UNIVERSITI SAINS MALAYSIA
GERAN PENYELIDIKAN UNIVERSITI PENYELIDIKAN
LAPORAN AKHIR

EFFECTS OF PERI-VITELLINE FLUID (PVF) OBTAINED FROM FERTILLIZED EGGS OF HORSESHOE CRAB ON CULTURED HUMAN DENTAL PULP STEM CELLS

PENYELIDIK

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DR. WAN FAIZIAH WAN ABDUL RAHMAN
DR. AHMAD SHANWANI MOHAMED SIDEK

2017

Project Code :
(for RCMO use only)

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RU GRANT FINAL REPORT FORM

Please email a softcopy of this report to rcmo@usm.my

A	**PROJECT DETAILS**
i	**Title of Research:** **Effect of Perivitelline Fluid (PVF) Obtained from Fertilized Eggs of Horseshoe Crab on Cultured Human Dental Pulp Stem Cells**
ii	**Account Number:**
iii	**Name of Research Leader: Prof Madya Dr T. P. Kannan**
iv	**Name of Co-Researcher:** 1. Dr. Azlina Ahmad 2. Dr. Khairani Idah Mokhtar@Makhtar 3. Dr. Anil Kumar Chatterjii
v	**Duration of this research:** **a) Start Date** : 15 December 2012 **b) Completion Date** : 14 December 2015 ***c) Duration*** : 42 months **d) Revised Date** (*if any*) : 14 June 2016
B	**ABSTRACT OF RESEARCH**
	(An abstract of between 100 and 200 words must be prepared in Bahasa Malaysia and in English. This abstract will be included in the Report of the Research and Innovation Section at a later date as a means of presenting the project findings of the researcher/s to the University and the community at large) **- Attached -**

C	BUDGET & EXPENDITURE
i	**Total Approved Budget** : **Total Expenditure** : **Balance** : **Percentage of Amount Spent (%)** : ***# Please attach final account statement (eStatement) to indicate the project expenditure***

ii Equipment Purchased Under Vot 35000

No.	Name of Equipment	Amount (RM)	Location	Status
		NIL		

 # Please attach the Asset/Inventory Return Form (Borang Penyerahan Aset/Inventori) – Appendix 1

D RESEARCH ACHIEVEMENTS

i Project Objectives *(as stated/approved in the project proposal)*

No.	Project Objectives	Achievement
1	To study the cytotoxic effect and population doubling time (PDT) of cultured DPSCs treated with and without PVF	100 % achieved
2	To study the morphological characeristics of cultured DPSCs treated with and without PVF	100 % achieved
3	To analyse the chromosomal aberrations of cultured DPSCs treated with and without PVF	100 % achieved
4	To determine the expression of genes involved in cell-cycle regulation in cultured DPSCs treated with and without PVF	100 % achieved

5	To identify the differentially expressed genes in cultured DPSCs treated with and without PVF	100 % achieved

ii Research Output

a) Publications in ISI Web of Science/Scopus

No.	Publication (authors,title,journal,year,volume,pages,etc.)	Status of Publication (published/accepted/ under review)
1	Marahaini Musa, Khadijah Mohd Ali, Thirumulu Ponnuraj Kannan, Azlina Ahmad, Nor Shamsuria Omar, Anil Chatterji, Khairani idah Mokhtar, Effect of Perivitelline Fluid obtained from Horseshoe Crab on the Proliferation and Genotoxicity of Dental Pulp Stem Cells, Cell Journal (Yakteh), 2015, 17, pp.253-263 **Impact factor : 1.105**	Published
2	Najian Ibrahim, Thirumulu Ponnuraj Kannan, Azlina Ahmad, Khairani Idah Mokhtar, Cell Viability and Expression of Cell Cycle Regulatory Genes in Stem Cells from Human Exfoliated Deciduous Teeth Treated with Perivitelline Fluid from Horseshoe Crab, *Marine Biotechnology* ***Impact factor : 3.062***	Submitted

b) Publications in Other Journals

No.	Publication (authors,title,journal,year,volume,pages,etc.)	Status of Publication (published/accepted/ under review)
1	Thirumulu Ponnuraj Kannan, Abdul Qawee Rani, Nur Izyan Azmi, Najian Ibrahim, Nor Shamsuria Omar, Azlina Ahmad, Khairani Idah Mokhtar, Effect of Perivitelline Fluid from Horseshoe Crab on the Expression of Cell Cycle Regulatory Genes in Human Dental Pulp Stem Cells, Archives of Orofacial Sciences, 2016, 11, pp.7-14	Published
2	Amanina Fatinah Binti Kamarudin, Najian Binti Ibrahim, Ahmad Aizat Abdul Aziz, Thirumulu Ponnuraj Kannan, Effect of Perivitelline Fluid from Horseshoe Crab on the Expression of COL1A1 in Dental Pulp Stem Cells, Archives of Orofacial Sciences, 2016, 11, pp.26-30	Published
3	Marahaini Musa, Thirumulu Ponnuraj Kannan, Azlina Ahmad, Khairani idah Mokhtar, Mini Review: Protein Components of Perivitelline Fluid (PVF) of Horseshoe Crabs & Its Applications in Medical Research, IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS), 2014, 9, pp.39-42.	Published

4	Najian Ibrahim, Nurul Fatihah Mohamad Nasir, Nur Izyan Azmi, Thirumulu Ponnuraj Kannan, Expression of odontogenic markers in stem cells from human exfoliated deciduous teeth treated with perivitelline fluid from horseshoe crab, Journal of Biomedical and Clinical Sciences	Submitted

c) Other Publications
(book,chapters in book,monograph,magazine,etc.)

No.	**Publication** (authors,title,journal,year,volume,pages,etc.)	**Status of Publication** (published/accepted/ under review)
	NIL	

d) Conference Proceeding

No.	**Conference** (conference name,date,place)	**Title of Abstract/Article**	**Level** (International/National)
1	1st International Conference on Medical and Health Sciences, 22-24 May 2013, Renaissance Hotel, Kota Bharu, Kelantan	Effect of perivitalline Fluid (PVF) from the fertilized eggs of horseshoe crab on the MRC-5 fibroblast cell line	International
2	UMTAS 2013 12th International UMT Annual Symposium " Advancements in Marine and Freshwater Sciences", 3-5 Dec 2013, Palm Garden IOI Resort, Putrajaya, Malaysia	Effect of perivitalline Fluid (PVF) from the Fertilized Eggs of Horseshoe Crab (*Tachypleus gigas Muller*) on the Proliferation of Stem Cells	National
3	ISSCR 2015 Annual Meeting, 24-27 June 2015, Stockholm, Sweden	Proliferative and Genotoxic Effect of Perivitelline Fluid from Horseshoe Crab on Human Dental Pulp Stem Cells	International
4	National Colloqium on Stem Cell Research, 7-8 March 2016, Perdana Hotel, Kota Bharu, Kelantan	Gene Expression Analysis of Odontogenic Markers in Dental Pulp Stem Cells Treated with Perivitelline Fluid from Horseshoe *Crab*	National
5	21st National Conference on Medical and Health Sciences, 17-18 Oct 2016, School of Dental Sciences,	Effect of Perivitelline Fluid on the Cell Cycle and Apoptotic Regulator Genes in Dental Pulp Stem Cells	National

	USM Kota Bharu, Kelantan		

 # Please attach a full copy of the publication/proceeding listed above

iii Other Research Ouput/Impact From This Project
(patent, products, awards, copyright, external grant, networking, etc.)

Networking: Universiti Malaysia Terengganu – The perivitelline fluid was provided by UMT.

E HUMAN CAPITAL DEVELOPMENT

a) Graduated Human Capital

Student	Nationality (No.)		Name
	National	International	
PhD	-	-	
MSc	-	-	
Undergraduate	1	-	1. Amanina Fatinah Binti Kamarudin

b) On-going Human Capital

Student	Nationality (No.)		Name
	National	International	
PhD	1	-	1. Nor Shamsuria Omar (under UMT)
MSc	1	-	1. Najian Binti Ibrahim
Undergraduate	-	-	

c) Others Human Capital

Student	Nationality (No.)		Name
	National	International	
Post Doctoral Fellow	-	-	
Research Officer	-	-	
Research Assistant	2	-	1. Nurul Fatihah Mohamad Nasir 2. Wan Nuraini Binti Wan Aziz
Others (.....)	-	-	

F	COMPREHENSIVE TECHNICAL REPORT
	Applicants are required to prepare a comprehensive technical report explaining the project. The following format should be used (this report must be attached separately): • Introduction • Objectives • Methods • Results • Discussion • Conclusion and Suggestion • Acknowledgements • References **- Attached -**

G	PROBLEMS/CONSTRAINTS/CHALLENGES IF ANY
	(Please provide issues arising from the project and how they were resolved) 1. I took over the project as the project leader since the previous project leader (Dr Khairani Idah Mokhta joined International Islamic University Malaysia. 2. One student Wan Nuraini Binti Wan Aziz joined as Research Assistant initially in this project with the ai of pursuing her Master program. However, subsequently, she joined IPPT, USM Penang. Hence, the was a delay in the progress of the project until the current Master student (Najian binti Ibrahim) enrolle under this project. 3. Also, since the breeding is seasonal in horseshoe crabs, it was sometimes difficult to collect the egg and was not as anticipated which caused further delay. However, the PVF was obtained subsequent which enabled smooth continuation of the project.
H	**RECOMMENDATION**
	(Please provide recommendations that can be used to improve the delivery of information, gra management, guidelines and policy, etc.) To fully elucidate the role of PVF in enhancing cell growth and proliferation as observed in this study, th research can be further extended to study high thoughput gene expression profiling analysis such a microarray. This will provide insights into exploring specific regulatory pathways that are affected/enhance when PVF treatment is introduced. Besides that, protein works such as western blot, flow cytometry, etc a also necessary to further confirm the treatments's impact in cell growth by looking into the production specific proteins.

Project Leader's Signature:

.....

Name : ASSOC. PROF. Dr. T.P. KANNAN
School of Dental Sciences
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan.
Malaysia.

Date : 11.04.2017

COMMENTS, IF ANY/ENDORSEMENT BY PTJ'S RESEARCH COMMITTEE

Good progress has been shown. End well.

.....

Signature and Stamp of Chairperson of PTJ's Evaluation Committee

Name : DR. AZLINA AHMAD
TIMBALAN DEKAN (PENYELIDIKAN & SISWAZAH)
Pusat Pengajian Sains Pergigian
USM Kampus Kesihatan
16150 Kubang Kerian Kelantan
Malaysia

Date : 16/4/17

.....

Signature and Stamp of Dean/ Director of PTJ

Name : PROF. DR. ADAM HUSEIN
DEKAN
Pusat Pengajian Sains Pergigian
USM Kampus Kesihatan
16150 Kubang Kerian, Kelantan

Date : 16/4/17

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RU GRANT
FINAL REPORT CHECKLIST

Please use this checklist to self-assess your report before submitting to RCMO.
Checklist should accompany the report.

NO.	ITEM	PLEASE CHECK (✓)		
		PI	JKPTJ	RCMO
1	Completed Final Report Form	✓		✓
2	Project Financial Account Statement (e-Statement)	✓		✓
3	Asset/Inventory Return Form *(Borang Penyerahan Aset/Inventori)*	NIL		–
4	A copy of the publications/proceedings listed in Section D(ii) (Research Output)	✓		✓
5	Comprehensive Technical Report	✓		✓
6	Other supporting documents, if any	✓		✓
7	Project Leader's Signature	✓		✓
8	Endorsement of PTJ's Evaluation Committee	✓		✓
9	Endorsement of Dean/ Director of PTJ's	✓		✓

UNIVERSITI SAINS MALAYSIA

BORANG PENYERAHAN ASET / INVENTORI

==========

A. BUTIR PENYELIDIK

1. NAMA PENYELIDIK :.....
2. NO STAF :.....
3. PTJ :.....
4. KOD PROJEK :.....
5. TARIKH TAMAT PENYELIDIKAN :.....

B. MAKLUMAT ASET / INVENTORI

BIL	KETERANGAN ASET	NO HARTA	NO. SIRI	HARGA (RM)
		NIL		

C. PERAKUAN PENYERAHAN

Saya dengan ini menyerahkan aset/ inventori seperti butiran B di atas kepada pihak Universiti:

.....

( ) Tarikh:

D. PERAKUAN PENERIMAAN

Saya telah memeriksa dan menyemak setiap alatan dan didapati :

☐ Lengkap
☐ Rosak
☐ Hilang : Nyatakan.....
☐ Lain-lain : Nyatakan .....

Diperakukan Oleh :

.....

Tandatangan
Pegawai Aset PTJ

Nama : .....
Tarikh : .....

***Nota :** Sesalinan borang yang telah lengkap perlulah dikemukakan kepada Unit Pengurusan Harta, Jabatan Bendahari dan Pejabat RCMO untuk tujuan rekod.

UNIVERSITI SAINS MALAYSIA
JABATAN BENDAHARI
KUMPULAN WANG UNIVERSITI PENYELIDIKAN (RU)
PENYATA PERBELANJAAN SEHINGGA 31 DISEMBER 2016

RM

Jumlah Geran :

Peruntukan DIS. 2012 :
(Tahun 1)

Peruntukan DIS. 2013 :
(Tahun 2)

Peruntukan DIS. 2014 :
(Tahun 3)

Ketua Projek : PROF MADYA DR. T.P KANNAN

Tajuk Projek: EFFECTS OF PERI-VITELLINE FLUID (PVF) OBTAINED FROM FERTILIZED EGGS HORSESHOE CRAB ON CULTURED HUMAN DENTAL PULP STEM CELLS

Tempoh : 3 Tahun (15/12/2012-14/12/2015)

No. Akaun :

Kwgan	Akaun	PTJ	Projek	Peruntukan Projek	Perbelanjaan Terkumpul sehingga Tahun lalu	Peruntukan Semasa	Tanggungan Semasa	Bayaran Tahun Semasa	Belanja Tahun Semasa	Baki Projek
1001	11000	PPSG	813077							
1001	14000	PPSG	813077							
1001	15000	PPSG	813077							
1001	21000	PPSG	813077							
1001	22000	PPSG	813077							
1001	23000	PPSG	813077							
1001	24000	PPSG	813077							
1001	25000	PPSG	813077							
1001	26000	PPSG	813077							
1001	27000	PPSG	813077							
1001	28000	PPSG	813077							
1001	29000	PPSG	813077							
1001	32000	PPSG	813077							
1001	35000	PPSG	813077							
1001	52000	PPSG	813077							

PUBLICATIONS

Original Article

Effects of Perivitelline Fluid Obtained from Horseshoe Crab on The Proliferation and Genotoxicity of Dental Pulp Stem Cells

Marahaini Musa, M.Sc.[1], Khadijah Mohd Ali, B.Sc.[1], Thirumulu Ponnuraj Kannan, Ph.D.[1, 2*], Ahmad Azlina, Ph.D.[1], Nor Shamsuria Omar, M.Sc.[1, 3], Anil Chatterji, Ph.D.[4], Khairani Idah Mokhtar, Ph.D.[5]

1. School of Dental Sciences, Universiti Sains Malaysia, Kelantan, Malaysia
2. Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia
3. Institute of Tropical Aquaculture (AQUATROP), University Malaysia Terengganu, Terengganu, Malaysia
4. National Institute of Oceanography (NIO), Dona Paula, India
5. Kulliyah of Dentistry, International Islamic University of Malaysia, Jalan Sultan Ahmad Shah, Pahang, Malaysia

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Email: kannan@usm.my

Received: 13/Sep/2014, Accepted: 30/Dec/2014

Abstract

Objective: Perivitelline fluid (PVF) of the horseshoe crab embryo has been reported to possess an important role during embryogenesis by promoting cell proliferation. This study aims to evaluate the effect of PVF on the proliferation, chromosome aberration (CA) and mutagenicity of the dental pulp stem cells (DPSCs).

Materials and Methods: This is an *in vitro* experimental study. PVF samples were collected from horseshoe crabs from beaches in Malaysia and the crude extract was prepared. DPSCs were treated with different concentrations of PVF crude extract in an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (cytotoxicity test). We choose two inhibitory concentrations (IC_{50} and IC_{25}) and two PVF concentrations which produced more cell viability compared to a negative control (100%) for further tests. Quantitative analysis of the proliferation activity of PVF was studied using the AlamarBlue® assay for 10 days. Population doubling times (PDTs) of the treatment groups were calculated from this assay. Genotoxicity was evaluated based on the CA and Ames tests. Statistical analysis was carried out using independent t test to calculate significant differences in the PDT and mitotic indices in the CA test between the treatment and negative control groups. Significant differences in the data were $P<0.05$.

Results: A total of four PVF concentrations retrieved from the MTT assay were 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). According to the AlamarBlue® assay, these PVF groups produced comparable proliferation activities compared to the negative (untreated) control. PDTs between PVF groups and the negative control were insignificantly different ($P>0.05$). No significant aberrations in chromosomes were observed in the PVF groups and the Ames test on the PVF showed the absence of significant positive results.

Conclusion: PVF from horseshoe crabs produced insignificant proliferative activity on treated DPSCs. The PVF was non-genotoxic based on the CA and Ames tests.

Keywords: Horseshoe Crabs, Proliferation, Genotoxicity, Mutagenicity.

Cell Journal(Yakhteh), Vol 17, No 2, Summer 2015, Pages: 253-263

Citation: Musa M, Mohd Ali K, Kannan TP, Azlina A, Omar NS, Chatterji A, Mokhtar KI. Effects of perivitelline fluid obtained from horseshoe crab on the proliferation and genotoxicity of dental pulp stem cells. Cell J. 2015; 17(2): 253-263.

MARINE BIOTECHNOLOGY
An International Journal Focusing on Marine Genomics, Molecular Biology and Biotechnology

Submissions Being Processed for Author KANNAN THIRUMULU PONNURAJ

Page: 1 of 1 (1 total submissions) Display 10 ▼ results per page.

Action	Manuscript Number	Title	Initial Date Submitted	Status Date	Current Status
Action Links	MBTE-D-17-00017	Cell Viability and Expression of Cell Cycle Regulatory Genes in Stem Cells from Human Exfoliated Deciduous Teeth Treated with Perivitelline Fluid from Horseshoe Crab	09 Apr 2017	09 Apr 2017	New Submission

Page: 1 of 1 (1 total submissions) Display 10 ▼ results per page.

<< Author Main Menu

Marine Biotechnology

Cell Viability and Expression of Cell Cycle Regulatory Genes in Stem Cells from uman Exfoliated Deciduous Teeth Treated with Perivitelline Fluid from Horseshoe Crab

--Manuscript Draft--

anuscript Number:	MBTE-D-17-00017
ll Title:	Cell Viability and Expression of Cell Cycle Regulatory Genes in Stem Cells from Human Exfoliated Deciduous Teeth Treated with Perivitelline Fluid from Horseshoe Crab
rticle Type:	Original Paper
unding Information:	Universiti Sains Malaysia (1001/PPSG/813077) Prof KANNAN THIRUMULU PONNURAJ

bstract: Perivitelline fluid (PVF) from the fertilized eggs of a horseshoe crab has been reported to support embryogenesis, enhance cell growth and differentiation which also led to organs regeneration in certain organisms. Our current study intended to investigate the role of PVF as a supplement in supporting stem cell growth in culture. 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. Next, expression of cell cycle regulatory genes in both groups of SHED were compared using reverse transcriptase polymerase chain reaction (RT-PCR) for 21 days. Our results suggest that PVF was effective in enhancing the viability of SHED as observed by the higher live cell percentage compared to control. CDKN2A, PTEN and TP53 expressed significantly higher in the treatment group compared to the control which proposes that PVF treatment enhances SHED growth and proliferation. MDM2 expression remained at low levels in the treatment group indicating that PVF treatment does not result in tumorigenic growth. Faint and low expression of apoptotic activator gene, BCL2L11 from day 1 until 14 with a sudden peak on day 21 was noted, demonstrating that overcrowding of SHED in the confined culture flask has induced the activation of BCL2L11 that activates cell death signalling pathways. Collectively, our findings suggest that PVF enhances cell cycle, proliferation and growth, do not result in tumorigenic growth in cell and promote higher cell viability in SHED.

orresponding Author:	KANNAN THIRUMULU PONNURAJ Universiti Sains Malaysia MALAYSIA
orresponding Author Secondary formation:	
orresponding Author's Institution:	Universiti Sains Malaysia
orresponding Author's Secondary stitution:	
rst Author:	Najian Ibrahim
rst Author Secondary Information:	
rder of Authors:	Najian Ibrahim KANNAN THIRUMULU PONNURAJ Azlina Ahmad Khairani Idah Mokhtar
rder of Authors Secondary Information:	

uthor Comments:	This is an outcome of a research study which mainly focuses on the role of perivitelline fluid claimed to have a potential to enhance cell proliferation.
uggested Reviewers:	MANOHARAN SHANMUGAM, PhD manshisak@yahoo.com Has worked on cell viability and expression analysis

THILAK PON JAWAHAR, PhD
ponthilak@yahoo.com
Is familiar with the expression studies.

Cell Viability and Expression of Cell Cycle Regulatory Genes in Stem Cells from Human Exfoliated Deciduous Teeth Treated with Perivitelline Fluid from Horseshoe Crab

Najian Ibrahim[1], Thirumulu Ponnuraj Kannan[1,2*], Azlina Ahmad[1], Khairani Idah Mokhtar[3]

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[2]*Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*

[3]*Kulliyyah of Dentistry, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia*

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Abstract

Perivitelline fluid (PVF) from the fertilized eggs of a horseshoe crab has been reported to support embryogenesis, enhance cell growth and differentiation which also led to organs regeneration in certain organisms. Our current study intended to investigate the role of PVF as a supplement in supporting stem cell growth in culture. 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. Next, expression of cell cycle regulatory genes in both groups of SHED were compared using reverse transcriptase polymerase chain reaction (RT-PCR) for 21 days. Our results suggest that PVF was effective in enhancing the viability of SHED as observed by the higher live cell percentage compared to control. *CDKN2A, PTEN* and *TP53* expressed significantly higher in the treatment group compared to the control which proposes that PVF treatment enhances SHED growth and proliferation. *MDM2* expression remained at low levels in the treatment group indicating that PVF treatment does not result in tumorigenic growth. Faint and low expression of apoptotic activator gene, *BCL2L11* from day 1 until 14 with a sudden peak on day 21 was noted, demonstrating that overcrowding of SHED in the confined culture flask has induced the activation of *BCL2L11* that activates cell death signalling pathways. Collectively, our findings suggest that PVF enhances cell cycle, proliferation and growth, do not result in tumorigenic growth in cell and promote higher cell viability in SHED.

Keywords: Horseshoe crab, Perivitelline fluid, Stem cells from human exfoliated deciduous teeth, Gene expression, Cell cycle, Cell viability

Introduction

Stem cells from human exfoliated deciduous teeth (SHED) has been widely identified as a population of stem cells that are of a highly proliferative ability (Miura et al. 2003). SHED are also one of the highly clonogenic cells that makes it capable of differentiating into a variety of cell types including neural cells, adipocytes, and odontoblasts (Kerkis et al. 2007). Several *in vivo* studies have also proven the ability of SHED to encourage bone formation (Seo et al. 2008), regenerate dentin (Sakai et al. 2010), and survive in mouse brain which even expresses neural markers (Sakai et al. 2012). SHED could be one of the pioneering candidates in the advancement of tissue engineering world as not only for their multipotent ability but they are a great alternative towards the invasive bone marrow derived mesenchymal stem cells (MSCs). Thus, exfoliated deciduous teeth hold great potential in providing resources for stem-cell therapies and tissue engineering. The key fundamentals to a successful tissue engineering are stem cells, morphogen, and a scaffold of extracellular matrix (Gandhi et al. 2011). The present study aims to investigate if perivitelline fluid (PVF) when introduced into SHED as a supplement, could give an impact to the gene expression of cell cycle regulatory genes as well as its cell viability.

Horseshoe crabs are terrestrial organisms that inhabit the world for more than 200 million years. They are a versatile creature as they have a much higher tolerance living in a broad range of temperatures and salinities compared to other marine organisms (Costlow Jr et al. 1966; Laughlin 1983). To date, they are only four main species of horseshoe crabs; *Limulus Polyphemus*, present in North America and the other three species, *Tachypleus tridentatus, Tachypleus gigas* and *Carcinoscorpius rotundicauda* found in the Southeast Asia (Mikkelsen 1988). In the past, humankind has discovered valuable biological resources obtained from the horseshoe crab. One of it is the well-known Limulus Amoebocyte Lysate (LAL) that has been used as an endotoxin tester in food and drugs (Jorgensen and Smith 1973).

PVF is derived from a fertilized egg of a horseshoe crab. PVF is the fluid that fills the space between the outer envelope and embryo of the horseshoe crab. It is considered to be valuable to many medicinal practices as the PVF contains many types of important primitive proteins which could supplement growth and proliferation of cells (Ghaskadbi et al. 2004). A number of studies have been conducted to investigate the use of PVF since the 1970's. Sugita and Sekiguchi reported that PVF contained important proteins, namely, hemagglutinins and hemocyanins that play a crucial role in the embryogenesis process (Shishikura and Sekiguchi 1984). PVF is also suggested to help with the vertebrate's embryogenesis as it influences the embryogenesis of chick at early stages

(Parab et al. 2004). PVF is also proposed to positively stimulate the development and differentiation of organs such as brain and heart. Further advancement of the potential of PVF in promoting organ regeneration and cell differentiation was discovered when PVF was found to contain lectin, a compound that promotes cardiac myocyte formation in chick embryos (Ghaskadbi et al. 2004). Crude extract of PVF has also been tested on stem cells previously to observe its effect in terms of cell proliferation as well as genotoxicity (Musa et al. 2015). The authors reported the IC_{50} and IC_{25} values as 26.887 and 14.093 mg/ml respectively, and a 102% and 102.5% cell viability as 0.278 and 0.019 mg/ml respectively and concluded the non-genotoxic effect of PVF.

