectoderm (Zhao *et al.*, 2002; Mezey *et al.*, 2003).

As a contrast, neural stem cell lines were shown to have the potential to develop into hematopoietic cells as well (Bjornson *et al.*, 1999). The concept of plasticity in ASCs remains an important aspect to be explored and needs further validation through more researches.

2.3.1 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) which are a type of ASCs that originate from the mesoderm holds a promising potential in the revolution of today's regenerative medicine due to its multipotent trait (Short *et al.*, 2003). Common biomarkers expressed by MSCs are CD73+, CD90+, CD105+, CD11b−, CD14−, CD19−, CD34−, CD45−, CD79a−, and HLA-DR (Dominici *et al.*, 2006). MSCs can differentiate into a group of tissues which includes tendons, bone, cartilage, ligaments, muscles, and neurons (Dominici *et al.*, 2006). The cases of dental carries, periodontal disease and tooth injury among children and adults have triggered the idea to regenerate tooth as an alternative treatment. Also, epithelial-MSCs have been shown to give rise to a mature tooth. The potential of MSCs to be chondrogenic, osteogenic, and adipogenic

in vitro has made MSCs a good candidate to be translated for therapeutic purposes in dental related injuries and diseases (Csaki *et al.*, 2007).

2.3.1(a) Dental pulp stem cells

One of the alternative sources to the classic BMMSCs is from dental tissues, called MSCs derived from dental tissues (DMSCs). There are a few types of DMSCs; dental pulp stem cells (DPSCs) isolated from dental pulp tissue of permanent teeth, stem cells from human exfoliated deciduous teeth (SHED) as shown in Figure 2.1, stem cells of apical papilla (SCAP), and periodontal ligament stem cells (PDLSCs) (Gronthos *et al.*, 2000; Miura *et al.*, 2003; Seo *et al.*, 2008). Due to their capacity to differentiate into multiple cell types (Gandhi *et al.*, 2011), recent studies have investigated the potential use of DMSCs in tissue engineering purposes. In addition, the availability of DMSCs in postnatal tissues makes it possible to provide endless opportunities to explore it for regenerative therapies (Gandhi *et al.,* 2011).

DPSCs are becoming an emerging tool in aiding the regenerative medicine as it has the multipotent capabilities to differentiate into different lineages of cells *in vitro* including odontoblasts, adipocytes, osteocytes, chondrocytes, and myocytes (Tatullo *et al.*, 2015). In a normal permanent tooth, DPSCs are responsible for regenerating the dentin layer of the teeth through complex series of mineralization process. More studies have been directed on exploring the potential of DPSCs in giving rise to multiple cell lineages. DPSCs have been portrayed to successfully differentiate into dentin *in vivo* as well as forming dentin-pulp like complex (Gronthos *et al.*, 2000). Gronthos *et al*. (2002) demonstrated the potential of DPSCs to form dense calcified

nodule. DPSCs also possess immune and anti-inflammatory properties which are necessary for successful allotransplantation experiments (Yan *et al.*, 2011). Moreover, the potential of DPSCs to differentiate into other cell lineages such as neuronal cells (Király *et al.*, 2009; Wang *et al.*, 2010) has also been demonstrated.

2.3.1(b) Stem cells from human exfoliated deciduous teeth

Another valuable resource of stem cells from dental tissues is SHED. SHED were discovered in 2003 by Dr. Songtao Shi and claimed to have greater differentiation potential than DPSCs which includes osteoblast-like, odontoblast-like cells, adipocytes, and neural cells (Miura *et al.*, 2003; Seo *et al.*, 2008; Nakamura *et al.*, 2009; Pivoriūnas *et al.*, 2009; Zheng *et al.*, 2009; Wang *et al.*, 2010). SHED has been shown to be able to generate viable amounts of bone and pulp/dentin complexes *in vivo* (Seo *et al.*, 2008; Zheng *et al.*, 2009; Sakai *et al.*, 2010). Similar to DPSCs, SHED also showed the potential to differentiate into osteogenic and adipogenic cells (Miura *et al.,* 2003). The most highlighted potential of SHED is their osteo-inductive capacity where successful bone forming cells were induced from SHED in the recipient murine cells following its transplantation *in vivo* (Miura *et al.,* 2003). Thus, these findings suggested that SHED could be involved in the bone formation during the eruption of permanent teeth. Furthermore, SHED is thought to have a huge potential in treating various bone related defects in the future due to its osteo-inductive potential.

