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Original Article

Effect of perivitelline fluid from horseshoe crab on the expression of cell cycle regulatory genes in human dental pulp stem cells

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Abstract 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 expression of cell cycle regulatory genes from human dental pulp stem cells (DPSCs) between different cell passages viz. 4, 5, 6. The cells were treated with a single dose of PVF (26.89 mg/ml) PVF. Gene expression was quantified for CDKNA2A, PTEN, MDM2 and TP53 genes using reverse transcriptase PCR. *CDKN2A* and *MDM2* expression for treated and untreated DPSCs, expressed a similar pattern of expression. The higher expression of *CDKN2A* showed that the treatment increased cell proliferation and prevented cell senescence. DPSCs with PVF treatment showed increased expression of *MDM2* at passage 4 and drastically declined expression at passage 5 and slightly increased at passage 6. *TP53* expression of DPSCs treated group showed a higher expression compared to untreated group. On the other hand, the expression of *PTEN* in DPSCs treated group started to increase from passage 5 to 6. However, on the whole, the *PTEN* expression was higher than the untreated group in all the passages studied here. The results showed that PVF could enhance cell cycle regulatory gene expression in DPSCs as indicated by the higher expression of all the genes considered in this study at different cell passages in the treated group compared to the untreated group. Mann Whitney test was utilized to determine the significance of cell cycle regulatory genes expression between treated and untreated group. Significant difference in expression of genes between the treated and untreated groups were found at all passages except for CDKN2A gene whereby, its expression was not significantly different at passage 5 though it did express slightly higher in PVF treated DPSCs.

Keywords: cell cycle, dental pulp stem cells, gene expression, horseshoe crab, perivitelline fluid.

Introduction

Horseshoe crabs belong to family Limulidae and are known as '*living fossil*' due to their origin 450 million years ago (Sadava *et al.,* 2011). Currently, there are only four living species of horseshoe crabs. *Limulus polyphemus* resides in the eastern coastline of North and Central America whereas, the other three inhabit the coastlines of Japan, India, and Indonesia.

For the past several decades, the horseshoe crab has had many uses for humans (Shuster *et al.,* 2003). In recent times, the biomedical properties of the horseshoe crab have assumed greater importance due to the unanticipated marketing of Limulus Amoebocyte Lysate (LAL) as an endotoxin tester in food, drug and pharmaceutical industries (Chatterji, 1994; Cooper, 1979). The lysate test has been proved to be a better substitute for the

rabbit vaccine test because of its efficiency in terms of quick results and costs (Chatterji, 1994).

The perivitelline fluid (PVF) refers to fluid that fills the perivitelline space (a space between the newly formed inner egg membrane and embryo) during early developmental stages of the horseshoe crab embryo (Sekiguchi, 1988; Punzo, 2000). PVF is reported to have increased the proliferation of beta cells which might be beneficial for insulin production in human beings (Ghaskadbi *et al.*, 2008). PVF of the developing embryo of horseshoe crab is known to show the presence of both anti- and proangiogenic activities useful in cardiac disorders and cancer therapies (Mirshahi *et al.,* 2005). PVF contains proteins such as hemagglutinins and hemocyanins which may play an important role during embryogenesis (Sugita and Sekiguchi, 1979; Shishikura and Sekiguchi, 1984). There is still lack of information on the individual components in PVF due to the variability in the different species of horseshoe crab which could contribute to the variations in the detected constituents. It is hypothesized that the proteins extracted from the PVF of horseshoe crab could produce proliferation enhancer activity (Ghaskadbi *et al.*, 2008; Srijaya *et al.*, 2013).

Dental pulp stem cells (DPSCs) can be found in permanent teeth within the ‘‘cell rich zone’’ of dental pulp. The dental pulp is considered a rich source of mesenchymal stem cells that are suitable for tissue engineering applications. It is known that dental pulp stem cells have the potential to differentiate into several types of cells and DPSCs are highly proliferative (Casagrande *et al.,* 2011). The usefulness of stem cells in clinical applications depends on their proliferation rate, differentiation potential, and accessibility (Yu *et al.,* 2009). DPSCs are capable of generating a tissue that has morphological and functional characteristics that closely resemble those of human dental pulp (Demarco *et al.,* 2010). Other studies have expanded the potential of these cells in the treatment of diseases and conditions such as muscular dystrophies, critical size bone defects, corneal alterations, spinal cord injury, and systemic lupus erythematosus (Nosrat *et al.*, 2001; Kerkis *et al.*, 2008; Seo *et al.*, 2008; Monteiro *et al.,* 2009; Ishkitiev *et al.,* 2010; Yamaza *et al.,* 2010). DPSCs have been perceived by many scientists as one of the highly proliferative mesenchymal stem cell (MSC) sources which have diverse potential to differentiate into several types of cells (Gronthos *et al.*, 2000; Shi and Gronthos, 2003). In a previous study, it was reported that crude PVF helps in the viability of DPSCs although the proliferative activity appears to be insignificant (Musa *et al.*, 2015).

