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Effect of Non-Thermal Atmospheric Pressure Plasma on Differentiation Potential of Human Deciduous Dental Pulp Fibroblast-like Cells

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Abstract: Human mesenchymal stem cells can differentiate into various cell types and are useful for applications in regenerative medicine. Previous studies indicated that dental pulp exfoliated from deciduous teeth is a valuable alternative for dental tissue engineering because it contains stem cells with a relatively high proliferation rate. For clinical application, it is necessary to rapidly obtain a sufficient number of cells in vitro and maintain their undifferentiated state; however, the abundance of stem cells in the dental pulp tissue is limited. Non-thermal atmospheric pressure plasma (NTAPP) has been applied in regenerative medicine because it activates cell proliferation. Here, we examined the effects of NTAPP to activate the proliferation of human deciduous dental pulp fibroblast-like cells (hDDPFs) in vitro. Compared with untreated cells, NTAPP increased cell proliferation by 1.3-fold, significantly upregulated well-known pluripotent genes for stemness (e.g., *Oct4*, *Sox2*, and *Nanog*), and activated the expression of stem cell-specific surface markers (e.g., CD105). Overall, NTAPP activated the proliferation of various mesodermal-derived human adult stem cells while maintaining their pluripotency and stemness. In conclusion, NTAPP is a potential tool to expand the population of various adult stem cells in vitro for medical applications.

Keywords: non-thermal atmospheric pressure plasma (NTAPP); human deciduous dental pulp fibroblast-like cells; regenerative medicine

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1. Introduction

Stem cells isolated from multiple tissues are used in regenerative medicine as they can differentiate into various tissues, including odontoblastic, chondrocytic, adipocytic, and osteoblastic cell lineages [1–4]. Previous in vivo studies demonstrated the differentiation of stem cells isolated from human dental pulp tissue into odontoblast-like cells lining the existing dentin surface [5] and the formation of a continuous layer of dentin-like tissue on the existing canal dentinal walls and mineral trioxide aggregate cement surfaces [6]. In addition, when stromal stem cells obtained from human dental pulp or bone fragments in vitro were transplanted into immunocompromised rats it resulted in the generation of a tissue structure with an integral blood supply similar to that of the human adult bone [7]. Stem cells from exfoliated human deciduous pulp show a higher proliferation rate than that of adult bone marrow stromal stem cells [8]. Similarly, fibroblast-like cells from exfoliated human deciduous dental pulp have a higher proliferation rate than that of those from permanent teeth [9]. Therefore, stem cells from exfoliated human deciduous pulp might be helpful in tissue regeneration, although those in the dental pulp tissue are present in minimal quantities. Some studies have reported that stem cells comprise 0.8% and 0.4% of human and mouse dental pulp, respectively [10,11]. Thus, it is necessary to proliferate cells and maintain the undifferentiated state of human dental pulp cells for clinical applications.

Various stimuli, such as drugs and incubation under hypoxia, have been used to activate pulp undifferentiation potential [12]; however, their use is currently limited because of safety and cost issues. Therefore, a safer and simpler alternative method is needed.

Recently, "plasma medicine", which involves the application of non-thermal atmospheric pressure plasma (NTAPP) for medical treatment, is a novel tool being applied in regenerative medicine. Plasma medicine aims at irradiating inorganic materials for surface modification or sterilization, whereas NTAPP combines irradiation at low temperatures and under atmospheric pressure, allowing the direct treatment of biological tissues for wound healing [13], selective apoptosis of cancer cells [14], and proliferation of pluripotent stem cells [15]. In the field of medical science, NTAPP has been studied worldwide for clinical applications. Although the information on the effects of direct plasma irradiation on the dental pulp and periapical soft tissues is limited, NTAPP is a simple, safe, and inexpensive tool that could be used in clinical dentistry.

In this study, we aimed to evaluate the effectiveness of NTAPP in activating the proliferation of undifferentiated human deciduous dental pulp fibroblast-like cells (hDDPFs). To this end, we examined the rate of cell multiplication in vitro, the expression of genes related to cell proliferation, and changes in mesenchymal stem cell (MSC) markers.

2. Materials and Methods

2.1. Cell Culture

Non-carious deciduous teeth (canine or molar) obtained from three healthy orthodontic patients at the pediatric dentistry in Osaka Dental University Hospital were kept in sterile 0.01 M phosphate-buffered saline (PBS; Wako Co., Ltd., Tokyo, Japan) and cut horizontally under sanitary conditions. The dental pulp tissue was gently removed, minced, and cultured in 35-mm tissue culture dishes, containing Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Wako, Tokyo, Japan), 100 U mL^{-1} penicillin (Life Technologies, Carlsbad, CA, USA), 100 μg mL^{-1} streptomycin (Life Technologies), and 4 mM L-glutamine (Life Technologies) at 37 °C in a humidified atmosphere of 5% CO_2. The culture medium was replaced with fresh medium every 3 days. hDDPFs isolated from the dental pulp tissues at passages 2–9 and expressed the markers CD105, CD44, and CD146 (data not shown) were used in this study. All experiments were approved by the Ethical Committee of Osaka Dental University (No. 111039). Informed consent was obtained from all study participants, and the study was conducted according to the principles of the Declaration of Helsinki.

2.2. NTAPP Stimulation Device

We used an argon-based NTAPP device in which the multi-gas plasma jet source has a columnar body similar to a pen and is connected to an AC power supply of 16 kHz and 9 kV and a gas cylinder (Plasma Concept Tokyo, Tokyo, Japan; Figure 1).