Besides that, the presence of various neural cell markers in SHED suggested that SHED could be differentiated into neuronal like cells under neurogenic condition (Miura et *al.,* 2003). This claim was supported when SHED were shown to survive

inside the mouse brain microenvironment for more than 10 days alongside the fact that they also expressed neural markers such as neurofilament M (NFM). Interestingly, this finding is consistent to what has been demonstrated for BMMSCs which also have the potential of differentiating into neural-like cells after *in vivo* transplantation into the brain of a rat (Azizi *et al.*, 1998).

2.3.1(c) Comparison between DPSCs and SHED

Significant distinct traits between DPSCs and SHED have been demonstrated by Miura *et al.* (2003) and higher proliferation rate has been reported in SHED compared to DPSCs. SHED generally act as the main building block for the formation of mineralized osteoclast and odontoblast tissue thus highlighting its great potential in aiding orofacial bone regeneration (Sonoyama *et al.*, 2008). The odontoblastic inductive potential of SHED has been shown in a previous study using Western blot analysis where the expression of bone markers, CBFA1, ALP, MEPE, and bone sialoprotein were persistent and increased in SHED under induced environment (Shi *et al.*, 2015). Besides that, the formation and accumulation of calcium in SHED were also demonstrated by Alizarin staining (Miura *et al.*, 2003).

Similar to DPSCs, the banking of SHED is a much simpler process than the BMMSCs. SHED are currently extensively studied as an alternative to BMMSCs as the collection process of BMMSCs from the patient could be painful and invasive which could result in trauma. SHED, on the other hand could become a more favourable choice for dental tissue engineering purposes as it can be easily obtained without any highly invasive procedure or surgery. SHED were also able to preserve its "stemness" even after

revival from cryopreservation which can make future test results more reproducible and valid (Hodgetts *et al.*, 2014).

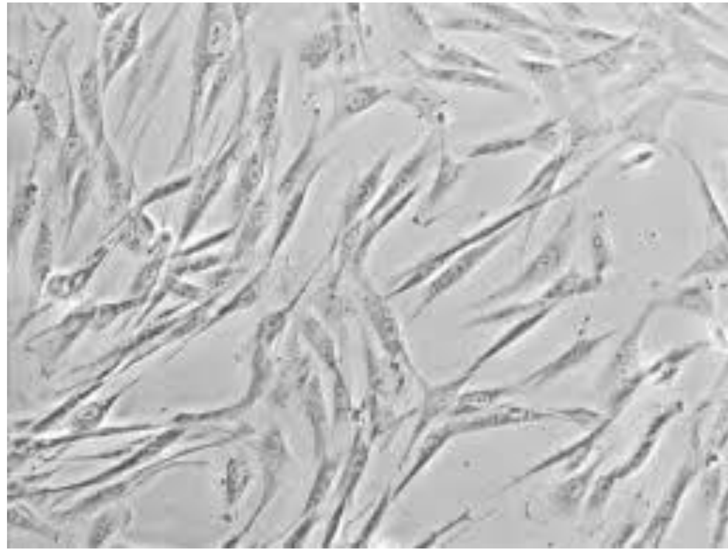

Figure 2.1 Spindle shaped morphology of stem cells from human exfoliated deciduous teeth under inverted microscope 10x magnification.

