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Original Article

Early morphology observation of human periodontal fibroblast cell line in the presence of human salivary exosomes

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Abstract Human salivary exosomes have been identified as a highly informative nanovesicle with clinical-relevant information for variation of diagnostic purposes. As a continued effort from previous studies on human salivary exosomes effect at gene expression level, this study is carried out to observe the morphology of human periodontal fibroblast (HPdLF) treated with exosomes cells under the same period of changes in genotypic level occurred. *In vitro*, HPdLF cells were cultured for 24 hours with 10 µg/ml of human salivary exosomes. The morphology of HPdLF cells was examined under inverted light microscopy and scanning electron microscopy (SEM) for both control samples and samples treated with human salivary exosomes, while the cell count was performed via trypan blue staining. There was no significant difference in the morphology under the inverted light microscopy and the cell number of HPdLF cells for both treated and untreated cells with exosomes. However, for SEM, the treated HPdLF with salivary exosomes showed slight observable changes on the filopodia, lamellipodia, cytoplasmic vesicles and the cytoskeleton of the cells. Even within a short period (24 hours) of culturing time for cells with human salivary exosomes, the samples showed minimal changes which positively suggested a simultaneous event of exchanging materials from human salivary exosomes to cells had occurred, hence, potentially proving that human salivary exosomes can enhance cell proliferation.

Keywords: Cell morphology; human periodontal ligament fibroblast cell; human salivary exosomes; scanning electron microscopy.

Introduction

The exosomes studies have recently expanded and opened more innovative approaches in development of tissue regeneration and diagnostic purposes. Exosomes, which have been identified in multiple biofluid sources such as human saliva (Kechik *et al*., 2018a; Kechik *et al.*, 2018b), are reported to contain not only important proteins and nucleic acids (Palanisamy *et al.*, 2010) but play profoundly significant role in intercommunication between cells, as a mediator of cell-to-cell transfer of genetic information (Behbahani *et al.*, 2016). A variety of proteins in exosomes such as heat shock proteins, cytoskeletal proteins, adhesion molecules, membrane transporter and fusion proteins have been an essential contributors in inducing genetic and epigenetic changes other than its ribonucleic acids contents (Behbahani *et al.*, 2016; Kobayashi *et al.*, 2014). Due to the rich potential of these exosomes, in prognosis, therapy, biomarkers and many more, other than the cancer studies (Pant *et al.*, 2012), researchers are also working hard to incorporate the function of exosomes to the tissue regeneration as well (Bjørge *et al.*, 2017).

Previous studies had applied human salivary exosomes on human periodontal ligament fibroblast (HPdLF) cells to find an alternative way to encounter the conventional periodontal therapies' limitations, in order to treat periodontitis, which is an inflammatory disease of teeth supporting tissues caused by microorganisms (Kagiya, 2016; Kechik *et al.*, 2018a). In general, tissue engineering in periodontal regeneration had been introduced with the aim to reform the lost tissue and to restore it to its original form including the bone structures with well-oriented periodontal ligament anchoring to the dental cementum, which common periodontal therapies for periodontitis such

as scaling, root planing and surgery failed to resolve (Iwata *et al.*, 2014). The combination of these two main components of biotechnologies and salivary diagnostic has shown a positive outcome. It is reported that human salivary exosomes significantly upregulate basic fibroblast growth factor (*bFGF*) gene expression level in HPdLF cells. The *bFGF* is one of the growth factors involved in wound healing, responsible for cell proliferation and is important in periodontal regeneration (Kechik *et al.*, 2018a).

Since human salivary exosomes have shown their effect on the HPdLF cells in gene expression level (Kechik *et al.*, 2018a), the present study was conducted to find out whether the effect of human salivary exosomes shows any effect on the cells under microscopic level as. Morphological observation between normal cells and cells treated with human salivary exosomes was carried out under light inverted microscope and scanning electron microscope (SEM). HPdLF cell criteria such as lamellipodia, filopodia, cytoplasmic vesicles and cytoskeleton were observed for any changes. Lamellipodia are flat extensions with thickness of 0.1-0.5 µm meanwhile filopodia are cylindrical or conical processes, usually 10-20 µm long with small diameter (Al-Hisayat *et al.*, 2012). Lamellipodia are projections which can be found in the leading edge of the cells by the assembly of focal adhesions when cell membranes are in contact with the substratum, consequently enabling the cells to migrate forward. Lamellipodia contains microspikes (the ribs of actin) (Nemethova *et al.*, 2008). The extension of microspikes will create filopodia (frontier lamellipodium) that protrude into an antennae-like shape to the surrounding environment. These slender cytoplasmic projections that extend beyond the leading edge of lamellipodia are initiated and elongated by the polymerization, convergence and crosslinking of actin filaments (Mattila and Lappalainen, 2008). As with lamellipodia, filopodia's main function also involve cell migration and wound healing. Cytoplasmic vesicles are membrane-limited structures derived from the plasma membrane or various intracellular membranes which function as storage, transport or in metabolism. Cytoskeleton function is to maintain the shape and internal organization of the cell, as well as to provide mechanical support (Mattila and Lappalainen, 2008).

