Ultrastructure changes in BMP-2-treated SHED cultured on glycerol preserved human amniotic membrane

Z. Faizuddin Osman[a], W. Zahari[a], T. P. Kannan[a], A. Ahmad[a], K. B. A. Amin Noordin[a*]

[a] School of Dental Sciences, Universiti Sains Malaysia, Kota Bharu 16150, Kelantan, Malaysia.

*Corresponding author, Email: kbariah@usm.my

Received: 14-07-20 Accepted: 10-03-21
Published: 09-04-21

ABSTRACT
SHED is known for its capabilities to extensive proliferation and multipotential differentiation. The utilization of natural scaffold such as amniotic membrane, for SHED differentiation into odontoblast-like cells offers great potential in dental regeneration. This study aims to investigate the ultrastructure transformation during differentiation of SHED into odontoblast-like cells of SHED on AM *in vitro* using scanning electron microscope (SEM). SHED was cultured on AM scaffold and treated with bone morphogenetic protein-2 (BMP-2) growth factor for 1, 7, 10 and 14 days, respectively**.** SHED cultured on AM without BMP-2 served as control. Formaldehyde-based protocol was used to fix the samples for imaging. Samples were gold-coated with sputter coating machine SCD 0005 and viewed by SEM FEG450 at 5000x magnification**.** SEM imaging showed the surface structure of AM was fibrous. Nanofibrous structure is known to facilitate interaction of the cells for adherence and infiltration into this scaffold. SHED displayed cell extensions with small finger-like or web-like projections at the very end of the cell after a day of BMP-2 treatment. On the following days, SHED lost the fibroblast-like morphology, became smaller and round-shaped. Emergence of odontoblast-like cell with columnar cell body and several processes were detected on day 7. By day 14, strong mineralization on the cell body was observed, suggesting a matured odontoblast. In control group, SHED appearance as fibroblast-like cell morphology remained up until day 14 of culture**.** In conclusion, morphological evaluation via SEM confirms AM support for a complete differentiation of SHED into odontoblast-like cells by day 14 of culture.

Keywords: Amniotic membrane; cell attachment; gold sputter-coating; natural scaffold; scanning electron microscope.

Cambios ultraestructurales en SHED tratado con BMP-2 cultivado en una membrana amniótica humana preservada con glycerol

RESUMEN
SHED es conocido por sus capacidades de proliferación extensiva y diferenciación multipotencial. El uso de andamiajes naturales, como la membrana amniótica, para la diferenciación del SHED en células similares a los de los odontoblastos ofrece un gran potencial en la regeneración dental. Esta investigación tiene por objetivo estudiar la transformación de la ultraestructura de SHED en AM *in vitro*, durante su proceso de diferenciación en células similares a los odontoblastos, utilizando la técnica de microscopía electrónica de barrido (MEB). Se cultivó SHED en un soporte de AM y se trató con un factor de crecimiento de proteína morfogenética ósea tipo 2 (BMP-2) durante 1, 7, 10 y 14 días, respectivamente. El SHED cultivado en AM sin BMP-2 sirvió como control. Se utilizó un protocolo basado en formaldehído para fijar las muestras para la obtención de imágenes. Las muestras se recubrieron con oro empleando la técnica de pulverización catódica SCD 0005 y se observaron mediante microscopía electrónica de barrido (MEB FEG450) con un aumento de 5000x. Las imágenes del MEB mostraron que la estructura de la superficie de AM era fibrosa. Se sabe que la estructura nanofibrosa facilita la interacción de las células para la adherencia e infiltración en este soporte. El SHED mostró extensiones de células con pequeñas proyecciones en forma de red en el extremo de la célula después de un día de tratamiento con BMP-2. En los días siguientes, el SHED perdió la morfología de fibroblastos, se volvió más pequeño y redondeado. El día 7 se detectaron diversos procesos y la aparición de una célula similar a un odontoblasto con cuerpo columnar. Para el día 14, se observó una fuerte mineralización en el cuerpo celular, lo que sugiere un odontoblasto maduro. En el grupo de control, la apariencia del SHED, como morfología celular similar a fibroblastos, se mantuvo hasta el día 14 del cultivo. En conclusión, la evaluación morfológica a través del MEB confirma que la AM sirve como andamio para una diferenciación completa del SHED en células similares a odontoblastos el día 14 del cultivo.

