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Original Article

Effect of perivitelline fluid from horseshoe crab on the expression of *COL1A1* in dental pulp stem cells

Amanina Fatinah binti Kamarudin[a,b,c], Najian binti Ibrahim[a], Ahmad Aizat Abdul Aziz[b], Thirumulu Ponnuraj Kannan[a,b]*

[a] School of Dental Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
[b] Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Kelantan, Malaysia.
[c] Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

* Corresponding author: kannan@usm.my

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Abstract Perivitelline fluid, extracted from the fertilized eggs of horseshoe crabs, has been reported to play a vital role in supporting embryogenesis as well as cell proliferation. The present study aims to evaluate the effect of PVF on the expression of *COL1A1* in human dental pulp stem cells (DPSCs). The cells were grouped into two; untreated (control) and treated with a single dose of PVF (0.019 mg/ml). Gene expression was quantified for *COL1A1* on day 1, 3 and 7 using reverse transcriptase PCR. The expression of *COL1A1* on day 3 of treated group with PVF was the highest though there was a decline of *COL1A1* expression on day 7. Mann Whitney test was utilized to determine the significance of *COL1A1* expression between treated and untreated groups. Significant difference in the expression of *COL1A1* was observed between the treated and untreated groups on day 3 though there was no significance in the expression on day 7. The present study indicates that PVF may have the potential to increase cell proliferation in human DPSCs.

Keywords: cell cycle, *COL1A1*, dental pulp stem cells, gene expression, horseshoe crab, perivitelline fluid.

Introduction

The perivitelline fluid (PVF) of the developing embryo of horseshoe crab is known to possess both anti- and proangiogenic activities useful in cardiac disorders and cancer therapies (Mirshahi *et al.*, 2005). PVF has been reported to contain proteins such as hemagglutinins and hemocyanins which may play a role during embryogenesis (Sugita and Sekiguchi, 1979; Shishikura and Sekiguchi, 1984). It has also been hypothesized that the proteins present in the PVF of horseshoe crab could enhance proliferation activity (Ghaskadbi *et al*., 2008; Srijaya *et al*., 2013).

Dental pulp stem cells (DPSCs) is the soft living tissue within teeth and have the ability to differentiate into variety of cell types such as adipocytes, osteocytes, chondrocytes, and myocytes as they are multipotent and highly proliferative (Casagrande *et al*., 2011). The application of stem cells clinically is based on various factors like proliferation rate, differentiation potential, and accessibility (Yu *et al*., 2009). Many researchers have perceived DPSCs to have a diverse potential to differentiate into several types of cells (Gronthos *et al*., 2000; Shi and Gronthos, 2003). DPSCs have the capability of generating a tissue that has morphological and functional characteristics closely resembling human dental pulp (Demarco *et al*., 2010). Crude PVF enhances the viability of DPSCs although the proliferative activity was insignificant (Musa *et al*., 2015). PVF is known to have increased the growth and differentiation of a chick embryo heart (Ghaskadbi *et al*., 2008).

The present study aimed to investigate the role of PVF in the expression of *COL1A1* (collagen type I alpha 1), a gene that regulates cell-cycle and proliferation. COL1A1 gene provides instructions for making a part of a large molecule called type I collagen. Type I collagen is the most

abundant form of collagen in the human body. Collagens are a family of proteins that play a role in strengthening and supporting many tissues in the body; cartilage, bone, tendon, skin, and the sclera of the eye (Dong and Lv, 2016). COL1A1 gene produces a component of type I collagen called the pro-α1(I) chain. Collagens begin as rope-like procollagen molecules which are each made up of three chains. Type I collagen is made of two pro-α1(I) chains and one pro-α2(I) chain. The pro-α2(I) chain is produced by the COL1A2 gene. Some of the diseases that are related to this gene due to the genetic mutations are Caffey disease, dermatofibrosarcoma protuberans, Ehlers-Danlos syndrome and osteogenesis imperfecta (Gensure *et al*., 2005).

Materials and methods

Study design

This was an in vitro experimental study carried out on DPSCs obtained from AllCells, USA (Cat. No. DP004F).

Perivitelline fluid

PVF was isolated and purified based on the previously described method (Chatterji *et al*., 1988) and the freeze-dried PVF was stored at -70˚C. The PVF was mixed with 1ml of phosphate buffered saline (PBS) (Invitrogen, UK) and further diluted to a single concentration of 0.019 mg/ml using mesenchymal stem cell (MSC) basal medium. This was then sterilized using a 0.25µm syringe filter (Sartorius, UK). The preparation of extract was made fresh for each experiment.