2.3.1(d) SHED cell line

SHED cell line that was used in this study was obtained commercially from AllCells (USA) (catalogue number DP004F). SHED was extracted from the pulp tissues of exfoliated deciduous teeth and hence considered as the most immature source of the dental stem cells. SHED showed the highest proliferative capacity compared to other sources of dental stem cells such as DPSCs and SCAP when grown in culture (Miura *et al.*, 2003). It was noted that the main characteristics of SHED cell line is their potential for multilineage differentiation and self-renewal capacity. SHED are able to differentiate into many types of cell lineages including osteoblasts, odontoblasts, neuronal-like cells, chondrocytes, adipocytes and pancreatic beta cells (Miura *et al.*, 2003; Seo *et al.*, 2008; Nakamura *et al.*, 2009; Pivoriūnas *et al.*, 2009; Govindasamy *et al.*, 2011).

Besides, the expression of ESCs and neural crest markers such as OCT4, SOX2, NANOG, p75 and SOX10 were also persistently observed in the cell line of SHED (Kerkis *et al.*, 2006; Riekstina *et al.*, 2009). Furthermore, SHED also express a wide variety of cell surface biomarkers that are involved in the regeneration of damaged tissues, namely CD13, CD44, CD90, CD146 and CD166, CD 105 which is similar to what was observed in BMMSCs (Pittenger *et al.*, 1999; Shi *et al.*, 2001; Gronthos *et al.*, 2002). A study also reported that SHED share the same immunomodulatory capabilities with BMMSCs by its lack of class-I HLA antigens' expression (Yamaza *et al.*, 2010). The traits and gene expression profiling of SHED were reported to be comparably similar to BMMSCs thus suggesting SHED as the best candidate for potential clinical applications in regenerative medicine.

2.4 Horseshoe crab

The oceans and seas serve as precious habitats for many living creatures. Marine organisms hold a crucial responsibility in biogeochemical processes for sustenance and give balance to the biosphere. They also provide humankind with a tremendous variety of natural products that are essential and functional for various drug development in treating diseases including cancers (Villa and Gerwick, 2010).

Generally, there are five main kingdoms (Monera, Protista, Fungi, Plantae and Animalia) with roughly 26 phyla altogether in the marine organisms' world. The marine world offers endless number of natural resources that are useful for many health and medical applications nowadays. It is noted that arthropods, a phylum belonging to the horseshoe crab has contributed to four-fifths of all marine animal

species with over 35,000 varieties (Cisne, 1974). Arthropods are characterized by their jointed limbs and their hard-outer shell which moult off as they mature through life.

Interestingly, there are a few marine organisms which were anticipated to be extinct. However, they still make it to this day, living and surviving normally. One of the famous arthropods "living fossil" is the horseshoe crab which belongs to the class Merostomata. Horseshoe crab is often referred to as the "living fossil" as the unique shape of its archaeal body has survived through 350 million years and the shape remains unchanged phenotypically (Chatterji and Abidi, 1994). This mud dwelling primitive arthropod called trilobite have lived since the Precambrian seas, approximately 600 million years ago (Shuster Jr, 1982) and has evolved into its present shape about 350 million years back. It can withstand the harshest situations from the estuarine and coastal shallow habitats. Its body system is specially made to be tolerable to a wide range of salinity, temperature, desiccation and submergence (Ehlinger and Tankersley, 2004). This is due to the different types of environment that a horseshoe crab will go throughout its entire life which resulted it to evolve. Generally, horseshoe crab prefers to live in a calm sea or an estuary with muddy sand bottom (Hicklin and Smith, 1984).

However, during mating season, they specifically migrate towards the sea shore to breed and lay eggs. Therefore, during these times, they are subjected to hurdle the abrupt changes of environmental conditions whereby they experience lower salinity than the littoral zone (Ehlinger and Tankersley, 2004). In fact, as soon as the eggs of horseshoe crab hatch, even the juveniles are naturally forced to experience the low saline conditions of the shore zone to significantly high saline conditions of the littoral

zone as they migrate towards the sea. Therefore, its ability to weather the fluctuating habitats' environment throughout its entire life has made horseshoe crab population to survive until this day.