Palabras claves: Membrana amniótica, adherencia celular, recubrimiento de oro por pulverización catódica, andamiaje natural, microscopio electrónico de barrido.

INTRODUCTION

Ultrastructural morphology analysis is one of the techniques of confirming the differentiation process of stem cells. Scanning electron microscope (SEM) is a widely used protocol to evaluate ultrastructure morphology and can be carried out using an establish methods such as formaldehyde based protocols. This technology is very useful in regenerative medicine, which is rapidly developing as a tool to repair and regenerate tissues using own body cells.

The essential properties of amniotic membrane (AM) as a scaffold in tissue engineering application are depicted in the biomaterial ability to support cell attachment, proliferation and differentiation. AM is a naturally derived scaffold, which is originally resides in the innermost layer of placenta. The structure of AM consists of a stromal layer, a thick basal lamina and a monolayer of endothelial cells.

In dental tissue engineering, the capability of stem cells from human exfoliated deciduous teeth (SHED) differentiation into odontoblast-like cells when cultured on AM in the presence of bone morphogenetic protein 2 (BMP-2) has yet to be described.

MATERIALS AND METHODS

Commercially sourced SHED (AllCells, USA) was cultured on de-epithelialized 2 cm x 2 cm glycerol-preserved γ-radiated AM in a culture dish containing 3 ml of media at different cell density for the respective days as the following; $2x10^5$ for day 1, $5x10^4$ for day 7, $2.5x10^4$ for day 10 and $1.5x10^3$ for day 14. Different numbers of cells were seeded during the initial culture to avoid over confluence of cells in the culturing flask. Prior to seeding the cells, AM scaffold was de-epithelialized using thermolysin. De-epithelialization of AM was achieved by using 125 μg/ml thermolysin at room temperature for 45 minutes. There were two groups, SHED cultured on AM scaffold (SA) and SHED cultured on AM with BMP-2 treatment (SAB). SA group was designated as control for this study.

On day 1, 7, 10 and 14 of culture, all groups were washed with phosphate-buffered saline (PBS). Then, they were subjected to formaldehyde-based protocol for SEM samples. Each AM scaffold was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 2 hours at room temperature. The membranes were washed again with PBS and immersed in 8% formaldehyde at 4°C. After 2 days, the membranes were dehydrated in 20, 30, 40, 50, 60, 70, 80, 90 and 100% graded alcohol for 10 min accordingly. Subsequently, the membranes were incubated in hexamethyldisilazane (HMDS) for 10 minutes, and later, were dried in a desiccator for 10 minutes. Samples were mounted on plastic microscope slides using high purity conductive double-sided adhesive carbon tapes. Prepared samples were gold-coated with sputter coating machine SCD 005 (Leica, Germany) and viewed by SEM FEG 450 (Quanta, Czechoslovakia).

RESULTS

SEM micrographs allow for observation of cell surface connections, formation of cell surface structures and mineral deposition on the surface of the cells. In this study, morphological appearance of SHED following BMP-2 treatment was carefully studied. Any phenotypic changes of the cells were noted in comparison with the control group. Physical interaction of SHED with the AM surface was revealed by SEM. Effects of BMP-2 growth factor on the phenotypic changes of the cells were observed on day 1, 7, 10 and 14 of culture.

The epithelial layer was successfully removed using thermolysin to provide an optimum surface for SHED establishment on the AM. As shown in figure 1, the structural organization of AM structure was carefully examined at 5000x magnification and showed that the surface structure of AM was fibrous and clearly visible. The nanofibrous structure is known to facilitate

interaction of the cells for adherence and infiltration into this scaffold.

