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Effect of horseshoe crab perivitelline fluid on the viability of stem cells from human exfoliated deciduous teeth and expression of cell cycle regulatory genes

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The effect of perivitelline fluid (PVF) (0.019 mg/ml) on the stem cell viability extracted from human exfoliated deciduous teeth was assessed using LIVE/DEAD viability/cytotoxicity. It showed that PVF enhanced viability as observed by higher live cell percentage and a greater number of viable cells in the treated group for 3 days. The expression of selected cell cycle regulatory genes investigated using reverse transcriptase polymerase chain reaction (RT-PCR) for 21 days showed that *CDKN2A, PTEN* and *TP53* expressed significantly higher in the treated group, suggesting that PVF enhanced SHED growth and proliferation. *MDM2* expression remained at low levels in the treated group indicating that PVF did not result in tumorigenic growth. Low expression of apoptotic activator gene, *BCL2L11* in treated group from day 1 until 14 with a sudden peak on day 21 was noted. This is deemed normal as the cells thrive to maintain homeostatic level at the 21st day of incubation.

[**Keywords**: Cell proliferation, Gene expression, Horseshoe crab, Perivitelline fluid, Stem cells from human exfoliated deciduous teeth]

Introduction

Stem cells from human exfoliated deciduous teeth (SHED) possess a high proliferative ability[1]. SHED are also one of the highly clonogenic cells that can be differentiated into many types of cells *viz.* adipocytes, neural cells and odontoblasts[2]. Several studies have also ascertained the ability of SHED to encourage bone formation[3], regenerate dentin[4], and survive in mouse brain which even expresses neural markers[5]. SHED could be one of the pioneering candidates in the advancement of tissue engineering world not only because of their multipotent ability, but also due to their great alternative against the invasive procedure involved in the mesenchymal stem cells (MSCs) derived from bone marrow. Thus, exfoliated deciduous tooth has a great potential as resources for stem-cell therapies and tissue engineering. The present study aims to investigate the cell viability as well as the cell cycle regulatory genes' expression of SHED due to the effect of perivitelline fluid (PVF).

Horseshoe crabs are terrestrial organisms that have inhabited the world for more than 200 million years. They are versatile creatures as they have a much higher tolerance living in a broad range of temperatures and salinities compared to other marine organisms[6]. The predominant species of horseshoe crabs are four in number. The one found in North America is *Limulus polyphemus* while *Tachypleus gigas*, *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda* are found in Southeast Asia[7]. In the past, humankind has discovered valuable biological resources obtained from the horseshoe crabs. One of it has been used in drugs and foods as a tester for endotoxin which is the well-known Limulus Amoebocyte Lysate (LAL)[8].

PVF is derived from the fertilized egg of a horseshoe crab. The fluid that occupies the space in between the embryo and the outer envelope of the horseshoe crab is the PVF. PVF comprises numerous types of primitive proteins which are important and are capable of supplementing the growth as well the cell proliferation[9]. Numerous studies have explored the usage of PVF since 1970's. Sugita & Sekiguchi[10] reported that PVF contained hemagglutinins and haemocyanins, proteins that play a crucial role in the process of embryogenesis. PVF is also suggested to help with the vertebrate's embryogenesis as it influences the embryogenesis of chick at early

stages[11]. PVF also positively stimulates the development as well as organ differentiation like the brain and heart. Later, it was found that PVF contained lectin promotes the formation of cardiac myocytes in the embryos of the chicks[9] which led to further the use of PVF in cell differentiation and organ regeneration. Stem cells were tested with the crude extract of PVF in order to observe the proliferation of cells as well as genotoxicity[12] and it was concluded that PVF had no genotoxic effect.

The PVF obtained from *Tachypleus gigas* (Malaysian horseshoe crabs) were treated on SHED to evaluate the cell cycle regulatory gene expression using reverse transcriptase polymerase chain reaction (RT-PCR). Cyclin-dependent kinase inhibitor 2A (*CDKN2A*), Bcl-2-like protein 11 (*BCL2L11*), phosphatase and tensin homolog (*PTEN*), mouse double minute 2 homolog (*MDM2*), and tumour protein p53 (*TP53*) were the genes selected for this purpose. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) was used to normalize the expression of genes considered in this study. Besides, comparison of live cell percentage and cell morphology of SHED with and without PVF treatment was made using the Live/Dead viability/cytotoxicity kit intended for mammalian cells. The concentration of PVF extract used in this study was 0.019 mg/ml based on a previous study[12].

