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The Effect of Human Salivary Exosome on The Basic Fibroblast Growth Factor and Collagen Type I Gene Expressions from Human Periodontal Fibroblast Cells

Abstract— Gene expression is the most fundamental level at which the genotype gives rise to phenotype. The development of human salivary exosomes has become one of the promising researches to improve cell-based tissue engineering but their functions in human periodontal ligament fibroblast (HPdLF) cells are not well studied. To study the effect of human salivary derived exosomes on the gene expression of HPdLF cells. *In vitro*, HPdLF cells were cultured for 24 hours with 10 µg/ml of human salivary exosomes. Determination of gene expression levels of basic fibroblast growth factor (*bFGF*) and collagen type I (*COL1*) in the presence and absence of human salivary exosomes in HPdLF culture was performed using quantitative reverse transcriptase polymerase chain reaction (RT-qPCR). Human salivary exosomes significantly upregulated *bFGF* gene expression but not *COL1* gene in HPdLF cells after 24 hours of culture. Human salivary exosomes are able to upregulate bFGF gene in HPdLF cells. Thus, they might have potential to be used as an alternative biomaterial in tissue engineering for periodontal regeneration.

***Keywords* — basic fibroblast growth factor, collagen, gene expression, human periodontal fibroblast cell, human salivary exosome**

1 INTRODUCTION

Periodontitis is an inflammatory disease of teeth supporting tissues caused by specific microorganisms or groups of specific microorganisms. It results in progressive destruction of the periodontal ligament and alveolar bone with periodontal pocket formation, gingival recession or both [1]. Plaque bacterial products and indirect damage through bacterial induction of the host inflammatory and immune response result in destruction of the tissues [2]. Periodontal disease has become a major health problem because it destroys the tooth supporting attachment apparatus.

Although the conventional periodontal therapies are already well-established, these methods have their limitations. Common periodontal therapies such as scaling, root planing, and periodontal surgery basically remove the bacterial pathogens and surrounding teeth's tissues that are infected. These therapies can stop the disease from worsening. However, they cannot restore the damaged tissue to its original form. Hence, tissue engineering in periodontal regeneration had been introduced with the aim to reform the lost tissue and restore it to its original form including the bone structures with well-oriented periodontal ligament anchoring to dental cementum [3].

Recently, the combination of emerging biotechnologies and salivary diagnostics has expanded the range of saliva-based diagnostics from oral cavity to the whole physiological system since most compounds found in blood are also present in saliva. Saliva can reflect the physiological state of the body including emotional, endocrinal and nutritional state, and metabolic variations. Therefore, it acts as a source to monitor the oral and systemic health [4]. Human saliva contains not only hormones and regular digestive enzymes but also important proteins [5][6]. Although exosomes have been identified in human saliva, their biochemical and biophysical characteristics are largely unknown. Nevertheless, exosomes secreted in human saliva contain proteins and nucleic acids that could be used for diagnostic purposes [7].

Exosomes are small vesicles, with a diameter range of 30-120 nm. They are secreted by all types of cultured cells and found in abundance in body fluids including blood, urine, ascites, amniotic fluid and cultured medium of cell cultures [8]. The exosome is a multi-protein

complex capable of degrading various types of ribonucleic acid (RNA) molecules. There is a growing interest in the clinical applications of exosomes due to their ability to be used in prognosis, therapy, biomarkers and many more functions. Exosomes contain cell and cell-state specific cargos of protein, mRNA, and miRNA. They could be transferred into recipient cells to modulate protein synthesis. It is also suggested that exosomes play a significant role in the intercellular communication by transferring both proteomic and genomic materials between cells [9]. Studies have shown that exosomes are selectively taken up by distal cells upon their release. They can reprogram the recipient cells due to their active molecular cargo [10].