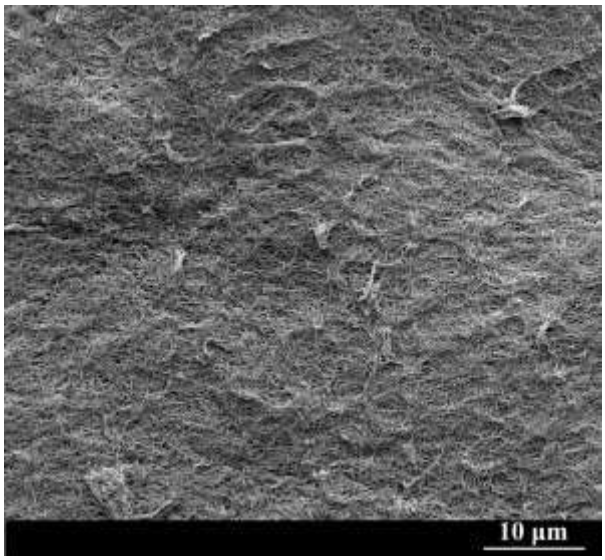

Fig. 1. SEM images showing the surface of the basal side of the AM at 5000x magnification. It displayed a dense and crossed fibrous structure on its surface.

Figure 2 presents the images obtained by scanning electron microscopy of SHED cultured on AM.

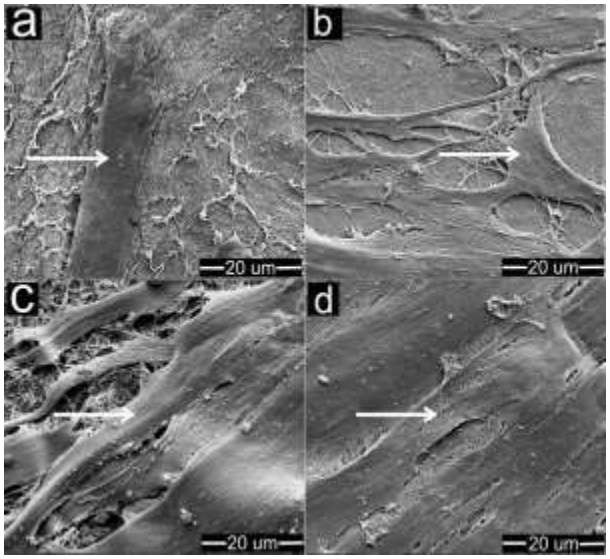

Fig. 2. SEM images of SHED cultured on AM on basal side of the membrane at day 1, 7, 10 and 14 in 5000x magnification. a) SHED (arrow) able to attach on AM surface at day 1; b) SHED (arrow) able to attach and proliferate on AM basal surface at day 7 with higher number of cells was observed; c) SHED (arrow) forms layer of cells on AM surface at day 10; d) SHED (arrow) shows cells confluence on AM surface at day 14.

SEM micrographs showed in figure 2 depict strong adherence of SHED to the AM surface since day 1 of culture. Further incubation of the cells showed progressive proliferation activities indicating successful interaction and support of AM with the cells. It can be clearly seen on day 10 (figure 2c) that nearly the entire AM surface had been covered with the cells. When the cells continued to proliferate, the cells seemed to grow overlapping each other while maintaining their fibroblast-like structure at all time points as shown in figure 3. Multiple layers of SHED covering the AM surface were observed in figure 2d.

In BMP-2 treated group, at magnification of 1000x, SHED appeared to be spread-out and well attached to the AM surface one day after BMP-2 treatment (figure 3). Similar with the control group in figure 2, the cells were seen in shape of fibroblast-like structure.