Marine bio resources produce a wide diversity of specific and powerful bioactive molecules and natural organic compounds namely, fatty acids, polysaccharides, polyethers, peptides, proteins, and enzymes (Haefner, 2003; Zhou *et al.,* 2013). The horseshoe crab known as the world's oldest living fossil has tremendous importance in pharmaceuticals, clinical and food industries. Horseshoe crab is one of the oldest existing living fossils which comprises four main species at present. *Limulus polyphemus* is found in North America and the other three species, *Tachypleus tridentatus, Tachypleus gigas* and *Carcinoscorpius rotundicauda* reside in Southeast Asia (Mikkelsen, 1988; Chatterji and Abdi, 1994). Horseshoe crab is also seen as a versatile organism, useful in the biomedicine field particularly as its blue blood has been widely integrated to be used for endotoxin tester in vaccines, drugs and injectables (Shuster *et al.,* 2003). Human beings have been utilizing horseshoe crabs for many purposes for the past several decades. Some of them have used the tail spines as spear tips and the body after grinding as fertilizers for fields and ponds (Chatterji and Abdi, 1994). Ancient remedy was also practiced traditionally in India by pricking the forehead with the horseshoe crab tailpiece as a pain reliever for various types of pains (Chatterji and Vijayakumar, 1988). Besides that, several Asian countries like India, Singapore, Malaysia and Borneo even enjoy horseshoe crab meat, eggs and appendages as a local delightful delicacy (Mikkelsen, 1988).

2.4.1 The blue blood of horseshoe crab

Researchers have found the blood of horseshoe crab to be useful in many pharmaceutical applications. The blood cells of *T. gigas* are known as amoebocytes and are famous due to its blue colour when exposed to air (Shuster, 1982). The amoebocytes are jam packed with huge refractile granules which contain many blood clotting factors. Therefore, the blood of horseshoe crab termed as Limulus Amoebocyte Lysate (LAL) has been actively extracted to be used as an endotoxin tester in food, drug and pharmaceutical industries (Mikkelsen, 1988; Chatterji and Abdi, 1994) as shown in Figure 2.2. LAL secretes a number of blood clotting factors and later on activates the blood clotting mechanism when it is exposed to small amounts of endotoxin or bacterial pyrogens (Levin and Bang, 1968). Degranulation of amoebocytes as well as the release of the coagulogens immediately takes place thus forming a gel or clot when even minute amount of endotoxin is present (Jorgensen and Smith, 1973).

An important serum protein called lactin is also found in the haemolymph of horseshoe crab that is very useful in the detection of gram positive bacteria families such as *Pseudomonadaceae*, *Enterobacteriaceae* (*Escherichia coli*), *Bacteriodaceae* and *Neisseriacea* (Mikkelsen, 1988). Pyrogen testing by haemolymph lysate has become a more preferred method than the classic rabbit vaccine test as it is more sensitive to small amount of endotoxins apart from giving quicker diagnostic result in the food, drug and pharmaceutical industries (Mikkelsen, 1988; Chatterji, 1994). The purified LAL has the capability of detecting one millionth of a billionth of a gram of endotoxin in less than 1 hour (Mikkelsen, 1988). Therefore, LAL has already been patented and

recognized by drug regulatory authorities and industry in most countries and are currently used as an end-product testing method for endotoxins associated in humans, animal injectables and drug products.

Figure 2.2 Extraction of blue blood of horseshoe crab as endotoxin tester for pharmaceutical purposes.

2.4.2 Perivitelline fluid of horseshoe crab

Another valuable biological resource found from the horseshoe crab that needs more exploration is PVF. Over the past decades, researchers have discovered PVF which is a fluid that is extracted from the space formed between the embryo and the egg shell of fertilized horseshoe crab as shown in Figure 2.3 (Punzo, 1999). It is rich in important proteins and amino acids that are crucial for early embryogenesis process (Sugita and Sekiguchi, 1979; Shishikura and Sekiguchi, 1984b). Studies have been carried out to purify and characterize the amino acid sequences in PVF of the horseshoe crab (Sugita and Sekiguchi, 1979; Shishikura and Sekiguchi, 1984a; Sekiguchi, 1988; Nagai *et al.*,

1999). It was found that the most potent proteins that were present in PVF were hemagglutinins and hemocyanins suggested to be important in supporting embryogenesis (Sugita and Sekiguchi, 1979; Shishikura and Sekiguchi, 1984a). It is considered as valuable to many medicinal practices as the PVF contains crucial primitive proteins which could supplement growth and proliferation of cells (Parab *et al.,* 2004). Hemocyanins are one of the most important proteins in PVF acting as oxygen-transporting glycoproteins among arthropods and molluscs. Based on the two-dimensional gel electrophoresis (2-DGE) proteomics analyses, it is proposed that hemocyanins might play significant role against bacterial, fungal and viral invasions as the amount of hemocyanins was significantly increased upon infection in virus-infected arthropods (Nagai *et al.*, 2001).