Due to the encouraging biological function of exosome in salivary fluid and the increasing number of the periodontal cases, the potential effect of exosomes on the morphology and expressions level of basic fibroblast growth factor (*bFGF*) and collagen type I (*COL1*) genes were explored in the current study. Both genes are responsible for the proliferation of human periodontal ligament fibroblast (HPdLF) cells. Understanding exosomes effects on both genes may provide insights in order to find a new approach in resolving oral health problem, particularly the periodontal diseases. As one of the growth factors involved in wound healing, *bFGF* gives pleiotropic effects on the cell while *COL1* is involved in the extracellular matrix (ECM) formation . Both elements are important in periodontal regeneration. Saliva is assumed to play an important role in influencing and manipulating the growth of periodontal cells [11]. With reference to previous researches made on salivary components and functions, exosomes derived from human saliva and its contents can be one of the new materials used in enhancing the development of periodontal cell evolution.

2 MATERIALS AND METHODS

2.1 Human salivary exosomes collection

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

The protocol for human salivary exosomes isolation was based on [12] on miRNA analysis on saliva. About 30 ml of the collected saliva sample was centrifuged at 6000xg for 20 minutes to remove cell debris. The saliva supernatants were made to pass through ultracentrifugation 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) [12][13]. The human salivary exosomes samples were submitted to SDS-PAGE, Western blot and Nanoparticle Tracking Analysis for purity confirmation before proceeding with cell culture treatment.

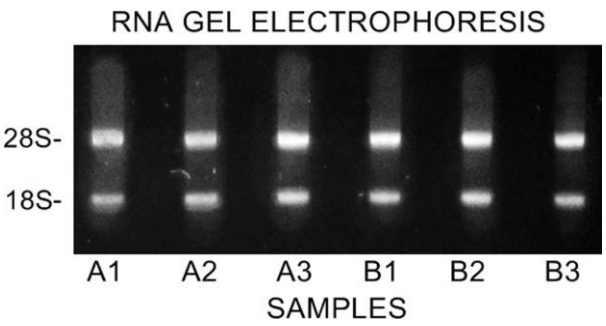

Figure 1: The RNA gel electrophoresis. This image shows the bands of all the samples, hence, confirming intact RNA samples for further investigation.

2.2 Cell culture and cell treatment with human salivary exosome

Human Periodontal Ligament Fibroblast (HPdLF) cells (LONZA, Switzerland) were cultivated in HyClone™ Minimum Essential Medium (MEM) Alpha Modification with L-glutamine, ribo- and deoxyribonucleosides (SH30265.01) (GE Healthcare Life Sciences, USA) and incubated at 37°C with 5% CO_2. HPdLF cells were seeded into 6 wells (90,000 cells/well) plate in its medium and divided into 2 groups (control and treatment) in triplicates and incubated for 24 hours. Control groups (A1, A2, and A3) were cultured using the regular medium while treatment groups (B1, B2, and B3) were treated with 10 µg of human salivary exosome. Both groups were cultured for another 24 hours in 37°C with 5% CO_2.

2.3 RNA extraction

RNA was extracted using RNeasy Plus Universal Kits (QIAGEN, Germany) according to manufacturer's instructions. The purity and the concentration of the total RNA extracted were checked by using the biophotometer and the quality was determined by agarose gel

electrophoresis (Bio-Rad, United States) according to the manufacturer's protocol.

2.4 Gene expression analysis

Single-stranded complementary DNA was synthesized from 1 µg RNA using a QuantiTect™ Reverse Transcription Kit (QIAGEN, Germany) according to the manufacturer's protocol. Real-time polymerase chain reaction (qPCR) was carried out using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, United States) and StepOnePlus™ Real-Time PCR System (Life Technologies). The PCR conditions were following the standard cycling mode primer T_m<60°C. The genes of interest for this study are the *bFGF* and *COL1* as both are the genes responsible for the proliferation of HPdLF cells. The reference gene that was used is glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and all reverse and forward primers of gene interests were from Integrated DNA Technologies (IDT, United States). Primers gene sequence used were listed as in Table I.

2.5 qPCR reaction analysis

The software used for analysis is the StepOnePlus Version 2.2.2. The standard curves for each gene were plotted. The efficiency values and R^2 values were automatically calculated by the software. The amplification plot was viewed and the relative quantification (ΔΔCT) values were obtained. The relative quantity of expression of $2^{-(\Delta\Delta C_T)}$ was calculated to analyze the results.