This study explored the effects of PVF obtained from Malaysian horseshoe crabs, *Tachypleus gigas* on SHED to evaluate their cell cycle regulatory gene expression by using reverse transcriptase polymerase chain reaction (RT-PCR). The genes selected were Cyclin-dependent kinase inhibitor 2A (*CDKN2A*), Bcl-2-like protein 11 (*BCL2L11*), Phosphatase and tensin homolog (*PTEN*), Mouse double minute 2 homolog (*MDM2*), and Tumor protein p53 (*TP53*). Glyceraldehyde-3-Phosphate Dehydrogenase (*GADPH*) was used as a housekeeping gene to normalize the expression of genes under study. Besides, comparison of live cell percentage of SHED with and without PVF treatment was made using the Live/Dead viability/cytotoxicity kit for mammalian cells. The concentration of PVF extract used in this study was 0.019 mg/ml based on the study by Musa et al (2015).

Materials and Methods

Cell line and culture

SHED from AllCells (USA, cat no. DP004F) were cultured in mesenchymal stem cell (MSC) basal medium (AllCells, cat no. MSC-002) with the addition of MSC stimulatory supplement (AllCells, cat no. MSC-003) and incubated at 37°C in a 5% CO_2 humidified incubator until confluence.

Perivitelline fluid

Fertilized eggs were collected from the horseshoe crabs that reside on a beach in Kuantan, Malaysia. The eggs were brought to Aquatrop Laboratory at the University Malaysia Terengganu (UMT), Malaysia and processed there. Eggs were incubated at a constant temperature of 29 ± 1°C in artificial incubators until they became transparent and showed the movement of trilobite larvae and processed further based on the method previously described (Chatterji et al. 1988). The freeze-dried PVF was stored at -70°C until use. For preparation of the PVF extract, the test sample was mixed with 1 ml of phosphate buffered saline (PBS, Invitrogen, UK) and further

diluted to 0.019 mg/ml using cell culture medium. The PVF extract was sterilized through a 0.25 µm syringe filter (Sartorius, UK). The extract was prepared fresh for each experiment.

Gene Expression Analysis

PVF treatment

The SHED were revived from the cryopreservation and subcultured twice before seeding for PVF treatment (0.019 mg/ml) at passage 6. The negative control group comprised of SHED without PVF treatment. Both the groups of SHED (treated and control) were incubated and harvested at day 1, 3, 7, 14 and 21.

Total RNA Extraction

Cells were trypsinized from culture flasks and prepared into a single suspension of cells. The cells were rinsed in PBS and the total RNA was extracted using commercial RNA extraction kit for mammalian cells, RNeasy Mini Kit from QIAGEN (Germany, cat no. 74104). The SHED were resuspended in lysis buffer and homogenized by passing through a QIAshredder column (QIAGEN, cat no. 79654). The homogenized lysate was then applied to the RNeasy column, rinsed repeatedly with a series of buffers and eluted into RNase-free deionized water. The total RNA was then quantified using UV spectrometry and checked for integrity and intactness by running them through the agarose gel electrophoresis.

cDNA Synthesis and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The 1st-strand cDNA from the total RNA was synthesized according to the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit from Epicentre (USA, cat no. MM070150). Next, RT-PCR was conducted as per the protocol (Table 1) using MyTaq™ HS Mix from Bioline (USA, cat no. BIO-25045). In each experiment, 1 µl of 3 µg total RNA were used for RT-PCR in a final reaction volume of 50 µl. Designed gene primers that were used in this study are listed in Table 2. The thermal cycling parameters were 95 °C for 1 min to activate the polymerase, followed by 35 cycles of 95 °C for 15 s, 54.2 °C for 10 s (*GADPH*); 58.0 °C for 15 s (*CDKN2A*); 59.8 °C for 15 s (*BCL2L11*); 58.0 °C for 15 s (*PTEN*); 57.5 °C for 15 s (*MDM2*); 57.0 °C for 15 s (*TP53*) and 72 °C for 10 s. The cDNA synthesis and RT-PCR reaction were performed in a Mastercycler nexus flat Thermal Cycler (Eppendorf, USA). The PCR products were stained with SYBR Green (1 µl), separated on an agarose gel (1.0%) and was visualized on a UV transilluminator (Biorad, USA). The images were photographed in an image analyser (Quantity One, USA). The experiments were carried out in triplicates and their band intensities were measured as

Average Density Value (ADV) with Quantity One 1-D Image Analysis software (Biorad, USA) and normalized to the ADV values of the housekeeping gene, Glyceraldehyde-3-Phosphate Dehydrogenase (*GADPH*). Mann Whitney test was carried out to determine the significance of expression of genes between control and treated group using SPSS software version 22.0.

Live/Dead Cell Viability Assay

Fluorescence Microscopy Protocol

The SHED were cultured as mentioned previously. Both the groups of SHED (treated and control) were cultured as sub confluent monolayers for 3 days on sterile glass coverslips which were placed in 6 well-plates. After 1, 2 and 3 days, the cells were washed with PBS twice to remove serum esterase activity in the media.

Live/Dead Cell Viability Assay

LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells from Thermo Fisher Scientific (USA, cat no. L3224) was used for this method. 100 µl of the combined LIVE/DEAD assay reagents dyes was added on the coverslips containing the cells. The cells were incubated for 45 minutes at room temperature in a covered dish. Following the incubation, 10 µl of the fresh LIVE/DEAD reagent dyes solution was added to a clean microscope slide. Using fine-tipped forceps, the wet coverslips were mounted quickly on the microscope slide. The coverslips were sealed with a clear nail polish to avoid evaporation. The labelled cells were viewed under the fluorescence microscope immediately with FITC (green emission) and propidium iodide (red emission) filters. Live cells were stained by Calcein AM dye which is membrane permeant and non-fluorescent until ubiquitous intracellular esterases remove ester groups and render the molecule fluorescent, thus generating green fluorescence. The Excitation (max) and Emission (max) are 494nm and 515nm wavelengths respectively for which FITC filters were used, whereas, dead cells were stained by Ethidium homodimer-1 that labels cells with compromised membrane, then binds to DNA with high affinity and produces red fluorescence. The Excitation (max) and Emission (max) are 528nm and 617nm thus emitting red signals (similar to propidium iodide).

Fluorescence Microplate Reader Protocol

The SHED were cultured as mentioned previously. Both groups of SHED (treated and control) were cultured in 96 well-plates for 3 days. 6 replicates were prepared for each group. The cells were analysed at day 1, 2 and 3 of incubation.

On each day of the test, the cells were washed with PBS twice to remove serum esterase activity in the media. At the last wash, PBS was added to cover the bottom of the wells. The cells were then treated with 200 µl LIVE/DEAD cytotoxic agents. The cells were incubated at room temperature for 45 minutes. Then, the fluorescence was measured using Fluorescence Microplate Reader at 645 nm and 530 nm as follows:

A. Fluorescence at 645 nm in the experimental cell sample, labelled with calcein AM and EthD-1 = F(645)sam

B. Fluorescence at 530 nm in the experimental cell sample, labelled with calcein AM and EthD-1 = F(530)sam

C. Fluorescence at 645 nm in a sample where all the cells are dead, labeled with EthD-1 only = F(645)max

D. Fluorescence at 645 nm in a sample where all the cells are dead, labeled with calcein AM only = F(645)min

E. Fluorescence at 530 nm in a sample where all (or nearly all) cells are alive, labeled with EthD-1 only = F(530)min

F. Fluorescence at 530 nm in a sample where all (or nearly all) cells are alive, labeled with calcein AM only = F(530)max

G. Fluorescence at 530 nm of the cell-free sample with or without dye added = F(530)0

H. Fluorescence at 645 nm of a cell-free sample with or without dye added = F(645)0

Dead cells are characterized by intense fluorescence at >600 nm and little fluorescence around 530 nm. The percentage of live cells was calculated from the fluorescence readings defined above as:

$$\% \text{ Live Cells} = \frac{F(530)sam - F(530)min}{F(530)max - F(530)min} \text{ X } 100\%$$

The percentage of dead cells was calculated from the fluorescence readings defined above as:

$$\% \text{ Dead Cells} = \frac{F(645)sam - F(645)min}{F(645)max - F(645)min} \text{ X } 100\%$$

Table 1: RT-PCR conditions for the different genes

Step	Temperature (°C)	Number of cycle(s)	Time
Initial denaturation	95	1	1 min
Denaturation	95	35	15 sec
Annealing	54.2 (*GAPDH*), 58.0 (*CDKN2A*), 59.8 (*BCL2L11*), 58.0 (*PTEN*), 57.5 (*MDM2*) 57.0 (*TP53*)		10 sec (*GAPDH*), 15 sec (*CDKN2A, BCL2L11, PTEN, MDM2, TP53*)
Extension	72		10 sec

Table 2: Primer sequences and the amplicon sizes of genes

Gene	Accession No.	Forward (5′-3′)	Reverse (5′-3′)	Product Size (bp)
GAPDH	NM_002046.5	CGACCACTTTGTCAAGCTCA	AGGGGAGATTCAGTGTGGTG	203
CDKN2A	NM_058195.3	GAGAACATGGTGCGCAGGTT	GCGCTGCCCATCATCATGA	219
BCL2L11	NM_138621.4	CAGCACCCATGAGTTGTGAC	CCTACACAAGAGAACCGCTG	401
PTEN	NM_000314.6	GCACAATATCCTTTTGAAGACC	AGTGCCACTGGTCTATAATCC	323
TP53	NM_001276760.1	GGAAGGAGACTTGCGTGTGG	GCTCTCGGAACATCTCGAAGCG	440
MDM2	NM_002392.5	GATTGGTTGGATCAGGATTCAG	CATCATTGTCCGCAACACATG	439

Results

Gene Expression Analysis

The expression of cell cycle (*CDKN2A, PTEN, TP53,* and *MDM2*) and apoptosis (*BCL2L11*) regulator genes were analysed for a period of 21 days at the interval of day 1, 3, 7, 14 and 21. The expression of these genes were normalized to the housekeeping gene, *GADPH*. The Average Density Values (ADV) were recorded in triplicates for each genes expressed in PVF treated and untreated SHED for 21 days, as tabulated in Table 3. Expression of the housekeeping gene, *GAPDH* was constant throughout for 21 days for both the control SHED and SHED treated with PVF (Figure 1a).

The expression of *CDKN2A, PTEN* and *TP53* in the PVF treated SHED appeared to be in a similar pattern (Figure 1b, 1d & 1f; Figure 2). It was observed that these 3 genes started to gradually increase in expression from day 3 onwards to day 14, and then slightly decreased on day 21. On the contrary, the expression of *CDKN2A, PTEN* and *TP53* in SHED control group gradually decreased from day 3 till the 21st day (Figure 2). The significance in the expression of the genes between both the groups of SHED were analysed by Mann Whitney test (Table 3). The expression of these genes were significantly different ($p<0.05$) starting from day 3 onwards until day 21 in the PVF treated SHED compared to the control. .

On the other hand, *MDM2* expressed relatively lower in the PVF treatment group than in the control (Figure 1e). *MDM2* was highly expressed in the control group at day 3 and 7 (Figure 3). The expression of *MDM2* however increased drastically in PVF treatment group at day 14 but then dropped significantly at day 21. Both groups showed low expression of *MDM2* at the end of the experiment (Figure 3).

BCL2L11 expression was either lesser or absent completely in the PVF treated group compared to the control from day 1 until day 14 (Figure 1c). However, at day 21, expression of *BCL2L11* drastically spiked in the treatment group while the expression in the control group remained low (Figure 4).

Table 3: Normalized average density values of gene expressions between control and perivitelline fluid treated SHED on day 1, 3, 7, 14 and 21

ADV / Genes	Day 1			Day 3			Day 7			Day 14			Day 21		
	Control	PVF Treated	p-value	Cont	Treat	p-value	Cont	Treat	p-value	Cont	Treat	p-value	Cont	Treat	p-value
CDKN2A	0.249345	0.607502	0.127	1.850009	0.315169	*0.050	2.190944	0.573587	*0.050	1.198459	1.888962	*0.050	0.126052	1.988341	*0.050
BCL2L11	0.512586	0.518572	0.827	0.631149	0.22442	*0.050	0.531562	0	*0.037	0.595326	0	*0.037	0.240928	0.989241	*0.046
PTEN	0.529433	1.046448	*0.050	1.345563	0.548422	*0.050	1.586935	0.671757	*0.050	0.589062	1.11622	*0.050	0.529277	0.772512	0.513
MDM2	0	0.552766	*0.037	2.221295	0.782414	*0.050	1.595363	0	*0.037	0.821188	2.04953	*0.050	0	0.420247	*0.037
TP53	0	0.558454	*0.037	0.459562	0.231912	*0.050	1.504731	0.340962	*0.046	0.642604	1.037007	*0.050	0	0.439084	*0.037

Cont: Control group; Treat: PVF treated group. *Significant difference of gene expression between control and PVF treated groups ($p \leq 0.05$).

Live/Dead Cell Viability Assay

This experiment was carried out to study the effects of PVF treatment with regard to the viability of SHED. The number of cells seeded onto the glass slides at the beginning of the experiment was kept constant for both groups which was 6000 cells. The cells were viewed on each day for 3 days. Live cells produced an intense uniform green fluorescence (ex/em ~495 nm/~515 nm) while dead cells emitted bright red-orange fluorescence (ex/em ~495 nm/~635 nm). As shown in Figure 5, the viability of cells between both groups were not significantly different at day 1 and 2. However, at day 3, a cluster of dead cells were detected in the control group as shown in image E in Figure 5. This could be due to the overcrowding of cells on the glass slide that led to cell senescence or death. However, in the treatment group, SHED appeared to be growing well and viable with minimum amount of dead cells compared to the control.

Table 4 shows the fluorescence readings at 645 nm and 530 nm in control and treated groups at 1, 2 and 3 days of incubation. The values of A, B, C, D, E, F, G and H were incorporated into the formula to obtain the percentage of live and dead cells. The percentage of live cells in control group decreased from day 1 to day 3, while the dead cells increased gradually from day 1 to 3 (Figure 6). In contrast, the percentage of live cells in SHED with PVF treatment remained higher than 100% until day 3, with a lower percentage of dead cells compared to the control group (Figure 6).

Table 4: Fluorescence readings at wavelengths of 645 nm and 530 nm in control and PVF treated SHED groups at 1, 2 and 3 days of incubation.

Groups / Readings	Control Day 1	Treatment Day 1	Control Day 2	Treatment Day 2	Control Day 3	Treatment Day 3
A	0.310	0.215	0.377	0.274	0.441	0.392
B	0.765	0.877	0.899	0.903	0.509	0.840
C	0.473	0.364	0.541	0.465	0.464	0.563
D	0.026	0.011	0.045	0.034	0.046	0.034
E	0.298	0.232	0.400	0.403	0.239	0.038
F	0.700	0.687	0.912	0.721	0.621	0.798
G	0	0	0	0	0	0
H	0	0	0	0	0	0

Please refer to the materials and methods section for the annotations of A, B, C, D, E, F, G and H.

Discussion

Investigating and comparing the expression of genes that regulates cell cycle is vital in grasping clues at the molecular level of how certain treatments affect the cell cycle and growth of cells. This information is important in order to determine the potential of PVF as a growth supplement for tissue engineering purposes in the future. PVF is abundant in important primitive proteins such as hemagglutinins, hemocyanins and lectins which are deemed important in supporting embryogenesis (Ghaskadbi et al. 2008; Shishikura and Sekiguchi 1984; Sugita and Sekiguchi 1979). PVF has also been widely studied previously and shown to support organ regeneration in chicks (Ghaskadbi et al. 2008), embryogenesis (Parab et al. 2004), and even gonadal development in red Tilapia (Srijaya et al. 2013). In the current study, genes which regulate cell cycle (*CDKN2A, PTEN, MDM2, TP53*) and apoptosis *(BCL2L11*) were selected. The expression of these genes were analysed in PVF treated and control SHED for a period of 21 days (day 1, 3, 7, 14 and 21).

CDKN2A gene encodes for tumour suppressor proteins which are p16(INK4a) and the p14(ARF) (Duro et al. 1995; Serrano et al. 1993). The tumour suppressor proteins produced by *CDKN2A* has specific roles that work together and share a common functionality in controlling cell cycle G1. *CDKN2A* has been found mutated or deleted in a wide variety of tumours such as head and neck squamous cell carcinoma (Cabanillas et al. 2013), melanomas (Monzon et al. 1998), and breast cancer (Dębniak et al. 2005). The functions of *CDKN2A* are often altered in cancers whereby it involves in significant reduction on amounts of the p16(INK4a) or p14(ARF) proteins. The absence of these tumour suppressor proteins will lead to cells growing and dividing without control; hence leading to the onset of cancer. Based on the results of our current study, *CDKN2A* expression was consistently present in the PVF treatment group. It was noted that *CDKN2A* expression increased gradually in PVF treatment group from day 3 to day 21. The expression in PVF treatment was also significantly higher as compared to the control group which portrayed a decline in expression as it approached the 21st day of incubation. Thus, this suggests that the proliferation activity of SHED are enhanced when PVF treatment was introduced.

PTEN is also one of the common tumour suppressor genes whereby its deregulation and disruption are often illustrated in many types of cancers such as Cowden disease and breast carcinomas just to name a few (Freihoff et al. 1999; Liaw et al. 1997). *PTEN* involved tightly with a series of signalling proteins; regulating a plethora of cellular processes including growth, survival, proliferation, motility, and polarity (Leslie and Downes 2004). *PTEN* encodes for lipid phosphatase which acts a ubiquitous regulator for the cellular PI (phosphoinositide) 3-

kinase signalling pathway that produces a second messenger; PtdIns(3,4,5)P3 (phosphatidylinositol 3,4,5-trisphosphate) (Leslie and Downes 2004). Evidences have shown that the signals emitted by PtdIns(3,4,5)P3 is vital in mediating downstream signalling in directing many cell physiology processes, namely, cellular proliferation, survival, growth and motility. The level of this lipid, PtdIns(3,4,5)P3 will commonly intensify prior to receiving external cellular stimuli such as growth factors. This is proven to be true when the results of this study showed the significant increase in *PTEN* expression when PVF treatment was given to SHED. We hypothesised that the PVF might contain growth stimulatory factors which explains the intensified expression of *PTEN* in treatment groups compared to the control.

Another tumour suppressor gene that was studied is *TP53*. Tumor suppressor p53 is a an important transcription factor that plays a central role in the regulation of cell cycle, apoptosis, DNA repair, senescence, and angiogenesis (Fridman and Lowe 2003; Teodoro et al. 2007; Vousden and Lu 2002). On the other hand, *MDM2* is an essential regulator of p53 in normal cells where p53 forms an autoregulatory feedback loop with the oncogene MDM2 proteins (Wu et al. 1993). Besides that, *MDM2* is a proto-oncogene whereby the encoded protein can promote tumor formation by targeting tumor suppressor proteins, p53, for proteasomal degradation. Overexpression or amplification of *MDM2* has been detected in large cases of different cancers and it was first found in sarcomas retaining wild-type p53 (Oliner et al. 1992). Our study showed low expression of *MDM2* in PVF treated group along the whole experiment time frame except for a sudden peak on day 14. This could probably be due to the actively increasing number of cells proliferating in the treatment group during that time. *MDM2* expression however remained at low levels again on day 21 which strongly suggests that PVF treatment does not lead to tumorigenic growth of cells in SHED. It is important to note that *MDM2* expression should be kept at low levels for overexpression of this gene could result to tumour growth as it inhibits DNA double-strand break repair mediated through a novel, direct interaction between Mdm2 and Nbs1 and independent of p53 (Zietz et al. 1998).

BCL2L11 is a member of BCL-2 protein family. BCL-2 family members can form hetero- or homodimers and act as anti- or pro-apoptotic regulators that are involved in a wide variety of cellular activities. Protein encoded by this gene contains a Bcl-2 homology domain 3 (BH3) which has been shown to interact with other members of the BCL-2 protein family and to act as an apoptotic activator (Hossini and Eberle 2008). Apoptosis is an essential cellular haemostatic mechanism that keeps cell population in balanced and prevents cell population from uncontrolled increase in growth (Kerr et al. 1972). If cell death is repressed, accumulation of cell will take place

Expression of odontogenic markers in stem cells from human exfoliated deciduous teeth treated with perivitelline fluid from horseshoe crab

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Abstract

Stem cells from human exfoliated deciduous teeth (SHED) makes an ideal resources for mesenchymal stem cells (MSCs) for cell-based regenerative therapies due to their ability to differentiate into wide range of cells lineages. Teeth regeneration requires regulated odontogenesis that will generate dentin. Previous studies has shown the potential of Perivitelline fluid (PVF) to act as a supplement material in enhancing proliferation of cells and promoting the generation of certain organs due to its high primitive protein content. This study analysed the expression odontogenesis markers in SHED treated with PVF (0.019mg/mL) on day 1, 3, and 7. The expression of dentin sialophosphoprotein (*DSPP*), dentin matrix acidic phosphoprotein 1 (*DMP-1*), osteopontin (*OPN*) and runt-related transcription factor 2 (*RUNX2*) were analysed at the transcript level in two groups of SHED which are PVF treated group (0.019mg/ml) and without PVF. All four genes expressed at higher level in treatment group as compared to control on all tested days. Significant difference in gene expression between the control and treated groups were found on all tested days except for *RUNX2* gene whereby its expression was not significantly different at day 7. Thus, it is suggested that PVF has the potential to enhance odontogenesis in SHED.

Keywords: Deciduous teeth, gene expression, horseshoe crab, odontogenesis, perivitelline fluid, stem cells.

Introduction

Stem cells from human exfoliated deciduous teeth (SHED) 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 (1). Besides its high capacity to differentiate, SHED markedly exhibit high proliferative trait which makes it an ideal source to obtain MSCs for cell-based regenerative therapies. It has the ability to yield human-specific odontoblast-like cells directly associated with a dentin-like structure (2). In order for successful regeneration of dentin to take place, SHED undergo a differentiation process called odontogenesis to form odontoblasts. The accumulation of odontoblasts then leads to the formation of dentin pulp through the mineralization process (3). However, SHED was reported to be unable to generate a complete dentin like structure *in vivo (*2).

The perivitelline fluid (PVF) from horseshoe crab has been adequately studied in many areas reflecting on its capability to promote many cell biological processes such as cell proliferation (4), angiogenesis (5), and gonadal development (6). PVF is rich with molecules such as haemagglutinins, haemocyanins and lectins which could stimulate growth and differentiation of cells (7). Studies by Ghaskadbi and colleagues (5) suggested that PVF contains peptides that could help to positively enhance differentiation of certain organs such as heart, brain and many more. It was further confirmed that the compound called lectins present in PVF play essential roles in stimulating embryogenesis at early stages by interacting with endogenous glycoproteins or *N*-acetylhexosamines (7). Hence, PVF was extracted at the trilobite larvae stage as it has been recognized as the early developmental stage of embryogenesis in horseshoe crab embryo and there is an abundance of proteins such as hemocyanins and hemagglutinins (8). In fact, PVF has been demonstrated to be non-toxic and non-mutagenic to dental pulp stem cells (DPSCs) which

reflects the potential of using PVF as a supplement in enhancing the proliferation effect of DPSCs (4).

Interestingly, the PVF concentration of 0.019 mg/ml has been shown to promote stem cell proliferation by 102.5% (4). Although many studies have implicated the potential of PVF as a material useful in many cell and tissue engineering applications, little is yet known in elucidating the capability of PVF in promoting odontogenic differentiation in SHED. Hence, this study aimed to elucidate the effect of PVF treatment towards the expression of odontogenic genes in SHED. This study has selected primary genes that were proven to be tightly involved in odontogenic process in dentin regeneration, namely, dentin sialophosphoprotein (*DSPP*), dentin matrix acidic phosphoprotein 1 (*DMP-1*), osteopontin (*OPN*) and runt-related transcription factor 2 (*RUNX2*) (7-12). The expression of these genes were compared between two groups which are PVF treated SHED and the control group without PVF treatment.

Materials and Methods

Cell culture

SHED from AllCells (USA, cat no. DP004F) were cultured in Mesenchymal Stem Cells (MSC) basal medium (AllCells, cat no. MSC-002) supplemented with fetal bovine serum (10%) and incubated at 37 ºC in a 5% CO2 humidified incubator until confluent. The SHED were revived from cryopreservation and sub-cultured twice before seeding for PVF treatment. A negative control group (SHED without PVF) was also included in this study. Both groups of SHED (treated and control) were incubated and were harvested at 3 distinctive days which were day 1, 3 and 7.

Perivitelline fluid

Collection of fertilized eggs from horseshoe crab was done from the nests on a sandy beach in Kuantan, Malaysia. Aquatrop Laboratory at Universiti Malaysia Terengganu (UMT),

Malaysia then processed the eggs. This was done by incubating the eggs at a constant temperature of 29 ± 1°C in artificial incubators. Once the eggs became transparent and showed the movement of trilobite larvae, further processing and purification steps were done as per the method described by Chatterji et al. (8). The PVF was freeze-dried and stored at -70 °C until further use. PVF was mixed with 1 ml of phosphate buffered saline (PBS, Invitrogen, UK) and further diluted to the concentration of 0.019 mg/ml using culture medium for the preparation of the sample. This was followed by sterilization using a 0.25 µm syringe filter (Sartorius, UK) (4).

Total RNA extraction

Two flasks of cells were trypsinized and prepared into a single-cell suspension for each period of incubation, namely, day 1, 3, and 7 for both groups which are the SHED seeded on a PVF supplemented media as well as the negative control in this study. The cells were rinsed in PBS and their total cell RNA was extracted using commercial RNA extraction kit (Invitrogen, Germany). The SHED were resuspended in lysis buffer and homogenized by passaging through a QIAshredder column (Qiagen, Germany). The homogenized lysate was then applied to the RNeasy column, rinsed repeatedly with a series of buffers and eluted into RNase-free deionized water.

cDNA synthesis by reverse transcription polymerase chain reaction

The 1st-strand cDNA from the total RNA was synthesized according to the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre, USA). Next, polymerase chain reaction (PCR) was conducted according to the conditions listed in Table II to analyse the gene expression profiles of cells under control and treated conditions. Primers that were used in this study were specifically designed to amplify the genes of interests as listed in Table I. The cDNA synthesis and reverse transcriptase PCR reactions were performed in a Mastercycler Nexus Flat Thermal Cycler (Eppendorf, USA).

