Isolated human dental pulp stem cell failed to be induced to swine valvular interstitial cell: The limitation of species differences

Pornthanate Seenak[1,2], Kantapich Kongpol[1,2], Phatiwat Chotimon[2,3], Boontharika Chuenjitkuntaworn[4], Arunya Jiraviriyakul[1,5] and Sarawut Kumphune[1,2,5*]

[1]Graduate Program in Biomedical Sciences, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok, 65000 [2]Biomedical Research Unit in Cardiovascular Sciences (BRUCS), Faculty of Allied Health Sciences, Naresuan University, Phitsanulok, 65000
[3]Department of Cardio-Thoracic Technology, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok, 65000
[4]Department of Oral Biology, Faculty of Dentistry, Naresuan University, Phitsanulok, 65000
[5]Department of Medical Technology Faculty of Allied Health Sciences, Naresuan University, Phitsanulok, 65000
*Corresponding author. E-mail address: sarawutk@nu.ac.th

Abstract

Valvular Heart Disease (VHD) commonly caused by heart valve tissue dysfunction, which consequently lead to heart failure, and worse of quality of lives of patients. As the heart valves tissue, cannot regenerate spontaneously, the valve replacement or reparative surgery is the most effective therapeutic approach of VHD and prevents the harmful cardiovascular complications. Recent report showed that mesenchymal stem cells derived from bone marrow (BMMSC) or adipose tissue (ADMSC) could potentially be induced into human valve interstitial cells (VICs). However, the potential of dental pulp stem cells (DPSCs), which is another source of mesenchymal stem cells, to be induced into valve interstitial cells has never been investigated. Therefore, this study aims to investigate the capability of the human DPSCs, isolated from human pulp tissue, on VICs induction. The third molat teeth of 3 participants were extracted and the dental pulp cells were isolated by explant outgrowth technique. The stem cells characterization was determined mesenchymal stem cell surface markers (CD29, CD44, CD90, and CD105) and hematopoietic stem cell marker (CD34 and CD117) by flow cytometry. The swine vulvular interestial cells (VICs) were isolated from swine heart valves tissue by enzymatic based cell isolation and characterized by determining the expression of α-smooth muscle actin, vimentin, and collagen type 1 by Western blot analysis. The differentiation induction ability of DPSCs to VICs was performed by trans-well co-culture technique.

The results showed that the isolated DPCs could highly express the mesenchymal stem cell surface markers (CD29, CD44, CD90 and CD105) and less expressed of the hematopoietic stem cell marker (CD34 and CD117). In addition, DPCs could be induced into osteogenic linage as the accumulation of calcium was observed by alizarin red staining. In addition, isolated swine valvular interstitial cells (VICs) were performed. This cell showed the expression of α-smooth muscle actin, vimentin, and collagen type 1, which were found to be less expressed in DPSCs. The induction of human DPSCs to swine VICs by culturing in transwell chamber could not induce the expression of those 3-specific proteins found in VICs. This unsuccessful data could possibly due to the different in the specie of cell source.

Keywords: Valvular heart disease, Dental pulp stem cell, heart valve, Swine valvular interstitial cells

Introduction

Valvular Heart Disease (VHD) commonly caused by heart valve tissue dysfunction, which consequently lead to heart failure, and worse of quality of lives of patients. According to the heart valves tissue cannot regenerate spontaneously, the valve replacement or reparative surgery is the most effective therapeutic approach of VHD and prevents the harmful cardiovascular complications (Heart Valve Surgery, 1 August 2015). The heart valve replacement surgery can be commonly achieved by using mechanical valve, bioprosthetic valve, and heart valve transplantation. It has been reported that approximately 275,000 valve replacements have been done each year worldwide (World Heath Statistics, 13 June 2015). However, the valves that have been widely used for the valve surgery still not provide sufficient functional restitution (Jordan et al., 2012). Moreover, the serious complications of the mechanical, bioprosthetic, and heart valve transplantation have been reported (Jordan et al., 2012; Khanna, Ross, & Monro, 1981). However, an autologous living valve replacement that produce hemodynamical functions, repairing/decreasing of ongoing tissue damage, and has long term durability and growth potential like those of the native heart valves, is an ideal therapeutic approach for heart valve transplantation. Therefore, stem cells derived- autologous heart valve cells in combination with possible potential in tissue engineering techniques could be a new way of VHD therapy.