3 RESULTS

The purity and the concentration of total RNA extracted for all samples and RNA Gel electrophoresis showed that all samples had a good quality RNA as shown in Fig. 1 and were deemed suitable to be proceeded for real-time PCR.

The relative quantification analysis method of $2^{-\Delta\Delta C_T}$ calculation (Comparative C_T) was used [14]. The threshold cycle (C_T) values obtained from real-time PCR instrumentation were imported into a spreadsheet and tabulated as shown in Table II. The change in expression of the *bFGF* and *COL1* target genes normalized to GAPDH was monitored from corresponding cDNA synthesized from each sample. The C_T values of the gene of interest (GOI) (*bFGF* and *COL1* genes) in both the experimental sample (s) and calibrator (c) (control sample) were adjusted in relation to a normalizer (norm) gene's (internal control gene, *GAPDH*) C_T from the same two samples. The resulting $\Delta\Delta C_T$ value is was incorporated to determine the fold changes in expression [14].

The analysis equations used is shown as follows:

$$\Delta C_{T\ sample} = C_{T\ GOI}\ s - C_{T\ norm}\ s$$
$$\Delta C_{T\ calibrator} = C_{T\ GOI}\ c - C_{T\ norm}\ c$$
$$\Delta\Delta C_T = \Delta C_T\ s - \Delta C_T\ c$$
$$\text{Fold change} = 2^{-\Delta\Delta C_T}$$

The technical replicates of PCRs were averaged to get the mean of C_T data before performing the $2^{-\Delta\Delta C_T}$ and the mean fold change ($2^{-\Delta\Delta C_T}$) of both *COL1* and *bFGF* gene expression were graphed in Fig. 2. The data were analyzed using Excel Analysis ToolPack of statistical Two Samples Student's T-Test to find the p-value of data using C_T Mean value of samples (Table II). From the Fig. 2, only *bFGF* genes significantly showed the up-regulation as the effect of the exosome on the cells.

4 DISCUSSIONS

The real-time PCR analysis endpoint is determined by a log-linear plot of the PCR signal versus the cycle number, the threshold cycle (C_T). In log-scale of ΔΔC_T values, positive value suggests up-regulation and the negative value suggests down-regulation. Since all C_T values are normalized to calibrator and the internal control gene, *GAPDH*, the comparative analysis of $2^{-(\Delta\Delta C_T)}$ value higher than 1.000 showing the upregulation of GOI. From the chart (Fig. 2), the values of fold change ($2^{-(\Delta\Delta C_T)}$) shows an increased pattern for the exosomes treated (experiment) samples for both *COL1* and *bFGF* genes. However, there is an only small difference for *COL1* fold change values between control and treated samples, compared to *bFGF* fold change values. Therefore, to support this result, all samples data were analyzed statistically to determine the significant values and only *bFGF* gene showed a significant value ($p<0.05$) (Fig. 2).

From the chart (Fig. 2), the insignificant value of *COL1* gene expression ($p>0.05$) shown by this study is suggested to be due to short incubation time. As published by other researchers, collagen induction by other factors such as hormones, only gave significant result after more than 24 hours of cell incubations. A research on the collagen production stimulated by tumor growth factor-beta 1 (*TGF-β1*) on fibroblast and myofibroblast cells on time-dependence (2-48 hours) showed that there was no increase in collagen production after long incubation (24-28