Materials and Methods

Cell line and culture

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

Perivitelline fluid

The horseshoe crab eggs (fertilized) were collected from a beach in Kuantan, Malaysia. The horseshoe crab eggs were brought to University Malaysia Terengganu (UMT) and were processed in the Aquatrop Laboratory and the eggs were incubated at 29±1 °C in artificial incubator. Once the eggs became transparent and the movement of trilobite larvae was seen, they were further processed according to the method described[13]. After collection of PVF, the horseshoe crab eggs were released back into the sea. The PVF which was freeze dried was stored at a temperature of -70 °C. A cell viability study was conducted by Musa and colleagues on dental stem cells (AllCells USA, cat no. DP004F) using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay[12]. They tested PVF at different concentrations by incubating the cells for a period of 72 hours and calculated the percentages of relative cell viability in comparison with control wells (cell culture medium without extracts). Of all the concentrations tested, they found that the concentrations of 0.019 and 0.278 mg/ml of PVF produced the highest cell viability of 102.5 and 102 %, respectively. Hence, 0.019 mg/ml concentration was selected to be used in this current study. In order to prepare the PVF extract, 1 ml of phosphate buffered saline (PBS) from Invitrogen, (UK) was added to the test sample and mixed which was further diluted to 0.019 mg/ml using the culture medium. A 0.2 µm syringe filter (Sartorius, UK) was used to sterilize the PVF extract. Fresh extract was prepared each time for the experiment.

Gene expression analyses protocol

PVF treatment

The SHED were revived from the cryopreservation and sub-cultured twice before seeding for PVF treatment (0.019 mg/ml) at passage 6. The negative control group comprised SHED without any 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

SHED were trypsinized from the flasks and prepared as a single suspension of cells. The cells were rinsed in PBS and a commercial kit for RNA extraction for mammalian cells, RN easy Mini Kit from QIAGEN (Germany, cat no. 74104) was used for extracting the total RNA. SHED were resuspended in alysis buffer and then homogenized using a QIA shredder column from QIAGEN (Germany, cat no. 79654). The lysate was applied to RN easy column and then rinsed repeatedly using a series of buffers. This was followed by elution of the lysate 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.

Synthesis of complementary DNA and reverse transcriptase polymerase chain reaction (RT-PCR)

The 1st-strand complementary DNA (cDNA) from the total RNA was synthesized using the MMLV

Reverse Transcriptase 1st-Strand cDNA Synthesis Kit from Epicentre (USA, cat no. MM070150). Next, RT-PCR was conducted as per the protocol using MyTaq™ HS Mix from Bioline (USA, cat no. BIO-25045). For each experiment, 1 µl (3 µg) of total RNA was used to run the RT-PCR in a 50 µl of final reaction volume. The gene primers that were designed and used in the current research are listed in Table 1. The thermal cycling parameters are as follows: 95 °C for 1 min for the activation of polymerase, 35 cycles of 95 °C for 15 sec, 54.2 °C for 10 sec (*GADPH*); 58.0 °C for 15 sec (*CDKN2A)*; 59.8 °C for 15 sec (*BCL2L11*); 58.0 °C for 15 sec (*PTEN*); 57.5 °C for 15 sec (*MDM2*); 57.0 °C for 15 sec (*TP53*) and 72 °C for 10 sec. The cDNA synthesis and RT-PCR were performed in a Mastercycler nexus flat Thermal Cycler (Eppendorf, USA). SYBR Green (1 µl) was used to stain the PCR products separated on 1.0 % agarose gel and was visualized on a UV transilluminator (Biorad, USA). The images were photographed in an image analyser (Quantity One, USA). All the experiments were performed thrice and their band intensities were measured as Average Density Value (ADV) with Quantity One 1-D Image Analysis software (Bio-Rad, USA) which was normalized to the ADV values of glyceraldehyde-3-phosphate dehydrogenase (*GADPH*). Mann Whitney statistical test was done to find out the significance of expression of genes between treated and control groups using SPSS software version 22.0.

Live/Dead cell viability assay

Fluorescence microscopy protocol

SHED were cultured as described previously. Both the groups of SHED (treated and control) were cultured as sub confluent monolayers for 3 days on glass cover slips (sterile) by placing in 6 well-plates. After 1, 2 and 3 days, PBS was used to wash the cells twice in order to eliminate serum esterase activity in the media.