PVF which was found to have increased the growth and differentiation of a chick embryo heart (Ghaskadbi *et al.*, 2008), has triggered the current study in assessing the role of PVF in enhancing cell viability and proliferation. Due to its proliferative potential, the current study was undertaken to investigate further on the role of PVF in the expression of genes that regulate cell-cycle, *CDKN2A*, *MDM2*, *PTEN* and *TP53*. Additionally, this study also serves to observe the expression pattern of cell cycle regulatory genes between different cell passages, namely 4, 5, 6.

Materials and methods

Perivitelline fluid

PVF was isolated and purified according to method previously prescribed (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 1ml of phosphate buffered saline (PBS) (Invitrogen, UK) and further diluted to a single concentration of 26.89mg/ml using culture medium. The PVF extract was sterilized through a 0.25µm syringe filter (Sartorius, UK). The extract was prepared fresh for each experiment.

Cell culture and harvesting

DPSCs from AllCells (USA, Cat. No. DP004F) 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 incubated at 37°C in a 5% CO_2 humidified incubator until confluence. The cells from passage 4 to 6 were treated with PVF. The cells of both groups were collected at passage 4, 5 and 6 by incubating the cells with trypsin for 1 to 2 minutes until all the cells were detached. Based on a previous study, cells from passage 4-6 is generally stable and the cells reached confluence faster (Sa-ngiamsuntorn *et al*., 2011).

RNA extraction

Total RNA was extracted from the cells using the commercially available kit (RNAeasy, QIAGEN, USA).

Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis

Primers that were used in this study are listed in Table 1. All these primer pairs were intentionally designed to span separate exons to ensure that the PCR products were from mRNA-generated cDNA, and not from genomic DNA (Xiao *et al.,* 1998; Gronthos *et al.*, 2000; Kim *et al.,* 2005). The respective genes were amplified using RT-PCR kit (Qiagen, Germany). The RNA extracts were analyzed by conventional RT-PCR using MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre, USA).

Polymerase chain reaction (PCR) amplification was performed with the total RNA obtained. The RNA was subjected to RT-PCR to detect the expression of genes controlling the cell-cycle such as CDKNA2A, PTEN and TP53 genes that act as tumor suppressor and MDM2 gene as negative regulator of the p53 tumor suppressor in the cell-cycle. The synthesis of DNA from an RNA template via reverse transcription produces complementary DNA (cDNA) which can be used directly as a template for the PCR. The amount of cDNA for each sample was standardized to 200 ng/µl. The thermal cycling parameters were 94 ºC for 1 min to activate the polymerase, followed by 30 cycles of 94ºC for 15s, 57ºC for 30s and 72ºC for 30s. The RT and PCR reaction were performed in a MJ Research PT2000 thermocycler PTC-200 Peltier Thermal Cycler (Biorad, USA). The PCR products were then separated on an agarose gel (1.5%) containing SYBR Green (0.125 µg/mL), visualized on a UV transilluminator. The level of expression of genes was determined and the housekeeping GAPDH gene was used as reference.

Gene expression analysis

DPSCs were cultured as mentioned previously. When confluent, the cells from passage 4-6 were treated with a single dose of PVF (26.89 mg/ml) and incubated for 24 hours at 37°C in a CO_2 incubator. This dose of PVF was selected based on previous study which showed the highest cell proliferation at 26.89 mg/ml of PVF (Musa *et al.*, 2015).

Statistical analysis

Mann Whitney test was carried out using SPSS software version 22.0 to determine the significance of individual gene expression between the control and treatment groups at passage 4, 5 and 6 respectively. The *p*-value was set at $p \leq 0.05$.