Heart valves tissue composes of 3 major types of cells includes interstitial cells, endothelial cells, and passenger cells (Itoh et al., 2009). The valve interstitial cells (VICs) are the most prevalent cells in the heart valve and commonly found in all three layers of the valve, which is responsible for maintaining the structural integrity, creation of an extracellular matrix, and healing of the valve(Itoh et al., 2009). So, it is interesting that VICs is the new denomination and used for build up the valve construct.

Nowadays, using of "Dental Pulp Stem Cell" (DPSC) becomes more interesting as alternative therapeutic approaches in many diseases and hold great potential as new therapeutic strategies for regenerating and repairing of damaged tissues or organs (Gomes et al., 2010; Gronthos, Mankani, Brahim, Robey, & Shi, 2000). DPSC was first described as Mesechymal Stem Cell (MSC) like odontogenic precursor cells with contains high proliferative potential and has been shown to be able to differentiate preferentially to osteoblasts (Laino et al., 2006). This type of stem cell was demonstrated to be useful in cardiovascular disease treatment, as DPSC showed the therapeutic potential for myocardial infarction (Gandia et al., 2008). Moreover, recent report showed that mesenchymal stem cells derived from bone marrow (BMMSC) or adipose tissue (ADMSC) could potentially be induced into human valve interstitial cells (VICs) (Duan et al., 2015). However, the induction of DPSC to VIC has never been investigated elsewhere. In the current study, we hypothesized that isolated human DPSC can differentiate/ or be induced to be heart valve interstitial cells (VICs), which can be used for further heart valve regeneration. Due to the difficulty of obtaining the healthy human heart valve in Thailand, therefore, this research project aim to set up the preliminary experiments using isolated swine heart valve to prove in concept of the hypothesis.

Methods and Materials

Isolation of human dental pulp stem cell and culture

Human-impacted third molars were obtained with written informed consent from Dental clinic service, faculty of dentistry, Naresuan University. All procedures were approved by Ethical committee of Naresuan University. To isolate DPSCs, normal human third molars were obtained from four young healthy patients (age 20-25). The teeth were immediately cracked open, and the pulp tissue was removed and processed. Pulp was minced into small fragments prior to digestion in a solution of collagenase type I at 37°C. After centrifugation, cells were seeded in culture flasks with growth medium Dulbecco's Modified Eagles Medium (DMEM) with low glucose supplemented with 15% fetal bovine serum

(FBS). Cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C with DMEM supplemented with 15% FBS. Non-adherent cells were removed 48 hours after the initial plating. The medium was replaced every 3 days. Cells were collected by trypsinization and sub cultured in growth medium.

Determination of the growth curve and population doubling time

The isolated DPSC cells, at a concentration of 1 x 10^5 cells/ml, were cultured in a 24-well plate with complete medium at 37 °C, 5% CO_2, and 95% O_2 for 7 days. The MTT cell proliferation assay was performed by incubating with 0.5 mg/ml MTT reagent for 2 h at 37 oC. After incubation, the excess MTT reagent was discarded and DMSO was added for solubilizing the formazan dye, which then was collected and the determined optical density (OD) was spectrophotometer at λ 490 nm. The data was used to generate the growth curve. In addition, cells were also harvested and counted every day for 7 days. Cell numbers were used to calculate the PDT by the following formula.

$$Td = (t2 - t1) \times \frac{\log(q2)}{\log(\frac{q2}{q1})}$$

q1= Quantity of the cells at start time. (1 unit/h)

q2= Quantity of the cells at the end. (1 unit/h)

t1= starting time (h)

t2= ending time (h)

Characterization of isolated human DPSCs (Huang, Snyder, Cheng, & Chan, 2008)

DPSCs were cultured in 75 cm^2 flask, until 80% confluence, cells were harvested by using a scraper. Cells were washed twice with 0.1% NaN_3, 5% FBS in PBS (FACs buffer) and centrifuged at 1,800 rpm 10 min. After that, DPSCs were counted; a 1 x 10^5 cells were transferred into sterile microcentrifuge tube. Supernatant was gently removed and added anti-human antibody conjugated with fluorescent CD44 FIT-c, CD90 PE-cy5, CD117 PE-cy5, CD 34 PE-cy5, and CD 29 PE in each tube incubated in the dark environment for 60 min on ice. Then, cooled FACs buffer was added for washing. DPSCs were centrifuged at 2,000 rpm for 10 min. DPSC were washed again with cooled FACs buffer and centrifuged at 2,000 rpm for 10 min. After that, supernatant was removed and re-suspended with 1 ml cooled FACs buffer. Cells were transferred into flow tube and analyzed by using flow cytometer.