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 cover slips containing the cells. The cells were then incubated for a period of 45 minutes in a covered dish at room temperature. Following the incubation, 10 µl of the LIVE/DEAD reagent dyes solution was then incorporated to a clean microscope slide. With the help of a fine-tipped forceps, the wet cover slips were then mounted quickly on the slide. This was followed by sealing the cover slips with a clear nail polish to avoid evaporation. The cells that were labelled were viewed under the fluorescence microscope immediately with fluoresce in isothiocyanate (FITC) (green emission) and propidium iodide (red emission) filters. The glass slides containing the cells were viewed thoroughly at all areas before the image was captured. The images of the cells were captured using Axio Vision 4.8 software. Live cells were stained by membrane permeant and non-fluorescent calcein AM dye until the ubiquitous intracellular esterase eliminate the ester groups rendering the molecule fluorescent which then generates the green fluorescence. For the Excitation (max) 494 nm and Emission (max) 515 nm wavelengths, 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) 528 nm and Emission (max) 617 nm emitted 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 when the test was conducted, the cells were washed using PBS twice in order 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

Table 1 — Primer sequences for the different genes and their amplicon sizes

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

200 µl LIVE/DEAD cytotoxic agents. Incubation of the cells was done for 45 min at room temperature. The fluorescence reading of cells were taken using SkanIt software 2.4.3 for Varioskan Flash. Then, the measurement of fluorescence was done using fluorescence micro plate reader at 645 nm and 530 nm as per the formula given by the manufacturer, Invitrogen, (USA).

Dead cells are characterized by intense fluorescence at > 600 nm and little fluorescence around 530 nm. The live cell percentage was calculated based on the fluorescence readings as given below:

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

The dead cell percentage was calculated based on the fluorescence readings as given below:

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

Results

Gene expression analyses

The gene expression of *CDKN2A*, *PTEN*, *TP53* and *MDM2* (cell cycle regulatory genes) and *BCL2L11* (apoptosis regulator gene) were analysed for a period of 21 days at day 1, 3, 7, 14 and 21. The expression of these genes was normalized to *GADPH*. The ADV was recorded in triplicates for each gene expressed in PVF treated and untreated SHED for 21 days (Table 2). The housekeeping gene, *GAPDH* showed constant expression throughout for 21 days for both the SHED treated with PVF and the control SHED (Fig. 1a).

The expression of *CDKN2A*, *PTEN* and *TP53* in the PVF treated SHED appeared to be in a similar pattern (Figs. 1b, d, f; and 2a – c). It was observed that these 3 genes started to gradually increase in expression from day 3 onwards to day 14, and 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 (Figs. 2a – c). The significance in the expression of the genes between both the groups of SHED were analysed by Mann Whitney test (Table 2). The expression of these genes showed significant difference ($p \leq 0.05$) beginning from day 3 until day 21 in the PVF treated SHED in comparison to the control. On the other hand, *MDM2* showed relatively lower expression in the PVF treated group than in the control group (Fig. 1e). *MDM2* was highly expressed in the control at day 3 and 7 (Fig. 2d). The expression of *MDM2* however increased drastically in PVF treated group at day 14 but then dropped significantly