Results

The genes controlling the cell cycle namely, *CDKN2A, PTEN, TP53* and *MDM2* were analyzed and normalized to the housekeeping gene, *GADPH*. The normalized gene expression was quantified as index intensity value, the results of which are shown in Table 2. Expression of *GAPDH* was constant for both the control DPSCs and DPSCs treated with PVF.

CDKN2A was expressed in a similar pattern in treated and untreated DPSCs. In both groups of DPSCs, expression of *CDKN2A* increased from passage 4 to 5, reached its peak at passage 5 and then declined at passage 6. The difference between treated and untreated group can be seen by the overall higher expression of *CDKN2A* in treated group compared to the untreated group at each passage number (Figure 1A).

MDM2 also showed a similar pattern of expression between both groups of DPSCs. Figure 1B shows the gene expression in both the groups. *MDM2* expression decreased from passage 4 to 5

and then increased at passage 6. *MDM2* expressed at a much higher level in PVF treated DPSCs as compared to the untreated group at all passages, similar to that of *CDKN2A.*

PTEN expression showed a different pattern between DPSCs treated with PVF and DPSCs untreated group. DPSCs treated group at passage 5 started to decrease and increased at passage 6. However, for DPSCs untreated group, passage 5 showed an increasing pattern at passage 5 and there was no change at passage 6 (Figure 1C). Besides that, as seen in Figure 1C, PVF treated DPSCs also showed higher expression of *PTEN* between all passages similar to that of the previous two genes described.

TP53 showed a higher expression in treated group compared to untreated group. Passage 5 of DPSCs treated with PVF showed the highest expression and then declined at passage 6. DPSCs in untreated PVF expressed much lower with a declining pattern where the gene expression decreased from passage 4 until passage 6. This is shown in Figure 1D.

Based on the Mann Whitney test that has been conducted, significant difference in expression was found in all genes between treated and untreated group. There was only one gene that did not express significantly different which is CDKN2A at passage 5. However, CDKN2A still did expressed higher in treated group compared to untreated group. As a whole, this suggested that the PVF treatment given onto DPSCs could enhance the cell growth of DPSCs.

Discussion

In this study, *GAPDH*, the housekeeping gene was expressed equally throughout the study period. This gene is an endogenous control gene which regulates gene expression. Constant and equal expression of this gene indicates that the RNA concentrations used in PCR had the same concentrations (Zhong and Simons, 1999). It is crucial to calibrate the *GAPDH* expression as different concentrations of RNA can give rise to different levels of gene expression.

Cyclin-dependent kinase inhibitor 2A is a gene which regulates $p16^{INK4a}$ and $p14^{ARF}$ proteins (Stott *et al.,* 1998; Wu *et al.,* 1999). It acts as a tumor suppressor, which means that it controls the cells from growing and dividing rapidly or in an uncontrolled way. The $p16^{INK4a}$ protein is a product of α transcript, binds to two other proteins called CDK4 and CDK6. These proteins help regulate the cell cycle, which is the cell's way of replicating itself in an organized, step-by-step fashion. CDK4 and CDK6 normally stimulate the cell to continue through the cycle and divide. However, binding of $p16^{INK4a}$ blocks CDK4's or CDK6's ability to stimulate cell cycle progression. In this way, $p16^{INK4a}$ controls cell growth and division. Meanwhile, the $p14^{ARF}$ protein, a product of β transcript, protects a different protein called p53 from being broken down as it can interact with E3 ubiquitin-protein ligase MDM2, which is a protein that is responsible for degradation of p53 (Stott *et al.,* 1998; Wu *et al.,* 1999). The p53 protein is an important tumor suppressor which is essential for regulating cell division and self-destruction (apoptosis). Protecting p53, $p14^{ARF}$ also helps in preventing tumor formation. Based on the results of this study, there was no significant difference of *CDKN2A* expression in treated group compared to untreated group although *CDKN2A* in DPSCs treated group from passage 4 to 6 showed higher expressions compared to untreated group. The higher expression of *CDKN2A* showed that the treatment increased cell proliferation and prevented cell senescence. At the same time, *CDKN2A* regulated cell cycle so that the cells do not lose the cell cycle control and become tumor. This has been supported by a study which reported that suppression of *CDKN2A* will prevent the cell from entering cell cycle phase and was associated with cell senescence (Baker *et al.*, 2011). For untreated group, passage 5 showed significantly higher expression of *CDKN2A* compared to passage 4 ($p<0.001$), and significant decrease of *CDKN2A* expression between passages 5 and 6. This indicated that DPSCs started to proliferate at passage 5 and became decreased in proliferation capabilities with increasing passaging number.