Based on previous researches, exosomes uptake by cells reported few different postulations. The mechanisms were as follow: 1) energy-dependent, receptor-mediated endocytosis (Svensson *et al.*, 2013; Tian *et al.*, 2013); 2) macropinocytosis (Fitzner *et al.*, 2011; Tian *et al.*, 2014); 3) direct fusion with plasma membrane or phagocytosis (Feng *et al.*, 2010); and 4) entrance via filopodia (Heusermann *et al.*, 2016). These multiple entry routes might even coexist in the same cell, depending on the targets and purposes. However, a deeper and exact understanding on the mechanisms of exosomes are currently still not clearly explained.

Therefore, with reference to previous researches on exosomes (Kechik *et al.*, 2018a; Kechik *et al.*, 2018b), the aim of the current research was to observe any morphological changes that occur within the same period of treatment that shows an effect on gene expression level.

Materials and methods

Human salivary exosomes collection

Human saliva samples were collected in accordance to the recommendations in the International Conference on Harmonization - Guidelines for Good Clinical Practice (ICH-GCP) and the Declaration of Helsinki (World Medical Association, 2001). The protocol was approved in 2013 by the Human Research Ethics Committee, Universiti Sains Malaysia (JEPeM) (FWA Reg. No: 00007781; IRB Reg. No: 00004494).

The human salivary exosome samples were the samples collected and processed from the previous reported research (Kechik *et al.*, 2018b). Therefore, the same protocol of saliva collection, exosome isolation and purity check procedures were applied. During collection, subjects were asked to refrain from eating, drinking, or using oral hygiene products for at least one hour prior to collection and were asked to rinse their mouth with water. After that, about 5 ml of unstimulated saliva was collected from each subject by using paper cup and transferred

into centrifuge tubes. About 30 ml of the saliva sample (pooled from 5 healthy male subjects) collected was centrifuged (Hettich Universal 32R Centrifuge, USA) at 6000xg for 20 minutes to remove cell debris. The saliva supernatants were made to pass through ultracentrifugation (Beckman Coulter Optima L-90K Ultracentrifuge, USA) at 110000xg for 2 hours at 4ºC. Following ultracentrifugation, the aqueous layer was removed and the pellet containing the exosomes was dissolved in 1 ml of phosphate buffer saline (PBS) (Bio-Rad, USA) (Michael *et al.*, 2010; Shahidan, 2011). The human salivary exosomes samples had undergone SDS-PAGE, Western blot, Nanoparticle Tracking Analysis and SEM as confirmation tests to proceed for cell culture treatment and all results are included as references (Kechik *et al.*, 2018a; Kechik *et al.*, 2018b).

Cell culture and cell treatment with human salivary exosomes

The HPdLF cells (LONZA, Switzerland) (CC-7049) were cultivated in HyClone™ Minimum Essential Medium (MEM) Alpha Modification with L-glutamine, ribo- and deoxyribonucleosides (α-MEM) (SH30265.01) (GE Healthcare Life Sciences, USA) at 37°C with 5% CO_2 in cell culture incubator (NuAire, USA). HPdLF cells were seeded into 6 wells plate (90,000 cells/well) in its media (2 ml/well) and divided into 2 groups (control and treatment). There were three wells prepared as technical replicates for each group and they were incubated for 24 hours. Control group (A1, A2 and A3) were cultured using the complete media (α-MEM) while treatment group (B1, B2 and B3) were treated with 10 µg/ml of human salivary exosomes in α-MEM. Both groups were cultured for another 24 hours in 37°C with 5% CO_2. The experiments' biological triplicates were carried out by repeating the same protocol using the three same sets at three separate times to prove reproducibility.