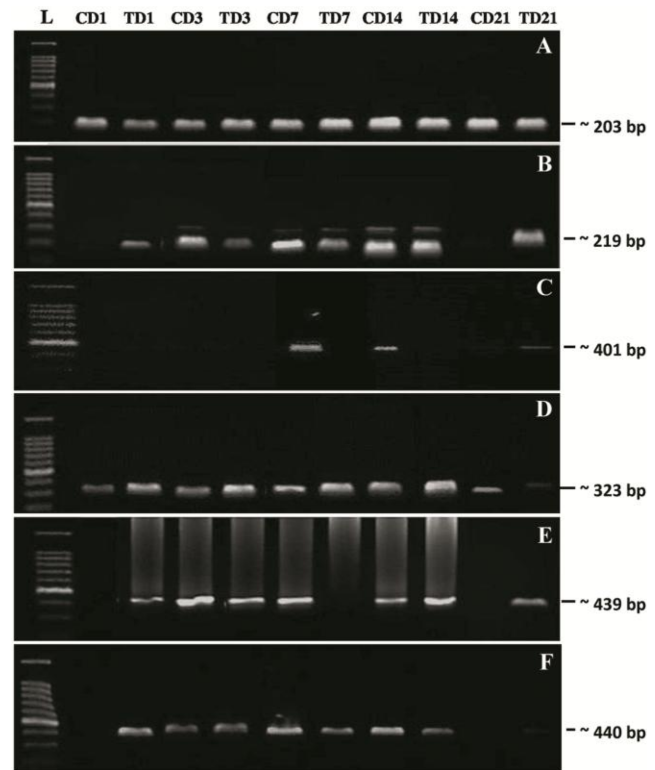

Fig. 1 — Expression of cell cycle regulatory genes in the control and perivitelline fluid treated SHED on day 1, 3, 7, 14 and 21 (A: *GAPDH*, B:*CDKN2A*, C: *BCL2L11*, D: *PTEN*, E: *MDM2*, and F: *TP53*). CD: Control SHED; and TD: PVF treated SHED

Table 2 — Normalized average density values (ADV) of gene expressions between control and perivitelline fluid treated SHED on day 1, 3, 7, 14 and 21

Genes	ADV Day 1			ADV Day 3			ADV Day 7			ADV Day 14			ADV Day 21		
	Control	Treated	*p*-value	Control	Treated	*p*-value	Control	Treated	*p*-value	Control	Treated	*p*-value	Control	Treated	*p*-value
CDKN2A	0.249	0.608	0.127	1.850	0.315	*0.050	2.191	0.574	*0.050	1.198	1.889	*0.050	0.126	1.988	*0.050
BCL2L11	0.513	0.519	0.827	0.631	0.224	*0.050	0.532	0	*0.037	0.595	0	*0.037	0.241	0.989	*0.046
PTEN	0.529	1.046	*0.050	1.346	0.548	*0.050	1.587	0.672	*0.050	0.589	1.116	*0.050	0.529	0.773	0.513
MDM2	0	0.553	*0.037	2.221	0.782	*0.050	1.595	0	*0.037	0.821	2.050	*0.050	0	0.420	*0.037
TP53	0	0.558	*0.037	0.460	0.232	*0.050	1.505	0.341	*0.046	0.643	1.037	*0.050	0	0.439	*0.037

Treated: PVF treated group; * Significant difference in gene expression between control and PVF treated groups ($p \leq 0.05$)