Gel electrophoresis

The PCR products were stained with SYBR Green (1 µl) and were separated on an agarose gel (1.0%). The bands were visualized on a UV transilluminator (Biorad, USA) and then photographed in an image analyzer (Quantity One, USA). The experiments were carried out in triplicates for each gene transcript in both groups for all 3 days. The band intensities were measured as Average Density Value (ADV) with Quantity One 1-D Image Analysis software (Biorad, USA) and normalized to the band intensities of the housekeeping gene, *GADPH.*

Statistical analysis

Mann Whitney test was carried out using SPSS software version 22.0 to determine the significance between the control and treatment groups. The *p*-value was set to $p \leq 0.05$.

Results

Expression of odontogenic markers, *DMP-1*, *DSPP*, *OPN*, and *RUNX2* were analysed by normalizing them to the housekeeping gene, *GAPDH*. The gene expression was quantified as ADV and shown in Figures 1 and 2. Expression of the genes were analysed on 3 distinctive days, day 1, 3 and 7. The expression of *DMP-1* showed a similar pattern of expression both in the control as well as PVF treated SHED where the expression increased from day 1 to day 3, followed by a decrease on day 7. This expression pattern was similar to the expression of *DSPP* and *RUNX2* for both the control and treatment groups. Nevertheless, *DMP-1*, *DSPP*, and *RUNX2* expression was higher in the PVF treated SHED compared to the control on all days (Fig. 2).

The expression of *OPN* in the control SHED also showed an increase from day 1 to day 3 followed by a decrease on day 7. Alternatively, the trend was different in the PVF treated groups where a declining pattern in the expression was observed from day 1 to day 7. However, the treatment group still showed higher expression of *OPN* compared to the non-treated SHED on all days (Fig. 2). The mean (SD) of ADV of the different genes were

calculated and shown in Table III. Statistical analysis conducted using Mann Whitney test generally showed significant difference between the expression of odontogenic genes in control and PVF treated group at all tested days.

Discussion

A number of markers have been well identified to be directly involved in odontogenic differentiation. These include *ALP, OPN, BSP, DMP-1* and *RUNX2 (*9). Four odontogenic specific genes were investigated in this study; *DSPP*, *DMP-1*, *OPN,* and *RUNX2* with *GAPDH* as the housekeeping gene. *GAPDH* showed equal and consistent expression throughout the 7 days in both groups of SHED, indicating that *GAPDH* is a suitable housekeeping gene to be used in the present study (10). The expression of *DMP-1, DSPP, RUNX2* and *OPN* was higher in PVF treated SHED as compared to the non-treated group on day 1, 3 and 7. These days were chosen as this study was done at a preliminary level whereby a period of 7 days is considered as sufficient to observe the initial expression pattern of genes at early differentiation process of SHED. Specific concentration of PVF (0.019mg/mL) was chosen based on a previous study which reported 102.5% cell viability of DPSCs when treated with PVF (4).

DSPP encodes for the non-collagenous protein in the dentin matrix. Study showed that mutation in *DSPP* led to cellular impairment as well as fluctuations in protein processing and/or transporting system during rapid dentin matrix formation (11), suggesting that *DSPP* holds an important role in regulating odontogenesis. The high expression of *DSPP* in PVF treatment cells showed that PVF may enhance the dentin matrix formation.

DMP-1 is crucial in the odontogenesis and proper mineralization of dentin. Previous study showed that *DMP-1* knockout mice had defects in odontogenesis and mineralization as it was observed to develop enlarged pulp chambers with increased width of the predentin zone and hypomineralization (12). The results of the present study showed that *DMP-1* was

highly expressed in PVF treatment cells. This suggests that PVF may promote odontogenesis and maintain proper mineralization of dentin in vitro in SHED.

Besides that, both *OPN* and *RUNX2* are thought to be necessary for osteoblast and odontoblast differentiation as well as help in regulating many bone- and tooth-related gene expressions (13-15). Thus, the increase in *OPN* and *RUNX2* expression in PVF treated cells indicated that PVF could contribute to osteoblast and odontoblast differentiation. Besides, the addition of PVF may also aid in refining the regulation of bone and tooth related gene expression.

Fluctuations in the expression of *DMP-1, DSPP* and *RUNX2* for both groups were also observed where the expression level suddenly dropped on day 7. *DMP-1, DSPP* and *RUNX2* have been suggested to be the markers which are highly involved in the early odontogenic differentiation (13, 16). Therefore, the observation from this study suggests that PVF could increase the expression of early odontogenic markers in SHED. Next, slightly different pattern of expression for *OPN* was exhibited where the expression in the PVF treatment group was seen to decline from day 1 to day 7. Perhaps the duration of treatment should be increased to 14 or 21 days to obtain a clearer picture on how PVF affects *OPN* expression. Nevertheless, in terms of comparing the *OPN* expression level between the control and treatment group, the treatment group still showed higher expression on all 3 days similar to the case of *DMP-1, DSPP* and *RUNX2.*

Based on the statistical analysis, there was significant difference in the expression of *DMP-1, DSPP, RUNX2* and *OPN* between the control and PVF treatment groups. Hence, this study throws light on the possibility of PVF to enhance odontogenic differentiation as well as dentin development as the odontogenic markers selected in this study were observed to be upregulated on all 3 days when PVF treatment was introduced to SHED. However, different concentrations of PVF should also be tested to fully exploit the potential of PVF to instigate odontogenesis at a higher level.

Conclusion

The upregulation of *DMP-1, DSPP, RUNX2* and *OPN* genes in the PVF treated SHED suggested that PVF (0.019mg/mL) possesses the potential in enhancing odontogenesis process that could lead to higher rate of dentin mineralization. It is also found that the difference in odontogenic markers expression in SHED is significant when PVF treatment was given. Hence, this could give us a clue that PVF may be effective in inducing odontogenesis at a higher level. However, further studies on more odontogenic specific genes with a longer time frame are necessary to elucidate the efficiency of PVF to be used as a supplement for dental pulp generation in SHED.

Table I: Primer sequences

Gene	Primer	Nucleotide Sequence 5′ to 3′	Melting Temp (°C)	Amplicon Size
GAPDH	Forward	CGACCACTTTGTCAAGCTCA	55.3	203 bp
	Reverse	AGGGGAGATTCAGTGTGGTG	56.6	
DSPP	Forward	TGTCGCTGTTGTCCAAGAAG	55.3	498 bp
	Reverse	ATTCTTTGGCTGCCATTGTC	53.9	
DMP-1	Forward	CAGGAGCACAGGAAAAGGAG	55.6	213 bp
	Reverse	CTGGTGGTATCTTGGGCACT	56.9	
OPN	Forward	CCCTTCCAAGTAAGTCCAACG	55.5	321 bp
	Reverse	GGATGTCAGGTCTGCGAAAC	56.0	
RUNX2	Forward	TCTTCACAAATCCTCCCC	52.6	230 bp
	Reverse	TGGATTAAAAGGACTTGGTG	51.3	

Table II: PCR conditions

Step	**Temperature (°C)**	**Cycle**	**Time**
Initial denaturation	95	1	1 minute
Denaturation	95	35	15 seconds
Annealing			
GAPDH	54.2		10 seconds
OPN	53.3		15 seconds
DMP-1	55.3		15 seconds
DSPP	52.7		15 seconds
RUNX2	58.2		15 seconds
Extension	72		10 seconds

Table III: Mean (SD) of the Average Density Values of the different genes

	DMP-1		*DSPP*		*OPN*		*RUNX2*		*GAPDI*	
DAY	**Control**	**PVF**	**Control**	**PVF**	**Control**	**PVF**	**Control**	**PVF**	**Control**	**PV**
1	1103.80	1721.30*	527.28	1379.58*	361.81	1414.90*	390.13	726.46*	784.51	65
	(21.047)	(44.783)	(49.352)	(13.229)	(8.677)	(4.442)	(1.499)	(4.698)	(4.909)	(6.
3	1276.45	1955.49*	1026.19	1751.75*	1006.47	1270.70*	1204.86	1412.83*	613.17	60
	(18.23)	(38.044)	(31.487)	(9.007)	(49.323)	(22.512)	(3.350)	(10.294)	(4.278)	(1
7	639.75	1631.70*	507.43	586.20*	676.23	1276.16*	628.22	688.75	1082.37	10
	(36.329)	(49.660)	(10.393)	(30.679)	(18.315)	(35.860)	(3.929)	(5.638)	(9.932)	(1

[a] Mean from triplicates of ADV values recorded for each genes on day 1, 3 and 7

*Indicates the significant difference in expression of respective genes between Control and PVF treated group on day 1, 3 and 7 ($p \leq 0.05$).

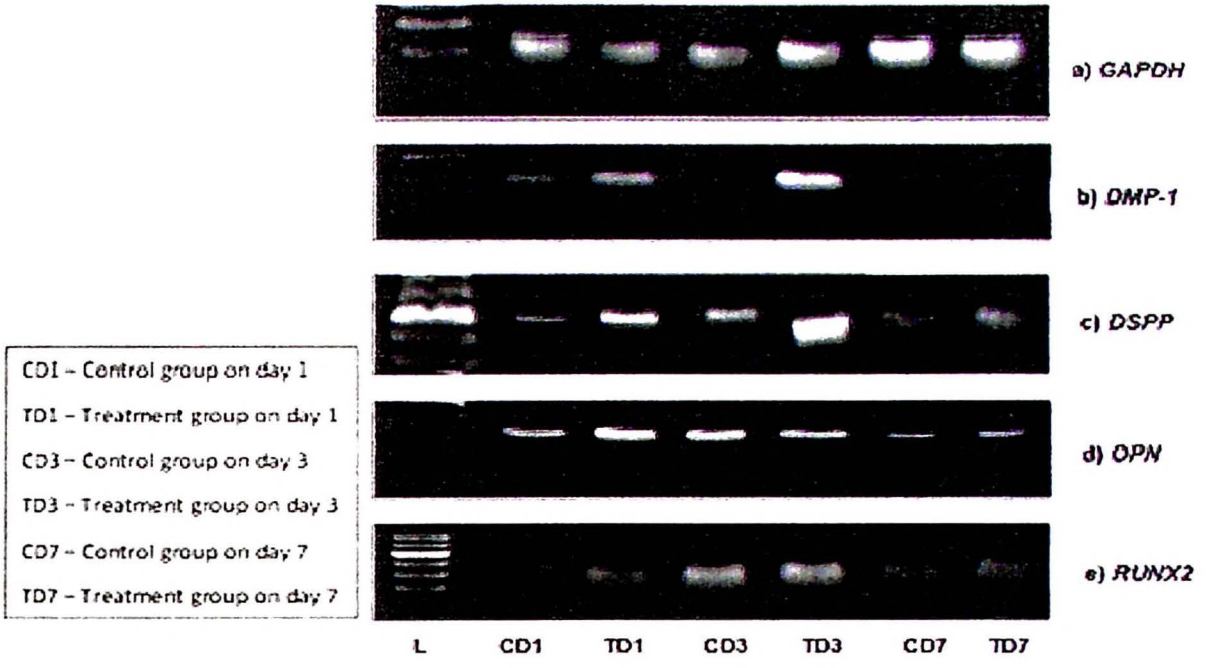

Figure 1: Gel image showing the bands of *GAPDH, DMP-1, DSPP, OPN* and *RUNX2* after RT-PCR amplification in control and PVF treated SHED at day 1, 3 and 7.

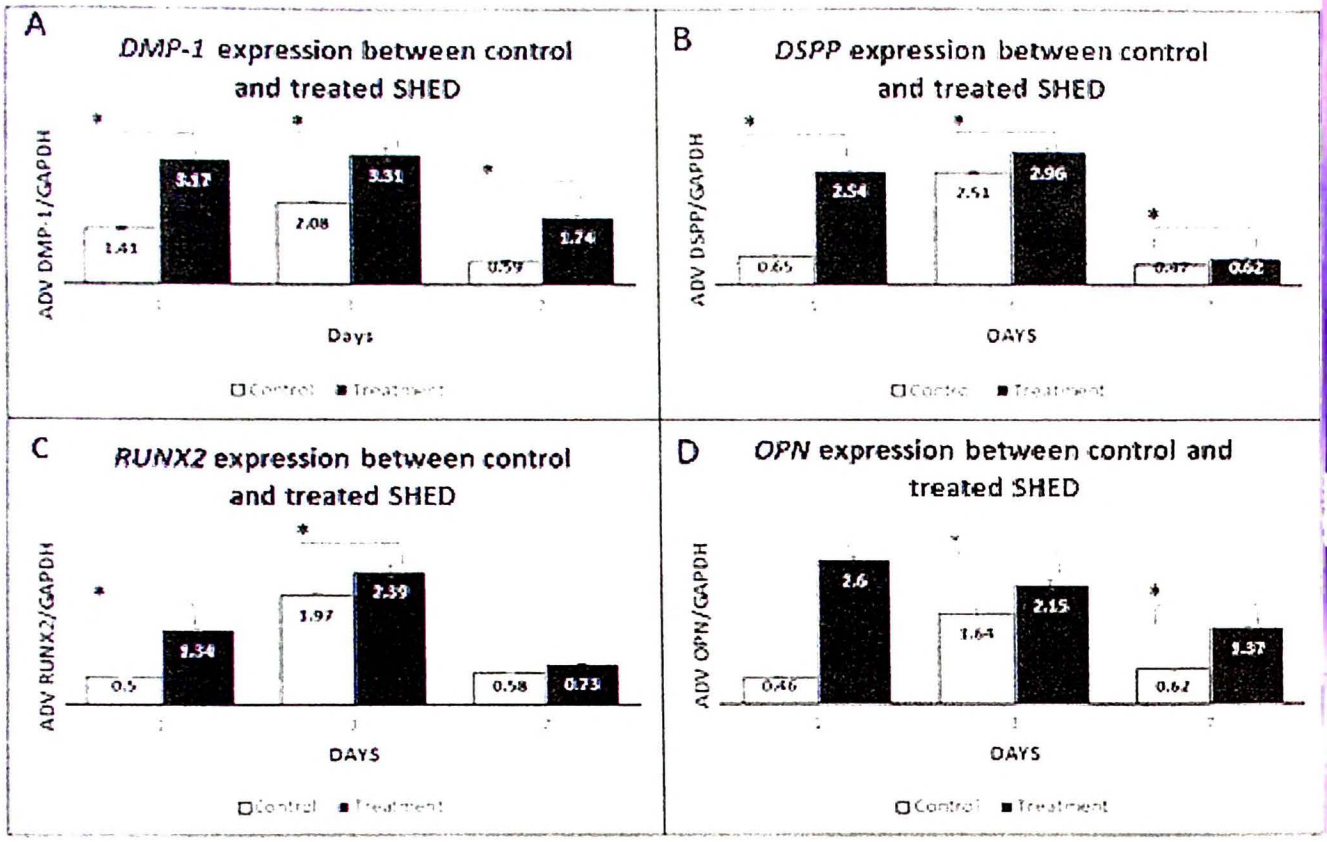

Figure 2: A, B, C and D show the normalized expression levels of *DMP-1, DSPP, RUNX2*, and *OPN* at day 1, 3 and 7 in control and PVF treated SHED. *Asterisks indicate significant difference between control and treated groups,*$p \leq 0.05$.

Acknowledgements

The authors would like to acknowledge the staff of the Craniofacial Sciences Laboratory, School of Dental Sciences, Universiti Sains Malaysia (USM), Malaysia and the research group at the Institute of Tropical Aquaculture (Aquatrop), Universiti Malaysia Terengganu (UMT), Malaysia for their technical support. This work was financially supported by Universiti Sains Malaysia Research University grant (1001/PPSG/813077).

Conflict of interest

There is no conflict of interest in the study.

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Figure Legends

Figure 1 – Gel image showing the bands of *GAPDH, DMP-1, DSPP, OPN* and *RUNX2* after RT-PCR amplification in control and PVF treated SHED at day 1, 3 and 7.

Figure 2 – A, B, C and D show the normalized expression levels of *DMP-1, DSPP, RUNX2*, and *OPN* at day 1, 3 and 7 in control and PVF treated SHED. *Asterisks indicate significant difference between control and treated groups,*$p \leq 0.05$.

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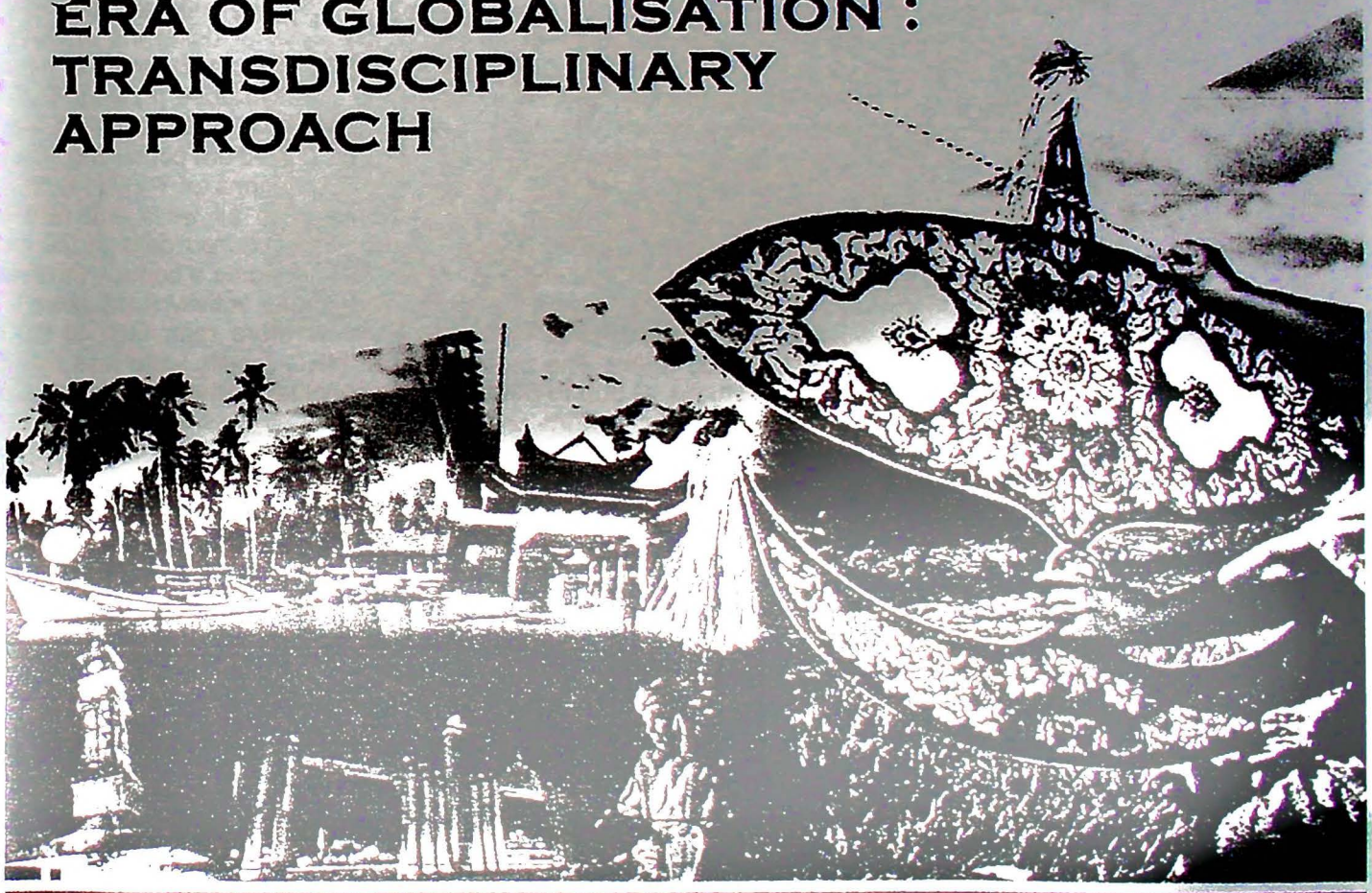

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FB10

Effect of Perivitelline Fluid (PVF) from the fertilized eggs of horseshoe crab on th proliferation of MRC-5 fibroblast cell line

Nor Shamsuria Omar, Faizah Shaharom, Anil Chatterji, Ahmad Sukari Halim & Khaira Idah Mokhtar

The perivitelline fluid (PVF) obtained from the fertilized eggs of the horseshoe c contains proteins such as hemagglutinin and hemocyanin which may exert impor effects during the process of embryonic development. Previous study had shown th constituent from this biological fluid can enhance growth and differentiation of c embryonic heart. PVF also shown some pro-angiogenic activity using the *in vivo* c embryonic chorioallantoic membrane (CAM) assay. Additionally, PVF might be impor during the process of wound healing. In this study, the effective dose (ED_{50}) of PVF determined on the proliferation of MRC-5 fibroblast cell line. MRC-5 fibroblast cell line 10^4 cells/well) was cultured in 96 well-plate using the growth medium containing 10% bovine serum (FBS) without L-glutamine. Different concentrations of PVF (10, 50, 200, 300, 400, 600, 700 and 800 µg/ml) were introduced to all wells and cells w incubated in a CO2 incubator at 37°C for 24 hours. The cultured cells in the gro medium with 10% FBS (without L-glutamine and PVF) was used as a control. Prolifera of the cells was measured using mitochondrial dehydrogenase activity (MTT assay). results showed that the proliferation of MRC-5 cells was more than 50% at the concentrations of 10 to 500 µg/ml. The proliferation of MRC-5 cells were less than when the PVF concentrations at 600 to 800 µg/ml. In conclusion, the most effective (ED_{50}) of PVF was 50 µg/ml where ~99% proliferation of MRC-5 cells was observed.

Marine and Environmental Sciences

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Effect of Perivitelline Fluid (PVF) from the Fertilized Eggs of Horseshoe C (Tachypleus gigas Muller) on the Proliferation of Steam Cells

Nor Shamsuria Omar[1,4], Faizah Shaharom[1*], Anil Chatterji[1], Mohd Effendy Abd Wa Ahmad Sukari Halim[3], Azlina Ahmad[4] and Thirumulu Ponnuraj Kannan[4] and Khaira Idah Mokhtar[4]

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Abstract

The perivitelline fluid (PVF) obtained from the fertilized eggs of the horseshoe crab con proteins such as hemagglutinin and hemocyanin which may have important role d embryogenesis. Previous study had shown that constituent from this biological fluid can enh growth and differentiation of chick embryonic heart. Additionally, PVF might also be impo during the process of wound healing. In this study, the effect of PVF on the proliferatio human mesenchymal stem cells (hMSC) and dental pulp stem cell (DPSC) cell lines determined. hMSC and DPSC cell lines ($1x\ 10^5$ cells/well) were cultured in a 96 well containing 100 ul of minimal essential medium (MEM) added with different concentratio PVF (5,10,15, 20, 25 and 30%).The culture plates were incubated in CO_2 incubator at 37 24 hours. After incubation period, 10 ul of Alamar Blue solution was added into each wel further incubated for 4 hours. The absorbance was measured using Elisa reader at a wavele of 570 nm for test and 600 nm for reference. The cultured cells containing MEM added fetal bovine serum (FBS) was used as a positive control and cells without PVF and FBS used as negative controls. The results showed that the cell proliferation of hMSC and DPSC were more than 90% for all concentrations of PVF. Decreased in concentrations of PVF en enhancement of cell proliferation. In conclusion, this result suggested that PVF of a horse crab posed a proliferative effect on the proliferation of human stem cells at a l concentration.

Introduction

Horseshoe crab is used as an example for organism that survived long time periods without significant changes in their anatomy, earning them the name of 'living fossils' (Eldredge Stanley, 1984). In the fossil record ancient horseshoe crabs are already known from Ordovician period (Rudkin et al., 2008; Van Roy et al., 2010), and modern forms which indistinguishable from recent species appear during Upper Jurassic (Sekiguchi and Sugita, Briggs et al., 2005). During the early developmental stages of the horseshoe crab embryo in the inner egg membrane is newly formed and a space between the inner egg membrane embryo, perivitelline space, is filled with perivitelline fluid (Chatterji, 1994).