Cell images viewing

The cell images were taken before and after 24 hours treatment with human salivary exosomes. Comparisons of images were done under inverted microscope (Axiovert 25 Inverted Microscope, Carl Zeiss, Germany) under the same magnifications. The light intensity was manually set to the same lamp voltage level and adjusted accordingly via control knob during observation. All images were observed under 10x of magnification with the focus and sharpness of images adjusted in manually by controlling the knob accordingly. The area of observation was chosen randomly, and the best images were selected to represent the average of the whole well.

Cell counts

Cell counts were carried out to compare the proliferation rate before and after exosomes intervention using Trypan Blue Solution (0.4%) (Sigma-Aldrich, USA) and were counted manually under microscope. The results were graphed by calculating the average for both control cells and cells treated with human salivary exosomes.

Scanning electron microscopy

To view cell under exosomes treatment, cells were cultured on poly-L-lysine glass slides that undergo the same procedure of cell culture and treatment. Procedure of cells fixation was based on Al-Hisayat *et al.* (2012) protocol and "General Sample Preparation for Scanning Electron Microscope Protocol" produced by Scanning Electron Microscope Laboratory, School of Health Sciences, Universiti Sains Malaysia with a slight alteration to suit the type of cell. Reagents used for this procedure were from Bio-Rad (USA).

After 24 hours of treatment period, all media were aspirated out from both control and treated cells. Samples were fixed with 1 ml of 2.5% glutaraldehyde for 30 minutes and then washed with PBS three times for 10 minutes each before continuing with the dehydration process. A dehydration series of 30%, 50%, 70% and 90% of ethanol were used for all samples for 30 minutes each. The samples were then fixed twice in 100% ethanol for 30 minutes each time. Samples were finally fixed in 1:1 of hexamethyldisilazane (HMDS) and 100% ethanol for 15 minutes and 100% HMDS for another 15 minutes. The samples underwent drying process by incubation in air-dryer for overnight before being mounted on an aluminium stub and coated with gold coating for viewing (Al-Hisayat *et al.*, 2012).

The samples were viewed under a FEI Quanta 450 Scanning Electron Microscope (FEI, USA) at 5.00 kV of high-voltage (HV)

and 10.6 mm of working distance (WD). The images were captured under 200x, 5000x, and 100000x magnifications and the changes in morphology for both groups were observed. The area of observation was chosen randomly, and the best images were selected to represent the results.

Results

Human salivary exosomes confirmation and concentration determination

As published on previous research, human salivary exosomes sample was confirmed by its morphology (round, vesicle form), correct size (30-120 nm) and protein markers (CD63) by SDS-PAGE, Western blot, Nanoparticle Tracking Analysis (NTA) and scanning electron microscope (SEM) (Kechik *et al.*, 2018a; Kechik *et al.*, 2018b), The concentration of human salivary exosome to be used on cell treatment (10 µg/ml) was based on the Bradford protein assay of the same previous research (Kechik *et al.*, 2018b), and the same concentration is being used on the gene expression level experiment (Kechik *et al.*, 2018a).

Cell morphology (inverted microscope) in the presence and absence of exosomes

Images of the human periodontal ligament fibroblast (HPdLF) cells cultured for 24 hours after the first cultivation and 24 hours after treatment with human salivary exosomes are shown in Fig. 1 and Fig. 2, respectively. The elongated shape of the cells was perfectly shown regardless before and after the exosome treatment.

Cell morphology (scanning electron microscope) in the presence and absence of exosomes

Fig. 3 shows the value of both control HPdLF cells and HPdLF cells after 24 hours of exosomes treatment. Under the SEM observation, the images of the control HPdLF cells and treated HPdLF cells under 200x magnifications (Fig. 4), 5000x magnifications (Fig. 5) and 100000x magnifications (Fig. 6, Fig. 7) showed some comparable cell features. The elongated shape of the cells was perfectly visible for both control and treated cells. However, the cell treated with exosomes also appeared to be rougher and wider in size. Cytoplasm showing more abundant cytoplasmic vesicles protruded on the treated cells (Fig. 5). The roughness of the fibroblast cytoplasm (as shown in Fig. 5) was due to the cytoplasmic vesicles or vacuoles, protruding as buds. Those vacuoles appeared larger in the cell treated with exosomes (Fig. 6). The features of lamellipodia and filopodia were more distinct in the cells treated with exosomes (Fig. 7).