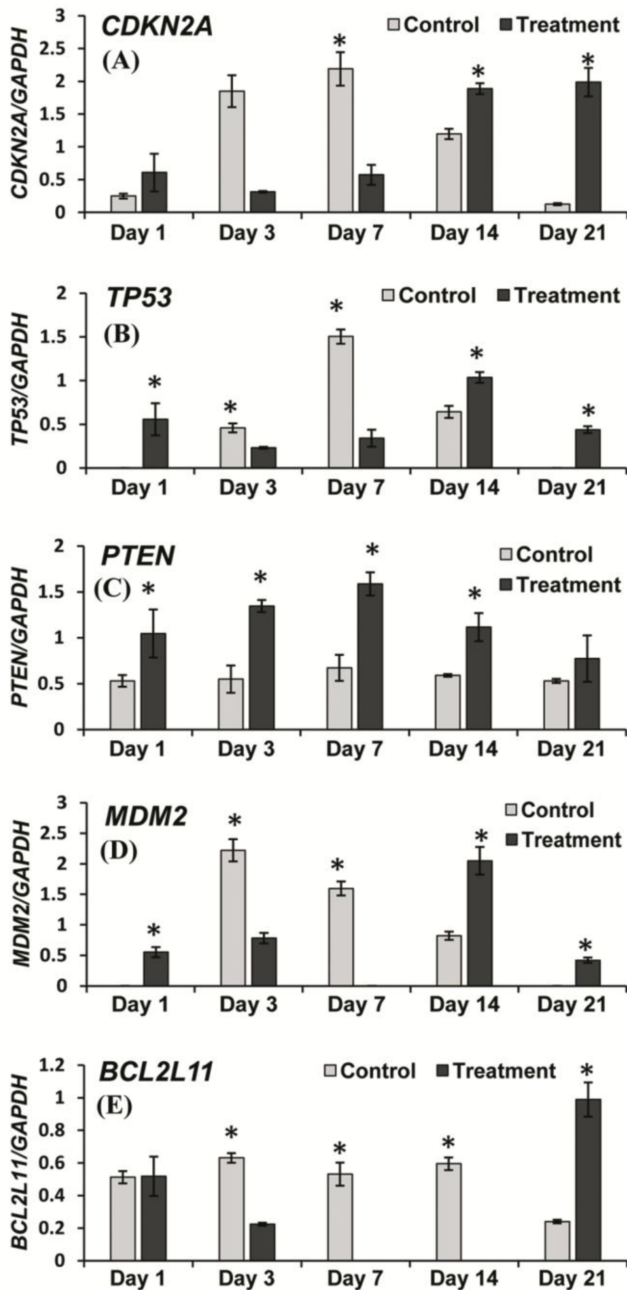

Fig. 2 — Normalized expression of cell cycle regulatory genes in control and perivitelline fluid treated SHED on day 1, 3, 7, 14 and 21 (A: *CDKN2A*, B: *TP53*, C: *PTEN*, D: *MDM2*, and E: *BCL2L11*). * shows significant difference between control and treated group at $p \leq 0.05$

at day 21. Both groups showed low expression of *MDM2* at the end of the experiment (Fig. 2d). *BCL2L11* expression was either lesser or absent completely in the group treated with PVF in comparison to the control group from day 1 until day 14 (Fig. 1c). However, at day 21, expression of *BCL2L11* drastically spiked in the PVF treated group while the expression in the control remained low as shown in Figure 2e.

Live/Dead cell viability

This experiment was carried out to study the effects of PVF treated on 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 were 6000 cells. The cells were viewed on each day for 3 days. Cells which were live produced an intense uniform green fluorescence (ex/em ~495 nm/ ~515 nm) while cells which were dead emitted bright red-orange fluorescence (ex/em ~495 nm/ ~635 nm). Figure 3 shows that the viability of cells between both groups did not exhibit any significant difference 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 3. This could be due to the overcrowding of cells on the glass slide that led to cell senescence or death. However, in the PVF treated group, SHED appeared to be growing well and viable with minimum number of dead cells compared to the control.

Table 3 shows the fluorescence readings at 645 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 percentage of live and dead cells. Live cell percentage in control group decreased from day 1 to 3, while the dead cells increased gradually from day 1 to 3 (Fig. 4). In contrast, the percentage of live cells in SHED with PVF treatment remained high which was above 90 % until day 3, with a lower percentage of dead cells in comparison to the control group as shown in Figures 4a & b.

Discussion

Investigating and comparing the expression of genes that regulates cell cycle is vital in grasping clues at the molecular level on how certain treatments affect the cell cycle and growth of cells. This is important 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, haemocyanins and lectins which are deemed important in supporting embryogenesis[10,11]. PVF has also been widely studied previously and shown to support organ regeneration in chicks[10] and even gonadal development in red tilapia[13].

In the current study, the genes *CDKN2A*, *PTEN*, *MDM2*, *TP53* and *BCL2L11* were selected because of