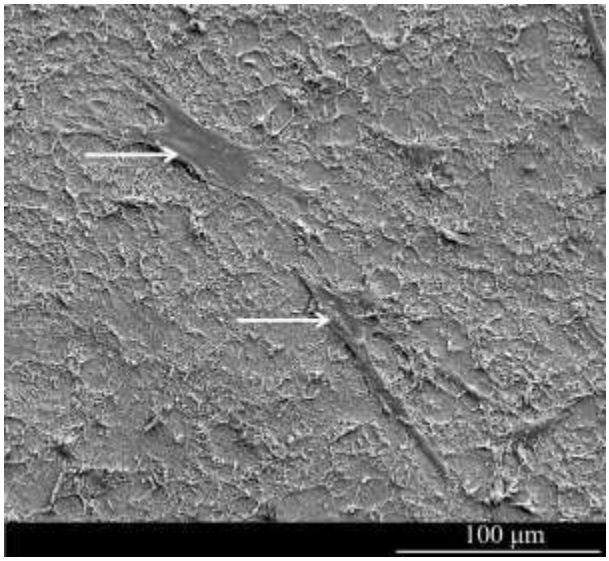

Fig. 3. SHED (arrows) were spread-out and well attached to the AM surface one day after BMP-2 treatment. Similar with the control group, the cells were seen in shape of fibroblast-like structure (1000x).

In a closer view at magnification of 5000x, the cells treated with BMP-2 displayed extensions further sprouting out small finger or web like projection at the very end of the cell (figure 4a), unlike the control group as shown in figure 2. Furthermore, it was evident that BMP-2 growth

factor enhanced SHED proliferation activities as compared to the control. On day 7 following the treatment, nearly all surface of the scaffold was covered with SHED. Moreover, it was observed that some cells started to lose the fibroblast-like morphology and became smaller and round-shaped figure 4(b-d). Emerging of phenotypic characteristics of odontoblast-like cell and a columnar cell body with several processes were detected at Day 7 as observed in figure 4b. The cells became rounder in shape with more structure of processes around the cell body on day 10. By day 14, a complete structure of odontoblast-like cells with strong cell induced mineralization on the cell body was observed. It is known that mineralization is one of the important criteria of mature odontoblast differentiation. The morphology of SHED transforming into odontoblast-like cells with mineral composition is shown in figure 4(d).

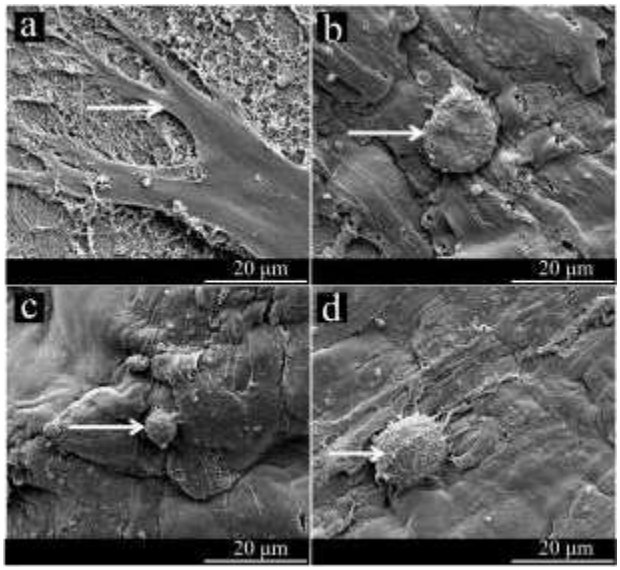

Fig. 4. Differentiation of SHED to odontoblast-like cells on AM scaffold at day 1, 7, 10 and 14 in 5000x magnification. a) after a day of BMP-2 treatment, SHED displayed cell extensions further sprouting out small finger or web like projection at the very end of the cell. On the following days of culture, SHED lost the fibroblast-like morphology and became smaller and round-shaped (b - d). Emerging of phenotypic characteristics of odontoblast-like cell and a columnar cell body with several processes were detected at Day 7 (b). By day 14, strong cell induced mineralization on the cell body was observed suggesting morphology of mature odontoblast (d).