Previous studies have shown that PVF could enhance cell growth and differentiation as well as in promoting the generation of certain organs (Ghaskadbi *et al.*, 2004; Ghaskadbi *et al.*, 2008; Srijaya *et al.*, 2013). In fact, the PVF has been tested on many types of stem cells acting as an additional treatment for the cell culture and positive results have been reported (Alam *et al.*, 2015; Musa *et al.*, 2015). Due to this, it is suggested that PVF could be used as a supplement for supporting cell growth in the future.

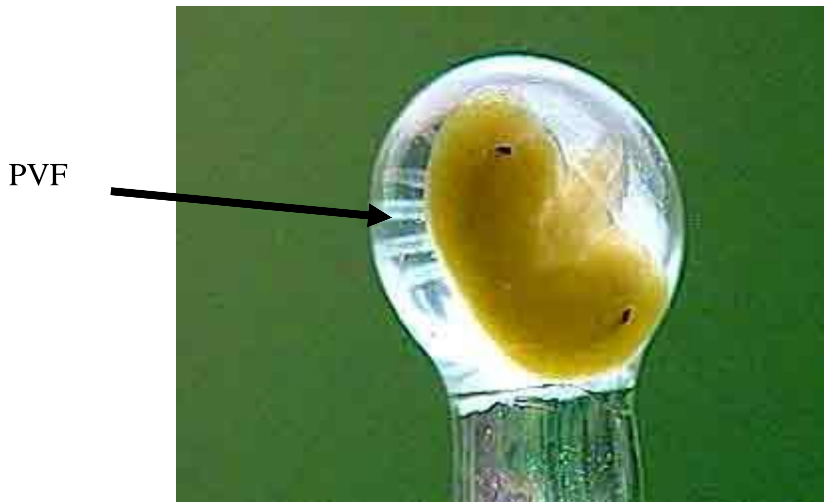

Figure 2.3 Perivitelline fluid of horseshoe crab which lies in between the outer envelope and the embryo of horseshoe crab (Chatterji *et al.,* 2004).

2.4.2(a) Perivitelline fluid as a treatment in cell culture

It is hypothesised that the hemocyanins and hemagglutinins in the PVF are responsible for giving a positive effect on the cardiac development in chick embryos (Ghaskadbi *et al.*, 2008). Treatment of PVF with a dose of 30 mg towards the gastrulating chick embryos resulted in 83% enlargement of the heart compared to the control. Not only the heart's size was enlarged, the PVF treatment was also effective in developing a normal functional heart as well (Ghaskadbi *et al.,* 2008). Furthermore, PVF was also able to induce angiogenesis as the enlarged heart chambers were reported to have more extensive blood vessel network resulting in a larger blood volume that could be pumped to whole body as compared to the control group (Ghaskadbi *et al.,* 2008). It was also noted that lectin, a molecule that is found in the purified PVF may also aid in the cardiac development in chick embryos (Ghaskadbi *et al.*, 2008). This may be due to the ability of lectins to induce cell agglutination and at the same time, act as a cellular and molecular mediator and recognition processes in a variety of biological

pathways (Singh *et al.*, 2011). Hence, lectin is also one of the promising candidates to be used as a therapeutic agent as they aid in the regulation of various cells via glycoconjugates and host-pathogen interactions and cell-cell communications (Singh *et al.*, 2011).