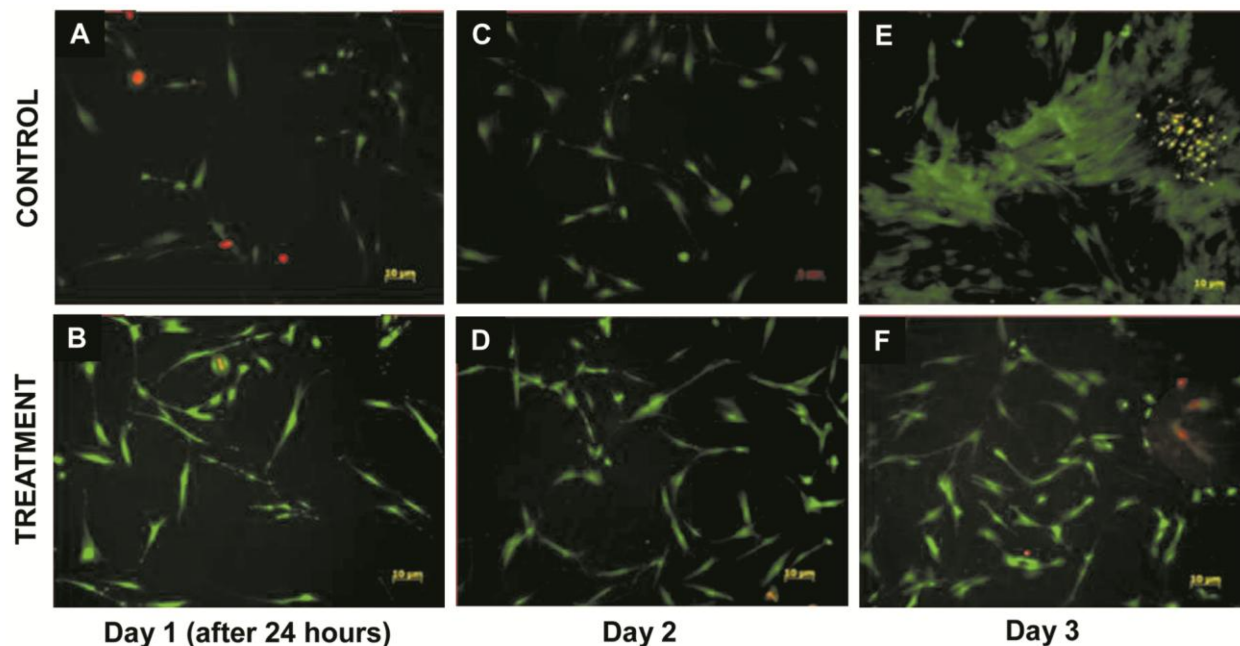

Fig. 3 — Fluorescence microscopy of SHED treated with perivitelline fluid (B, D, F) and control (A, C, E) at day 1, 2 and 3, scale bar = 5 µm and 10 µm. Live and dead cells are shown by the green and red-orange fluorescence respectively

Table 3 — Fluorescence readings at wavelengths of 645 nm and 530 nm in control and perivitelline fluid treated SHED groups at day 1, 2 and 3 of incubation

Groups Readings	Control Day 1	Treated Day 1	Control Day 2	Treated Day 2	Control Day 3	Treated Day 3
A	0.010	0.140	0.077	0.274	0.149	0.424
B	0.765	0.960	1.099	0.757	1.347	0.741
C	0.073	1.664	0.251	1.515	0.413	1.199
D	0.005	0.110	0.034	0.214	0.067	0.341
E	2.055	0.050	1.940	1.780	1.767	1.616
F	0.700	0.967	0.912	0.721	1.215	0.657
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

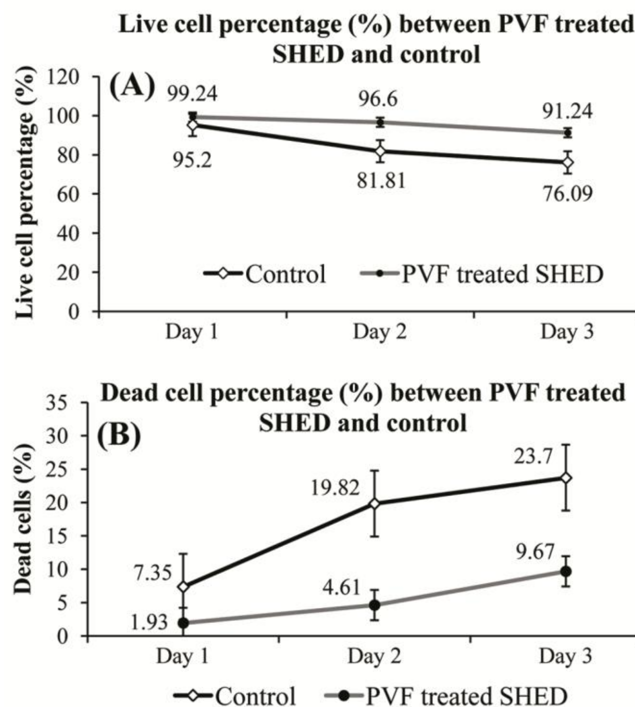

Fig. 4 — Percentage of live (A) and dead (B) cells in control and perivitelline fluid treated groups for 3 consecutive days (Values from Table 3 were incorporated into the given formula to obtain the percentage (%) of live and dead cells)