Cell culture and harvesting

DPSCs were cultured and maintained in T75 culture flasks with filtered caps (Nunc™, Denmark) using MSC basal medium (AllCells, Cat no. MSC-002) supplemented with MSC stimulatory supplement (AllCells, Cat. No MSC-003). They were incubated at 37˚C in 5% CO2 humidified incubator until the cells reached 70% confluence. Cells of passage 9 were set constant for the current experiment. When the cells reached 70% confluence, they were seeded according to treatment and control groups for day 1, 3 and 7. The number of cells seeded for day 1, 3 and 7 flasks of both groups were 500000, 200000 and 100000 respectively. The amount of cells seeded for each tested day was optimized to have consistent number of cells at the time of harvesting. The cells of both groups were collected on day 1, 3 and 7 by incubating the cells with trypsin for 1 to 2 minutes until all the cells were detached and then the cell lysate was centrifuged at 1000 RPM for 10 minutes to collect the cell pellets.

RNA extraction

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

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

The forward primer for COL1A1 gene was 5′-ACAGTGATTGAATACAAAACCA-3′ and the reverse primer was 5′-GTGGAGAAAGGAGCAGAAAG-3′. The annealing temperature (Ta) was 58°C with a product size of 496 bp. The *COL1A1* was amplified using RT-PCR kit (Qiagen, Germany). The RNA extracts were subjected to conventional RT-PCR using MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre, USA). The RNA was subjected to RT-PCR to detect the expression of COL1A1 gene. The complementary DNA (cDNA) synthesized from RNA template via reverse transcription was used directly as a template for PCR. cDNA (200 ng/µl) was used in the current experiment. The thermal cycling parameters were 95 ºC for 1 min to activate the polymerase, followed by 35 cycles of 95ºC for 30s, 58ºC for 30s and 72ºC for 30s. The final extension was added to extend the template at 72ºC for 2 min. The gradient PCR and PCR reactions were performed in a MJ Research PT2000 thermocycler PTC-200 Peltier Thermal Cycler (Biorad, USA). The PCR products were run on an agarose gel (1.0%) containing SYBR Green (0.125 µg/mL) and loading dye and then visualized on a UV transilluminator.

Gene expression analysis

The expression level of *COL1A1* was determined by recording the Average Density Value (ADV) reading of the bands

on day 1, 3 and 7 and normalized to the ADV of the housekeeping gene, GAPDH. The samples were run in triplicates to obtain an average reading of each group. A single dose of 0.019 mg/ml of PVF was employed based on a previous study (Musa *et al*., 2015) who reported higher cell viability at this concentration.

Statistical analysis

Mann Whitney test using SPSS software version 22.0 was employed to determine the statistical significance between the control and treatment groups at days 1, 3 and 7 respectively. The *p*-value was set at $p \leq 0.05$.

Results

The expression of COL1A1 gene was analysed and normalized to the housekeeping gene, GADPH. The normalized gene expression was quantified as ADV as shown in Fig. 1. The gene expression of GAPDH remained constant in the treated and untreated DPSCs. *COL1A1* was expressed in a different pattern in treated and untreated DPSCs. On day 1, there was no expression of COL1A1 gene in the PVF treatment group but, it started to express on day 3 which was the highest and then declined on day 7. On day 7, the expression of untreated group was higher than the treated group with PVF (Fig. 2). Based on the Mann Whitney U test conducted, there was a significant difference in the expression on day 3 while for day 7, no significant difference in the expression was noticed.

Discussion

GAPDH, the housekeeping gene considered in the present study expressed equally throughout the experiments. Constant and equal expression of GAPDH, gene, the endogenous control gene indicates that the RNA concentrations used in PCR had the same concentrations (Zhong and Simons, 1999). Based on the results of the present study, there was no expression of *COL1A1* both in the untreated and treated groups on day 1 which indicates that *COL1A1* is not expressed in the early stages of proliferation of DPSCs. However, the expression in both groups on day 3 in both the groups shows that *COL1A1* expression is enhanced by prolonged exposure to PVF. Also, this is evidenced by the increased expression in the treated group compared to the untreated group. Moreover, significant difference ($p \leq 0.05$) in *COL1A1* expression was detected between the untreated and treated group.

On day 7, the expression of *COL1A1* in both the groups declined. The decline of *COL1A1* expression on the 7th day might suggest that this gene was not strongly expressed yet in the early stages of growth of DPSCs. Grottkau *et al*. (2010) had reported the absence of *COL2A1* expression in DPSCs from day 1 until day 14 when cultured in a standard culture media (without any supplement). However, the expression of this gene gradually increased when the DPSCs were cultured in supplemented media specific to osteogenesis, chrondogenesis and adipogenesis (Grottkau *et al*., 2010). There was no statistical significance between the treated and untreated groups on day 7 ($p=0.275$). The present study though covering only until day 7 indicates that PVF may have the potential to enhance the expression of the cell cycle regulatory gene *COL1A1* in DPSCs and hence may hypothesize that this may be due to the stimulatory growth factors present in PVF. However, further study for a longer duration is necessary to completely understand the role of PVF in enhancing the expression of *COL1A1*.