DISCUSSION

SHED is postnatal stem cells capable of extensive proliferation and multi-potential differentiation [1]. Growing evidences show that SHED differentiation can be directed into odontoblast-like cells under stimulation of various growth factors and scaffolds. Nevertheless, such potential using AM scaffold and BMP-2 growth factor is yet to be elucidated. This study attempted to visualize the ultrastructure of SHED cultured on AM and differentiated into odontoblast-like cells using SEM. For the first time, SEM investigation confirmed odontoblast-like cells phenotype following BMP-2 treatment on SHED. It is very important to notice that based on the SEM images, morphological transformation of SHED into odontoblast-like structures was observed within 14 days of culture.

Scaffold is an essential component in successful tissue engineering triad, which consists of scaffold, stem cells and growth factor. An ideal scaffold should provide functionality with suitable environment for cell attachment, proliferation, migration and differentiation. In addition, cell adhesion to scaffold is also considered as an important parameter in evaluating the suitability of the scaffold for tissue engineering. The interaction between cells and natural scaffolds is a complex process involving numerous aspects such as behavior of the cell, material surface properties and environmental factors. To date, study has shown that chemical composition and topography of the surface could accelerate cell adhesion, proliferation, migration and differentiation [2]. Cells adhesion and proliferation on scaffolds define the early success of the cell-scaffold interaction and influence the subsequent behavior of the target cells.

AM is an ideal scaffold for tissue engineering due to its flexibility, enrich with growth factors [3], low immunogenicity and easy to process. AM, the inner layer of the fetal membrane, consists of a double layer of epithelium, basement membrane and avascular stroma [4]. AM used in this study is a gamma-radiated glycerolized type of membrane. The use of glycerolized AM is

preferable as it is easier to store compared to cryopreserved AM which requires expensive storage in -80°C. In this study, AM used for SHED scaffold was treated with thermolysin to help in de-epithelialization of the membrane. The general consensus is that intact epithelium hinders the uniform expansion of cultured cells, and may reduce the formation of strong integrin-based adhesion complexes such as hemidesmosomes with the basement membrane [5] is indeed a critical step, which in turn will not assist in the attachment of SHED onto AM scaffold. The removal of the amniotic epithelial cells in order to allow the *ex vivo* cultured epithelial cells to interact with the underlying basement membrane [6]. Therefore, the ability to remove the epithelial cell layer whilst maintaining the integrity of the underlying basement membrane were the main considerations when using thermolysin as the denuding agent for AM in this study. De-epithelialization of AM using thermolysin is important to prepare the basement membrane for cell attachment and proliferation.

Morphology and structure of the AM scaffold were observed under SEM, as shown in figure 1 at 5000x magnification. AM scaffold on the basement membrane side showed a uniformed fibrous structure across the surface. The fibrous structure of the AM helps in SHED attachment onto the scaffold, which is important for the bioresorbability of the material and plays an important role in the odontogenic conductivity of the scaffold. According to Wang et al. nanofibrous surface helps in cell seeding, migration and growth as well as cell proliferation and differentiation [7].

The basement side of the membrane was used in this study as the surface for seeding SHED on AM scaffold due to its superiority in comparison with epithelial and stromal sides of the AM. It was made up of Type IV, V and VII collagen in addition to fibronectin and laminin [8]. These elements help in cell attachment to the scaffolds. To the best of our knowledge, there are no previous reports studying the efficacy of AM as scaffold for odontogenic differentiation and in this study the potential use of AM as scaffold in dental regeneration and details regarding the odontoblast differentiation on AM platform will be further elaborated below.