The perivitelline fluid contains proteins such as hemagglutinins and hemocyanin, wh may have an important role during embryogenesis (Nagai *et al.*, 1999). Previous study

Abstract & Proceeding

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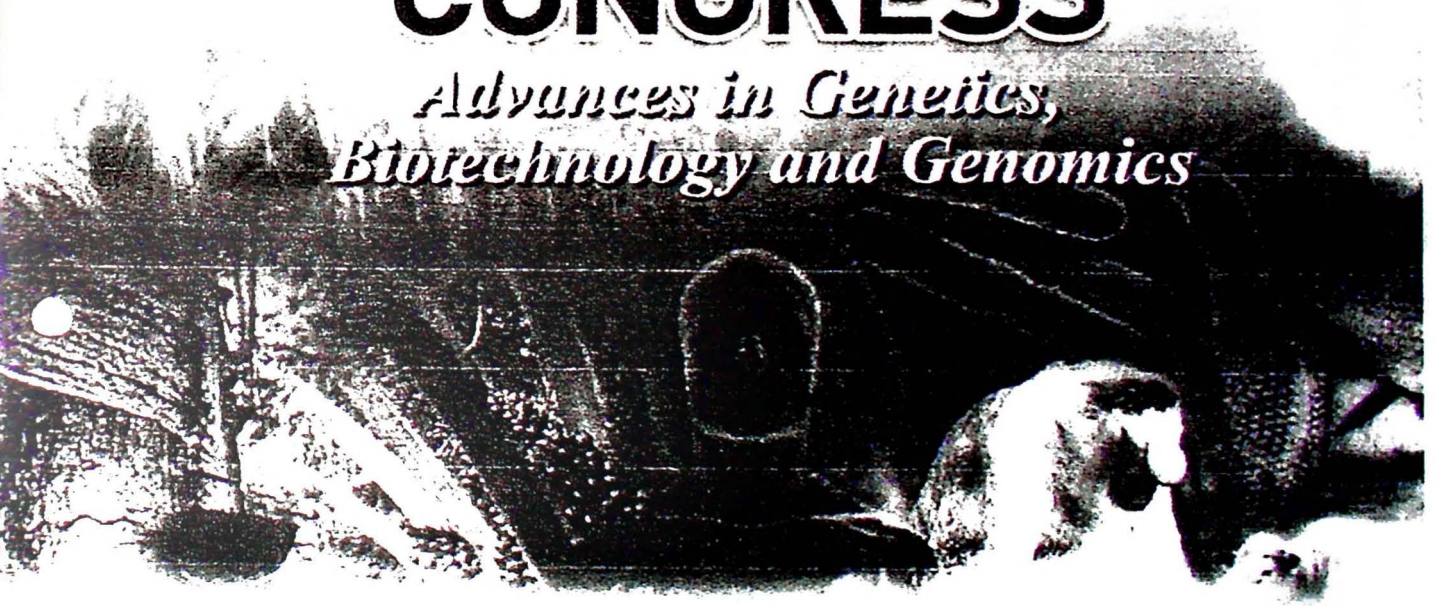

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Cell Proliferation and Cytotoxicity Study of Peri-Vitelline Fluid (Pvf) Obtained From Fertilized Eggs of Horseshoe Crab on Human Dental Pulp Stem Cells

[illegible]

ABSTRACT

Horseshoe crab which is known as living fossil is identified as one of the best-known living creature in this earth. This organism can be found in the coastline of various countries including Malaysia, Japan and Indonesia. Horseshoe crab possesses many clinical and medical applications. The objectives of the study are to evaluate the cell proliferation and cytotoxicity of peri-vitelline fluid (PVF) from horseshoe crab on dental pulp stem cells (DPSCs) using MTT assay and Alamar Blue test. Fertilized eggs of the horseshoe crab (*Tachypleusgigas*) were collected from the nests located on the sandy beach at Balok, Kuantan, Malaysia. The eggs were then incubated and cleaned before the peri-vitelline fluid (PVF) was collected. For the testing, the freeze dried PVF was mixed and diluted with phosphate buffer saline (PBS). For MTT assay (cytotoxicity test), 2 x 10[illegible] of DPSCs were seeded in 96-well plate and treated with different concentrations of PVF (45, 22.5, 11.25, 5.625, 2.813, 1.406, 0.703, 0.352 mg/ml) for 72 hours. IC[illegible] and IC[illegible] values were recorded from triplicate tests. Alamar Blue test (proliferation assay) was performed whereby 1 x 10[illegible] of DPSCs were seeded and treated with concentrations of PVF that produced IC[illegible] and IC[illegible]. Other concentrations of PVF which produced higher percentages of cell viability compared to the control were also included. Experiment was conducted in triplicates for 10 consecutive days. In MTT assay, higher concentration of PVF produced lower cell viability and the more diluted PVF produced greater percentage of cell viability. The determination of IC[illegible] and IC[illegible] was performed using an Excel add-in ED50V10. The values of IC[illegible] and IC[illegible] are 26.667 and 14.093 mg/ml respectively. From Alamar Blue assay, PVF produced greater cell proliferation as compared to the control. In conclusion, PVF shows promising results to enhance the proliferation of DPSCs.

FP8

Comparative Study on the Genetic Diversity of Probosc[illegible] (*Nasalis larvatus*) from Selected Populations in Malays[illegible]

[illegible]

ABSTRACT

Proboscis Monkey (*Nasalis larvatus*) is a large and sexually dim[illegible] primate that specifically belongs to subfamily Colobinae. Th[illegible] endemic to the island of Borneo and classified as Totally Protect[illegible] Wild Life Protection Ordinance Sarawak due to often defor[illegible] conversion that had highly restricted their nature habitat. Primat[illegible] mainly focus on their living behaviors and diet with fewer stu[illegible] genetic diversity especially in Sarawak. This research study is a[illegible] genetic diversity of *N. larvatus* population from Sarawak and S[illegible] control region segment. Non-invasive sampling was applied [illegible] collecting fecal as the major samples source. Out of a total of [illegible] 29 unique haplotypes were identified. Eighteen haplotypes wer[illegible] the Sarawak and 14 haplotypes were from Sabah populations. [illegible] were shared within population from Sarawak and Sabah. Popula[illegible] retained higher haplotypes diversity and nucleotide diversity o[illegible] population.

Keywords: genetic diversity, proboscis monkey, D-loop control [illegible] Sarawak

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PROLIFERATIVE AND GENOTOXIC EFFECT OF PERIVITELLINE FLUID FROM HORSESHOE CRAB ON HUMAN DENTAL PULP STEM CELLS

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Kannan Thirumulu Ponnuraj[1], Marahaini Musa[1], Khadijah Mohd Ali[1], Ahmad Azlina[1], Nor Shamsuria Omar[3], Anil Chatterji[2], Khairani Idah Mokhtar[3]

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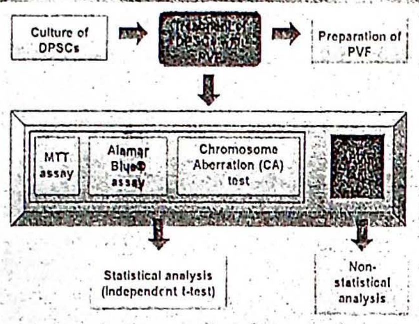
ABSTRACT

The aim of this study was to evaluate the effect of perivitelline fluid (PVF) on the proliferation, chromosome aberration and mutagenicity of dental pulp stem cells (DPSCs). Crude extracts were prepared from the PVF collected from horseshoe crabs. For cytotoxicity test (MTT assay), the DPSCs were subjected to different concentrations of PVF crude extract. Two inhibitory concentrations (IC_{50}=26.887mg/ml and IC_{25}=14.093mg/ml) and two other concentrations (0.278mg/ml and 0.019mg/ml) which produced higher cell viability were selected for downstream experiments. The proliferative effect was assessed using AlamarBlue® assay for a period of 10 days and the population doubling time (PDT) were also calculated based on this assay. Chromosome aberration and Ames tests were employed for the evaluating the genotoxicity. Statistical analysis was carried out using independent t-test to assess the significant differences (p<0.05). AlamarBlue® assay showed that PVF groups produced comparable proliferation activity to negative (untreated) control. The PDTs between PVF groups and negative control were also insignificantly different (p>0.05). Both the chromosome aberration test and Ames test did not show any significant differences in the PVF treated groups. Hence, it can be concluded that PVF from horseshoe crabs produced insignificant proliferative activity and was non-genotoxic on DPSCs based on chromosome aberration and Ames tests.

INTRODUCTION

- Perivitelline fluid (PVF) has the capacity to induce cell proliferation thus potentially serving as a valuable supplement to the stem cells.
- The PVF contains proteins such as hemagglutinins and hemocyanins which may play an important role during embryogenesis (Sugita *et al.*, 1979; Shishikura *et al.*, 1984).
- The present study aimed to evaluate the effect of PVF on the proliferation, chromosome aberration and mutagenicity of dental pulp stem cells (DPSCs).
- Research has shown that DPSCs have the potential to differentiate into a various types of other cells including cardiomyocytes for repairment of damaged cardiac tissue following a heart attack, neurons for generation of nerve and brain tissue, myocytes for muscle repairment and osteocytes for bone generation (Kawashima 2012).
- This study might prove beneficial in exploring new possibilities of using PVF as a new proliferation agent for DPSCs for its therapeutic application.

METHODS



RESULTS

MTT assay

Alamar Blue® assay

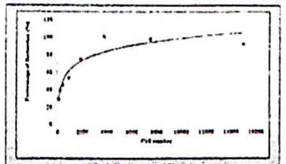


Fig 1: Standard curve of DPSCs

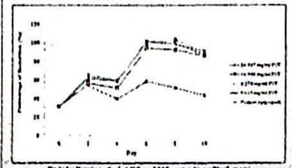


Fig 2: Proliferation study of PVF on DPSCs using AlamarBlue® assay

Table 1. Population doubling time (PDT) of cells treated with PVF

Group	PDT (SD) Hours
PVF (26.887 mg/ml)	28.45 (10.60)
PVF (14.093 mg/ml)	12.39 (1.28)
PVF (0.278 mg/ml)	13.09 (3.67)
PVF (0.019 mg/ml)	12.31 (2.04)
Untreated	13.02 (2.58)

Table 2: Mitotic index (MI) of DPSCs treated with PVF

Groups	Hours	Mean Mitotic Index (SD) (%) a	
		Without S9	With S9
PVF (26.887 mg/ml)	4	3.65 (0.07)	3.69 (0.14)
	24	3.35 (0.29)	-
PVF (14.093 mg/ml)	4	3.90 (0.14)	3.55 (0.21)
	24	3.30 (0.00)	-
PVF (0.278 mg/ml)	4	3.89 (0.14)	4.20 (0.14)
	24	3.60 (0.14)	-
PVF (0.019 mg/ml)	4	3.70 (0.28)	3.80 (0.29)
	24	3.45 (0.07)	-
MMC b	4	1.15 (0.21)*	-
	24	1.25 (0.21)*	-
CP b	4	-	1.70 (0.29)*
Negative control (culture medium) c	4	3.55 (0.14)	4.45 (0.14)
	24	3.30 (0.28)	-

a Mean from triplicate tests
b Positive controls Mitomycin C (MMC) at a concentration of 0.1 µg/ml for 4 hours and 0.05 µg/ml for 24 hours without S9 mix and Cyclophosphamide monohydrate (CP) at a concentration of 10 µg/ml for 4 hours with S9 mix
c MSC basal medium
* p < 0.05, mitotic index is significantly different as compared to negative control

DISCUSSION

MTT assay

- IC_{25} and IC_{50} values were 14.093 and 26.887 mg/ml respectively and the selected concentrations of PVF which produced higher cell viability were 0.278 and 0.019 mg/ml which produced 102% and 102.5% of viability of treated DPSCs respectively (Figure 1).
- More concentrated PVF produced greater cytotoxic effect as compared to diluted extract which might be due to the differences in the pH of the extract. More acidic/

Alamar Blue®

- The differences in the proliferation of DPSCs of treatment groups and negative control seen in AlamarBlue® assay were contributed by the concentrations of PVF (Fig. 2 and 3). Study showed that the metabolism of cells in organized environment is majorly associated with the intercellular metabolic interactions between different types of cells (Park *et al.*, 2004; Choi *et al.*, 2008). Enhancement of *in vitro* growth and activity of mesenchymal stem cells (MSCs) can be achieved by supplementing proteins and factors in culture medium, mimicking the physiologic environment which would lead to optimal proliferation and differentiation activity.
- No significant differences were found in PDT between treatment and negative control groups despite slight reductions in the DT (doubling time) of the PVF groups (Table 1). This indicates that the treated cells divide at almost the same rate as the untreated cells. It seems that the components in the crude extract of the PVF do not promote cell division process and thus no significant decrease in the PDT (population doubling time) was produced.
- The differences in the PDTs of treatment groups were observed due to the effect of various PVF extracts' concentrations. A concentrated PVF extract which produced the IC_{50} value (reduction in the 50% of cell population) produced a higher PDT compared to other PVF and negative control groups.
- This indicates slower rate of cell division in the treated cells due to high concentration of PVF. This effect may be due to the reduction in the pH of the extract which does not serve as an optimum condition for the cells to proliferate, as compared to much diluted PVF extract.

Chromosome Aberration test

- No significant chromosomal aberrations were found in PVF groups (Figure 4). This shows the non-genotoxic effect of PVF. This differed from positive control groups (MMC and CP) where various aberrations were observed. The significant reduction in the MI in positive controls groups as compared to the negative control also indicates the cytotoxic effect of MMC and CP to the treated cells (Table 2).
- Two treatment durations (4 and 24 hours) and different concentrations of positive controls were applied in the present study as reported previously by Hori *et al.* (1993). The cytotoxicity and genotoxicity of both positive controls in CA test have been reported by other studies (Tohamy *et al.*, 2012; Brüsehafer *et al.*, 2014).

Ames test

- For the Ames test, even though the standard concentration values suggested by Mortelmans and Zeiger (2000) ranged from 313 µg/ ml to 5000 µg/ ml, the concentration of 14.09 mg/ml of PVF (IC50) which was obtained from the initial cytotoxic study was used.
- This is further explained in OECD guidelines (1997) which states that testing for concentrations of material at more than 5 mg/plate may be considered when evaluating substances containing substantial amounts of potentially mutagenic impurities. It has also stated the requirement for at least five different analysable concentrations of the test substance to be included in Ames test which justifies the usage of different concentrations of PVF extract.

CONCLUSIONS

- Though there has been a slight increase in the viability of cells, yet, there was no significant proliferative activity on DPSCs treated with crude PVF from horseshoe crabs.
- The absence of chromosomal aberrations and also insignificant reduction in the MI values in the treated cells as compared to the negative control as well as the negative results from Ames test indicate the non genotoxic nature of the PVF of horseshoe crabs.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Mrs. Asiah Abu Bakar, School of Dental Sciences, Universiti Sains Malaysia (USM), Institute of Tropical Aquaculture (AQUATROP), Universiti Malaysia Terengganu, Malaysia for their technical support. This work was supported by USM Research University grant (1001/PPSG/813077).

REFERENCES

An Unveiling of

STEM CELL INNOVATION

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Dear Kannan Thirumulu Ponnura,

Your abstract, submission #795, entitled PROLIFERATIVE AND GENOTOXIC EFFECT OF PERIVITELLINE FLUID FROM HORSESHOE CRAB ON HUMAN DENTAL PULP STEM CELLS, has been scheduled for a poster presentation at the ISSCR Annual Meeting, 24-27 June, 2015.

You will be presenting during Poster Presentation II, to be held **Thursday Jun 25 2015, 18:00 - 20:00**

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On behalf of the International Society for Stem Cell Research, we are pleased to welcome your participation in the ISSCR2015 Annual Meeting. This international conference will be held 24-27 June 2015 in Stockholm, Sweden.

International Society for Stem Cell Research
ISSCR2015 Annual Meeting
24-27 June 2015
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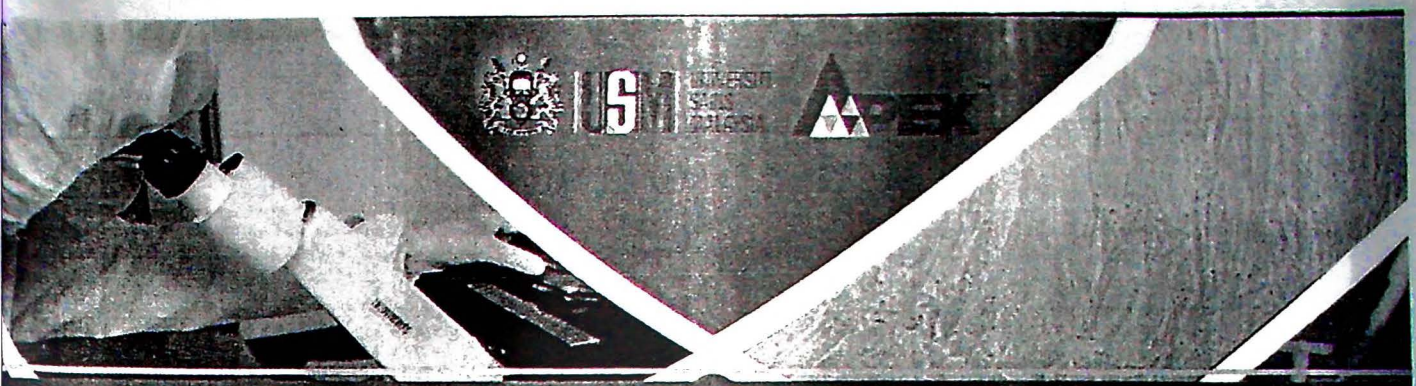
The meeting will take place at the Stockholmsmässan Convention Center. Delegates will learn the latest advances in stem cell research and regenerative medicine through lectures, symposia, exhibits, and networking events. The conference will feature over 120 speakers in plenary and concurrent sessions and more than 1,400 posters presented in the exhibition hall.

We look forward to seeing you in Stockholm.

Best regards,

Nancy Witty
Chief Executive Officer



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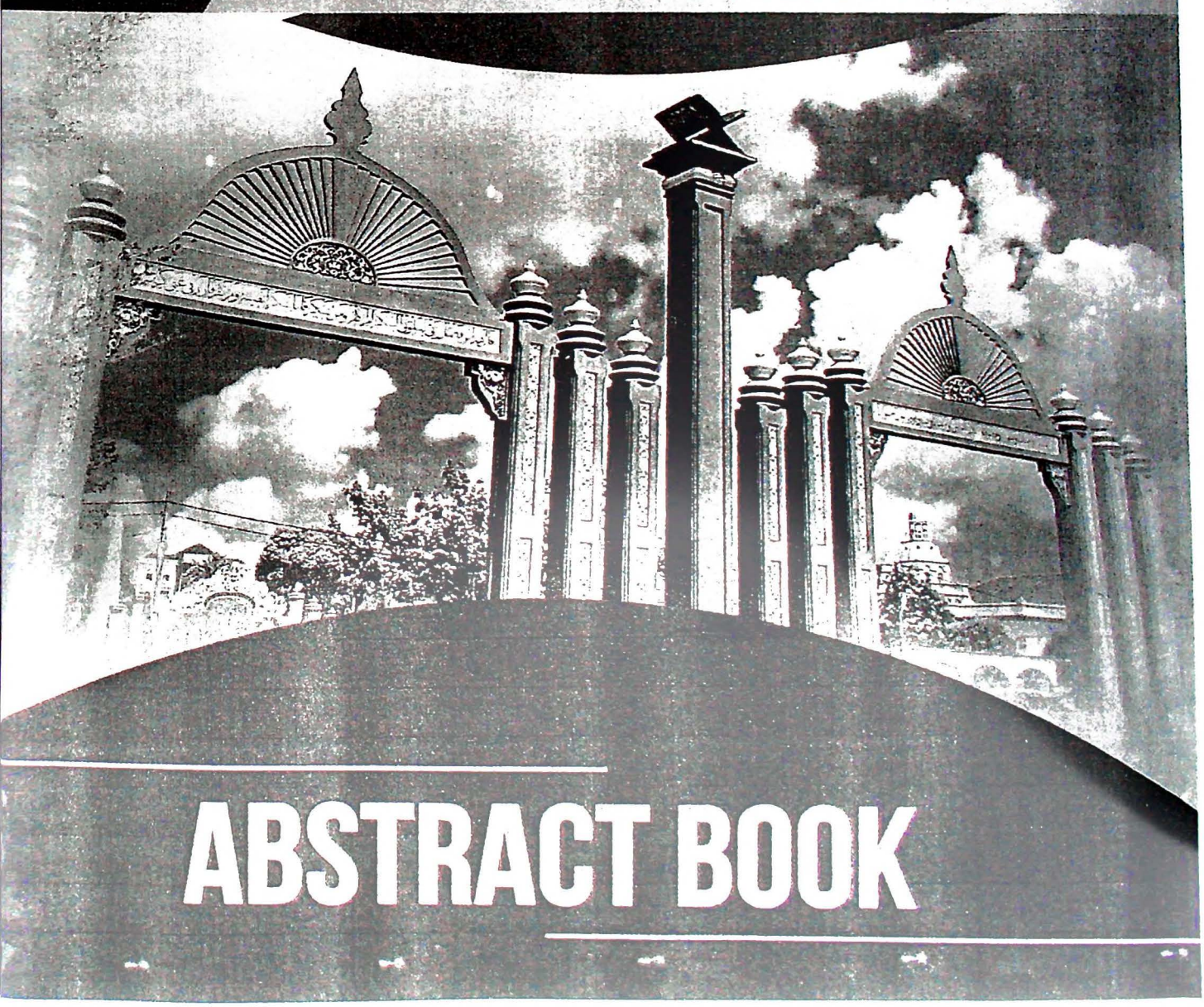

ABSTRACT BOOK

O-7

Expression of protein stem cell markers by stem cells from human extracted deciduous teeth at different passages

Muhammad Fuad Hilmi Yusof[1], Siti Nurnasihah Md Hashim[1], Nurul Hidayat Yusoff[1], Ahmad Azlina[1,2], Thirumulu Ponnuraj Kannan[1,2], Khairul Bariah Ahmad Amin Noordin[1], Suzina Sheikh Ab Hamid[3], Khairani Idah Mokhtar[4]

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Introduction: Stem cells from human extracted deciduous teeth (SHED) are postnatal stem cells originated from the neural crest. These multipotent stem cells express typical mesenchymal stem cells (MSCs) surface markers and lack of the expression of endothelial, epithelial and hematopoietic markers. These properties led to a development of progressive methods to isolation and characterization of SHED for therapeutic applications in regenerative medicine.

Objective: To determine the expression of SHED markers at passages 10 and 15.

Methods: SHED was purchased from AllCells (USA) and cultured in α-MEM media. The cells were harvested at 2 different passages that were 10 and 15. The respective lysates were investigated for the expression of MSC specific cell surface antigens which include CD44, CD73, CD90, and CD105 using flow cytometry in order to verify the stemness of these cells. CD45, CD11b, CD34, CD19, and HLA-DR were markers for hematopoietic, endothelial, and epithelial cells and were used as the negative MSC markers.

Results: All our cells from both passage numbers positively expressed MSC specific cell surface markers. The presences of endothelial, epithelial and hematopoietic markers, on the other hand, were undetected.

Conclusion: The presence of MSC markers and lack of expression of the negative markers suggested that SHED colonies were homogenous MSCs. Thus, SHED at passage 15 was able to maintain its stemness similar to the one in passage 10.

O-8

Gene expression analysis of odontogenic markers in dental pulp stem cells treated with perivitelline fluid from horseshoe crab

Najian Ibrahim[1], Nurul Fatihah Mohamad Nasir[2], Nur Izyan Azmi[1], Thirumulu Ponnuraj Kannan[1,3]

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Introduction: Dental pulp stem cells (DPSCs) possess infinite self-renewal and multipotent differentiation ability and functions mainly in regenerating dentin. In order to sustain a healthy tooth development, odontogenesis needs to be regularly maintained. Perivitelline fluid (PVF) from the fertilized eggs of horseshoe crab has been reported to enhance proliferation of cells and promotion of the generation of certain organs in certain organisms due to its richness in proteins and important amino acids.

Objective: The aim of this research was to study the effect of PVF in the odontogenic gene expression in DPSCs.

Methods: The odontogenic genes selected were dentin sialophosphoprotein (*DSPP*), dentin matrix acidic phosphoprotein 1 (*DMP-1*), osteopontin (*OPN*) and runt-related transcription factor (*RUNX2*). *GAPDH* was used as the house keeping gene. Their expressions were analysed in DPSCs both with (0.019mg/ml) and without the treatment of PVF on day 1, 3 and 7. The gene expression was analysed using conventional polymerase chain reaction followed by 1% agarose gel electrophoresis. Mann Whitney test was utilized to determine the significance between the treated and untreated groups.

Results: All the four genes, *DMP-1*, *DSPP*, *RUNX2* and *OPN* expressed at a higher level as compared to control (untreated) on day 1, 3 and 7 though there was no statistical significant difference.

Conclusion: PVF has the potential to enhance odontogenesis in DPSCs as revealed by the increased expression of odontogenic markers, *DMP-1*, *DSPP*, *RUNX2* and *OPN*.

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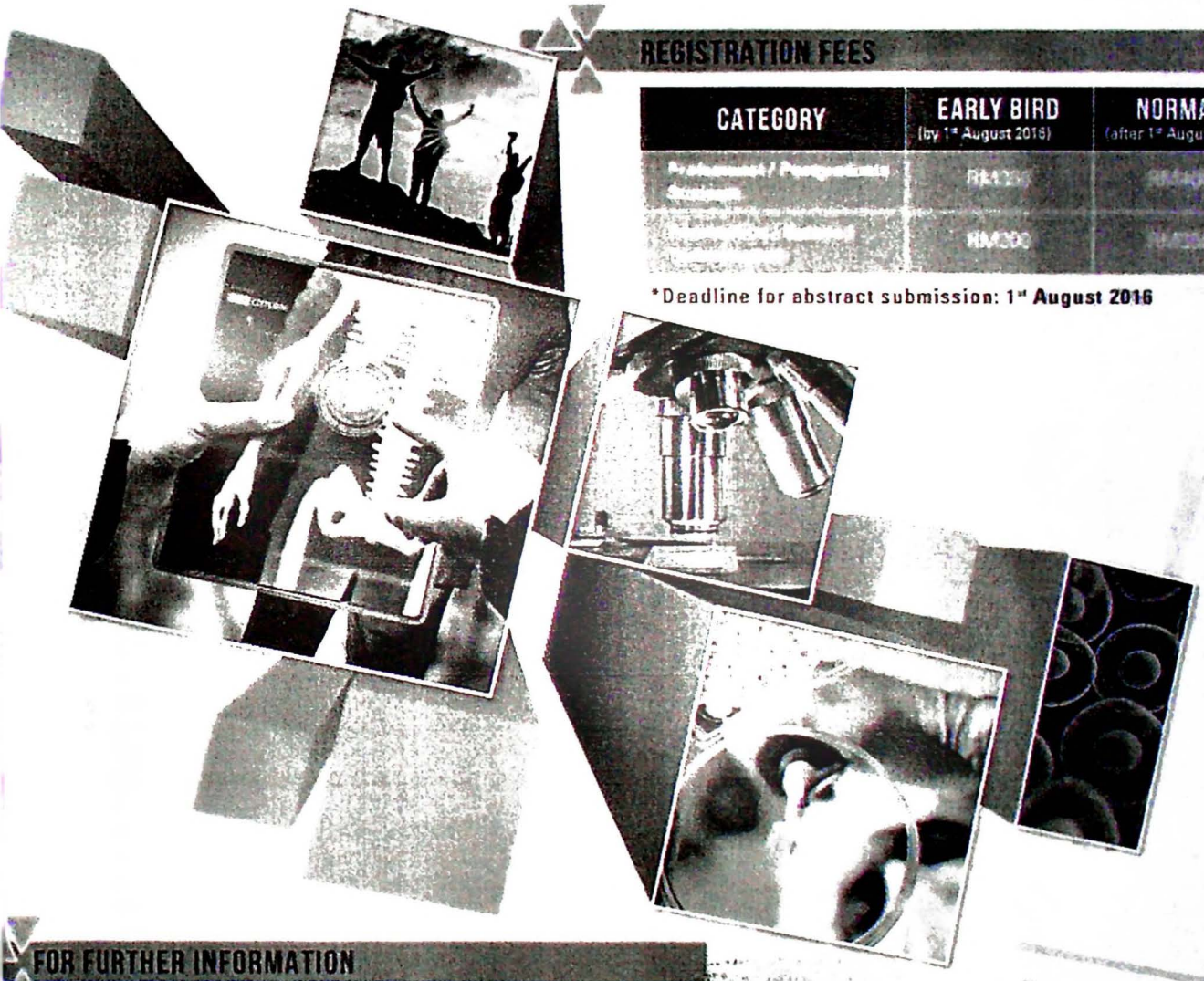

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OB19

Effect of perivitelline fluid on the cell cycle regulatory genes in dental pulp stem cells

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Introduction: Perivitelline fluid (PVF) from the fertilized eggs of horseshoe crab has been reported to act as a supplement in enhancing many types of cell proliferation and differentiation. Dental pulp stem cells (DPSCs) are one of the best candidates to support regenerative medicine therapies due to their multipotent capabilities, similar to mesenchymal stem cells (MSCs), besides being less invasive in terms of extraction as compared to the classic bone marrow MSCs.