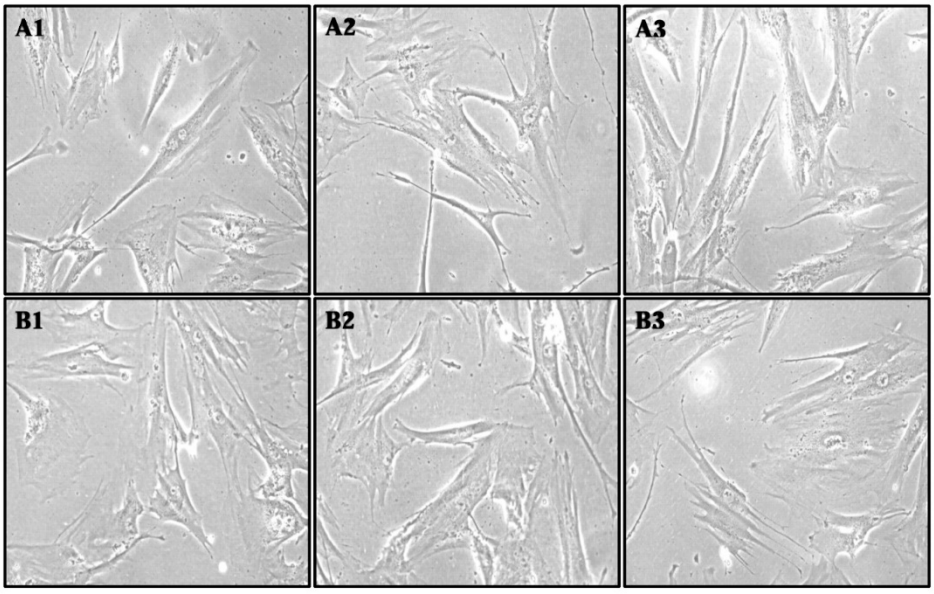

Fig. 1 The HPdLF cells 24 hours' post seeding under 10x magnifications. The morphology of HPdLF cells were viewed under inverted microscope. The initial number of cells seeding was 90,000 cells/well. The elongated shape of the cells was perfectly visible. All wells were observed to be in the same confluence percentage at this time.

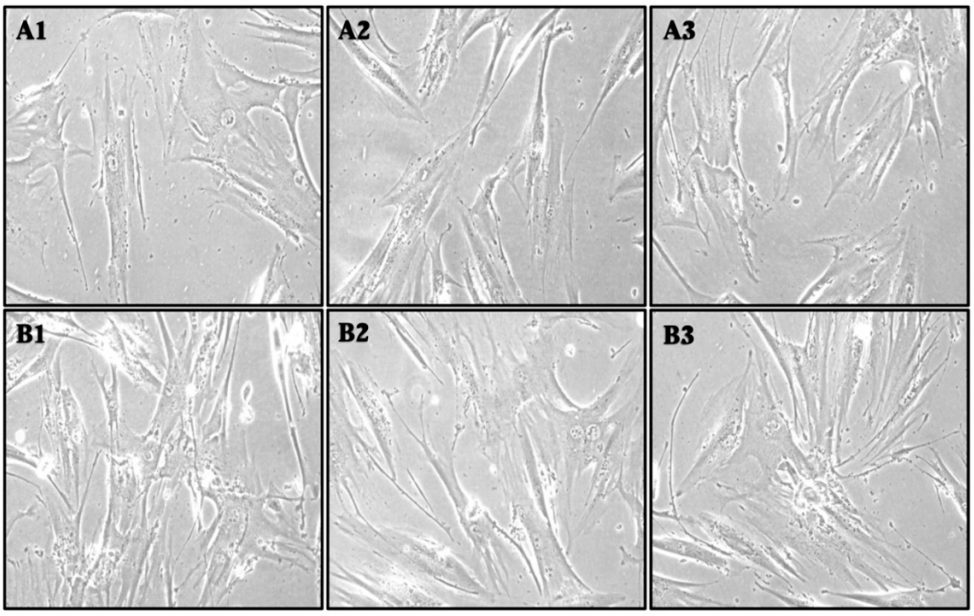

Fig. 2 The (HPdLF cells 24 hours' post treatment with exosomes under 10x magnifications. The A line wells (A1, A2 and A3) acted as the control meanwhile the B line wells (B1, B2 and B3) were treated with 10 µg/ml exosomes. The HPdLF cells showed elongated and spindle shape morphology in all groups. There was no difference in the morphology for all wells. Both groups also were observed to be in the same confluence percentage.