SEM imaging of SHED showed a clear attachment of the cells on AM surface while maintaining its fibroblastic morphology (figure 2). The flattened shape of SHED was a sign of good and healthy cells compared to rounded form of the cells, which may indicate non-uniform cell morphology. The cell adhesion of SHED seeded on AM scaffold was visualized within 24 hours following cell seeding (figure 2a). On day 7 of seeding, it was observed the cells had spread well, had a close contact with the surface of the AM scaffold, and had migrated to cover the surface of the membrane (figure 2b). In addition, fibroblastic morphology of SHED with normal spindle-shaped cells on the covered membrane surface was observed. The fibroblastic morphology remains in the observation until day 14 without any changes of the morphology and characteristics of the SHED (figure 2d). This result suggests that although SHED is really capable of differentiating into multiple types of cells, but when seeded on AM scaffold without growth factor treatment, the cells did not undergo cells differentiation process. These findings are consistent with those found in previous study [9], who evaluated the odontogenic potential of SHED when seeded in human tooth slice/scaffolds and cultured *in vitro* or implanted subcutaneously into immunodeficient mice. Their results showed that SHED cultured in deproteinised tooth slice/scaffolds, or scaffolds without a tooth slice, did not express odontogenic markers. Notably, blockade of BMP-2 signaling inhibited the expression of markers of odontoblastic differentiation by SHED cultured in tooth slice/scaffolds.

The adhesion, proliferation and migration of SHED and its differentiation throughout the 14 days of observation on nanofibrous surface of AM demonstrates that AM scaffold provides an ideal extracellular matrix (ECM) for the regeneration of odontoblast-like cells. These findings

were consistent with previous studies [7] showing that poly-L-lactide (PLLA) scaffolds with similar nanofibrous architecture like the one on AM served as a better scaffold surface for dental pulp stem cells (DPSCs) proliferation and the production of ECM. A previous study also demonstrated that nanofibrous architecture enhanced protein adsorption, including fibronectin and vitronectin, contributing to pre-osteoblast cell attachment [10].

It is noteworthy to add that AM properties assist in supporting SHED survival, growth and differentiation on AM scaffolds. The basement membrane, also known as the basal lamina, consists of the lamina lucida and the lamina dense, and is supported by an underlying morphologically distinct extracellular stromal matrix. It acts as a mechanical scaffold and the key basement membrane proteins also act as regulators in epithelial morphogenesis, growth, proliferation, and differentiation and in preventing apoptosis [6]. Collagen is a major component of AM and it has been shown that this material drives the viability of the cells and supported the odontoblastic differentiation of DPSCs *in vitro* [11]. Collagen-based scaffolds also have potential towards the application in dental pulp tissue engineering, for the similarity of having collagen as a major component in both collagen-based scaffolds and dental pulps. Notably, collagen has been shown to support DPSCs survival and growth [12].

After several washes before taking the sample for SEM viewing, it was observed from SEM images that SHED cells were well attached to the scaffold (figure 3). A possible interpretation of the enhanced cell attachment and growth could be that filopodia played an important role in biological processes and nanofibrous architecture could have altered the mode of anchorage, allowing filopodia to anchor more tightly. In addition, the local mass transport condition in the nanofibrous scaffolds is likely better than those in solid-walled architecture. The nanofibrous pore walls might improve nutrient/oxygen supply to and metabolic waste removal from the attached cells, contributing to better extracellular environment for DPSCs growth and ECM production [7].

Based on our results, it was noted that morphological changes of SHED occurred between day 7 to day 14 following BMP-2 growth factor treatment suggesting formation of odontoblast-like cells formation (figure 4). The formation of cell morphology with highly proliferative characteristics was observed for up to 14 days. The appearance of abundant fibroblastic layer of SHED also appeared to change during the later stage of culture at day 7 onwards, and the presence of mineral aggregates on the AM surface showing the mineralization of odontoblast. These findings are consistent with studies of DPSCs cultured on 3D calcium phosphate surface [13]. During the formation of abundant fibrillar collagenous protein, the highly proliferative cell morphology observed for up to 14 days also appeared to change during the later stage of culture, as indicated by the presence of mineral aggregates on the 3D calcium phosphate surface [13].