MDM2 gene is also known as E3 ubiquitin-protein ligase Mdm2. This gene mediates ubiquitination of p53/TP53 that leads to degradation process by proteasome, which can promote tumor formation (Freedman *et al.*, 1999). This gene is predominantly expressed in nucleoplasm and its interaction with $p14^{ARF}$ also helps in localization of both proteins to the nucleolus. In the treated group, expression of DPSCs at passage 4 was highly significant ($p<0.01$) compared to passage 5, and passage 6 showed significant difference ($p<0.05$) as compared to passage 5 of untreated group. The higher expression of *MDM2* in passage 4 and passage 6 treated groups might promote the cells to become cancerous. In the untreated group, passages 5 and 6 showed highly significant difference ($p<0.01$) compared to passage 4 where low level expression of this gene maintained the condition of normal cells.

TP53 is an important regulator of the cell cycle, apoptosis, DNA repair and cellular senescence (Teodoro *et al.,* 2007). In this study, for treated group, there was significant difference ($p<0.05$) of *TP53* expression between passages 5 and 6. Meanwhile, for untreated group, there was only significant difference between passages 4 and 6. The *TP53* expression for both conditions of cells has been linked with *MDM2* expression. The expressions of MDM2 and TP53 genes have been found to regulate each other's functions through an auto-regulatory feedback loop (Shangary and Wang, 2008). Upon activation, *TP53* promotes transcription of the MDM2 gene and in turn, the MDM2 protein will inhibit P53 activity. This supported our findings that at passage 5 of treated group, expression of *MDM2* was lower compared to passage 6 and simultaneously, the expression of *P53* was found to be inversely proportional to *MDM2* expression.

PTEN known as *MMC-1* or *TEP-1* has been identified as a tumor suppressor and involved in the regulation of many cell processes such as growth, adhesion, migration, invasion, and apoptosis (Yamada and Araki, 2001). Our results showed that only passage 44 of untreated group was significantly different ($p<0.05$) compared to passages 5 and 6. The treated group showed upregulation of *PTEN* compared to untreated group. This might indicate that the cells proliferated and overexpression of this gene could prevent mutation leading to tumor formation.

PVF and its potential to enhance many cell biological processes have been anticipated in a number of studies previously. One of them showed that PVF had the ability to stimulate the development and differentiation of certain organs including brain and heart by promoting angiogenesis (Ghaskadbi *et al.,* 2008). Besides that, PVF was suggested to positively influence the early development of gonads in red Tilapia fingerlings by resulting in significantly higher gonadal weight (Srijaya *et al.,* 2013). Moreover, a study has shown that PVF stimulated cell differentiation by triggering the human bone marrow stem cells to turn into cardiomyocytes (Alam *et al.*, 2015). They also reported a more intense and significantly higher expression of myosin in the cells which were cultured with PVF-8 and hence postulated that PVF had the potential to supplement cellular differentiation. Another study has reported that the PVF crude extract was non-genotoxic to DPSCs and hence can be considered for further biomedical applications (Musa *et al.,* 2015).

Cell proliferation and growth involve a variety of signalling pathways which are regulated by a series of growth factors produced by genes that are known to regulate cell cycle pathways (Cooper, 2000). The current study observed the expression of cell cycle regulatory genes, *CDKN2A* and *MDM2*, and tumor suppressor markers, *TP53* and *PTEN* in treated and untreated DPSCs at different passages. Based on the pattern of gene expression, it can be deduced that DPSCs started to proliferate at passage 5 and then decline as the passage number increased. Based on the above findings that PVF treated group showed significantly higher expression of all studied genes at cell passage 4, 5 and 6 in comparison to the untreated group, it can be concluded that PVF has the potential to enhance cell cycle regulatory gene expression in DPSCs. This indicates that the PVF may contain important growth stimulatory components that could support cell growth.