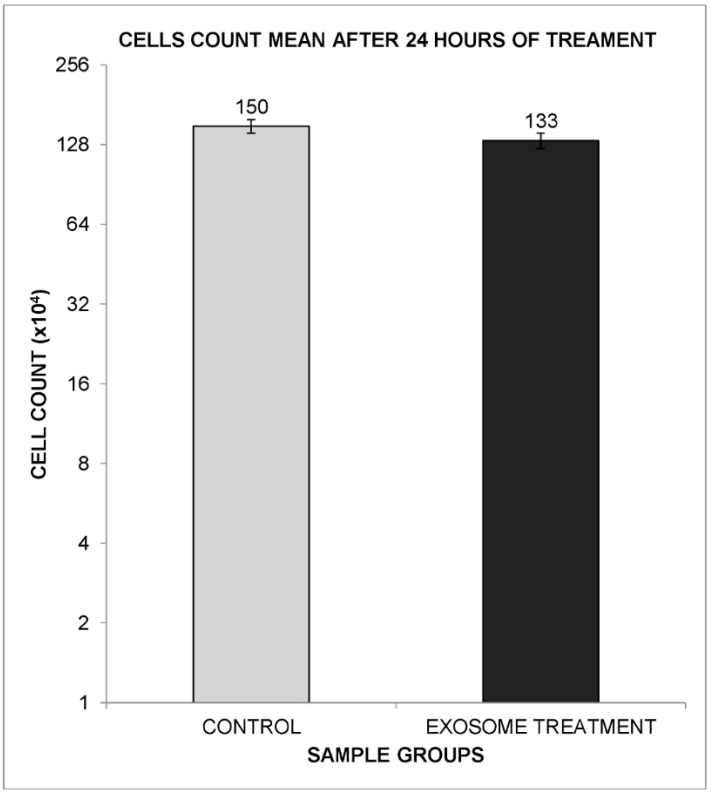

Fig. 3 The graph of the cell counts after 24 hours' post treatment with exosomes. The human periodontal ligament fibroblast (HPdLF) cell counts were done manually with Trypan Blue staining under inverted microscope. Initial cell seeding number was 90,000 cells/well. (Data represent the mean value ± SEM; EXCEL 2016: ANOVA: Single Factor Statistical Analysis, $p>0.05$).

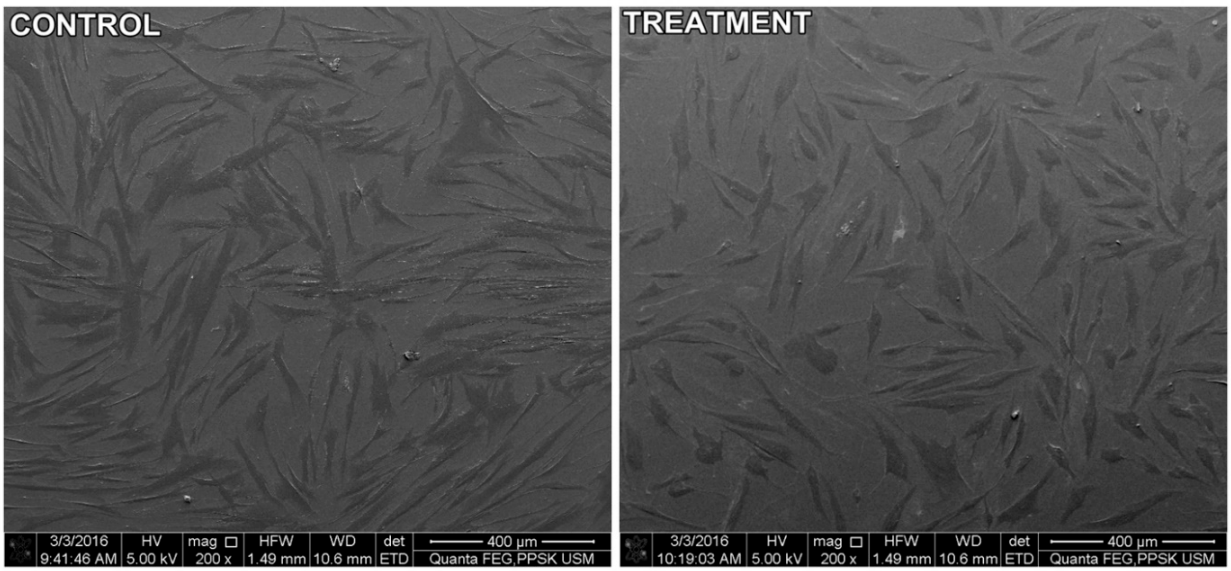

Fig. 4 The representative images of HPdLF cells 24 hours after exosomes treatment under 200x magnifications of scanning electron microscope (SEM). Both control and treatment show no distinct difference in morphology. The elongated shape of the cells was perfectly shown for both control and treated cells.