Studies have also shown that the peptides and proteins present in the PVF of horseshoe crab could also induce and enhance early gonadal maturity in red tilapia (Srijaya *et al.*, 2013). Positive influence in the early development of gonads in red tilapia fingerlings have been demonstrated which resulted in significantly higher gonadal weight and Gonado-somatic index (GSI) in the PVF treated group compared to the control. It was reported that the PVF helped with the gonad maturation in tilapia as the ovaries in PVF treated group appeared to be thicker and weighed double than the ones without PVF injection (Srijaya *et al.,* 2013). In fact, the ovaries of the PVF treated females showed abundant developing oocytes compared to the ovaries of the control (Srijaya *et al.*, 2013). Thus, it was hypothesized that PVF contains peptide molecules which are effective in enhancing growth, gametogenesis as well as spawning in vertebrate models (Srijaya *et al.,* 2013).

More recently, a study was conducted to test the potential of PVF in stimulating cell differentiation by triggering the BMMSCs to turn into cardiomyocytes (Alam *et al.,* 2015). Based on the fluorescence-activated cell sorting (FACS) analysis, they suggested that the optimum dose of 0.1 mg/ml from the eighth fraction (PVF-8) showed highest activity on the differentiation of BMMSCs into myocytes. This study was further strengthened and verified with various tests such as protein sequencing by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) which

showed the presence of 122 amino acids, followed by the identification of myocytes in the bone marrow stem cell culture by the expression of myosin using immunohistochemical and FACS analyses. Therefore, this study confirmed that the PVF-8 treatment on BMMSCs gave a more intense and significantly higher expression of myosin compared to the cells that were cultured with vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (b-FGF). It is also important to note the ability of PVF treatment to maintain the CD 34+ phenotype in BMMSCs for prolonged duration as well increasing the total number of CD 34+ cells in culture (Alam *et al.*, 2015). Thus, these findings prove that PVF has the potential to supplement cellular differentiation (Alam *et al.,* 2015).

Crude extract of PVF has also been tested on stem cells derived from human dental pulp to observe its effect in terms of cell proliferation as well as genotoxicity (Musa *et al.,* 2015). This study purposed four PVF crude extract concentrations from the MTT assay which represents 26.887 mg/ml (IC_{50}), 14.093 mg/ml (IC_{25}), 0.278 mg/ml (102% cell viability) and 0.019 mg/ml (102.5% cell viability). They concluded that the PVF crude extract was non-genotoxic to the DPSCs and hence can be considered safe to be used for further biomedical applications as it did not result in any significant chromosome aberrations. From their study, they also found that the cytotoxic effect of PVF was inversely proportional to the viability of DPSCs noting that the PVF crude extract could be one of the agents effective in inducing cell viability and proliferation if used in small specific amount as suggested.

2.4.2(b) Properties of PVF contents

The benefits and advantages of PVF specifically from horseshoe crab has been explored in the literature review section. However, there is dearth of studies concerning the effect of PVF as a treatment on stem cells besides one study by Alam *et al.* (2015) that used human bone marrow stem cells. They had demonstrated enhancement in the differentiation of bone marrow stem cells into cardiomyocytes when PVF treatment was given.

PVF in drosophila is found to surround the embryo by the ventral formation which helps in the embryonic dorsal-ventral polarity activity (Stein and Nüsslein-Volhard, 1992). The PVF in drosophila appeared to be important in aiding the formation of a ligand for the Toll protein which results in the dorsalization of embryos in the presence of serine proteolytic activity (Stein and Nüsslein-Volhard, 1992). PVF from the horseshoe crab contains proteins named hemagglutinins and hemocyanins which play a crucial role in supporting the process of horseshoe crab embryogenesis, especially during the third embryonic molting (Shishikura and Sekiguchi, 1984).

Hemocyanin which acts as the biological macromolecule in oxygen-transportation is present abundantly in PVF which exhibits bacterial, fungal and viral protections towards many invertebrates (Nagai *et al.*, 2001). A study reported that the chitin present in invertebrates acted as the scaffold for hemocyanin binding thus resulting in exoskeleton wound healing from the phenol oxidase activity (Nagai *et al.*, 2001). Besides that, PVF from other molluscs have been reported to be responsible for the