Objective: To analyse the expression of cell cycle regulatory genes in DPSCs treated with PVF.

Methodology: DPSCs were treated with 0.019 mg/ml of PVF for a period of 14 days. Concurrently, another group comprised of untreated DPSCs. The cells were harvested on day 1, 3, 7 and 14. RNA was extracted from the cells and converted to cDNA. Polymerase chain reaction was carried out using specific primers for *PTEN, CDKN2A* and *BCL2L11. GAPDH* was used as the house keeping reference gene. Gene expression was analysed using gel electrophoresis and statistical analysis was done using Mann-Whitney test.

Results: There was significant upregulation of *PTEN* and *CDKN2A* on all days in treated groups compared to untreated groups. *BCL2L11* expressed rather weakly on day 3 and 7 followed by absence of expression on day 14 in the treated group as compared to the untreated.

Conclusion: Based on the study, it can be concluded that PVF enhances cell proliferation and inhibits apoptosis of DPSCs.

OB20

Evaluations of bacterial growth an adhesion of *Streptococcus mutans* o different types of tooth coloured restorati materials

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Introduction: Bacterial adhesion to the surfa of dental restorative materials is one of th important parameter in the aetiology secondary caries formation. *GtfB* and *gbp* genes play a significant factor in early adhesi of *S. mutans* on the tooth surface and are central significance in biofilm formation in or cavity.

Objective: To study the effect of the nanofill and micron-sized materials on the adhesion *S. mutans* by evaluating the bacterial gro and gene expression analysis.

Methodology: Resin Modified Glass Ionom Cement (RMGIC); Ketac™ N100 (nanofill RMGIC, 3M ESPE, USA) and Fuji II™ L (microfilled RMGIC, GC Corporation, Japa and composites resin; Filtek™ Z350 (nanofill composite, 3M ESPE, USA) and Filtek™ Z2 (microhybrid composite, 3M ESPE, USA) we packed in acrylic mould and light-cured th polished with Sof-Lex discs. All the materia were cultured with *S. mutans* and observe under Scanning Electron Microscope (SEM analyzed for bacterial growth by densitome and gene expression analysis by using re time PCR at several incubation times.

Results: Within the same group, nanofill materials showed less accumulation of *mutans* compared to micron-sized materia RMGIC groups gave a lower *S. mutan's* gro compared to composite resin group at all t incubation times. Nanofilled RMGIC gav significantly lower of expression levels of g and *gbpB* genes compared to other materia $p<0.05$.

Conclusion: Nanofilled materials capable combating the accumulation of *S. muta* compared to micron-sized materials. Betwe nanofilled materials, nanofilled of RMGIC h better performance in reducing *S. muta* adhesion on the materials compared nanofilled of composite.

COMPREHENSIVE TECHNICAL REPORT

Effect of Perivitelline Fluid (PVF) obtained from Fertilized Eggs of Horseshoe Crab on Cultured Human Dental Pulp Stem Cells

Abstract

Horseshoe crab is one of the oldest existing living fossils which comprises of four main species at present day. *Limulus polyphemus* is found in North America and the other three species, *Tachypleus tridentatus, Tachypleus gigas* and *Carcinoscorpius rotundicauda* resides in Southeast Asia. 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. Interestingly, over the past decades, researchers have explored a new material called perivitelline fluid (PVF) from the egg of a fertilized horseshoe crab which is rich in important primitive proteins and amino acids that are crucial for embryogenesis. Previous studies have shown that PVF has the ability to enhance cells' growth and differentiation as well as promoting generation of certain organs. PVF has been tested on many types of cells, stem cells particularly and positive results have been reported. Hence, it is suggested that PVF could be used as a supplement for supporting cell growth. PVF was extracted from the species of *Tachypleus gigas.* The current study analysed the effect of PVF on stem cells from human exfoliated deciduous teeth (SHED). First, IC_{25} (26.887 mg/ml) and IC_{50} (14.093 mg/ml) of PVF was determined by using MTT assay. To add, we also found that the reduction in PVF concentration led to an enhancement of cell proliferation based on the Alamar Blue assay; which suggest that PVF treatment is only needed in a minute concentration for it to be able to give an impact towards the proliferation of cell. Therefore, we have chosen the concentration of 0.019 mg/ml of PVF to be used in gene expression analysis as it produced 102.50% cell viability according to the MTT assay done. Besides that, PVF is reported to be non-mutagenic and non-toxic towards SHED hence it is hypothesized as a safe treatment to be used in future clinical practice. Several sets of genes has been chosen for gene expression analysis. Odontogenic markers (*DMP-1, DSPP, RUNX2* and *OPN*) in SHED have shown enhancement in their expression when PVF (0.019 mg/ml) treatment was introduced. This suggest that PVF could be effective in supporting dentin regeneration in SHED. Next, PVF has potential in enhancing cell growth and proliferation as shown by the upregulated expression cell cycle regulator (*MDM2, CDKN2A, TP53, PTEN*) and apoptotic regulator genes (*BCL2-L11*) in SHED. Finally, Live/Dead cell viability assay done on SHED treated with PVF (0.019 mg/ml) showed higher viability and percentage of live cells in the treated group. In conclusion, PVF is a great material to be used as a supplement for cell growth for tissue engineering in the future and continuous study should be conducted to further explore its potential.

Kesan Perivitelline Fluid (PVF) daripada Telur Belangkas terhadap Stem Sel Pulpa Gigi Manusia

Abstrak

Belangkas adalah salah satu daripada hidupan fossil paling lama yang masih hidup sehingga kini di mana ia terdapat empat spesis utama, *Limulus polyphemus* yang terdapat di Amerika Selatan dan tiga spesis lain iaitu, *Tachypleus tridentatus, Tachypleus gigas* dan *Carcinoscorpius rotundicauda* yang terdapat di Asia Tenggara. Belangkas sering dilihat sebagai organisma yang versatil malah berguna dalam bidang biomedikal. Darah biru belangkas telah banyak diaplikasikan untuk digunakan sebagai assay endotoxin di dalam vaksin, dadah dan suntikan. Menariknya, para penyelidik telah menerokai bahan biologikal daripada belangkas yang bernama perivitelline fluid (PVF) daripada telur belangkas yang kaya dengan protein dan amino acid penting untuk embriogenesis. Kajian yang terdahulu telah menunjukkan kemampuan PVF untuk meningkatkan perkembangan dan pembezaan sel dan juga membantu regenerasi sesetengah organ di dalam sesetengan organisma. Rawatan PVF telah diperkenalkan kepada berbagai jenis sel, stem sel terutamanya dan keputusan positive telah dilaporkan. Oleh itu, PVF telah diramalkan berguna sebagai suplimen untuk membantu perkembangan sel. PVF diekstrak daripada spesis *Tachypleus gigas.* Kajian terkini telah menganalisa kesan PVF terhadap stem sel daripada gigi bongsu manusia. Pertama, konsentrasi IC_{25} (26.887 mg/ml) dan IC_{50} (14.093 mg/ml) PVF telah dikenalpasti melalui assay MTT. Tambahan pula, kami mendapati bahawa pengurangan rawatan PVF telah menghasilkan kepada peningkatan sel proliferasi berdasarkan assay Alamar Blue yang telah dilakukan. Oleh itu, kami telah memilih konsentrasi 0.019 mg/ml rawatan PVF untuk digunakan dalam analisa gen ekspresi kerana konsentrasi ini telah memeberi 102.50% daya maju cell. Selain itu, PVF juga dilaporkan untuk tidak mutagen dan tidak toksik terhadap SHED sekaligus membuatkan ia selamat sebagai rawatan untuk amalan klinikal di masa hadapan. Beberapa gen telah dipilih iaitu Odontogenic markers (*DMP-1, DSPP, RUNX2* and *OPN*). Kesemua gen tersebut menunjukkan peningkatan ekspressi di dalam SHED apabila rawatan PVF (0.019 mg/ml) diperkenalkan. Oleh itu, boleh diramalkan bahawa PVF merupakan rawatan yang efektif dalam menyokong regenerasi dentin dalam SHED. Selain itu, PVF mempunyai potensi dalam meningkatkan pertumbuhan dan perkembangan sel yang terbukti dengan peningkatan ekspressi gen yang bertanggungjawab dalam pusingan sel (*MDM2, CDKN2A, TP53, PTEN*) dan apoptosis (*BCL2-L11*) dalam SHED. Akhir sekali, Live/Dead cell viability assay yang telah dilakukan terhadap SHED dengan rawatan PVF menunjukkan peningkatan daya maju sel dan peratus sel hidup berbanding kumpulan yang tiada rawatan PVF. Secara konklusi, PVF adalah

bahan yang bernilai untuk digunakan sebagai suplimen untuk pertumbuhan sel di dalam dunia kejuruteraan tisu di masa hadapan.

15th December 2012 to 14th June 2016

Effects of Peri-Vitelline Fluid (PVF) Obtained from Fertilized Eggs of Horseshoe Crab on Cultured Human Dental Pulp Stem Cells

Objective: The main objective was to study the effects of peri-vitelline fluid (PVF) on cultured human dental pulp stem cells from deciduous teeth (SHED).

Work done (36 months): The process to isolate and collect the test material (PVF) has been performed by Aquatrop, UMT. Fertilized eggs of the horseshoe crab (*Tachypleus gigas*) were collected from the nests located on the sandy beach at Balok (Kuantan), Malaysia. The eggs then were incubated at constant temperature of 29 ± 1° C and cleaned before the vitelline membrane of the embryo was pierced and the liquid (PVF) was collected after centrifugation. The collected PVF was freeze dried and stored at -80°C prior to the analysis. For the testing, the freeze dried PVF was mixed and diluted with 1ml phosphate buffer saline (PBS). Further dilution of PVF sample was performed by adding complete culture medium Alpha-MEM (α-MEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin antibiotic solution, 2 mM L-Glutamine and 100 µM of ascorbic acid. Same complete medium was used for the culture of selected cell lines (SHED) in the present study.

The evaluation of IC_{50} and IC_{25} was performed using cytotoxicity test (MTT assay) whereby SHED were treated with different concentration of PVF (45, 22.5, 11.25, 5.625, 2.813, 1.406, 0.703, 0.352 mg/ml). Experiments were conducted in triplicates. Collection of data and analysis of results of MTT assay have been completed. The preliminary study on the effect of PVF on proliferation of SHED using Alamar Blue assay also was performed whereby the cells were treated with different concentrations of PVF (5, 10, 15, 20, 25 and 30%) without the addition

of FBS. The proliferation of PVF-treated cells was compared with cells incubated with culture medium (Alpha-MEM) in addition of 10% FBS.

Prior to the proliferation test, the standard curve of SHED was constructed. The study of cell proliferation using Alamar Blue test using different concentrations of PVF is already completed. SHED were treated with IC_{50}, IC_{25} values and concentrations with higher cell viability compared to control in MTT assay for 10 days. The concentrations tested were as stated below:

Table 1: The concentrations of tested PVF for Alamar Blue assay

Group	Concentration of PVF (mg/ml)
IC_{50}	26.887
IC_{25}	14.093
102 %*	0.278
102.50 %*	0.019
Negative control	0.000

After the addition of Alamar Blue reagent, cells were incubated for 3 hour prior to the absorbance reading at 570 and 600 nm. The percentages of reduction (% Red) were calculated using this formula below:

% Red = [(117.216) A570- (80.586) A600] / [(155.677) A'600-(14.652) A'570] x 100%

After the collection of % Red data, the population doubling time (PDT) of treated cells at log phase of growth were determined using this formula:

PDT = 1/r

Where, multiplication rate (r): 3.32 (log N_H – log N_I) / (t_2 – t_1); N_I: cell number at 96 hour (Day 4); N_H: cell number at 144 hours (Day 6)

For statistical analysis of PDT, independent t-test was selected to study the effect of PVF on treated cells when compared to negative control where significant difference was valued at $p < 0.05$.

The morphological characteristics of cells treated with PVF have been studied using phase-contrast microscope for the period of 10 days. Negative control was also included in the study. Chromosome aberration test (CA) has been completed. SHED cells were treated with IC50, IC25 and two PVF concentrations which produced 102% and 102.5% of cell viability in MTT assay (Table 1) concurrent with positive and negative controls. Two treatment conditions were applied: with and without addition of S9 metabolic activation. Depending on the condition of the treatment, different positive controls were used: mitomycin C for treatment without S9 and cyclophosphamide monohydrate for treatment with S9. Negative control which included in this test was culture medium (Alpha-MEM) without addition of PVF. The scoring of mitotic indices for each group (PVF groups, negative and positive controls) are recorded. Numerical and structural aberrations of the chromosome were recorded. The formula used to calculate the mitotic index is as follow:

Mitotic Index (MI)= (Total number of metaphase/total number of cells) × 100%

Where total number of cells= 1000 cells

Results

MTT assay

The result of MTT assay for SHED treated with PVF was presented in Figure 1. The determination of IC_{50} and IC_{25} was performed using an Excel add-in (ED50V10). From the software, the value of IC_{50} and IC_{25} are 26.887 and 14.093 mg/ml.

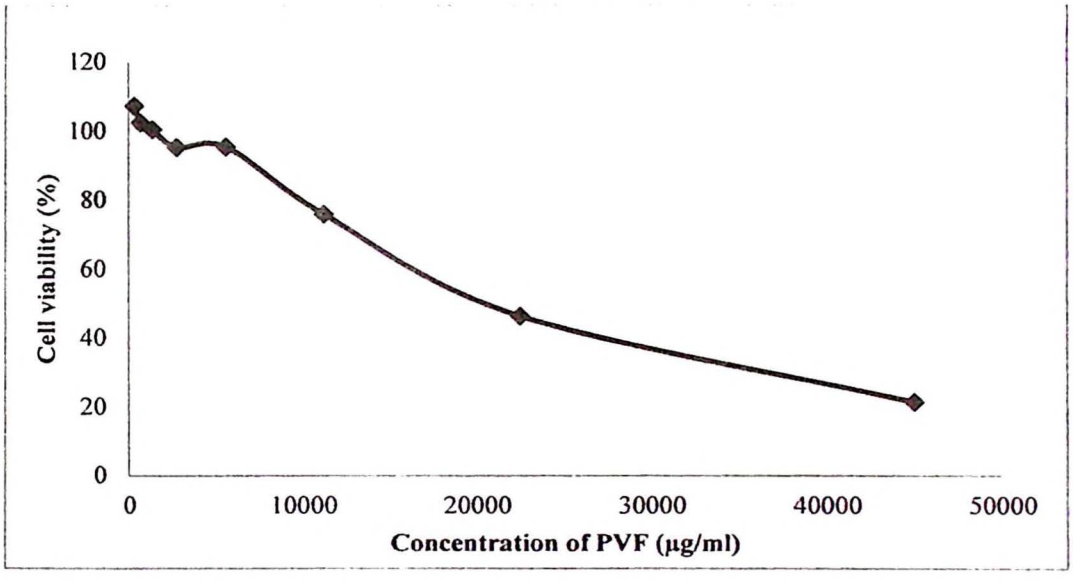

Figure 1: MTT assay result of SHED treated with PVF

Alamar Blue assay

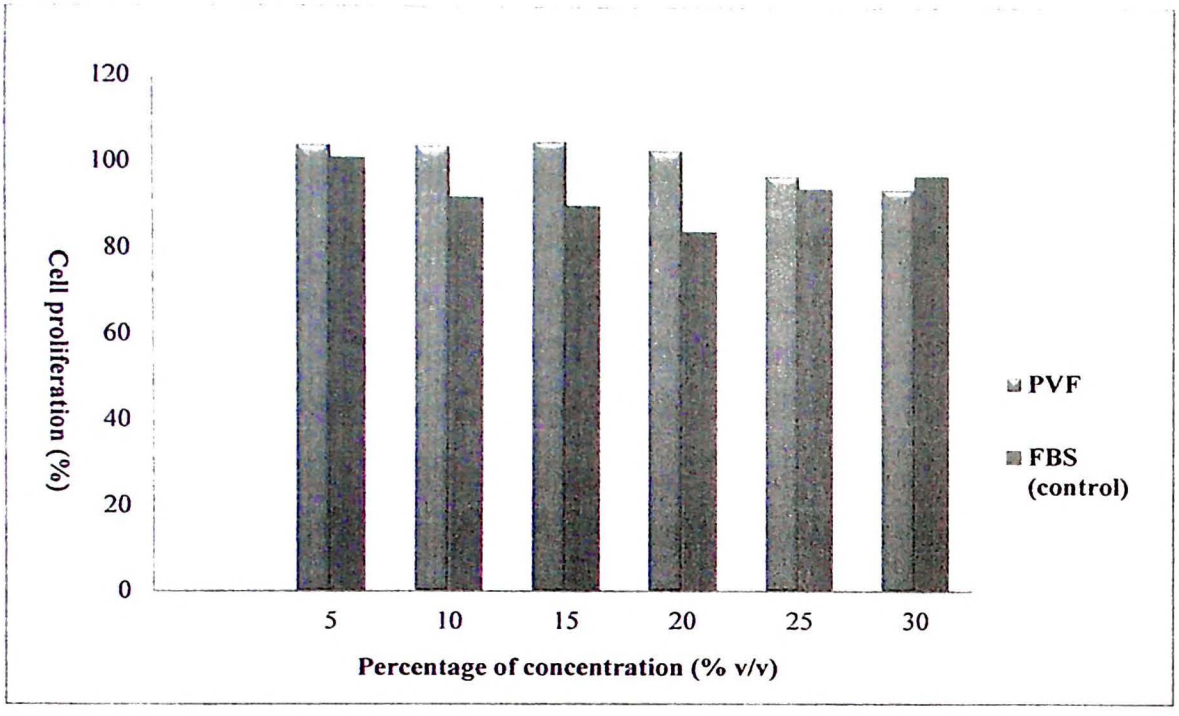

Figure 2: Preliminary Alamar Blue assay result of SHED treated with PVF as compared to SHED treated with culture medium in addition of FBS

The results of preliminary Alamar Blue assay in Figure 2 shows that the SHED treated with selected concentrations of PVF produced more than 90% in cell proliferation, higher than control (culture medium in addition of FBS). Reduction in the concentrations of PVF resulted in enhancement of cell proliferation.

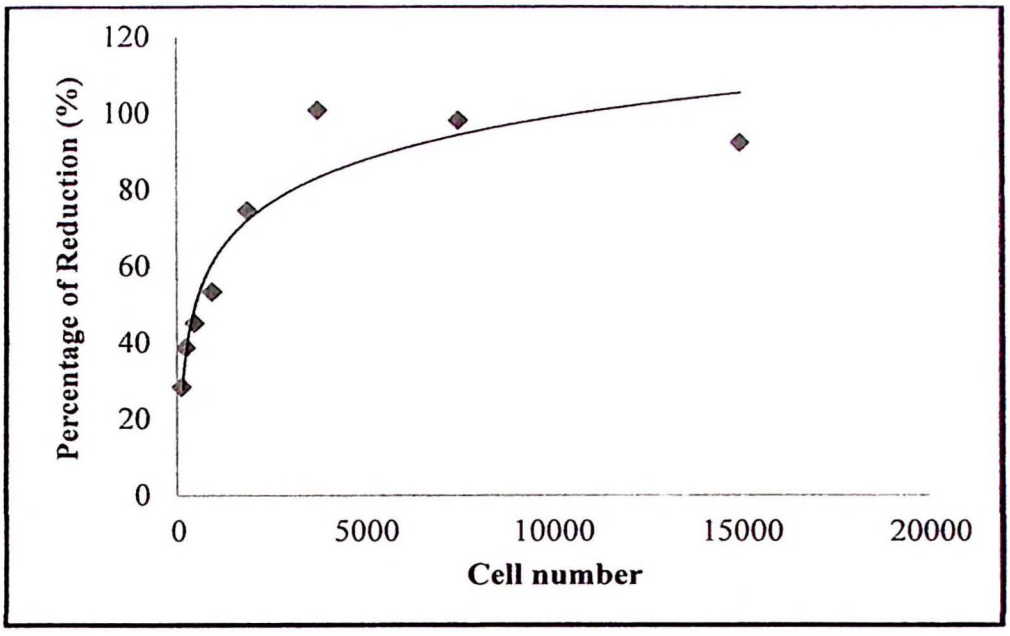

Figure 3: Standard curve of SHED

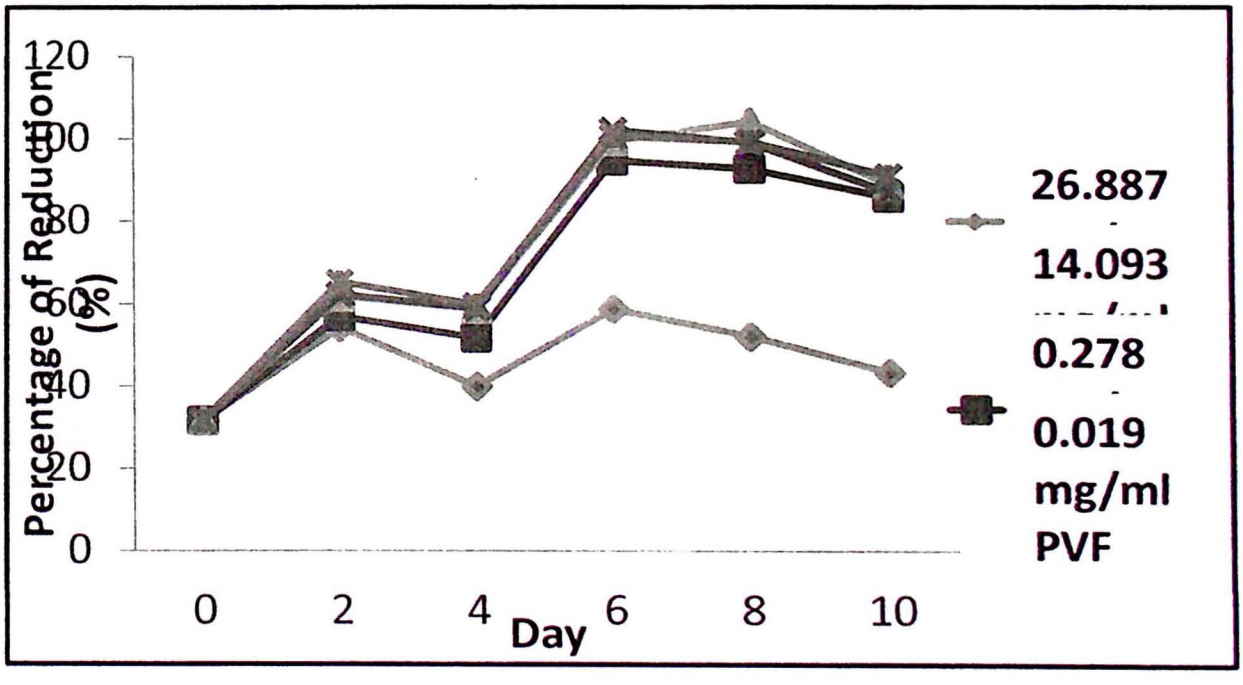

Figure 4: Proliferation effect of PVF on SHED using Alamar Blue assay

Figure 3 shows the standard curve for SHED's growth. The graph clearly indicates that higher cell number produced greater % of Red. Figure 4 reveals the % Red cells treated with different

concentrations of PVF where 0.278 and 0.019 mg/ml PVF produced greater cell proliferation as compared to the negative control at Day 8 and 6 respectively. Meanwhile, the cells treated with IC_{50} concentration of PVF produced no increase in cell proliferation over time. SHED treated with IC_{25} indicated enhancement in cell proliferation over time especially at the end of the treatment (Day 10), comparable to IC_{25}, IC_{50} and negative control.

Table 2: The PDT of cells treated with PVF

Concentrations of PVF (mg/ml)	PDT (hours)
26.887	28.45
14.093	12.39
0.278	13.09
0.019	12.31
Negative control	13.02

Table 2 shows the PDT of cells treated with PVF. Cells treated with 14.093, 0.278 and 0.019 mg/ml PVF shows comparable PDT with negative control whereas a slight difference in PDT of cells treated with 26.887 mg/ml PVF was observed when compared to the negative control. However, results of independent t-test revealed no significant difference in PDT of all treatment groups when compared to the negative control ($p > 0.05$).

Chromosome aberration test

In the case of the CA test, we observed insignificant differences in the MI between the PVF groups and negative control. On the other hand, both positive control agents (MMC and CP) demonstrated lower MI values compared to other groups. Those percentages of MI were

significantly different when compared to the untreated (negative) control (reduction in more than 50% of MI; Table 3). No dose relationship was observed.

Table 3: Mitotic index (MI) of SHED treated with perivitelline fluid (PVF)

Groups	Hours	Mean mitotic index (SD) (%) [a]	
		Without S9	With S9
PVF (26.887 mg/ml)	4	3.65 (0.07)	3.60 (0.14)
	24	3.25 (0.21)	-
PVF (14.093 mg/ml)	4	3.90 (0.14)	3.55 (0.21)
	24	3.50 (0.00)	-
PVF (0.278 mg/ml)	4	3.80 (0.14)	4.20 (0.14)
	24	3.60 (0.14)	-
PVF (0.019 mg/ml)	4	3.70 (0.28)	3.60 (0.28)
	24	3.45 (0.07)	-
MMC [b]	4	1.15 (0.21)*	-
	24	1.25 (0.21)*	-
CP [b]	4	-	1.70 (0.28)*
Negative control (culture medium) [c]	4	3.50 (0.14)	4.40 (0.14)
	24	3.30 (0.28)	-

[a] Mean from triplicate tests

[b] Positive controls mitomycin C (MMC) at a concentration of 0.1 µg/ml for 4 hours and 0.05 µg/ml for 24 hours without S9 mix and cyclophosphamide monohydrate (CP) at a concentration of 10 µg/ml for 4 hours with S9 mix.