In the current study, the phenotype of the differentiated odontoblast-like cells from SHED on AM could be visualized using SEM for the first time. This study showed the essential role of BMP-2 growth factor in influencing the differentiation process *in vitro*. The appearance of phenotypic characteristics such as columnar cell body with several processes, and formation of mineral further proved the odontoblastic differentiation. Morphological evaluation via SEM confirms complete differentiation of SHED into odontoblast-like cells by 14 day of culture. These findings are consistent with a study which showed the appearance of spherical structures on cells compatible with matrix vesicles and mineralization nodules produced by osteoblasts differentiated from DPSCs at 14 days of culture with osteogenic culture medium [2]. The ability to mineralize is an essential phase in the cell-induced dentin formation process which can only be performed by odontoblast, besides indicating a physiological development of the cells [14]. In our 3D construct it is suggested that the role of BMP-2 not only

induce odontogenic differentiation but the growth factor also induce mineralization of the odontoblast-like structure.

In vitro SEM analysis of AM scaffolds seeded with SHED showed that cells had spread well on their surfaces. Most of the cells had multiple filopodia attached on the cell body. This is in agreement with the results of [15] and [16]. In addition, after 14 days AM scaffolds were loaded with SHED and incubated in odontogenic differentiation media, abundant formation of collagen-like structures attaching the cells to the scaffolds was detected. It was also demonstrated the presence of many mineralized odontoblast-like cells on the membrane surface. On the other hand, the AM scaffolds loaded with SHED and incubated in plain media in the absence of BMP-2 growth factor revealed no odontoblast-like cells, mineralized signs and collagen-like structures.

This study demonstrates a potential application of SHED cultured on AM with BMP-2 growth factor for odontoblast differentiation in dental regenerative medicine. AM scaffold enhanced the odontogenic differentiation of SHED and mineralization *in vitro* with the presence of BMP-2 growth factor and is a promising scaffold for dentin regeneration. The results suggested that the SHED on AM scaffold underwent complete odontoblastic differentiation with the stimulation of BMP-2 within 14 days of culture.

CONCLUSIONS

Stimulation of AM as a scaffold for SHED differentiation into odontoblast-like cells with the assistance of BMP-2 growth factor may provide a great platform for further exploration of possible clinical application in the future. Nanofibrous structure of AM scaffold greatly helps the attachment and adhesion of SHED cells on the surface. Differentiation of SHED into odontoblast-like cells complete in 14 days of culture as demonstrated from the ultrastructure images. In conclusion, these studies confirmed for the first time that complete differentiation process of SHED into odontoblast-like cells occur via BMP-2 stimulation within 14 days. This observation was supported via ultrastructure findings of the study.

ACKNOWLEDGEMENT

This study was supported by Research University Grant 1001.PPSG.812127 and Universiti Sains Malaysia Postgraduate Assistant Scheme.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

[1] Miura M., Gronthos S., Zhao M., Lu B., Fisher L. W., Robey P. G., Shi S. (2003) “SHED: stem cells from human exfoliated deciduous teeth” *Proc. Natl. Acad. Sci.* 100(10):5807-5812. DOI: 10.1073/pnas.0937635100.

[2] Jiménez N. T., Munévar J. C., González J. M., Infante C., Perdomo S. J. (2018) “In vitro response of dental pulp stem cells in 3D scaffolds: A regenerative bone material” *Heliyon*, 4(9):e00775. DOI: 10.1016/j.heliyon.2018.e00775.

[3] Yatim R. M., Kannan T. P., Ab Hamid S. S. (2016) “Effect of gamma radiation on the expression of mRNA growth factors in glycerol cryopreserved human amniotic membrane” *Cell Tissue Bank*, 17(4):643-651. DOI: 10.1007/s10561-016-9576-3.