Osteogenic induction of isolated DPSCs

Human dental pulp stem cell were cultured in complete DMEM supplemented with 15% fetal calf serum, contained with 50 µg/ml ascorbic 2-phosphate, 10 nM dexamethasone, and 10 mM β glycerol phosphate in complete DMEM medium in 6 wells plate (Huang et al., 2008). Osteogenic induction medium was replaced every 3 days. After day 7, 14, 21 and 28, cells were were fixed with 4 % PFA for 30 min and stained with 40 mM alizarin red for 30 min at room temperature and observed under microscope.

Isolation of swine valve Insterstitial cell (Gould & Butcher, 2010)

Swine aortic heart valves were taken from male swine hearts. Primary VICs were prepared from trimmed valve cusps. Valve cusps were incubated with cold collagenase solution (600 U/ml in DMEM culture medium supplemented with 10% FBS, 5,000 units of penicillin and streptomycin) for 5-10 min at 37°C. After that, a dry sterile swap was used for removing the VECS gently until feel smoother. After isolation of the VECs, aortic valve cusps were immediately incubated with collagenase solution (600 U/ml in DMEM culture medium supplemented with 10% FBS, 5,000 units of penicillin and streptomycin) for 5-10 min at 37°C for 16 hours. After 16 h, the degraded cusps were gently re-suspended

and homogenized solution was centrifuged at 1,000 rpm for 5 mins for 2 times. After that, primary VICs were seeded on culture flask.

Characterization of isolated human VICs by determining the expression of α-smooth muscle actin, vimentin, and collagen type 1 (Butcher & Nerem, 2006; Latif et al., 2015)

Isolated human VICs were seeded on 6-well plate until reached 80% confluence or more. Cellular protein extraction was performed by adding 2X SDS sample buffer containing 10% (v/v) β-mercaptoethanol and boiled for 5-10 min. Protein extract was separated under SDS-Polyacrylamide gel electrophoresis and transferred to PVDF membrane. The PVDF membrane was probed with the primary antibodies against α-smooth muscle actin, vimentin, and collagen type 1, followed by horseradish peroxidase-conjugated secondary antibody. The antibody-antigen complexes were visualized by enhanced chemiluminescence and detected by gel documentation.

Induction of isolated human dental pulp stem cell to isolated swine valve interstitial cell (Butcher & Nerem, 2006; Latif et al., 2015)

Isolated human DPSCs and swine VICs was Co-cultured using transwell chamber. After that, the DPSCs at concentration of 1 x 10^4 cells/ml were seeded in the lower chamber and cultured for another 12 h for cell attachment and spreading. Later, the isolated VICs at the concentration of 1 x 10^5 cells/ml in a volume of 0.5 ml was added to upper chamber. Then cells in transwell chamber were incubated for 7, 14, 21 and 28 days with upper chamber compartment change every 3 days. After day 7, 14, 21 and 28, DPSCs in each group was used to determine the expression of α-smooth muscle actin, vimentin, and collagen type 1 by Western blot analysis.

Statistical analysis

Data was presented in Mean ± Standard Error of Mean (S.E.M) and was analyzed by Graph Pad Prism 5.0. The different of coefficient of variance (CV) in each group was analyzed by ANOVA or t-test. A p value < 0.05 was considered as statistically significant.

Results and Discussions

Isolation and characterization of human dental pulp stem cells (hDPSCs)

The human dental pulp stem cells (hDPSCs) were obtained from third molars of healthy subject. After the pulp tissue was removed and digested with collagenase type II, the pulp tissue was place on culture dish and the DPSCs was explant outgrowth as showed in Fig. 1A and 1B. The results showed that the morphology of isolated DPSCs was in fibroblast-like cells with high rate of proliferation. The calculated population doubling time as 34.65 h and the growth curve showed the sigmoid curve with the linear regression equation y = 0.2574x - 0.1482 (R^2=0.9422) (Fig. 1C). Isolated cells were subcultured and determined for the expression of cell surface molecular markers of stem cells by flow cytometry. The results showed that isolated human DPSCs expressed stem cell surface markers including CD29 (76.09%), CD44 (89.08%), CD90 (97.99%), CD105 (66.84%), when compared to isotype control. These cell surface molecules are specifically indicated that isolated DPSCs expressed mesenchymal stem cell characteristics (Fig. 2A). In contrast, DPSCs were negatively expressed hematopoietic stem cell surface markers CD34 (27.81%), CD117 (18.77%), when compared to isotype control as shown in Fig. 2B. These results suggested that isolated DPSCs expressed mesenchymal stem cell characteristics, but not hematopoietic stem cell characteristics.