[c] MSC basal medium

***p<0.05, MI is significantly different compared to the negative control.**

Regardless of the treatment conditions (with and without S9 mix), different concentrations of PVF and duration of the treatment (4 and 24 hours), there was an absence of significant gross aberration in the chromosomes of the treated cell lines with PVF extract compared to the negative control. In contrast, multiple chromosomal abnormalities were seen in the both

positive control groups (MMC and CP). The observed aberrations included chromosomal gaps, breaks, dicentrics, loss of centromeres and endoreduplications. Figure 5 shows the representative metaphase spreads from all groups.

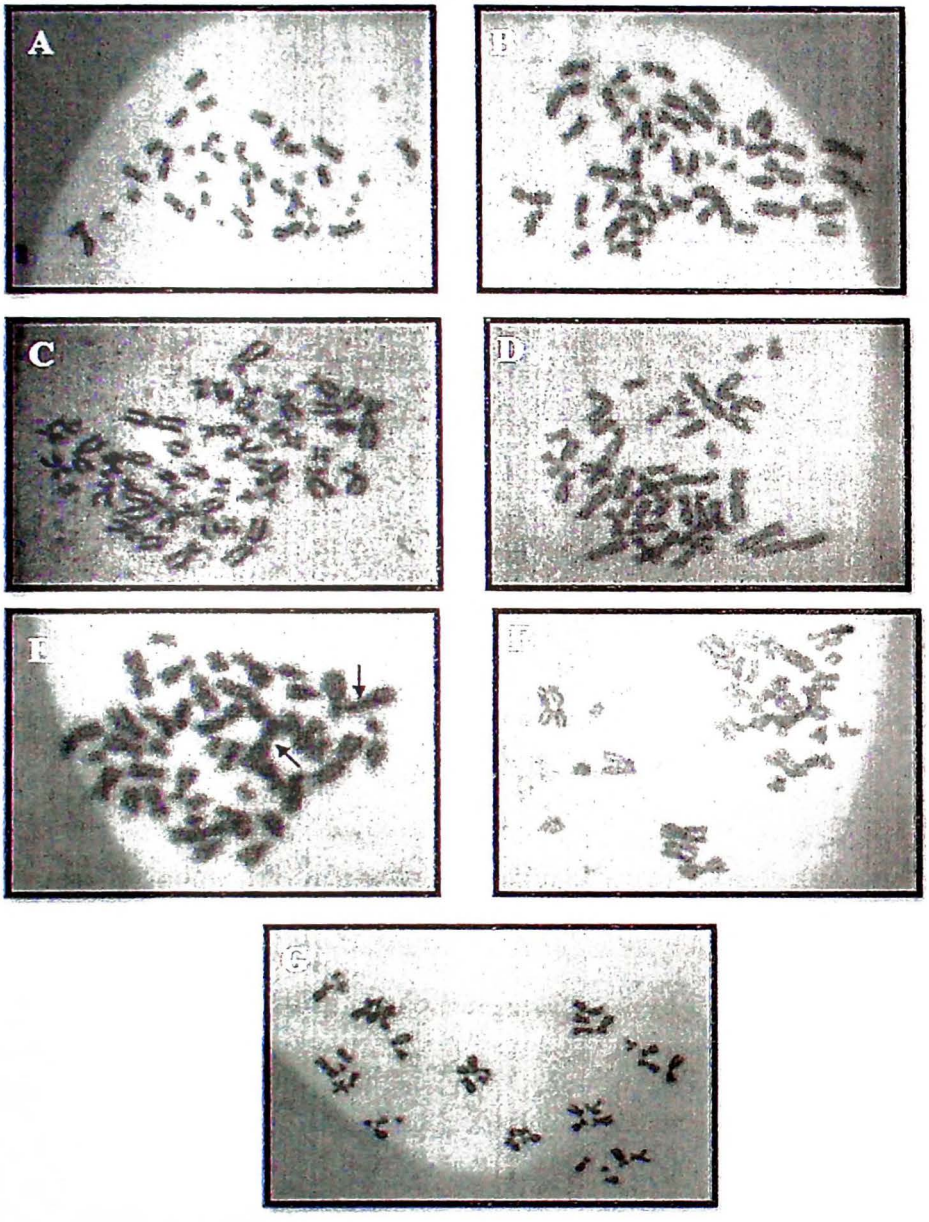

Fig 5: Representative images of cells treated with A. 26.887 mg/ml perivitelline fluid (PVF), B. 14.093 mg/ml PVF, C. 0.278 mg/ml PVF, D. 0.019 mg/ml PVF, E. Mitomycin C (MMC), F. cyclophosphamide monohydrate (CP) and G. Negative control. No significant chromosome aberrations (CA) were observed in PVF groups and negative control. Arrows show the gaps in the chromosome of cells treated with MMC. Formation of endoreduplication was seen in cells treated with CP.

Ames test

In all triplicate tests, the results showed that the number of revertant colonies (TA98 and TA100 strains) treated with various PVF concentrations were less than 2-fold of the positive control in both treatment conditions (presence and absence of S9 mix; Table 4). The interpretations of these results were based on previously mentioned non-statistical analyses. No dose-response relationship was observed.

Table 4: Ames results of perivitelline fluid (PVF)

Group	Average number of colonies [a]			
	TA98		TA100	
	With S9	Without S9	With S9	Without S9
PVF (14.093 mg/ml)	9 (2.08)	13 (10.12)	22 (9.61)	27 (10.41)
PVF (7.045 mg/ml)	9 (3.51)	16 (6.93)	32 (8.54)	24 (4.04)
PVF (3.523 mg/ml)	8 (1.15)	18 (8.54)	23 (5.03)	25 (4.04)
PVF (1.761 mg/ml)	9 (2.89)	15 (5.29)	21 (4.73)	24 (3.21)
PVF (0.881 mg/ml)	11 (3.61)	16 (7.94)	26 (3.51)	24 (9.71)
Positive control [b]	37 (11.14)	65 (11.37)	92 (12.06)	175 (21.36)
Negative control (ddH_2O)	10 (3.51)	12 (4.04)	28 (4.58)	27 (3.46)

[a] Average from triplicate tests

[b] Positive controls: 4-nitro-o-phenylenediamine (4-NoPD) and sodium azide (NAN3) for strains TA98 and TA100, respectively.

Morphology of cells treated with PVF for 10 days

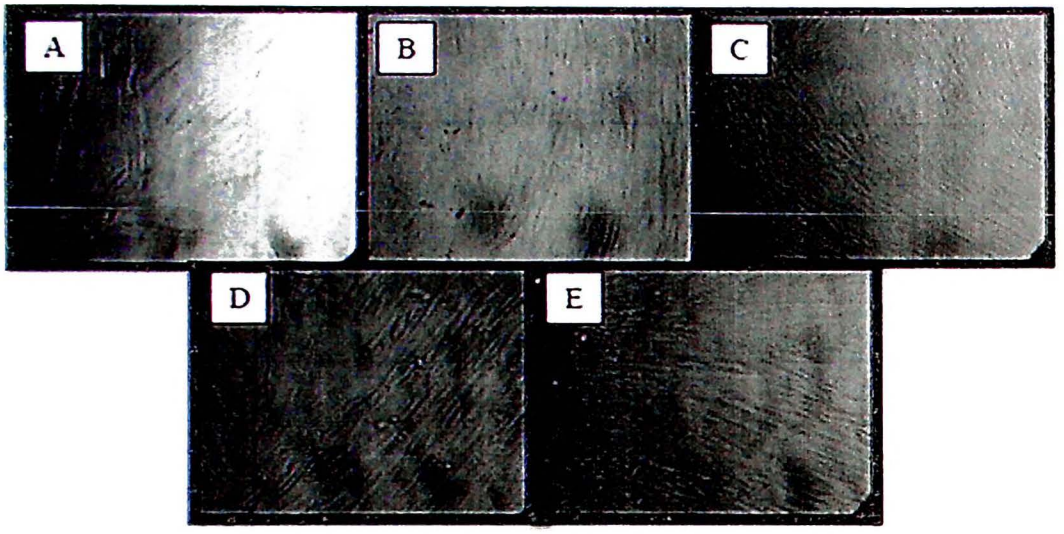

Figure 6. Morphology of cells treated with PVF for 10 days. A) 26.887 mg/ml PVF; B) 14.093 mg/ml PVF; C) 0.278 mg/ml PVF; D) 0.019 mg/ml PVF and E) Negative control

The morphology of cells treated with different concentrations of PVF is shown in Figure 6. Confluence cells were observed in all treatment groups except for 26.887 mg/ml PVF group.

The Expression of Cell Cycle Regulatory Genes in Human Dental Pulp Stem Cells between Different Passages

The genes controlling the cell cycle namely, *CDKN2A, PTEN, TP53* and *MDM2* genes were analysed and normalized to the housekeeping gene, *GADPH.* The gene expression was quantified as average density value (ADV), the results of which are shown in table 5. Expression of *GAPDH* was constant for both the control SHED and SHED treated with PVF. *CDKN2A* and *MDM2* expression for both the SHED with and without PVF treatment groups expressed a similar pattern expression but a different ADV value. The expression of *CDKN2A*

in the untreated SHED at passage 4 had the lowest expression, reached its peak at passage 5 and declined at passage 6 whereas, SHED in PVF showed slightly increased expression in passages 4 and 5 and then decreased at passage 6 (Figure 7A). SHED with PVF treatment showed increased expression of *MDM2* at passage 4 and drastically declined expression at passage 5 and slightly increased at passage 6. Meanwhile, for untreated SHED, *MDM2* expression lowered by half compared to treated cells. Passage 5 of untreated group showed a declined trend from passage 4 and slightly increased at passage 6 (Figure 7B).

TP53 expression of SHED treated group showed a higher expression compared to untreated group. Passage 5 of SHED treated with PVF showed the highest expression and declined at passage 6. SHED in untreated PVF expressed much lower with a declining pattern where the expression decreased from passage 4 until passage 6. This is shown in Figure 7C.

PTEN expression showed a different pattern between SHED treated with PVF and SHED untreated group. SHED treated group at passage 5 started to decrease and increased at passage 6. However, for SHED untreated group, passage 5 showed an increasing pattern at passage 5 and there was no change at passage 6 (Figure 7D).

Table 5. Average density value (ADV) of different genes expressed in SHED with and without treatment of PVF

Gene	Passage	Treated			Average	Untreated			Average
CDKN2A	4	1.56	1.9	1.96	1.8	0.6	0.85	0.85	0.8
	5	1.5	2.25	2.36	2	1.53	1.72	1.55	1.6
	6	1.7	1.77	1.9	1.8	1	1.09	0.9	1
MDM2	4	3.04	2.8	3.1	3	1.6	1.5	1.6	1.6
	5	1.6	1.5	1.63	1.6	0.69	0.9	0.8	0.8
	6	2.57	1.9	1.6	2	1.2	0.9	0.87	1
PTEN	4	3.2	2.95	3.78	3.3	0.86	1.1	1.05	1
	5	2.48	3.3	3.3	3	1.9	2.09	2	2
	6	4	3.38	3.2	3.5	2.3	2.27	1.5	2
TP53	4	2.6	3.3	4.1	3.3	1.27	1.2	1.4	1.3
	5	2.96	3.9	4.1	3.6	0.8	1.18	1.04	1
	6	2.47	1.76	1.84	2	0.8	0.45	0.56	0.6

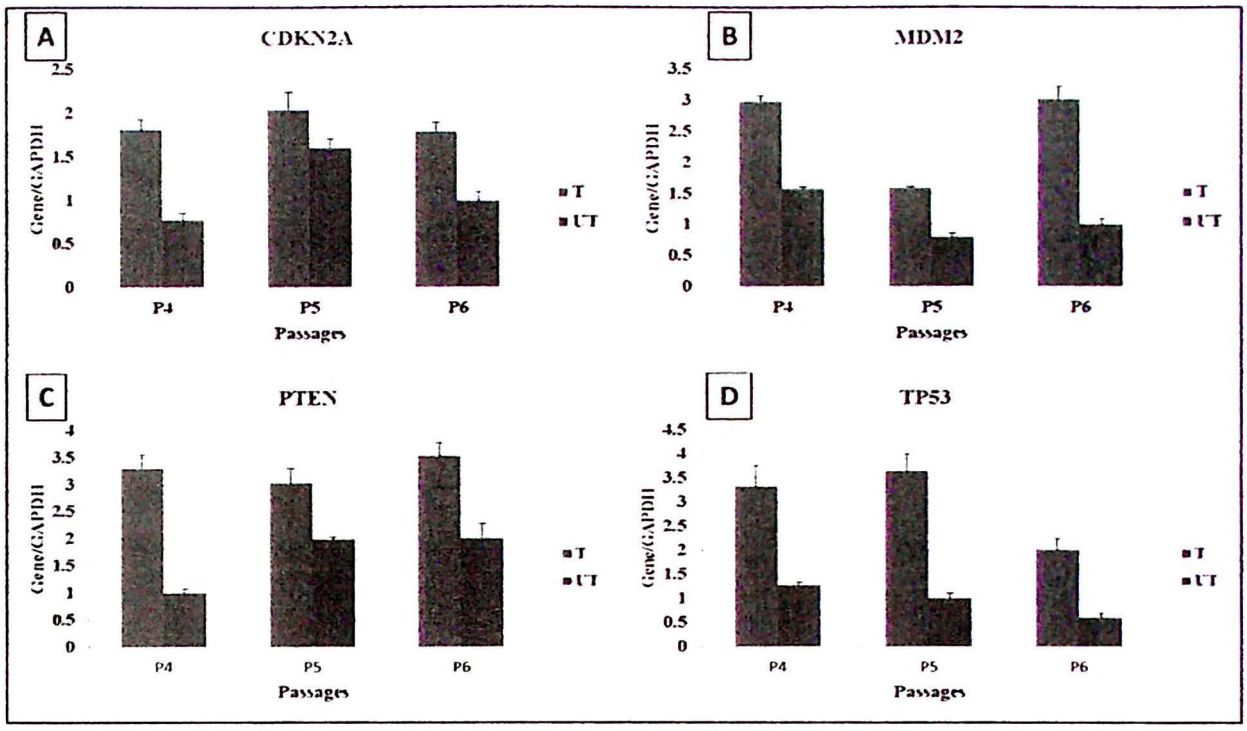

Figure 7. Comparison of gene expression between treated and un-treated groups at P4, P5 and P6. A, B, C, D: Comparison of *CDKN2A, MDM2, PTEN, TP53* gene expression respectively between treated and un-treated groups at P4, P5 and P6

Gene Expression Analysis of Odontogenic Markers in Stem Cells from Human Exfoliated Deciduous Teeth Treated with Perivitelline Fluid from Horseshoe Crab

Table 6 shows the primer sequences for the odontogenic markers of *GAPDH*, *DSPP*, *DMP-1*, *OPN* and *RUNX2*. The PCR conditions are shown in Table 7.

Table 6. Primer sequences

Gene	Primer	DNA Sequence 5′ to 3′	Melting Temp (°C)	Amplicon Size
GAPDH	Forward	CGA CCA CTT TGT CAA GCT CA	55.3	203 bp
	Reverse	AGG GGA GAT TCA GTGTGGTG	56.6	
DSPP	Forward	TGT CGCTGT TGT CCA AGA AG	55.3	498 bp
	Reverse	ATT CTTTGG CTG CCATTG TC	53.9	
DMP-1	Forward	CAG GAG CAC AGG AAA AGG AG	55.6	213 bp
	Reverse	CTG GTG GTATCTTGG GCA CT	56.9	
OPN	Forward	CCCTTC CAA GTA AGT CCA ACG	55.5	321 bp
	Reverse	GGATGT CAG GTC TGC GAA AC	56.0	
RUNX2	Forward	TCTTCACAAATCCTCCCC	52.6	230 bp
	Reverse	TGGATTAAAAGGACTTGGTG	51.3	

Table 7. PCR conditions

Step	Temperature (°C)	Cycle	Time
Initial denaturation	95	1	1 min
Denaturation	95	35	15 sec
Annealing	54.2 (*GAPDH*),		10 sec (*GAPDH*),
	53.3 (*OPN*),		15 sec (*OPN*, *DMP-1*,
	55.3 (*DMP-1*),		*DSPP*, *RUNX2*)
	52.7 (*DSPP*),		
	58.2 (*RUNX2*)		
Extension	72		10 sec

Expression of odontogenic markers, *DMP-1*, *DSPP*, *OPN*, and *RUNX2* were analysed by normalizing them to the housekeeping gene, *GADPH*. The integrity and intactness of RNA samples are shown in figure 8.

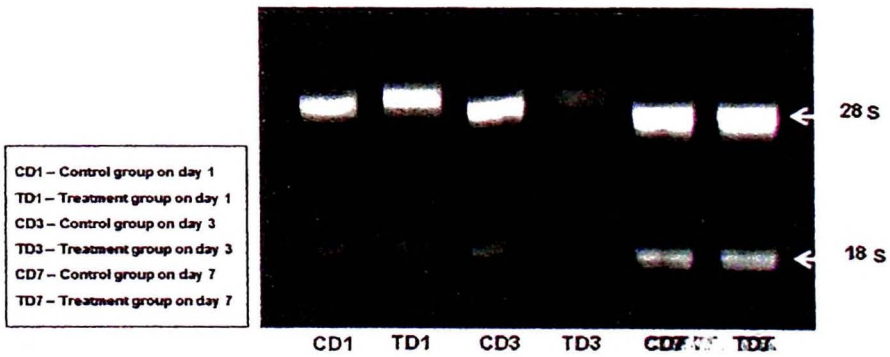

Figure 8. RNA bands of Control and Treated SHED harvested on Day 1, 3 and 7

The gene expression was quantified as ADV and shown in figure 9 and 10. Expression of the genes were analysed on 3 distinctive days, day 1, 3 and 7. The expression of *DMP-1* showed a

similar pattern of expression both in the control as well as PVF treated SHED where the expression increased from day 1 to day 3, followed by a decrease on day 7. This expression pattern was similar to the expression of *DSPP* and *RUNX2* for both the control and treatment groups. Nevertheless, *DMP-1*, *DSPP*, and *RUNX2* expression was higher in the PVF treated SHED compared to the control on all days (Fig. 10).

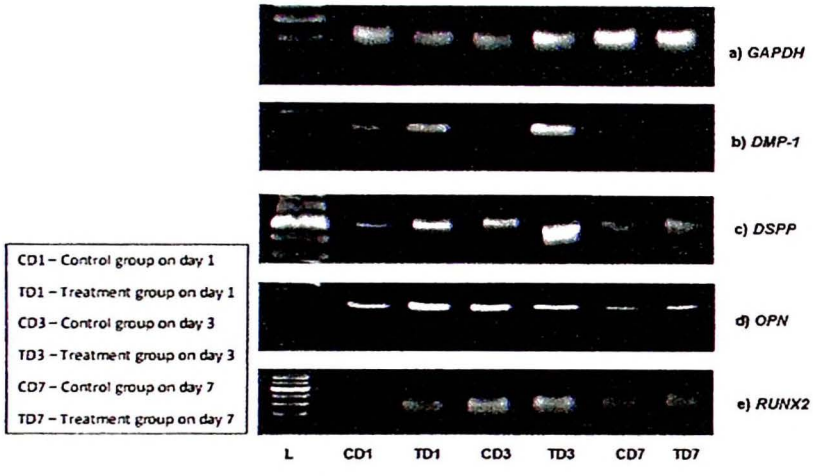

Figure 9. Gel image showing the bands of *GADH*, *DMP-1*, *DSPP*, *OPN* and *RUNX2* PCR amplification in control and PVF treated SHED at day 1, 3 and 7

The expression of *OPN* in the control SHED also showed an increase from day 1 to day 3 followed by a decrease on day 7. Alternatively, the trend was different in the PVF treated groups where a declining pattern in the expression was observed from day 1 to day 7. However, the treatment group still showed higher expression of *OPN* compared to the non-treated SHED on all days (Fig. 10).

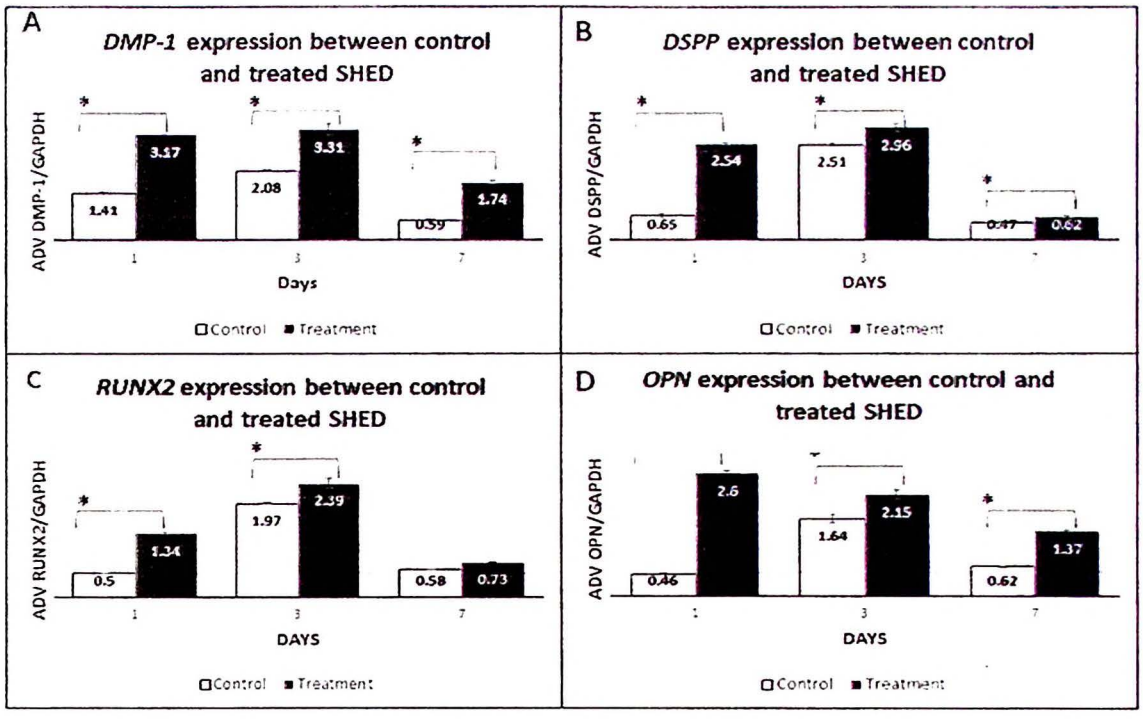

Figure 10. Normalized expression levels of *DMP-1, DSPP, RUNX2* and *OPN* at day 1, 3 and 7 in control and PVF treated SHED

The mean (SD) of ADV of the different genes were calculated and shown in table 8. Statistical analysis conducted using Mann Whitney test showed no significant difference between the expression of genes in control and PVF treated group.

Table 8. Mean (SD) of the ADV of the different genes

	DMP-1		*DSPP*		*OPN*		*RUNX2*		*GAPDH*	
DAY	**Control**	**PVF**	**Control**	**PVF**	**Control**	**PVF**	**Control**	**PVF**	**Control**	**PVF**
1	1103.80	1721.30	527.28	1379.58	361.81	1414.90	390.13	726.46	784.51	653.8
	(21.047)	(44.783)	(49.352)	(13.229)	(8.677)	(4.442)	(1.499)	(4.698)	(4.909)	(6.93
3	1276.45	1955.49	1026.19	1751.75	1006.47	1270.70	1204.86	1412.83	613.17	603.5
	(18.23)	(38.044)	(31.487)	(9.007)	(49.323)	(22.512)	(3.350)	(10.294)	(4.278)	(11.3
7	639.75	1631.70	507.43	586.20	676.23	1276.16	628.22	688.75	1082.37	1007
	(36.329)	(49.660)	(10.393)	(30.679)	(18.315)	(35.860)	(3.929)	(5.638)	(9.932)	(1.67

*Significant at $p < 0.05$.

Six Months Extension Period

Work done (6 months): During the 6-month's extension period, we have done gene expression analysis and cell viability test using the Live/Dead kit for mammalian cells. For both these objectives we also selected the same concentration of PVF (0.019 mg/ml) to be used for the whole experiment. The concentration of PVF was chosen based on the proliferation test of SHED with the addition of PVF that had been done using MTT assay. From this, we have chosen the PVF concentration of 0.019 mg/ml as it resulted to 102.5% cell proliferation towards SHED.

Gene expression analysis were done on 2 groups; control and PVF treated SHED. We selected genes that are crucial in cell cycle and apoptotic regulation which are *PTEN, CDKN2A, MDM2, COL1A1, TP53*, and *BCL2L11*. The housekeeping gene that were used in this study was *GAPDH*. The gene expression of respective genes was recorded up to 21 days. First, SHED were cultured in sterile T75 cm^2 cell culture flasks in CO_2 incubator under 37'C. The cells were maintained using MSC media supplemented with penstep antibiotics. Passage 6 of SHED were used for the whole experiment to act as the control variable. The cells were then divided into 2 groups, control and treated. The PVF were prepared freshly at each usage whereby it was diluted with 1 ml media and then sterilized using 22µm syringe filter. The PVF was then further diluted to 0.019 mg/ml before incorporating it into the media of the treated SHED group. The cells of both groups were grown up till 21 days. A few days of cells harvesting were selected which are day 1, 3, 7, 14 and 21. After the cells were harvested, RNA of the cells was collected using the QIAGEN RNA extraction kit for mammalian cells. The RNAs of all samples were read through the UV spectrometer to check its purity. The RNAs were also run through gel electrophoresis to determine the intactness of the RNA. Next, specific DNA primers were designed and gradient Polymerase Chain Reaction (PCR) were conducted using

the control SHED and all gene primers. Gradient PCR was conducted to optimize the gene primers so that it will give out clear, specific bands of the gene itself. A few criteria that has been taken into consideration while optimizing the gene primers were the annealing time during PCR, the time and voltage used during gel electrophoresis and the amount of PCR product loaded in the gel wells. Once all the genes have been optimized, all cells sample were run with the specific primers and gene expression were analysed using the gel electrophoresis method. Triplicates were done for each sample.