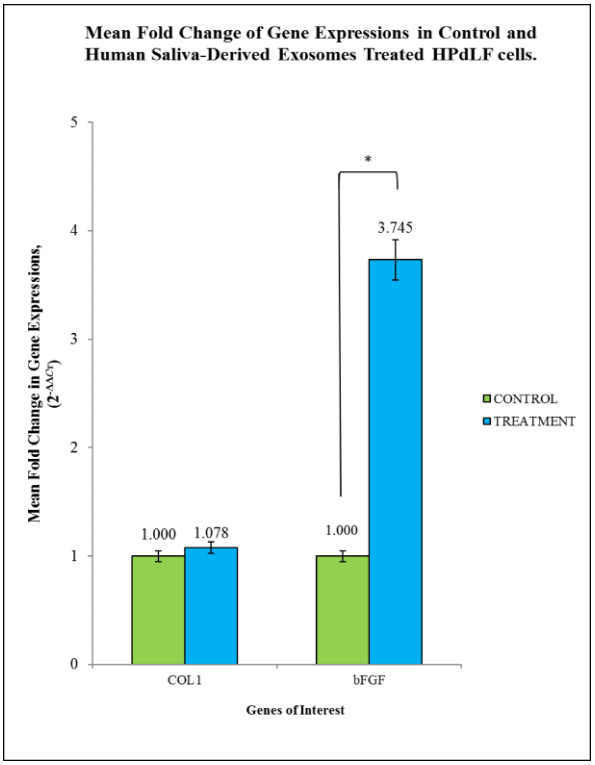

Figure 2: The gene expression results showing mean fold change in gene expression ($2^{-\Delta\Delta C_T}$) values of the control versus treatment by the genes of interest. *p<0.05, significant difference.

hours) at low concentrations (<1 ng/ml), or after short incubation (2-4 hours) at high concentrations (1-15 ng/ml). Even though the collagen production increased after a long incubation at high concentration, the maximum increase was only observed after 48 hours [15]. Another study using *TGF-β1*-containing exosomes from injured epithelial cells on fibroblasts showed that the *COL1* expression was significantly increased after 24 hours and 48 hours of cell incubations [16]. These reports supported our findings that collagen gene might not give significant difference within only 24 hours of exosomes treatment. Despite the insignificant increase of *COL1* gene expression level, our study was able to prove the significant increase in *bFGF* gene level within just 24 hours. Hence, these findings suggest that exosomes may influence the cell proliferation and might indirectly be involved in wound healing.

The results of human salivary exosomes affecting the genes of interests (*bFGF* and *COL1*) obtained from the current study were concurrent with the results obtained from other studies that used exosomes from other sources [6][17][18]. This study paves a way to venture into the effect of human salivary exosomes on HPdLF cells. Therefore, regardless of the exosome's origins (sources), their general effects on genes expressions (downregulate or upregulate) are used to support our findings. As an example, oligodendroglial exosomes affect the gene expression in neuron cells by downregulated the immediate early response 3 (*Ier3*), VGF nerve growth factor inducible (*Vgf*), and brain-derived neurotrophic factor (*Bdnf*) on transduction pathways in neurons after exosome treatment [18]. Another study done on the exosomes from human monocytes resulted in the increase of the gene expression of the osteogenic markers runt-related transcription factor 2 (*RUNX2*) and bone morphogenetic protein-2 (*BMP-2*) [6]. Meanwhile, tumor-derived exosomes and other types of body fluids derived exosomes; (amniotic fluid exosomes, liver cirrhosis ascites exosomes, and malignant ascites exosomes of ovarian cancer patients) were analyzed to give effect on the THP-1 cells gene expression as well [17].

The effects shown by exosomes on the gene expressions of cells proved the involvement of exosomes in intercellular communication and RNA/mRNA/miRNA information transfer, despite the different origins of the exosomes [9][19]. There is cell to cell signaling and information transfer between exosomes and cells. Some of the exosomes effects on the gene expression may include involvement in cell maturation and differentiation [6][18] or associated with immunosuppressive mechanisms induction [17].

Even though the effect was only shown by two types of genes in the gene expression analysis, this could still be an indicator for further study. The fact that this study was basically performed under 24 hours of exosome treatment and the effect can already be observed and analyzed should be taken into consideration as a positive sign for researchers to continue revealing the potential of the human salivary exosome. The development of human salivary exosome research enables to improve the cell-based tissue engineering for periodontal regeneration. However, the mechanism by which exosomes may regulate genes expression and its role in exosome-induced proliferation require further investigation.

Likewise, the results obtained from this study suggested that human salivary exosomes may affect periodontal regeneration and may be involved in wound healing as positively shown by the upregulation of the genes studied.

5 CONCLUSION

Human salivary exosomes affect the gene expressions of *bFGF* and *COL1* in HPdLF cells through upregulation of the genes. Therefore, suggesting the involvement of exosomes in the periodontal cells regeneration.

CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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