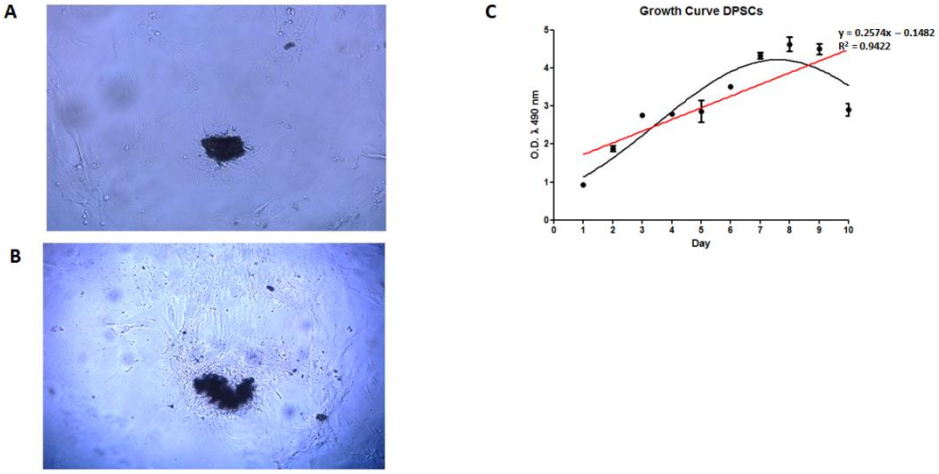

Figure 1. Microscopic examination of isolated human dental pulp stem cells (A) the morphology of expand out growth cells (10X). (B) the morphology of expand out growth cells (20X). (C) The growth curve of isolated DPSCs showed the rate of cell proliferation characteristic of isolated human DPSCs and the linear regression of growth curve.

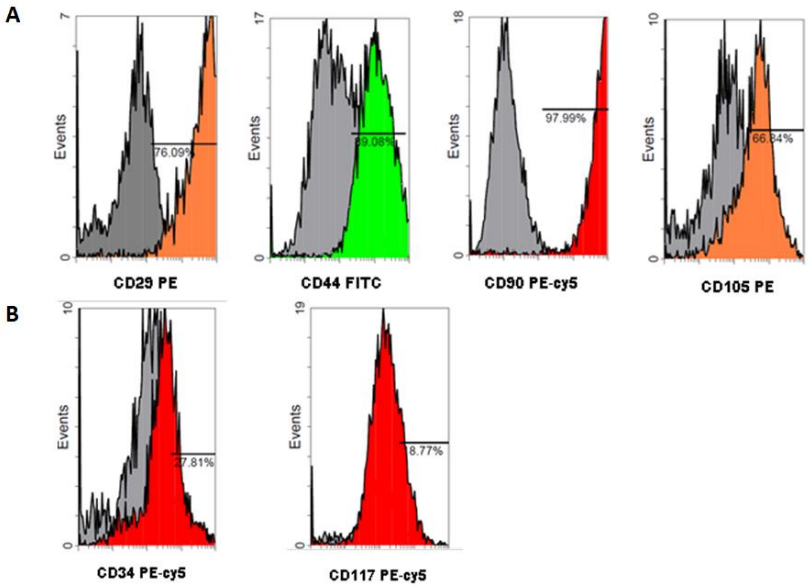

Figure 2. Flow cytometric analysis of the expression of cell surface molecules specific for mesenchymal stem cell characteristics (CD29, CD44, CD90, CD105), and hematopoietic stem cell markers (CD34 and CD117).