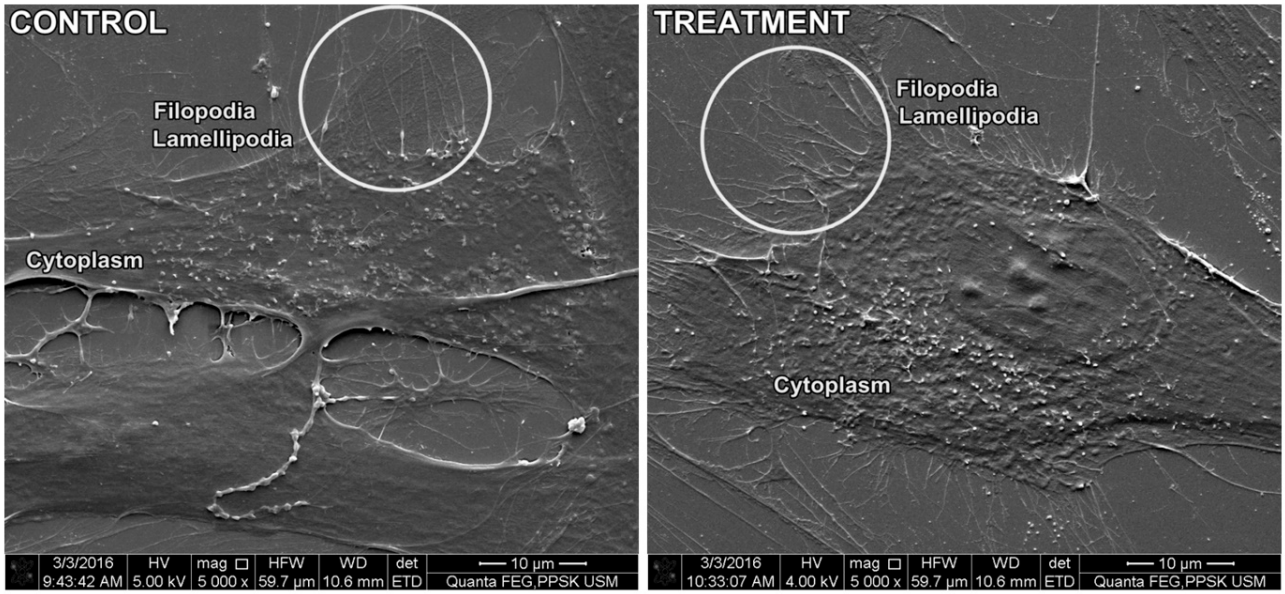

Fig. 5 The representative images of human periodontal ligament fibroblast (HPdLF) cells 24 hours after exosomes treatment under 5000x magnifications of scanning electron microscope (SEM). The filopodia and lamellipodia were more abundant in cell treated with exosomes. The surface of HPdLF cells treated with exosomes appeared to be rougher and wider in size. Cytoplasm showing more abundant cytoplasmic vesicles protruded on the treated cells.

