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

Abstract- Stem cells from human exfoliated deciduous teeth (SHED) makes an ideal source 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 have 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 of odontogenesis markers in SHED treated with PVF (0.019 mg/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 (0.019 mg/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.

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1 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]. 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 and brain. 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 was compared between two groups which are PVF treated SHED and the control group without PVF treatment.

2 METHODS

2.1 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 foetal bovine serum (10%) and incubated at 37 ºC in a 5% CO_2 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 days 1, 3 and 7. Passage 6 was chosen for the current study.

2.2 Perivitelline fluid

Collection of fertilised 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 sterilisation using a 0.25 µm syringe filter (Sartorius, UK) [4].

2.3 Total RNA extraction

Two flasks of cells were trypsinised and prepared into a single-cell suspension for each period of incubation, namely, days 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 RNA extracted was checked for its purity using an UV spectrophotometer at the absorbance ratio of 260 and 280 nm. All samples showed a purity of >1.8. The SHED were resuspended in lysis buffer and homogenised by passaging through a QIAshredder column (Qiagen, Germany). The homogenised lysate was then applied to the RNeasy column, rinsed repeatedly with a series of buffers and eluted into RNase-free deionised water.

Table I: Primer sequences

Gene	Primer	Nucleotide Sequence 5´ to 3´	Melting Temp (ºC)	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	

2.4 cDNA synthesis by reverse transcription polymerase chain reaction

The 1st-strand cDNA from the total RNA was synthesised 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. 200 ng/µl of cDNA template was used for the RT-PCR reactions. 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).

2.4 Gel electrophoresis

The PCR products (3 µl) were stained with SYBR Green (1 µl) and separated on an agarose gel (1.0%). The bands were visualised on a UV transilluminator (Biorad, USA) and then photographed in an image analyser (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.*

2.5 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 $p \leq 0.05$.

3 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 days 1 to 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 (Figure 2). The expression of *OPN* in the control SHED also showed an increase from days 1 to 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 days 1 to 7. However, the treatment group still showed higher expression of *OPN* compared to the non-treated SHED on all days (Figure 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.

Table II: PCR conditions

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

4 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 days 1, 3 and 7. These days were chosen in this preliminary study where a period of 7 days is considered as sufficient to observe the initial expression pattern of genes during the early differentiation process of SHED. Specific concentration of PVF (0.019 mg/mL) was chosen based on a previous study which reported 102.5% cell viability of dental pulp stem cells from deciduous teeth when treated with PVF [4].

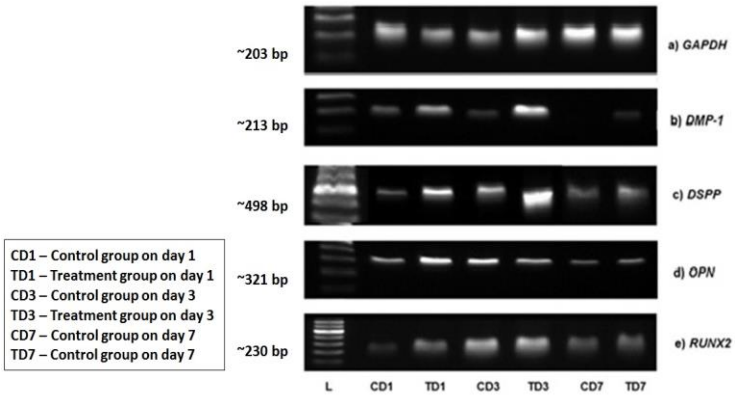

Figure 1: Gel image showing the bands of *GAPDH, DMP-1, DSPP, OPN* and *RUNX2* after RT-PCR amplification in control and PVF treated SHED at days 1, 3 and 7.

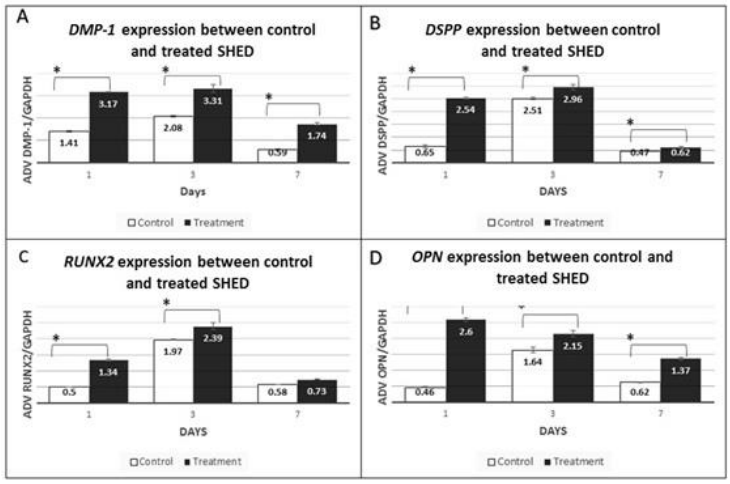

Figure 2: A, B, C and D show the normalized expression levels of *DMP-1, DSPP, RUNX2*, and *OPN* at days 1, 3 and 7 in control and PVF treated SHED. *Asterisks indicate significant difference between control and treated groups, *p ≤ 0.05.

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

DAY	*DMP-1*		*DSPP*		*OPN*		*RUNX2*		*GAPDH*	
	Control	**PVF**	**Control**	**PVF**	**Control**	**PVF**	**Control**	**PVF**	**Control**	**PVF**
1	1103.80 (21.047)	1721.30* (44.783)	527.28 (49.352)	1379.58* (13.229)	361.81 (8.677)	1414.90* (4.442)	390.13 (1.499)	726.46* (4.698)	784.51 (4.909)	653.86 (6.936)
3	1276.45 (18.23)	1955.49* (38.044)	1026.19 (31.487)	1751.75* (9.007)	1006.47 (49.323)	1270.70* (22.512)	1204.86 (3.350)	1412.83* (10.294)	613.17 (4.278)	603.52 (11.378)
7	639.75 (36.329)	1631.70* (49.660)	507.43 (10.393)	586.20* (30.679)	676.23 (18.315)	1276.16* (35.860)	628.22 (3.929)	688.75 (5.638)	1082.37 (9.932)	1007.90 (1.672)

[a] Mean from triplicates of ADV values recorded for each gene 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 ($p \leq 0.05$).

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 mineralisation of dentin. Previous study showed that *DMP-1* knockout mice had defects in odontogenesis and mineralisation as it was observed to develop enlarged pulp chambers with increased width of the predentin zone and hypomineralisation [12]. The results of the present study showed that *DMP-1* was highly expressed in PVF treated cells. This suggests that PVF may promote odontogenesis and maintain proper mineralisation 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*. Since, OPN proteins are mainly secreted during the formation of odontoblasts, the lower expression of OPN in this study might indicate that SHED still consists of population of undifferentiated cells.

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.

5 CONCLUSION

The upregulation of *DMP-1, DSPP, RUNX2* and *OPN* genes in the PVF treated SHED suggested that PVF (0.019 mg/mL) possesses the potential in enhancing odontogenesis process that could lead to higher rate of dentin mineralisation. 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.

CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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