In addition to stem cell specific surface markers expression, identification of the potency of stem cell to differentiate into various types of tissue is the most common and reliable way to identify their multipotency. Mesenchymal stem cell can differentiate into adipocytes, osteoblasts, myocytes, and chondrocytes *in vivo* and *in vitro* [1,4]. In this report, the isolated human DPSCs were determined for their potency to differentiate into osteogenic cells by culturing the DPSCs in osteogenic induction medium for 7 days, 14 days, 21 days, and 28 days and stained with alizarin red for determining mineralization ability. The results showed that culture of DPSCs in osteogenic induction medium for 21 days and 28 days could clearly detect the positive alizarin red staining(Fig. 3). These results suggested that isolated DPSCs not only expressed stem cell surface molecules, but also expressed the stem cell characteristic of multipotency. Therefore, these isolated DPSCs could be used in further experiments.

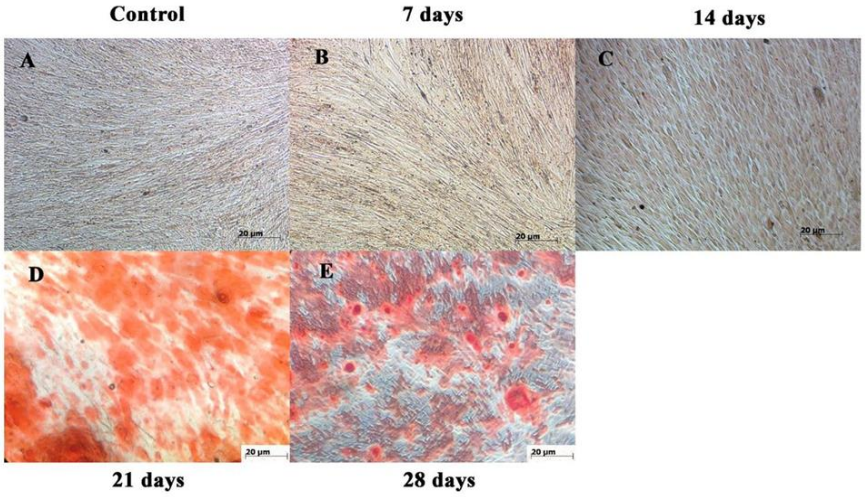

Figure 3. Alizarin red staining in isolated human DPSCs. Cells were cultured in osteogenic induction medium for 7, 14, 21 and 28 days. After incubation periods, cells were stained with Alizarin red for detecting calcium deposition (mineralization).

In addition, protein markers specifically expressed in VICs such as α-smooth muscle actin, vimentin, collagen type 1, was also determined in isolated hDPSCs in attempt to measure the basal expression level of these proteins before performing the induction to VICs. The results showed that isolated hDPSCs could express these 3 proteins in very low level (Fig 5A).

Isolation and characterization of swine valvular interstitial cells (sVICs)

Due to the difficulty of obtaining the healthy human heart valve in Thailand, therefore, this research project aim to set up the preliminary experiments using isolated swine heart valve. Heart valves were dissected and isolated for VICs by enzymatic digestion. Isolated swine VICs showed the fibroblast-like morphology with long spindle shape (Fig 4A, 4B). After obtaining homogenous cell populations, cells were cultured and extracted for cellular proteins, which was then be determined for the expression of specific protein markers including α-smooth muscle actin, vimentin, collagen type 1. The results showed that isolated swine VICs could strongly expressed α-smooth muscle actin, vimentin, collagen type 1 (Fig.4C).