The PVF has been reported to play a crucial role in promoting embryogenesis and vasculogenesis as it contains important primitive types of proteins such as hemagglutinins and hemocyanin (Sugita and Sekiguchi, 1979; Shishikura and Sekiguchi, 1984). Besides that, PVF also contains peptides which have the potential of influencing differentiation of specific organs (Ghaskabdi *et al*., 2008). This was indicated by the enlargement of the chick embryo's heart tissue as well as the development of new blood vessels in the presence of PVF treatment (Ghaskadbi *et al*., 2008). Hence, further research on human adult stem cells from various origins using PVF is necessary to elucidate more information. Also, studies encompassing different genes and at prolonged time points with regard to gene expression analysis between PVF treated and control samples are deemed necessary.

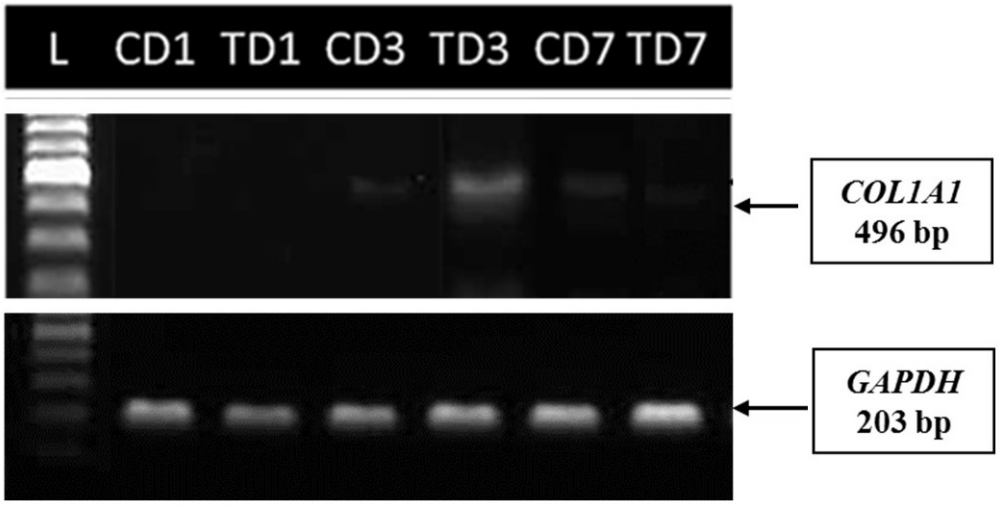

Fig. 1 Gel image showing the expression of *COL1A1* and the housekeeping gene, GAPDH in untreated control DPSCs (CD1, CD3 and CD7) and PVF treated DPSCs (TD1, TD3 and TD7) at day 1, 3 and 7 respectively. Lane L: 100 bp ladder.