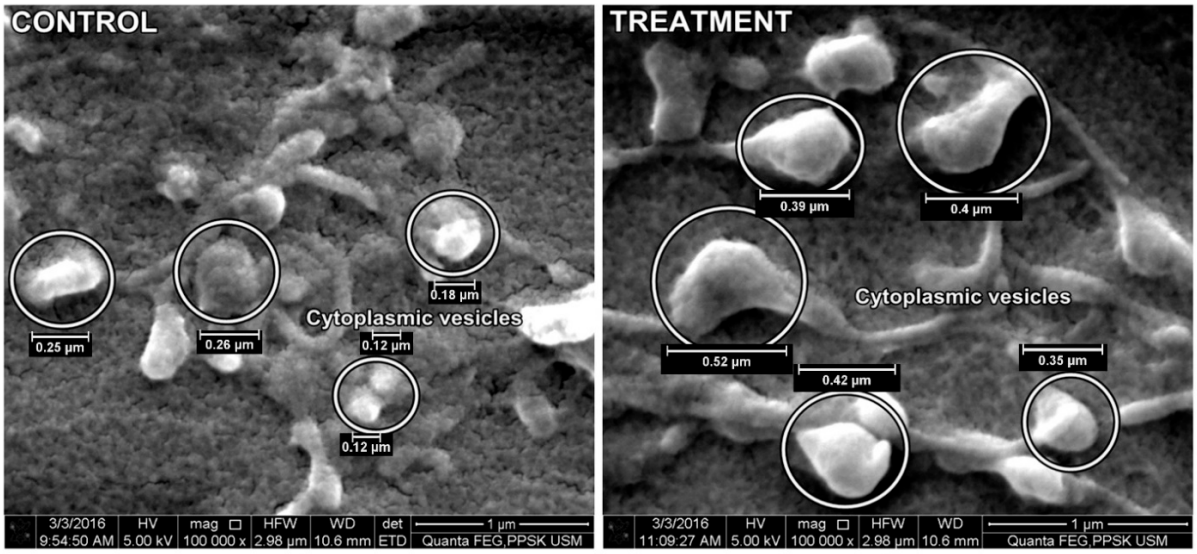

Fig. 6 The representative images ofHPdLFcells 24 hours after exosomes treatment under 100000x magnifications of scanning electron microscope (SEM). The roughness of the fibroblast cytoplasm (shown in Fig. 5) was due to the cytoplasmic vesicles or vacuoles protruding as buds. Those vacuoles appeared larger in the cell treated with exosomes.

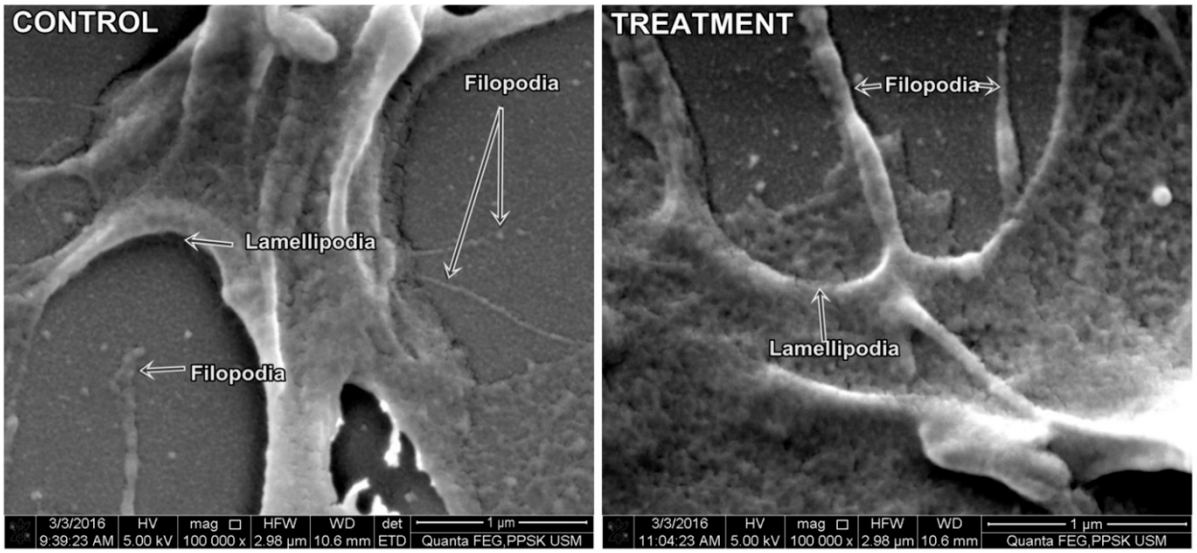

Fig. 7 The representative images of HPdLF cells 24 hours after exosomes treatment under 100000x magnifications of scanning electron microscope (SEM). The lamellipodia and filopodia were observed. As shown in Fig. 5, the features of lamellipodia and filopodia were more distinct in the cell treated with exosomes

Discussion

From the observation of cells under inverted microscope after 24 hours of treatment, human salivary exosomes showed no distinct effect on the morphology of the HPdLF cells (Fig. 1 and Fig. 2) and no significant changes (p>0.05) in cell number (Fig. 3). However, as further analysis was carried out under the scanning electron microscope (SEM) to look at the ultrastructural changes of the HPdLF cell surface, a few notable changes were observed. The SEM revealed that under 100,000x magnifications, the sample treated with exosomes showed minimal differences that can be observed with the morphology criteria. The treated sample showed more distinct and richer filopodia and lamellipodia. Both filopodia and lamellipodia are involved

in cell growth and proliferation as they function in cell migration (Mattila and Lappalainen, 2008; Small *et al.*, 2002). The protrusion of filopodia is a method for cells to respond to the outer materials of the cells or as the inner response of the cells themselves (Mattila and Lappalainen, 2008).