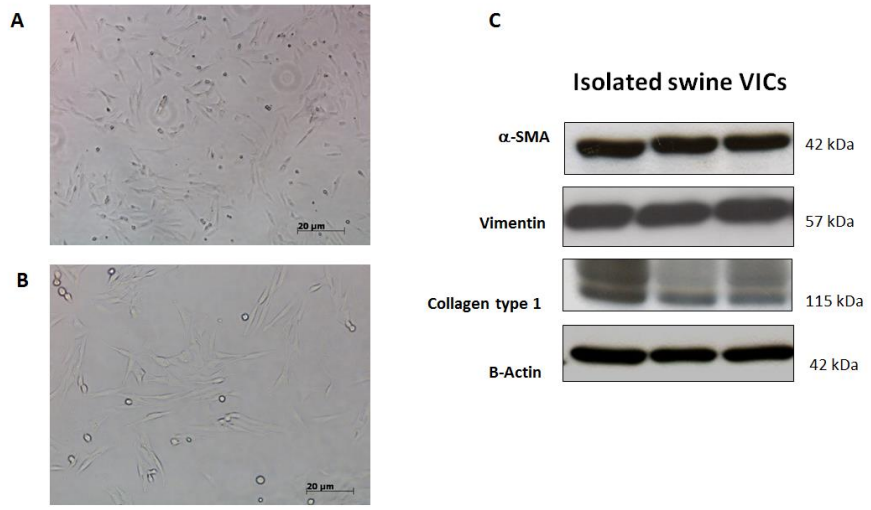

Figure 4. Microscopic examination of isolated swine valvular interstitial cells (VICs) (A) the morphology of VICs cells with 10X magnification. (B) the morphology of VICs cells with 20X magnification. (C) The Western blot analysis showed the expression of cellular proteins extracted from isolated swine VICs including α-smooth muscle actin, vimentin, collagen type 1, with loading control of β-actin.

Induction of human dental pulp stem cells to swine valvular interstitial cells

As the results in this study showed that isolated human DPSCs presented the Mesechymal Stem Cell (MSC) characteristics with contained high proliferative potential and could differentiate. Therefore, DPSCs were then induced to express VICs characteristics by culturing these 2 types of cells in transwell chamber for 4 weeks. At the end of incubation periods, DPSCs were extracted for cellular protein and determined the expression of VICs proteins including α-smooth muscle actin, vimentin, collagen type 1 by Western blotting. The results showed that cultured DPSCs with VICs for 4 weeks could not be induce to expression the VICs specific proteins. There was only a slight increase in collagen type 1 protein in indueced DPSCs. However, the overall expression of these cellular proteins was not different from the basal level expressed in DPSCs (Fig. 5). These results suggested the unsuccessful of stem cells induction to valvular interstitial cells.

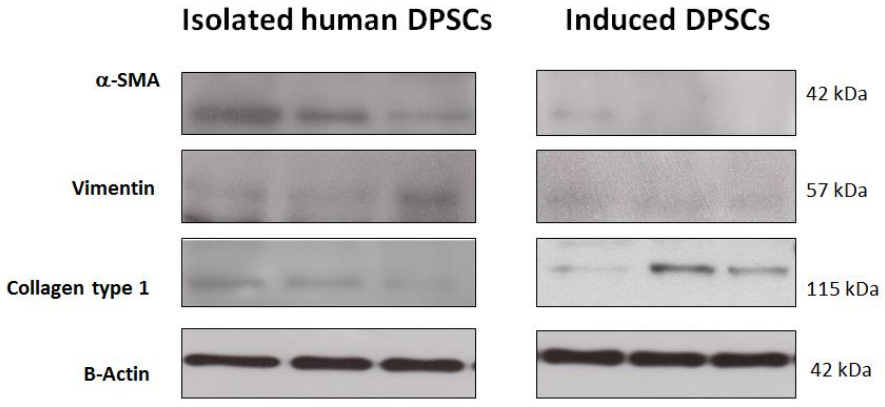

Figure 5. The Western blot analysis showed the expression of cellular proteins extracted from isolated isolated human DPSCs and DPSCs cultured with swine VICs in transwell chamber. The cellular proteins were extracted and mearing the n expression of α-smooth muscle actin, vimentin, collagen type 1, with loading control of β-actin.

The findings in this study was different from the recent reported by Duan B. et al. in 2015 that mesenchymal stem cells derived from bone marrow (BMMSC) or adipose tissue (ADMSC) could potentially be sources for tissue-engineered heart valves (TEHV), and could be induced into paediatric human aortic valve interstitial cells (pHAVIC) in relevant 3D environments (Duan et al., 2015). Interestingly, this study was the only study to show that MSC could be induced into heart valve cells. Since, DPSCs is also categorized in MSC group, therefore, it could potentially be induced into heart valve cells as well. However, there are no any study report the potency of DPSCs to be induced to heart valve cells and its application for tissue-engineered heart valves. The factors that could possibly lead to the limitations of our study could be the source of valvular tissues. Since, we could not obtain any healthy human valvular tissue, in contrast the sample in Duan B. et al was obtained the healthy whole heart valvular tissue from 12-year-old patient undergoing cardiac transplant for a myocardial contractility deficiency(Duan et al., 2015), which is quite difficul in our experimental settings that most of the patients who went to heart valve operation, particularly in Thaialnd, were valvular tissue with pathological conditions. Therefore, in this study, we could not obtain any human valvular tissue that qualified for VICs isolation. Although postmortem biopsy of human heart valve could possibly be another choice, but freshly isolation of heart valve is also impossible in this study. These points are considered as a limitation of this study. Therefore, in this study model was designed to use swine heart valve tissue for VICs isolation, just in case to be a VICs model for pre-liminary study. Another interesting issue is the method of induction. In Duan B. et al study, the research cultured cells in three-dimensional (3D) bioactive hybrid hydrogels with material modulus comparable to that of aortic heart valve leaflets. This microenvironment could be a significant factor for heart valve cells induction. The most important point that is inevitably mentioned could be the different in specie of the cells, as in this study we used isolated human DPSCs to be induced into swine VICs. The different in specie could possibly be a major drawback of this work. Therefore, using the specie matched cells, such as swine VICs and swine DPSCs, could provide more information and more possible for induction. Furthermore, detection of cellular differentiation could be performed not only at the protein level by Western blot analysis; however, gene expression level by real-time PCR could provide more information.