[4] Van Herendael B. J., Oberti C., Brosens I. (1978) “Microanatomy of the human amniotic membranes. A light microscopic, transmission, and scanning electron microscopic study” *Am. J. Obstet. Gynecol.*, 131(8):872-880. DOI:10.1016/s0002-9378(16)33135-0.

[5] Mamede A. C., Carvalho M. J., Abrantes A. M., Laranjo M., Maia C. J., Botelho M. F. (2012) “Amniotic membrane: from structure and functions to clinical applications” *Cell Tissue Res.* 349(2):447-458. DOI: 10.1007/s00441-012-1424-6.

[6] Hopkinson A., Shanmuganathan V. A., Gray T., Yeung A. M., Lowe J., James D. K., Dua H. S. (2008) "Optimization of amniotic membrane (AM) denuding for tissue engineering" *Tissue Eng. Part C Methods* 14(4):371-381. DOI: 10.1089/ten.tec.2008.0315.

[7] Wang J., Ma H., Jin X., Hu J., Liu X., Ni L., Ma P. X. (2011) "The effect of scaffold architecture on odontogenic differentiation of human dental pulp stem cells" *Biomaterials* 32(31):7822-7830. DOI: 10.1016/j.biomaterials.2011.04.034.

[8] Malhotra C., Jain A. K. (2014) "Human amniotic membrane transplantation: Different modalities of its use in ophthalmology" *World J. Transplant* 4(2):111-121. DOI: 10.5500/wjt.v4.i2.111.

[9] Casagrande L., Demarco F. F., Zhang Z., Araujo F. B., Shi S., Nör J. E. (2010) "Dentin-derived BMP-2 and odontoblast differentiation" *J. Dent. Res.* 89(6):603-608. DOI: 10.1177/0022034510364487.

[10] Woo K. M., Chen V. J., Ma P. X. (2003) "Nano-fibrous scaffolding architecture selectively enhances protein adsorption contributing to cell attachment" *J. Biomed. Mater. Res. A* 67(2):531-537. DOI: 10.1002/jbm.a.10098.

[11] Cavalcanti B. N., Zeitlin B. D., Nör J. E. (2013) "A hydrogel scaffold that maintains viability and supports differentiation of dental pulp stem cells" *Dent. Mater.* 29(1):97-102. DOI: 10.1016/j.dental.2012.08.002.

[12] Gebhardt M., Murray P. E., Namerow K. N., Kuttler S., Garcia-Godoy F. (2009) "Cell survival within pulp and periodontal constructs" *J. Endod.* 35(1):63-66. DOI: 10.1016/j.joen.2008.09.020.

[13] Nam S., Won J.-E., Kim C.-H., Kim H.-W. (2011) "Odontogenic differentiation of human dental pulp stem cells stimulated by the calcium phosphate porous granules" *J. Tissue Eng.* 2011:812547. DOI: 10.4061/2011/812547.

[14] Neunzehn J., Pötschke S., Hannig C., Wiesmann H.-P., Weber M.-T. (2017) "Odontoblast-like differentiation and mineral formation of pulpsphere derived cells on human root canal dentin in vitro" *Head Face Med.* 13(1):23. DOI: 10.1186/s13005-017-0156-y.

[15] Yasser S., Nagy N. B., Marei M. K. (2012) "In Vitro Characterization of Stem Cells from Human Exfoliated Deciduous Teeth (SHED)" *Maced. J. Med. Sci.* 5(4):389-396. DOI: 10.3889/MJMS.1857-5773.2012.0249.

[16] Yang X., Van der Kraan P. M., van den Dolder J., Walboomers X. F., Bian Z., Fan M., Jansen J. A. (2007) "STRO-1 selected rat dental pulp stem cells transfected with adenoviral-mediated human bone morphogenetic protein 2 gene show enhanced odontogenic differentiation" *Tissue Eng.*13(11):2803-2812. DOI: 10.1089/ten.2006.0439.