Since filopodia and lamellipodia extended from the actin cytoskeleton of the cells, the increase in the number of their formations showed a higher level in actin contents and actin meshwork. Actin basically functions in intracellular transport. The contractility of the actin-based parts of the cells control the cell shape changes and direct involvement of cells from cell division to cell migration and wound healing process (Pollard and Cooper, 2009).

Fig. 5 showed the abundance of filopodia and lamellipodia in cells treated with exosomes. A higher number of filopodia and lamellipodia may also increase the effectiveness of the fibroblast attachment to surfaces. Therefore, from Fig. 7, we can observe better attachment of the cells onto the surface in the treated sample. This criterion is important especially in relation to the quality of wound healing and tissue regeneration. Cells are required to attach onto the root surfaces in the process of healing and regeneration of periodontal tissue (Ramseier *et al.*, 2012). However, as the current study was just a preliminary study, which utilised one concentration of human salivary exosome (10 µg/ml), this result could only suggest the potential of exosomes in positively influencing cell attachment. A further quantification of cell attachment tests should be carried out to solidify this hypothesis.

In endodontic surgery, the fibroblast migration attachment and orientation are vital for the healing process as it initiates the cellular functions (Al-Hisayat *et al.*, 2012). Anchorage dependent cell such as HPdLF cell requires cell adhesion and attachment formation for survival (Khalili and Ahmad, 2015; Sharifi *et al.*, 2016).

SEM also showed the lumpier surface on the cytoplasm of the treated cells due to the cytoplasmic vesicles or vacuoles (Fig. 5). As the magnification for observation increased, the treated cells showed abundance of vesicles and the cells appeared larger in treated exosomes sample compared to the control sample (Fig. 1). The size of the cells can be compared based on the SEM scales provided on the images. The formation of the cytoplasmic vesicles could be due to the phagocytosis process. During this process, filopodia folds to embrace the foreign materials around, which is then followed by invagination of the enclosed plasma membrane (Masci *et al.*, 2016). During this phase, the cells were in contact with exosomes particles from the surrounding and phagocyte them into the cells. The larger sizes of the vesicles indicate the increased materials ensnared by the filopodia and in this case, they might be exosomes themselves. This theory is supported by a research done by Heusermann *et al.* (2016) on the exosomes uptake by cells. Their research managed to prove the recruitment of exosomes (conditioned medium originated exosomes) into cell body (human primary fibroblast) by surfing on filopodia as well as by the grabbing and pulling motions of filopodia (Heusermann *et al.*, 2016).

In addition, the fibroblast cytoplasm itself is filled with vacuoles containing varying materials with different consistency and density. Some of the materials in the vacuoles are related to the actual continuity to collagen fibril (Masci *et al.*, 2016). Therefore, this also proposes that the particular phase visualizes the moment where the vacuoles are conveying the inner complex endogenous material from the cells to the extracellular matrix environment. The cytoplasmic vesicles are likely to be the Golgi-associated vesicles delivering the materials such as hydrolytic enzymes into the collagen containing vacuoles for additional processing (Masci *et al.*, 2016). However, the larger-sized vesicles from the present results could indicate that the cell has received or contained more endogenous materials.

This morphological finding could further explain our previous report on the upregulation of the basic fibroblast growth factor (*bFGF*) gene expression of HPdLF in the presence of human salivary exosomes (Kechik *et al.*, 2018a). The effect of human salivary exosomes on the gene level was proven by the morphological observation

where the process of infusion of exosomal contents into the target cells took place.

Conclusion

The minor differences showed by the scanning electron microscope (SEM) images may not suffice to account for the effect of human salivary exosomes on human periodontal ligament fibroblast (HPdLF). However, within 24 hours of culturing the cells with human salivary exosomes, the samples had already shown some respective observable changes that could suggest intercommunication between exosomes and cells in exchanging materials had occurred effectively, which supported the gene expression level upregulation (Kechik *et al.*, 2018a). With some extended research, human salivary exosomes can be fully proven as having the capabilities to enhance the cell proliferation and therefore, could contribute to tissue regeneration in their full potentials.

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