their significant role in regulating cell cycle as well as in apoptosis, respectively. *CDKN2A* regulates cell cycle arrest at G1 stage encoding two important tumour suppressor proteins, namely p16(INK4a) and p14(ARF)[14] which play a crucial part in the retinoblastoma (Rb) protein and p53 pathways, respectively[15]. p16(INK4a) as well as p14(ARF) work differently at the end of G1 phase in initiating cell cycle rest, thus halting the cells from entering S phase[16]. Moreover, the relationship between *CDKN2A*, *MDM2* and *TP53* in regulating cell cycle progression at G1 checkpoint is crucial to be understood as these three genes are tightly interconnected to one another. As reported in previous studies it is well known that the p14(ARF) protein which is produced by *CDKN2A* promotes activation

of p53 protein, which in turn leads to inactivation of MDM2 protein[17,18]. Also, *TP53* is often activated as a response to DNA damage[19]. This is followed by the increase in *MDM2* levels as both genes have a negative autoregulatory feedback. This phenomenon results in G1 arrest[20]. Therefore, expression of *MDM2* at a higher level would incline to apoptosis[20]. *PTEN*, on the other hand, catalyses dephosphorylation of 3′ phosphate of the inositol ring in PIP3 which results in the biphosphate product PIP2 (PtdIns(4,5)P2). The dephosphorylation step plays an important role as it results in the inhibition of AKT signalling pathway[21]. Yamada & Araki[21] have also demonstrated the significance of *PTEN* in mediating cellular senescence as well as the control of cell growth. The expression of apoptotic regulator gene, *BCL2L11*, which encodes for BH3 proteins functions as apoptotic activator when it interacts with other sets of proteins from the BCL-2 family. Previous studies have demonstrated that over expression of BIM protein will trigger apoptosis by the activation of cell death pathways that requires other caspases known as BH3 proteins[22].

The expression of these genes was analysed in PVF treated and control SHED at passage 6 for a period of 21 days (day 1, 3, 7, 14 and 21). In general, low passage and high passage can affect the phenotype and gene expression characteristics of cells. Also, high passage cell lines do not represent reliable models of original source tissue[23]. Sa-ngiamsuntorn and colleagues[24] reported that generally, cells from passage 4-6 are stable with the cells reaching confluence faster. Hence, passage 6 was selected for the current study. The choice of number of days was based on previous studies that have conducted gene expression analyses on day 1, 3, 7, 14 and 21[refs. 25,26] since such a sequence would enable in evaluating the pattern of gene expression at different time points between the experimental and control groups.

CDKN2A gene encodes tumour suppressor proteins which are p16(INK4a) and p14(ARF)[27]. The tumour suppressor proteins produced by *CDKN2A* have specific roles that work together and function in controlling cell cycle G1. The product of α transcript, p16INK4a, inhibits phosphorylation of Rb protein caused by the cyclin-dependent kinases, namely CDK4 and CDK6 leading to cell cycle arrest in G1, while, p14ARF, the product of the human *CDKN2A*β transcript, causes cell cycle arrest at both G1 and G2/M by activating a p53 response which results in the elevated levels of MDM2 and p21CIP1[ref. 17]. *CDKN2A* has been mutated or deleted in a many types of tumours such as head and neck squamous cell carcinoma[28]. The functions of *CDKN2A* are often altered in cancers whereby it involves in significant reduction on amounts of p16(INK4a) or p14(ARF) proteins. 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 the current study, C*DKN2A* expression was consistently present in the PVF treated group. It was noted that *CDKN2A* expression increased gradually in PVF treated group from day 3 to day 21. The expression in PVF treated group 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 is enhanced in the presence of PVF.

PTEN is also one of the common tumour suppressor genes whereby its deregulation and disruption are often illustrated in various types of cancers such as Cowden disease and breast carcinomas[29,30]. *PTEN* is involved tightly with a series of signalling proteins; regulating a plethora of cellular processes including growth, survival, proliferation, motility, and polarity[31]. *PTEN* encodes lipid phosphatase a ubiquitous regulator for cellular PI (phosphoinositide) 3-kinase signalling pathway producinga second messenger, PtdIns(3,4,5)P3 (phosphatidylinositol 3,4,5-trisphosphate)[31]. Evidences have shown that the signals emitted by PtdIns(3,4,5)P3 is vital in mediating downstream signalling in directing many cell physiological processes, namely, proliferation of cells, cell survival, growth as well as motility. The level of the 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 hypothesise that the PVF might contain growth stimulatory factors which explain the intensified expression of *PTEN* in treated groups compared to the control.