Live/Dead Viability test was also conducted on the SHED of 2 groups, control and PVF treated. This experiment also uses the same concentration of PVF as mentioned above. The objective of this experiment was to analyse the cells viability of SHED based on its morphology when treated with and without PVF under fluorescence microscopy. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually non-fluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. Firstly, SHED were cultured on 18 mm sterile glass coverslips for 2 to 3 days until acceptable cell confluency is achieved. The cells were then washed twice with PBS to remove and dilute serum esterase activity which may be present in the media. The cells were then treated with the cytotoxic agent dyes using recommended concentration. The cells were left incubated for 30-45 minutes under room temperature. Following incubation, the glass coverslip was mounted onto the microscope slide. The coverslip was then sealed with clear nail polish to prevent evaporation. The labelled

cells were viewed under the fluorescence microscopy immediately. Triplicates were done for each sample.

Another cell viability analysis was conducted by using fluorescence microplate to determine the percentage of live and dead cells of SHED treated with PVF of such concentration. The cells were cultured in 96 well plates for 3 days. The cells were tested on day 1, 2 and 3. On each tested day, the cells were washed with PBS twice and then treated with cytotoxic dyes for 30-45 minutes. The fluorescence in the experimental and control cell samples were measured at 645nm and 530 nm. From the results of the fluorescence, we can calculate the percentage of live and dead cells as below:

A. Fluorescence at 645 nm in the experimental cell sample, labelled with calcein AM and EthD-1 = F(645)sam
B. Fluorescence at 530 nm in the experimental cell sample, labelled with calcein AM and EthD-1 = F(530)sam
C. Fluorescence at 645 nm in a sample where all the cells are dead, labeled with EthD-1 only = F(645)max
D. Fluorescence at 645 nm in a sample where all the cells are dead, labeled with calcein AM only = F(645)min
E. Fluorescence at 530 nm in a sample where all (or nearly all) cells are alive, labeled with EthD-1 only = F(530)min
F. Fluorescence at 530 nm in a sample where all (or nearly all) cells are alive, labeled with calcein AM only = F(530)max

G. Fluorescence at 530 nm of the cell-free sample with or without dye added = F(530)0

H. Fluorescence at 645 nm of a cell-free sample with or without dye added = F(645)0

Dead cells are characterized by intense fluorescence at >600 nm and little fluorescence around 530 nm. The percentage of live cells can be calculated from the fluorescence readings defined above as:

$$\% \text{ Live Cells} = \frac{F(530)sam - F(530)min}{F(530)max - F(530)min} \text{ X } 100\%$$

The percentage of dead cells can be calculated from the fluorescence readings defined above as:

$$\% \text{ Dead Cells} = \frac{F(645)sam - F(645)min}{F(645)max - F(645)min} \text{ X } 100\%$$

Results

Gene Expression Analysis

Table 1: The PCR conditions used in the experiment. The annealing temperatures for each gene were optimized.

Step	Temperature (°C)	Cycle	Time
Initial denaturation	**95**	**1**	**1 min**
Denaturation	**95**	**35**	**15 sec**
Annealing	**54.2 (*GAPDH*),**		**10 sec (*GAPDH*),**
	58.0 (*CDKN2A*),		**15 sec (*CDKN2A*,**
	59.8 (*BCL2L11*),		***BCL2L11*, *PTEN*,**
	58.0 (*PTEN*),		***MDM2*, *TP53*)**
	57.5 (*MDM2*)		
	57.0 (*TP53*)		
Extension	**72**		**10 sec**

Table 2: The designed DNA primer sequences and their amplicon sizes of genes under analysis

Gene	Accession No.	Forward (5'-3')	Reverse (5'-3')	Produc Size
CDKN2A	NM_058195.3	GAGAACATGGTGCGCAGGTT	GCGCTGCCCATCATCATGA	219
BCL2L11	NM_138621.4	CAGCACCCATGAGTTGTGAC	CCTACACAAGAGAACCGCTG	401
PTEN	NM_000314.6	GCACAATATCCTTTTGAAGACC	AGTGCCACTGGTCTATAATCC	323
MDM2	NM_002392.5	GATTGGTTGGATCAGGATTCAG	CATCATTGTCCGCAACACATG	439
TP53	NM_001276760.1	GGAAGGAGACTTGCGTGTGG	GCTCTCGGAACATCTCGAAGCG	440
GAPDH	NM_002046.5	CGACCACTTTGTCAAGCTCA	AGGGGAGATTCAGTGTGGTG	203

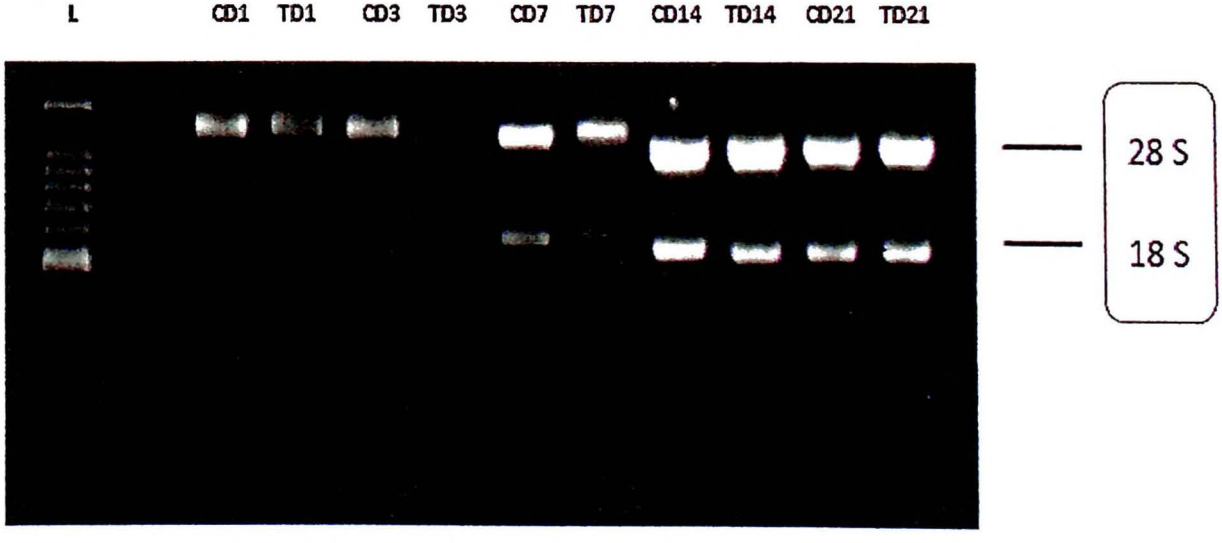

Figure 1: 28S and 18S RNA bands of control and PVF treated SHED groups on day 1, 3, 7, 14 and 21. Both these bands signifies that the RNAs extracted for the experiment is intact and viable for downstream analysis.

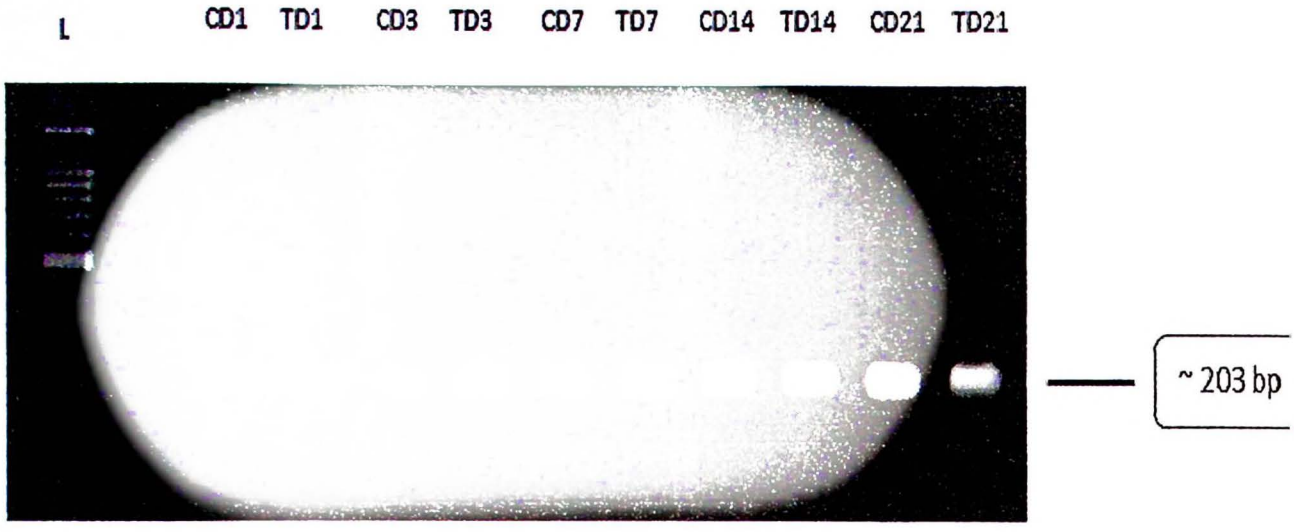

Figure 2: The expression of housekeeping gene, *GAPDH* between control and PVF treated SHED on day 1, 3, 7, 14 and 21.

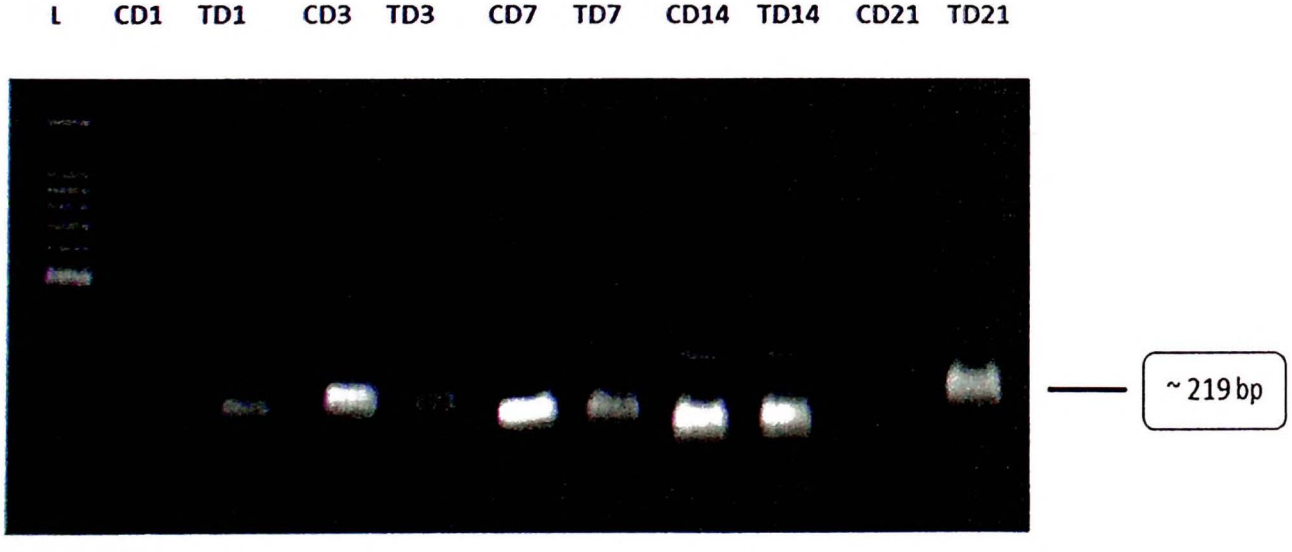

Figure 3: The expression of *CDKN2A* between control and PVF treated SHED on day 1, 3, 7, 14 and 21.

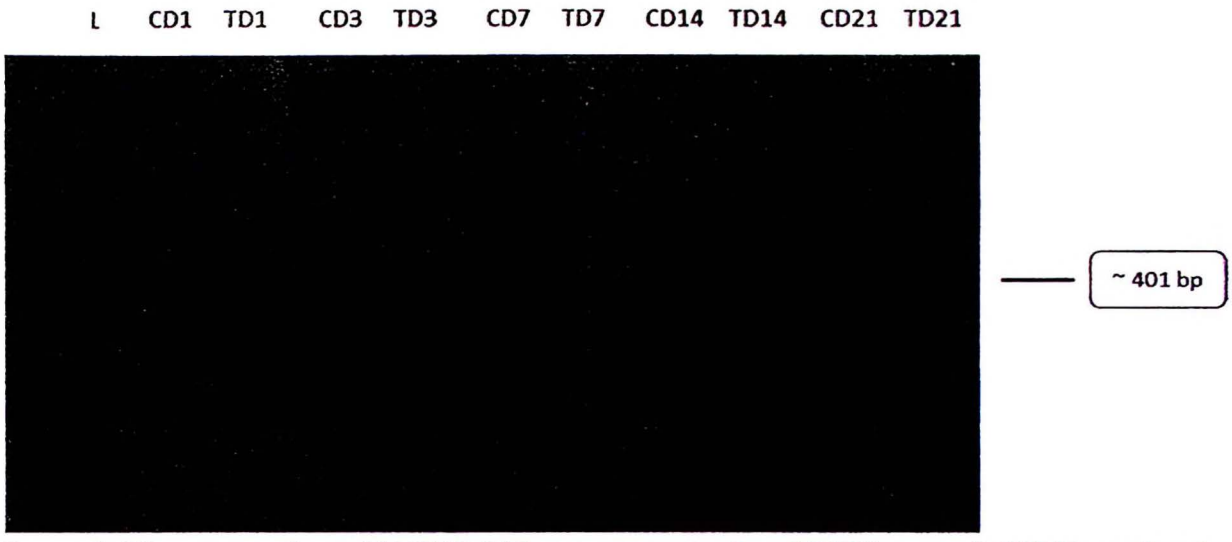

Figure 4: The expression of *BCL2L11* between control and PVF treated SHED on day 1, 3, 7, 14 and 21.

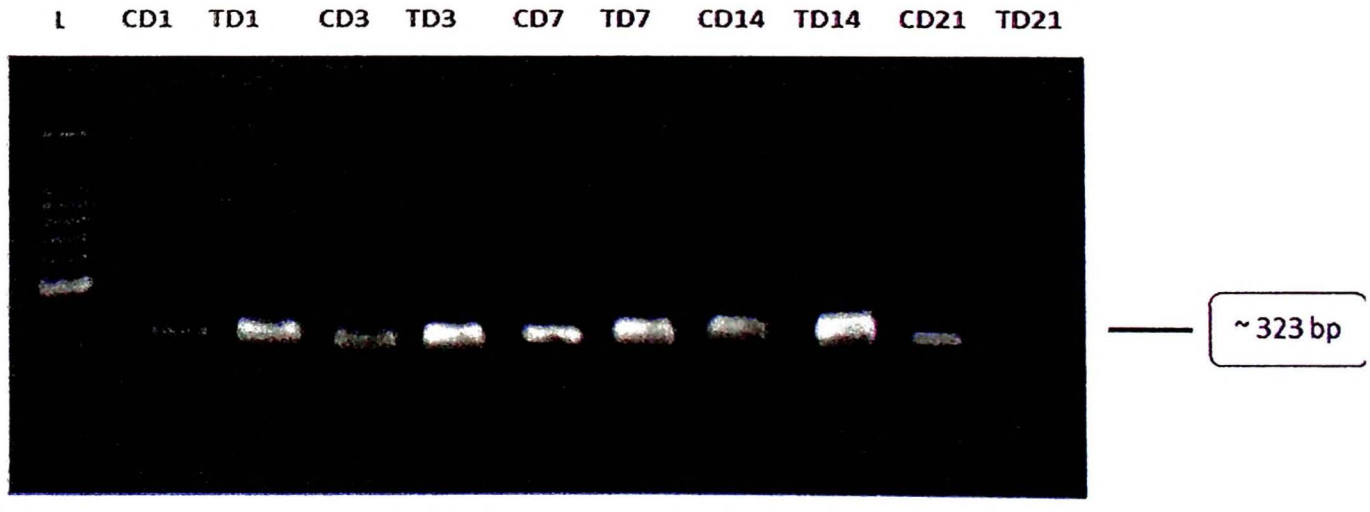

Figure 5: The expression of *PTEN* between control and PVF treated SHED on day 1, 3, 7, 14 and 21.

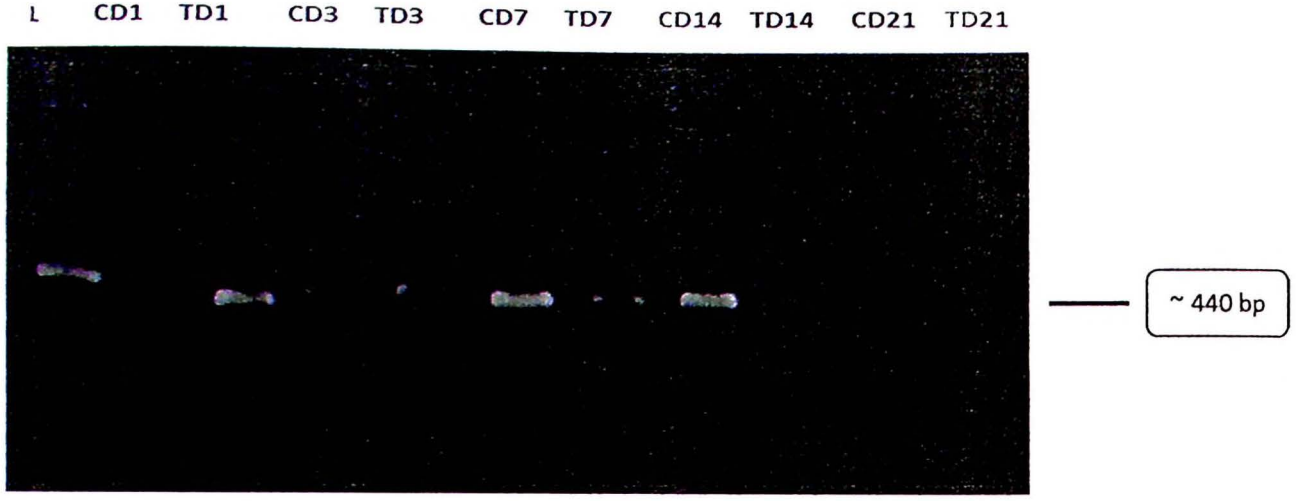

Figure 6: The expression of *TP53* between control and PVF treated SHED on day 1, 3, 7, 14 and 21.

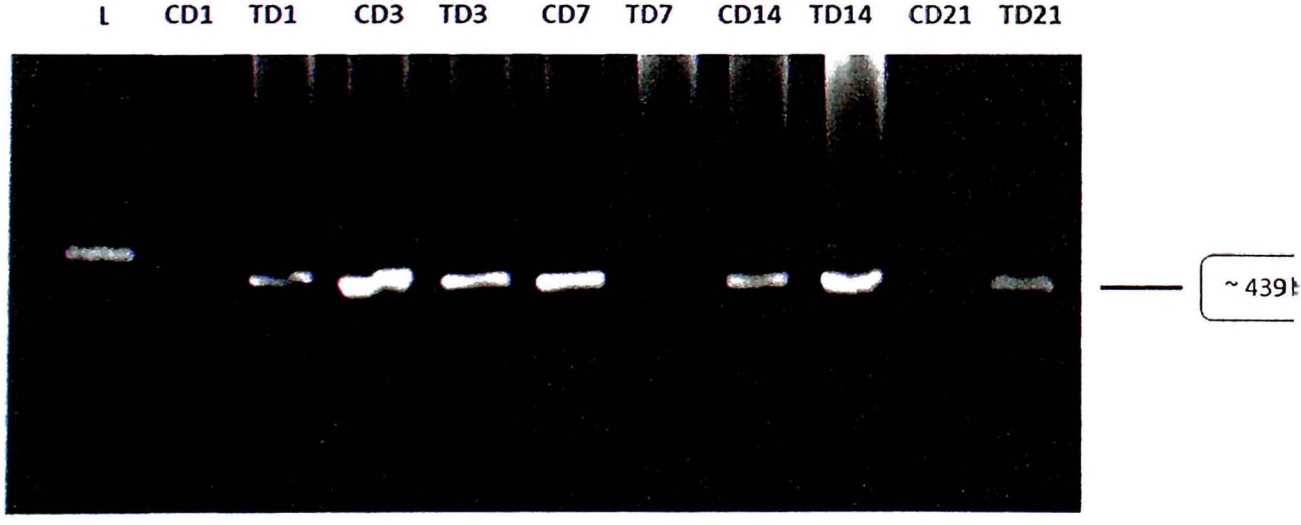

Figure 7: The expression of *MDM2* between control and PVF treated SHED on day 1, 3, 7, 14 and 21.

Table 3: The normalized ADV values of gene expressions between Control and Treated groups at day 1, 3, 7, 14 and 21. Asterisks sign shows the significance of expression between control and treated groups at p-value of $p \leq 0.05$.

ADV / Genes	Day 1			Day 3			Day 7			Day 14			Day 21		
	Con	Trt	p-value	Con	Trt	p-value	Con	Trt	p-value	Con	Trt	p-value	Con	Trt	p-value
CDKN2A	0.249345	0.607502	0.127	1.850009	0.315169	*0.050	2.190944	0.573587	*0.050	1.198459	1.888962	*0.050	0.126052	1.988341	*0.050
BCL2L11	0.512586	0.518572	0.827	0.631149	0.22442	*0.050	0.531562	0	*0.037	0.595326	0	*0.037	0.240928	0.989241	*0.046
TP53	0	0.558454	*0.037	0.459562	0.231912	*0.050	1.504731	0.340962	*0.046	0.642604	1.037007	*0.050	0	0.439084	*0.037
MDM2	0	0.552766	*0.037	2.221295	0.782414	*0.050	1.595363	0	*0.037	0.821188	2.04953	*0.050	0	0.420247	*0.037
PTEN	0.529433	1.046448	*0.050	1.345563	0.548422	*0.050	1.586935	0.671757	*0.050	0.589062	1.11622	*0.050	0.529277	0.772512	0.513

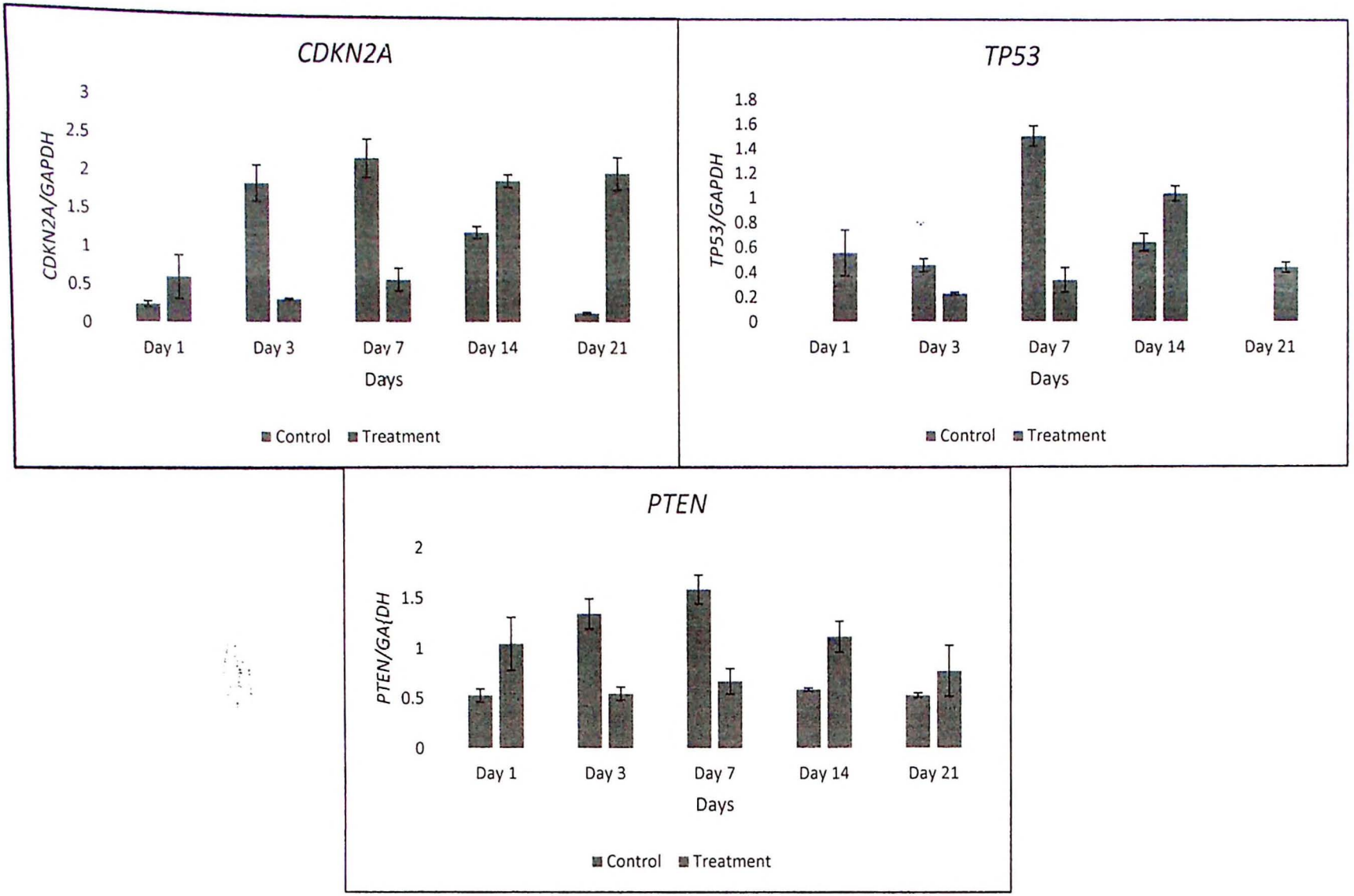

Figure 8: Normalized expression of *CDKN2A, TP53* and *PTEN* between control and treatment groups on day 1, 3, 7, 14 and 21.