Table 1 Primer sequences of different genes

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Ta	Product size
CDKN2A	GAGAACATGGTGCGCAGGTT	GCGCTGCCCATCATCATGA	57	219
PTEN	GCACAATATCCTTTTGAAGACC	AGTGCCACTGGTCTATAATCC	57	323
TP53	TCCCTTCCCAGAAAACCTAC	CTATCTGAGCAGCGCTCATG	57	265
MDM2	GTGTAGAATTTGAAGTTGAATCT	TGAAGTGCATTTCCAATAGTC	57	185
GAPDH	CCCATCACCATCTTCCAGGA	TTGTCATACCAGGAAATGAGC	60	631

Table 2 Index intensity value of different genes expressed in DPSCs with and without treatment of PVF

Gene	Passage	Treated			Average	Untreated			Average
CDKN2A	4	1.56	1.90	1.96	1.80	0.60	0.85	0.85	0.80
	5	1.50	2.25	2.36	2.00	1.53	1.72	1.55	1.60
	6	1.70	1.77	1.90	1.80	1.00	1.09	0.90	1.00
MDM2	4	3.04	2.80	3.10	3.00	1.60	1.50	1.6	1.60
	5	1.60	1.50	1.63	1.60	0.69	0.90	0.8	0.80
	6	2.57	1.90	1.60	2.00	1.20	0.90	0.87	1.00
PTEN	4	3.20	2.95	3.78	3.30	0.86	1.10	1.05	1.00
	5	2.48	3.30	3.30	3.00	1.90	2.09	2.00	2.00
	6	4.00	3.38	3.20	3.50	2.30	2.27	1.50	2.00
TP53	4	2.60	3.30	4.10	3.30	1.27	1.20	1.40	1.30
	5	2.96	3.90	4.10	3.60	0.80	1.18	1.04	1.00
	6	2.47	1.76	1.84	2.00	0.80	0.45	0.56	0.60

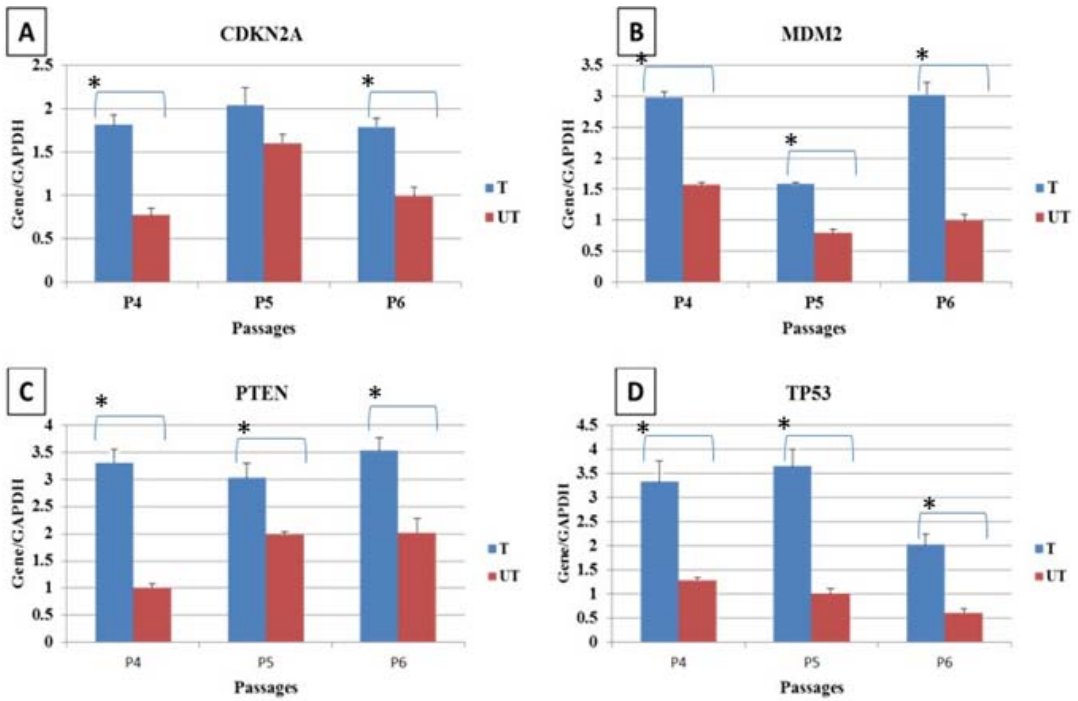

Figure 1 Comparison of (A) *CDKN2A*, (B) *MDM2*, (C) *PTEN*, (D) *TP53* normalized gene expressions between treated and untreated groups at Passages 4, 5 and 6. Asterisks indicate significant difference between treated and untreated groups, $*p \leq 0.05$.

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