Conclusion

In summary, the present study demonstrates isolated human dental pulp stem cells contain mesenchymal stem cell characteristics and differentiation potential. This study could successfully isolate swine valvular interstitial cells and characterized these isolated cells by various specific proteins. However, the induction of isolated DPSCs to VICs could not successfully achieve in these experimental settings.

Acknowledgements

This study was financially supported by Naresuan University Research endowment fund and National Research Council of Thailand (NRC) Grant I.D. number R2558B048.

References

Heart Valve Surgery. Retrieved 1 August, 2015, from http://www.mayoclinic.org/heart-valve-surgery/.

World Heath Statistics. 2008. Retrived 13 June, 2015, from http://www.who.int/entity/whosis/whostat/EN_WHS08_Full.pdf.

Butcher, J. T., & Nerem, R. M. (2006). Valvular endothelial cells regulate the phenotype of interstitial cells in co-culture: effects of steady shear stress. *Tissue Eng*, *12*(4), 905-915.

Duan, B., Hockaday, L. A., Das, S., Xu, C., & Butcher, J. T. (2015). Comparison of Mesenchymal Stem Cell Source Differentiation Toward Human Pediatric Aortic Valve Interstitial Cells within 3D Engineered Matrices. *Tissue Eng Part C Methods, 21*(8), 795-807.

Gandia, C., Arminan, A., Garcia-Verdugo, J. M., Lledo, E., Ruiz, A., Minana, M. D., et al. (2008). Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction. *Stem Cells, 26*(3), 638-645.

Gomes, J. A., Geraldes Monteiro, B., Melo, G. B., Smith, R. L., Cavenaghi Pereira da Silva, M., Lizier, N. F., et al. (2010). Corneal reconstruction with tissue-engineered cell sheets composed of human immature dental pulp stem cells. *Invest Ophthalmol Vis Sci, 51*(3), 1408-1414.

Gould, R. A., & Butcher, J. T. (2010). Isolation of valvular endothelial cells. *J Vis Exp*(46).

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

Huang, A. H., Snyder, B. R., Cheng, P. H., & Chan, A. W. (2008). Putative dental pulp-derived stem/stromal cells promote proliferation and differentiation of endogenous neural cells in the hippocampus of mice. *Stem Cells, 26*(10), 2654-2663.

Itoh, A., Krishnamurthy, G., Swanson, J. C., Ennis, D. B., Bothe, W., Kuhl, E., et al. (2009). Active stiffening of mitral valve leaflets in the beating heart. *Am J Physiol Heart Circ Physiol, 296*(6), H1766-1773.

Jordan, J. E., Williams, J. K., Lee, S. J., Raghavan, D., Atala, A., & Yoo, J. J. (2012). Bioengineered self-seeding heart valves. *J Thorac Cardiovasc Surg, 143*(1), 201-208.

Khanna, S. K., Ross, J. K., & Monro, J. L. (1981). Homograft aortic valve replacement: seven years' experience with antibiotic-treated valves. *Thorax, 36*(5), 330-337.

Laino, G., Carinci, F., Graziano, A., d'Aquino, R., Lanza, V., De Rosa, A., et al. (2006). In vitro bone production using stem cells derived from human dental pulp. *J Craniofac Surg, 17*(3), 511-515.

Latif, N., Quillon, A., Sarathchandra, P., McCormack, A., Lozanoski, A., Yacoub, M. H., et al. (2015). Modulation of human valve interstitial cell phenotype and function using a fibroblast growth factor 2 formulation. *PLoS One, 10*(6), e0127844