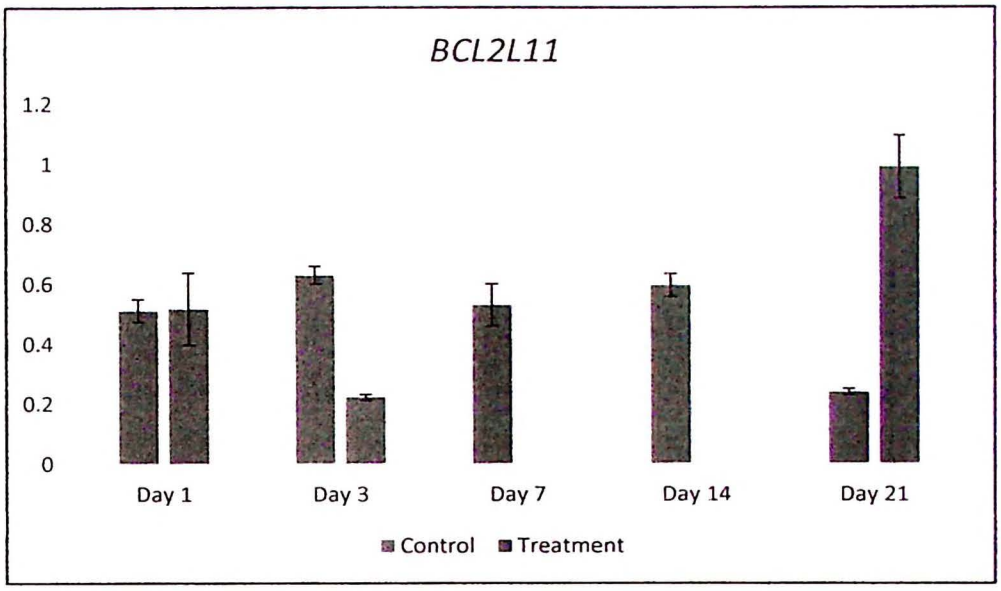

Figure 9: Normalized expression of *BCL2L11* between control and treatment groups on day 1, 3, 7, 14 and 21.

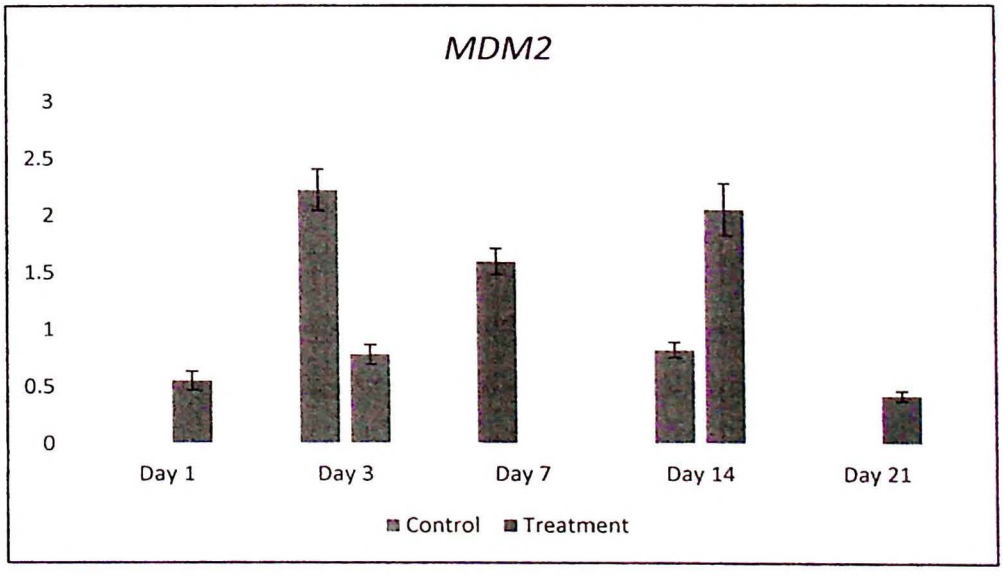

Figure 10: Normalized expression of *MDM2* between control and treatment groups on day 1, 3, 7, 14 and 21.

Discussion

The cell cycle and apoptosis regulator genes which are CDKN2A, BCL2L11, PTEN, TP53 and MDM2 genes were analysed and normalized to the housekeeping gene, *GADPH*. The gene expression was quantified as Average Density Value (ADV), as tabulated in Table 3. Expression of *GAPDH* was constant throughout 21 days for both the control SHED and SHED treated with PVF.

The passage of SHED in this experiment was kept constant which is passage 6. The expression of *CDKN2A, PTEN* and *TP53* in the PVF treated SHED appeared to be in a similar pattern (Figure 8). It was observed that these 3 genes started to gradually increase in expression from day 3 onwards to day 14, and then slightly decreased on day 21. On the contrary, the expression of *CDKN2A, PTEN* and *TP53* in SHED without the addition of PVF treatment seems to gradually decrease from time (Figure 8). Furthermore, Mann Whitney test has been conducted as well to determine the significance of the differential expression between both groups (Table 3). The differences that the PVF treatment acted on the expression of these three genes were significant starting from day 3 onwards till day 21. Hence, it is hypothesized that *CDKN2A, PTEN* and *TP53* which play crucial roles in regulating cell cycle has been affected in terms of its expression when PVF treatment was introduced into the culture media. Higher expression of these genes in PVF treatment shows that the cell growth and regulation of SHED are significantly enhanced. It also signifies that the dosage of PVF (0.019 mg/ml) is significant enough to give a difference in enhancing the cell cycle in SHED.

On the other hand, *MDM2* is observed to express relatively lower in the PVF treatment group than in the control. As shown in Figure 9, *MDM2* is highly expressed in the control group at day 3 and 7 (Figure 10). Both groups showed low expression of *MDM2* at the end of the

experiment (Figure 10). It is important that *MDM2* expression to be kept at low levels as overexpression of it could result to tumour growth as it inhibits DNA double-strand break repair mediated through a novel, direct interaction between Mdm2 and Nbs1 and independent of p53.

BCL2L11 is a member of BCL-2 protein family. Protein encoded by this gene contains a Bcl-2 homology domain 3 (BH3) which has been shown to interact with other members of the BCL-2 protein family and to act as an apoptotic activator. The current study shows that *BCL2L11* expression was rather lower or absent completely in the PVF treated group compared to the control from day 1 until day 14. This suggest that SHED in PVF treatment are actively growing with very minute induction of apoptosis. However, at day 21, expression of *BCL2L11* drastically spiked in the treatment group (Figure 9) while the expression in the control remained low which indicates that overcrowding of cells in the confined culture flask treatment group at day 21 induces apoptosis/cell death as suggested by (Eisenhoffer et al., 2012).

Live/Dead Cell Viability Test

Materials and Methods

Part 1: Fluorescence Microscopy

Cell culture

SHED from AllCells (USA, cat no. DP004F) were cultured in mesenchymal stem cell (MSC) basal medium (AllCells, cat no. MSC-002) supplemented with fetal bovine serum (10%) and incubated at 37°C in a 5% CO2 humidified incubator until confluent. The SHED were revived from the cryopreservation and sub-cultured twice before seeding for PVF treatment after the 6th passage of the cells. A negative control group which is SHED without PVF was also

included in this study. Both groups of SHED (treated and control) were cultured on sterile glass coverslips as sub confluent monolayers for 3 days. They were cultured in 6 wells plates. After 1, 2 and 3 days, the cells were washed with PBS twice to remove or dilute serum esterase activity in the media.

Performing the Viability Assay

100 µl of the combined LIVE/DEAD assay reagents were added on the coverslips containing the cells. The cells were incubated for 30-45 minutes at room temperature in a covered dish. Following the incubation, 10 µl of the fresh LIVE/DEAD reagent solution were added to a clean microscope slide. Using fine-tipped forceps, the wet coverslips were mounted quickly on the microscope slide. The coverslips were sealed with a clear nail polish to avoid evaporation. The labelled cells were viewed under the fluorescence microscope immediately with filters for FITC (blue emission) and propidium iodide (red emission).

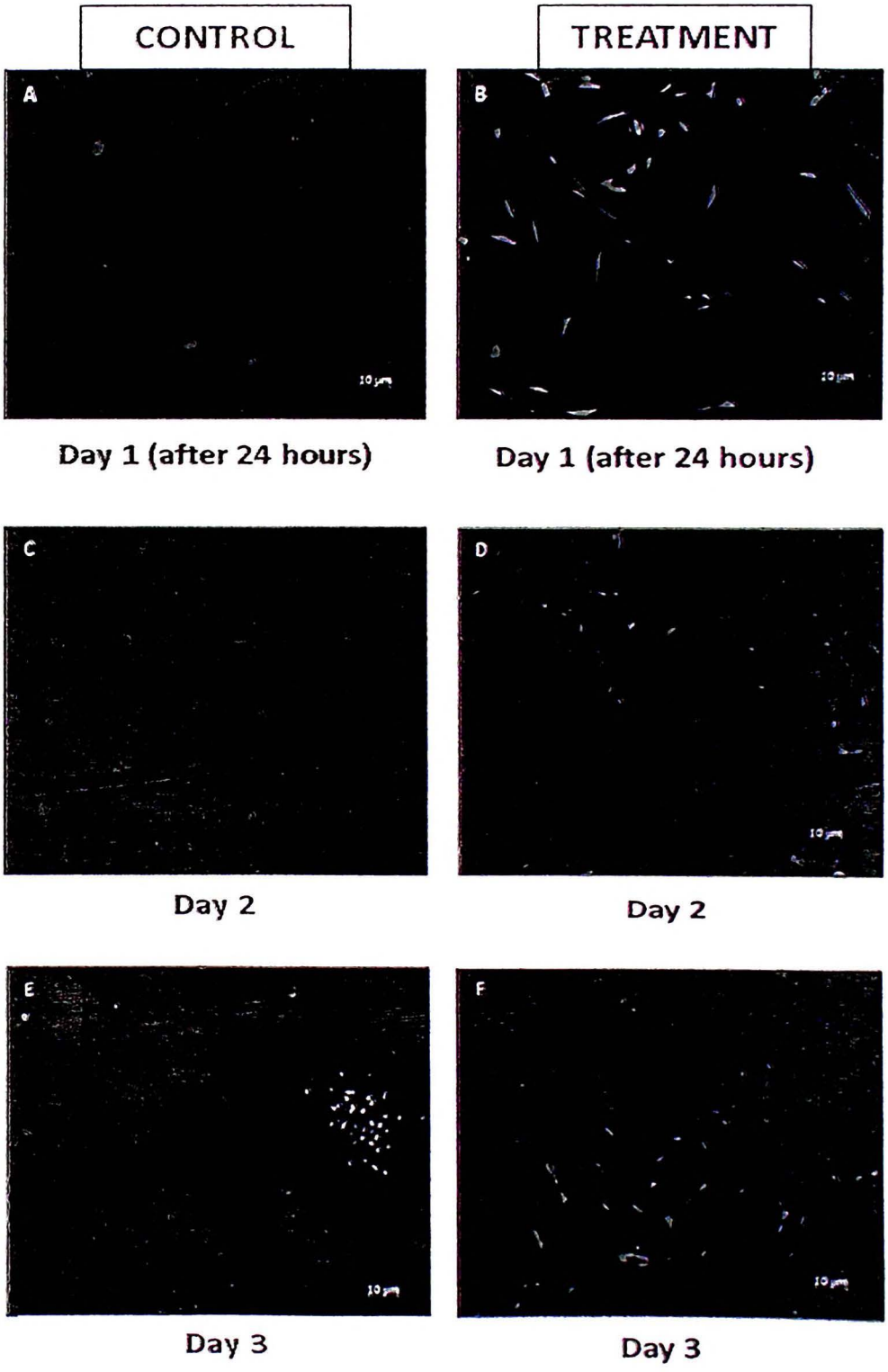

Figure 11: Fluorescence microscopy of SHED treated with (left) and without Perivitelline Fluid (PVF) treatment (right) at day 1, 2 and 3. Live and dead cells were labelled by the green and red-orange fluorescence respectively.

Discussion

This experiment shows the effects of PVF treatment onto the viability of SHED. The number of cells seeded onto the glass slides at the beginning of the experiment is kept constant for both groups which is 6000. As shown in Figure 11, the viability of cells between both groups are not significantly different at day 1 and 2. However, on day 3, a cluster of dead cells could be seen in the control group. This could be due to the overcrowding of cells. However, in the treatment group, SHED appeared to be growing well and viable with minimum number of dead cells.

Part 2: Fluorescence Microplate Reader

Cell culture

SHED from AllCells (USA, cat no. DP004F) were cultured in mesenchymal stem cell (MSC) basal medium (AllCells, cat no. MSC-002) supplemented with fetal bovine serum (10%) and incubated at 37°C in a 5% CO2 humidified incubator until confluent. The SHED were revived from the cryopreservation and sub-cultured twice before seeding for PVF treatment after the 6^{th} passage of the cells. A negative control group which is SHED without PVF was also included in this study. Both groups of SHED (treated and control) were cultured in 96 well plates for 3 days. 6 replicates were prepared for each group. The cells were analysed at day 1, 2 and 3 of incubation.

Performing the Viability Assay

At each day of test, the cells were washed with PBS twice to remove or dilute serum esterase activity in the media. At the last wash, PBS was added to cover the bottom of the wells. The cells were then treated with 200 µl LIVE/DEAD cytotoxic agents.

The cells were incubated at room temperature for 45 minutes. Then, the fluorescence was measured at 645 nm and 530 nm as follows:

A. Fluorescence at 645 nm in the experimental cell sample, labelled with calcein AM and EthD-1 = F(645)sam
B. Fluorescence at 530 nm in the experimental cell sample, labelled with calcein AM and EthD-1 = F(530)sam
C. Fluorescence at 645 nm in a sample where all the cells are dead, labeled with EthD-1 only = F(645)max
D. Fluorescence at 645 nm in a sample where all the cells are dead, labeled with calcein AM only = F(645)min
E. Fluorescence at 530 nm in a sample where all (or nearly all) cells are alive, labeled with EthD-1 only = F(530)min
F. Fluorescence at 530 nm in a sample where all (or nearly all) cells are alive, labeled with calcein AM only = F(530)max
G. Fluorescence at 530 nm of the cell-free sample with or without dye added = F(530)0
H. Fluorescence at 645 nm of a cell-free sample with or without dye added = F(645)0

Dead cells are characterized by intense fluorescence at >600 nm and little fluorescence around 530 nm. The percentage of live cells can be calculated from the fluorescence readings defined above as:

$$\% \text{ Live Cells} = \frac{F(530)sam - F(530)min}{F(530)max - F(530)min} \text{ X } 100\%$$

The percentage of dead cells can be calculated from the fluorescence readings defined above as:

$$\% \text{ Dead Cells} = \frac{F(645)sam - F(645)min}{F(645)max - F(645)min} \text{ X } 100\%$$

Table 4: Fluorescence readings at 645 nm and 530 nm in control and treated groups at 1, 2 and 3 days of incubation. The annotation of A, B, C, D, E, F, G and H can be referred to the materials and methods section above.

Groups / Readings	Control Day 1	Treatment Day 1	Control Day 2	Treatment Day 2	Control Day 3	Treatment Day 3
A	0.310	0.215	0.377	0.274	0.441	0.392
B	0.765	0.877	0.899	0.903	0.509	0.840
C	0.473	0.364	0.541	0.465	0.464	0.563
D	0.026	0.011	0.045	0.034	0.046	0.034
E	0.298	0.232	0.400	0.403	0.239	0.038
F	0.700	0.687	0.912	0.721	0.621	0.798
G	0	0	0	0	0	0
H	0	0	0	0	0	0

Table 5: Calculated percentage (%) of live and dead cells in control and treated groups for 3 days. Values from Table 4 were incorporated into the given formula to obtain the percentage (%) of live and dead cells.

Groups / %	Day 1		Day 2		Day 3	
	Control	Treatment	Control	Treatment	Control	Treatment
Live	116.17	141.75	97.46	157.23	70.68	105.53
Dead	63.53	57.79	66.94	55.68	94.50	67.67

Discussion

The percentage of live cells in control group descended from day 1 to day 3, while the dead cells increased gradually from day 1 to 3. In contrast, the percentage of live cells in SHED with PVF treatment remained higher than 100% until day 3, with a lower percentage of dead cells compared to the control. It can be hypothesized that the PVF treatment of the 0.019 mg/ml dosage has allow enhancement of SHED viability. To add, this data also agrees to the live and dead fluorescence microscopy images of SHED above, showing higher viability of cells in the presence of PVF.

Conclusion

Based on the studies that have been carried out, a few conclusions can be made. We have found that the IC_{50} and IC_{25} concentration of PVF are 26.887 and 14.093 mg/ml respectively using MTT assay. Alamar Blue assay conducted on SHED with selected concentrations of PVF produced more than 90% in cell proliferation higher than control. It is also noted that reduction in the concentrations of PVF has resulted in enhancement of cell proliferation. Therefore, we selected a single concentration of PVF (0.019 mg/ml) from MMT assay for downstream analyses as it gave 102.50% towards SHED cell viability. PVF concentration of 0.019 mg/ml

resulted in the upregulation of *DMP-1, DSPP, RUNX2* and *OPN* genes SHED thus suggested that PVF (0.019mg/mL) possesses the potential in enhancing odontogenesis process that could lead to higher rate of dentin mineralization. It is also found that the difference in odontogenic markers expression in SHED is significant when PVF treatment was given. Hence, this could give us a clue that PVF may be effective in inducing odontogenesis at a higher level. However, further studies on more odontogenic specific genes with a longer time frame are necessary to elucidate the efficiency of PVF to be used as a supplement for dental pulp generation in SHED. Besides that, enhanced cell cycle and growth in SHED was also reported as indicated by the significant increase in expression of *CDKN2A, PTEN* and *TP53* genes. We also conclude that PVF treatment did not cause any tumorigenicity as supported by the *MDM2* expression that remained low and controlled all through 21 days of incubation. PVF treatment also showed higher cell viability towards SHED as shown by fluorescence microscopy and microplate reader. As a conclusion, PVF from horseshoe crab is a great material that has huge prospect as a supplement for stem cell culture. Therefore, continuous exploration of PVF in different areas and aspects are deemed necessary in the future.

Acknowledgements

We would like to acknowledge the staff of the Craniofacial Sciences Laboratory, School of Dental Sciences, Universiti Sains Malaysia (USM), Malaysia and the research group at the Institute of Tropical Aquaculture (Aquatrop), Universiti Malaysia Terengganu (UMT), Malaysia for their technical support. This work was financially supported by Universiti Sains Malaysia Research University grant (1001/PPSG/813077).

Appendix 1

Gantt Chart of Research Activities

Activity	2012-2013				2013-2014				2014-2015			
	Dec-Feb	March-May	Jun-Aug	Sept-Nov	Dec-Feb	March-May	Jun-Aug	Sept-Nov	Dec-Feb	March-May	Jun-Aug	Sept-Nov
Isolation and purification of PVF	100% completed →											
Sub-culture and maintaining of SHED Cell-line		100% completed →										
Cell proliferation assay (MTT assay) to find the ED_{50} of cells in different concentrations of PVF			100% completed →									
Morphological characteristics analysis of cultured cells in PVF treated and untreated condition					60% completed →							
Chromosomal aberration analysis of cultured cells in PVF treated and untreated condition					100% completed →							
Gene expression analysis of genes involved in cell-cycle regulation in cultured cells of PVF treated and untreated condition between different passages							100% completed →					

Gene expression analysis of odontogenic genes in treated and control SHED											100 % completed →

Gantt Chart of Research Activities During 6 Months Extension Period

Project Activities	2016					
	Jan	Feb	March	April	May	June
Cell Culture and PVF Treatment	100% completed →					
Optimization of primers	100% completed →					
Gene expression analysis			100 % completed →			
Live/Dead Confocal Cell Imaging					100% completed →	
Submission of papers for publication and conferences					100% completed →	

OTHER SUPPORTING DOCUMENTS

Rujukan :
Tarikh KK/PTJPK/PPEND/PRO 1383
Kepada 04 286
11 Januari 2015
: Bendahari

PEJABAT PENDAFTAR
KAMPUS KESIHATAN
UNIVERSITI SAINS MALAYSIA
KENYATAAN GAJI STAF SAMBILAN PROJEK/GERAN (SPG)
[Penamatan Perkhidmatan]

MAKLUMAT PERIBADI

1. Nama :
2. No. Kad Pengenalan :
3. Tarikh Lahir :
4. Taraf Perkahwinan :
5. Jabatan Bertugas : Ketua Projek : PUSAT PENGAJIAN SAINS PERGIGIAN
   Staf Projek : PUSAT PENGAJIAN SAINS PERGIGIAN

MAKLUMAT GAJI

1. Jawatan & Gred Gaji : Pembantu Penyelidik - SPM (N17)
2. Tajuk Projek/Geran : Effects Of Peri-Vitelline Fluid (PVF) Obtained From Fertilized Eggs Of Horseshoe Crab On Cultured Human Dental Pulp Stem Cells
3. No. Akaun Projek/Geran : 1001/PPSG/813077
4. Jenis Projek/Geran : RUI (Individual)
5. Gaji : RM 820.38 Sebulan
6. Jenis Gaji : Bulanan
7. No. Ahli KWSP :
8. Kadar Caruman KWSP : 11%
9. Tarikh Mula Projek/Geran : 15 Disember 2012
10. Tarikh Tamat Projek/Geran : 14 Disember 2015
11. **Baki geran** :
12. **Tarikh Kuatkuasa Lantikan** : **31 Disember 2014**
13. **Tarikh Tamat Lantikan** : **31 Disember 2014**
14. Nama Bank : Bank Islam Malaysia Berhad
15. No. Akaun Bank : 03102020042317

IMBUHAN TETAP	AMAUN	BAYARAN BANTUAN	AMAUN
1. Khidmat Awam	RM 115 Sebulan	1. Sara Hidup	RM 250 Sebulan

CATATAN

Penamatan perkhidmatan sebagai Pembantu Penyelidik gred N17 berkuatkuasa mulai hingga 31 Disember 2014.

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1. Pembayaran Emolumen ini adalah tertakluk kepada status akaun/ kewangan terkini projek.
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   b) **Pelantikan kurang 3 bulan** - **Hospital Universiti Sains Malaysia (HUSM) sahaja**

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4. Syarat-syarat lain adalah seperti yang termaktub dalam surat tawaran dan Lampiran A.
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PERAKUAN

Segala maklumat di atas telah diperakukan benar, disemak dan disahkan oleh **PROFESOR MADYA DR. T. P. KANNAN** Ketua *Projek/Geran ini pada* **11 Januari 2015.** *Ketua Projek/Geran boleh turut disabitkan di bawah Akta 605 sekiranya terdapat sebarang* maklumat yang kurang/tidak tepat.
Tarikh perakuan penerimaan oleh **WAN NURAINI BINTI WAN AZIZ** pada **11 Januari 2015.**

(AZHARUDDIN ABDUL AZIZ)
Penolong Pendaftar
b/p Pendaftar

s.k. PROFESOR MADYA DR. T. P. KANNAN
Ketua Projek
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SEMESTER ENROLLED	:	2011/12-1
TOTAL SEMESTER	:	11
PROGRAMME STRUCTURE	:	RESEARCH
PROGRAMME NAME	:	DOCTOR OF PHILOSOPHY
FIELD OF STUDY	:	CELL AND MOLECULAR BIOLOGY
FACULTY / INSTITUTE	:	AKUATROP
MAIN SUPERVISOR	:	PROFESOR EMERITUS DR. FAIZAH BINTI MOHD SHAROUM
CO SUPERVISOR	:	1. PROFESOR DR MOHD. EFFENDY BIN ABD.WAHID 2. PROF. MADYA DR. THIRUMULU PONNURAJ KANNAN 3. PROF. DR. AHMAD SUKARI HALIM 4. PROF. MADYA DR. KHAIRANI IDAH MOKHTAR

CODE	COURSE TITLE	CREDIT
SCM6999	PH.D. THESIS	12

Total Credit : 12

ENDORSEMENT BY POSTGRADUATE MANAGEMENT CENTRE

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DATE : 28-MAR-2017

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Tarikh : 22/2/2016

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Mod Pengajian	:	Penyelidikan
Bidang Penyelidikan	:	Stemcell
Tajuk Penyelidikan	:	Study Of The Effect Of Perivitteline Fluid From Horseshoe Crab On The Expression Of Cell Cycle Regulatory Genes And Anti/pro Apoptotic Genes As Well As Cells' Viability Of Human Dental Pulp Stem Cells
Status Pencalonan	:	Penuh Masa
Tempoh Pencalonan	:	Min : 12 bulan / 2 semester Mak : 36 bulan / 6 semester
Tarikh Luput Tawaran	:	22/2/2017
Syarat Tawaran	:	
Penyelia Utama (PU)	:	PROFESOR MADYA DR. T. P. KANNAN
Penyelia Bersama (PB)	:	
	:	
Tempat Pendaftaran	:	Institut Pengajian Siswazah, Bangunan PPSP Kampus Kesihatan, Universiti Sains Malaysia Kubang Kerian, Kelantan

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Tarikh : 16 Mac 2016

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Tarikh Kuatkuasa Pencalonan	: 1 April 2016
Tarikh Maksimum Pencalonan	: 31 Mac 2019
Jenis Pencalonan	: Penuh Masa
Bidang/ Penkhususan	: Stemcell

*Anda diwajibkan untuk mendaftar di Pejabat Am Pusat Pengajian. Anda tidak dibenarkan untuk meneruskan pengajian anda di Pusat Pengajian sehingga anda berdaftar.

Sila maklumkan kepada kami dengan kadar segera sekiranya berlaku pertukaran alamat surat-menyurat untuk mengelakkan sebarang kesulitan. Puan juga dipohon untuk menyatakan nombor pendaftaran di dalam semua urusan surat-menyurat dengan pihak kami.

Sekian.

(SITI SRI MAS BINTI ISMAIL)
Penolong Pendaftar
E-mel : srimas@usm.my

s.k Dekan, Pusat Pengajian Sains Pergigian
(PU) Profesor Madya Dr. T.P.Kannan