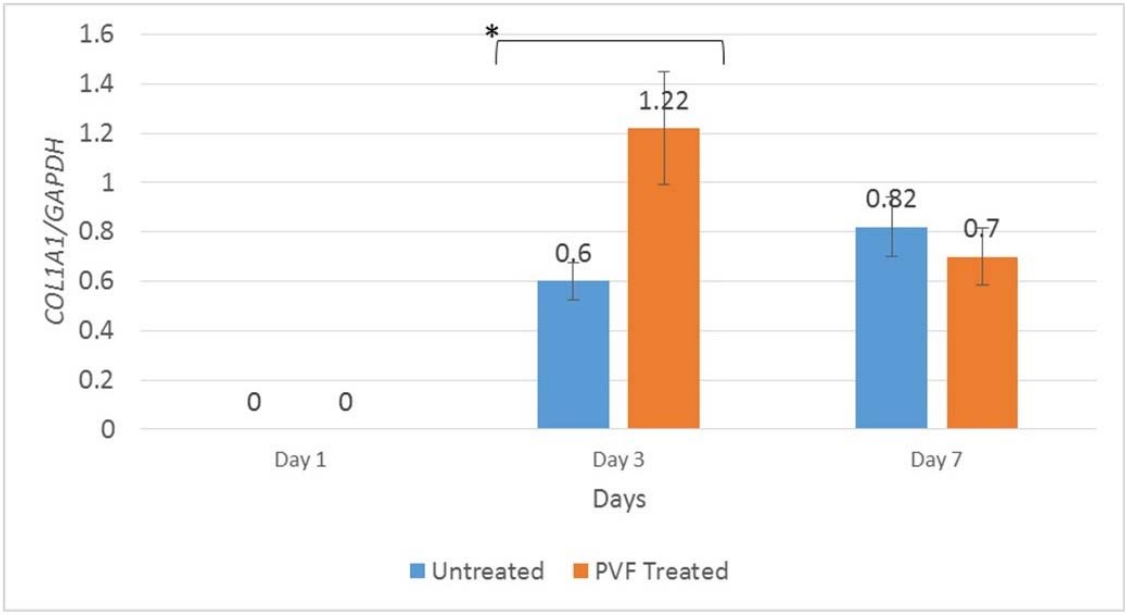

Fig. 2 Normalized index gene expressions between PVF treated and untreated DPSC groups on day 1, 3 and 7. Asterisk indicates significant difference between treated and untreated groups *$p \leq 0.05$. The values represent the mean of triplicates of each experiment.

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References

Casagrande L, Cordeiro MM, Nör SA, Nör JE (2011). Dental pulp stem cells in regenerative dentistry. *Odontology*, **99**(1): 1-7.

Chatterji A, Vijayakumar R, Parulekar AH, (1988). Growth and morphometric characteristic in the horseshoe crab, *Carcinoscorpius rotundicauda* (Latreille) from Canning (West Bengal), India. *Pakistan J Sci Indian Res,* **31**(5): 352-353.

Demarco FF, Casagrande L, Zhang Z, Dong Z, Tarquinio SB, Zeitlin B *et al.* (2010). Effects of morphogen and scaffold porogen on the differentiation of dental pulp stem cells. *J Endod,* **36**(11): 1805-1811.

Dong C, Lv Y (2016). Application of collagen scaffold in tissue engineering: recent advances and new perspectives. *Polymers*, 8(2): 42-62.

Gensure RC, Mäkitie O, Barclay C, Chan C, DePalma SR, Bastepe M *et al.* (2005). A novel *COL1A1* mutation in infantile cortical hyperostosis (Caffey disease) expands the spectrum of collagen-related disorders. *J Clin Invest,* **115**(5): 1250-1257.

Ghaskadbi S, Patwardhan V, Chakraborthy M, Agrawal S, Verma MK, Chatterjee A *et al.* (2008). Enhancement of vertebrate cardiogenesis by a lectin from perivitelline fluid of horseshoe crab embryo. *Cell Mol Life Sci,* **65**(20): 3312-3324.

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000). Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. *Proc Natl Acad Sci USA,* **97**(25): 13625-13630.

Grottkau BE, Purudappa PP, Lin YF (2010). Multilineage differentiation of dental pulp stem cells from green fluorescent protein transgenic mice. *Int J Oral Sci*, **2**(1): 21-27.

Mirshahi M, Mirshahi P, Soria J, Alam H, Therwath A, Chatterji A (2005). Peri-vitelline fluid of the fertilized eggs of the Indian horseshoe crab (*Tachypleus gigas*, Müller) helps in maintaining the original phenotype of CD34+ stem cells and enhances colony formation *in vitro*. In: *Horseshoe Crab in Asia*. Saarbrücken: Lambert Academic Publishing.

Musa M, Khadijah MA, Kannan TP, Ahmad A, Nor Shamsuria O, Chatterji A, Khairani IM (2015). Effects of perivitelline fluid obtained from horseshoe crab on the proliferation and genotoxicity of dental pulp stem cells. *Cell J,* **17**(2): 253-263.

Shi S, Gronthos S (2003). Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J Bone Miner Res*, **18**(4): 696-704.

Shishikura F, Sekiguchi K (1984). Studies on perivitelline fluid of horseshoe crab embryo. II. Purification of agglutinin-binding substance from the perivitelline fluid of *Tachypleus gigas* embryo. *J Biochem,* **96**(3): 629-636.

Srijaya TC, Pradeep PJ, Hassan A, Chatterji A, Shaharom F (2013). Effect of peri-vitelline fluid from horseshoe crab embryo in enhancing the early gonadal development in red tilapia. *Biol Forum,* **5**(2): 1-7.

Sugita H, Sekiguchi K (1979). Protein components in the perivitelline fluid of the embryo of the horseshoe crab, *Tachypleus tridentatus*. *Dev Biol,* **73**(2), 183-192.

Yu J, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin II *et al.* (2009). Human induced pluripotent stem cells free of vector and transgene sequences. *Science,* **324**(5928): 797-801.

Zhong H, Simons JW (1999). Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. *Biochem Biophys Res Commun,* **259**(3): 523-526.