Another tumour suppressor gene that was studied is *TP53*. Tumour suppressor p53 is an important transcription factor that has an important role in regulating cell cycle, DNA repair, apoptosis, senescence, and angiogenesis[32-34]. However, *MDM2* is

a necessary regulator of p53 in the case of normal cells where p53 creates an autoregulatory feedback loop with the oncogene MDM2 proteins[35]. Besides that, *MDM2* is a proto-oncogene whereby the encoded protein promotes tumour formation by targeting the tumour suppressor p53 proteins for proteasomal degradation. The amplification or over expression of *MDM2* has been found in large cases of various cancers and was first detected in sarcomas that retain the wild type p53[36]. 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 PVF treated 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 as over expression of this gene could result in tumour growth. This is due to the fact that it can inhibit the DNA double-strand break repair that is mediated through a novel direct interaction occurring between the MDM2 and Nbs1, independent of p53[37].

BCL2L11 is a member of BCL-2 protein family. TheBCL-2 family members can create hetero- or homodimers acting as an anti- or pro-apoptotic regulator which are involved in a variety of cellular activities. The protein encoded by this gene has a Bcl-2 homology domain 3 (BH3) that has been made known to interact with the other members of BCL-2 protein family as well as to act as an apoptotic activator[38]. Apoptosis is an essential cellular haemostatic mechanism that keeps cell population in balance and prevents cell population from uncontrolled increase in growth[39]. If cell death is repressed, cell accumulation takes place that could lead to many types of cancers and autoimmune diseases[40,41]. Based on the results of our study, *BCL2L11* expression in PVF treated group was maintained at low levels from day 1 to 3 but disappeared completely on day 7 and 14. Low expression levels of *BCL2L11* at an earlier stage of incubation suggest that the cells are actively proliferating at the moment with minimal signs of cell death. However, on the 21st day, a sudden spike in *BCL2L11* expression was detected in PVF treated group. This could be due to the overcrowding of cells in the confined culture flask treated group at day 21 which might induce the process of apoptosis/cell death as suggested[40]. Their findings suggested that during homeostatic turnover, the growth as well as the division of epithelial cells on confined substratum could result in overcrowding which could eventually lead to their extrusion causing death due to the loss of survival factors[42].

Besides analysing the gene expression profiles, the cell viability of SHED using fluorescence microscopy and fluorescence microplate method is also investigated in the current study. The LIVE/DEAD® Viability/Cytotoxicity Assay Kit was employed in this study to conduct the experiment because it provides an effortless, reliable and sensitive fluorescence-based method in order to determine the viability of both adherent and non-adherent cells. The kit contains two probes, namely calcein AM and ethidium homodimer-1 that distinguishes the live cells from the dead cells by producing intense uniform green fluorescence in cells that are live (ex/em ~495 nm/ ~515 nm). On the other hand, dead cells are characterized by the production of a bright red-orange fluorescence (ex/em ~495 nm/ ~635 nm). The findings of this study suggested that incubating the SHED with PVF for 3 days gave higher cell viability in comparison to the control as shown by higher percentage of live cells. Besides that, the fluorescence microscopy images showed viable SHED for all 3 days of incubation with minimal signs of cell death.

Conclusion

Treatment with 0.019 mg/ml of PVF on SHED enhanced cell cycle and growth as shown by a significant increase in the expression of CDKN2A, PTEN and TP53 genes. Also, PVF treatment did not cause any tumorigenicity as supported by the *MDM2* expression that remained low and controlled throughout the 21 days of incubation. In contrast, it is also hypothesized that due to the actively proliferating SHED with PVF treatment inside a confined culture flask environment for a longer period might cause the cells to undergo apoptosis as shown by the sudden spike in *BCL2L11* expression on 21st day of incubation. PVF treatment also showed higher cell viability of SHED as shown by fluorescence microscopy and microplate reader. It can be concluded that PVF from horseshoe crab can serve as a beneficial material in stem cell culture and regenerative medicine.

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Conflict of Interest

Authors declare that there is no conflict of interest in this study.

Author Contributions

First author (IN) contributed for data collection, laboratory analysis, review of literature, data processing and drafting the manuscript; other authors (TPK, AA & IMK) contributed for review of research methodology, data analysis, manuscript review and editing. All authors have read and approved the final version of the manuscript.

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