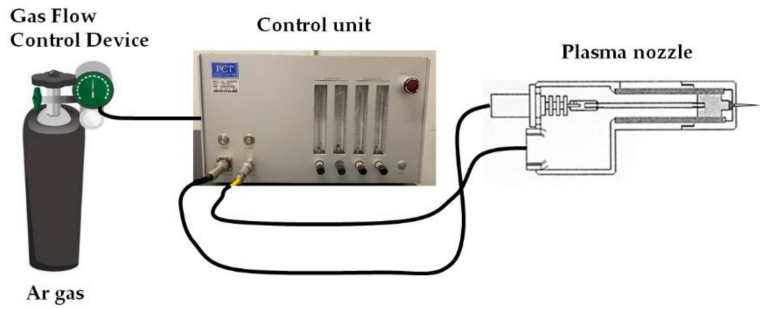

Figure 1. Argon-based non-thermal atmospheric pressure plasma (NTAPP) stimulation device used in this study. The multi-gas plasma jet source has a columnar body like a pen connected to an AC power supply of 16 kHz and 9 kV and a gas cylinder.

2.3. Cell Proliferation Assay

2.3.1. Investigation of NTAPP Irradiation Conditions

To investigate the effects of NTAPP, we first examined the cell proliferative potential after NTAPP irradiation. To expose cells to NTAPP, 1×10^4 cells seeded in 24-well culture plates were incubated for 96 h. The cells were exposed to the indicated doses of NTAPP (2.7 standard L/min, 20 V) for 10, 20, 30, and 40 s every hour, and the number of times was 1, 2, 3, and 4. The distance between the NTAPP stimulation device and cells was fixed to 2 cm, and 0.5 mL of medium was used. Then, the cells were further incubated for 72 h. After that, cell proliferation was evaluated using a Cell Titer96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Briefly, hDDPFs were cultured in 24-well plates at 1×10^4 cells per well for 96 h at 37 °C under a humidified atmosphere of 5% CO_2 and then treated with or without the indicated dose of NTAPP (2.7 standard L/min^{-1}, 20 V). The NTAPP-treated cells and non-treated cells (control) were further incubated for 72 h. Next, 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate were added to the cultures. Absorbances at 490 nm and 690 nm were measured using a Spectra Max5 microplate reader (Molecular Devices, Downingtown, PA, USA).

2.3.2. Effects of NTAPP on Culture Medium

Some studies have reported that NTAPP irradiation alters the culture medium and affects the cells [16,17]. Then, we wondered whether the cell proliferation induced by NTAPP was the direct effect of NTAPP, or the indirect outcome of the medium modified by NTAPP, or both.

To answer this question, we experimented with four groups. (Table 1) Groups 1 and 2 were not irradiated, whereas groups 3 and 4 were irradiated 3 times with NTAPP for 20 s. Immediately after irradiation, the cell culture media of groups 2 and 3 were exchanged. The cell proliferation was evaluated using the Cell Titer96 Aqueous One Solution Cell Proliferation Assay kit and the results were compared with the results of the earlier cell proliferation assay.

Table 1. Irradiation conditions for cells and culture medium.

Group	Cell	Medium
1	NTAPP(-)	NTAPP(-)
2	NTAPP(-)	NTAPP(+)
3	NTAPP(+)	NTAPP(-)
4	NTAPP(+)	NTAPP(+)

2.4. Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

We examined the expression of *Oct4*, *Sox2*, and *Nanog* to determine whether hDDPFs irradiated with NTAPP 3 times for 20 s each time could maintain proliferative capacity and pluripotency. We also studied the expression of *Sox9*, a marker of pluripotency in undifferentiated neural crest cells, and that of *ALP*, a marker of pluripotency in undifferentiated osteoblasts. Complementary DNA (cDNA) was isolated from NTAPP-treated cells and non-treated cells after 72 h of incubation using a Cells-to-CT 1-Step TaqMan kit (Thermo-Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. RT-PCR was performed using a Step One Plus system (Thermo-Fisher Scientific) in a total volume of 20 µL, consisting of 5 µL Master Mix, 13 µL RNase-free water, 0.5 µL *Oct3/4* (Hs01654807_s1), *Sox2* (Hs01053049_s1), *Nanog* (Hs02387400_g1), *Sox9* (Hs00165814_m1), or *ALP* (Hs01029144_m1) primers, 0.5 µL glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Hs02758991_g1) primers and 1 µL cDNA. In the TaqMan Gene Expression Assay (Life Technologies), the thermal conditions were as follows: 50 °C for 5 min, followed by

40 cycles at 95 °C for 20 s, 95 °C for 3 s, and 60 °C for 30 s. Gene expression in multiplex reactions was quantified using the comparative Ct method with normalization of the amount of the target (FAM) to endogenous GAPDH (VIC) expression. The relative expression levels were normalized to GAPDH expression.

2.5. Flow Cytometry

To detect stem cell-specific surface markers, NTAPP-treated cells and non-treated cells were detached with Accutase (Innovative Cell Technologies, San Diego, CA, USA), washed with FACS buffer (PBS, 1% FBS, 0.1% NaN_3 sodium azide), and centrifuged for 5 min at 1000 rpm and 4 °C. Cells were incubated with an optimal dilution of fluorescein-conjugated monoclonal antibodies (anti-CD44-allophycocyanin [APC], anti-CD105-fluorescein isothiocyanate [FITC], and anti-CD146-phycoerythrin [PE]; Biolegend, San Diego, CA, USA) for 1 h on ice. In total, 10,000 stained cells per assay were evaluated using BD FACSVerse (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo (BD Biosciences). To evaluate the fluorescence intensity, we measured the value of ΔMFI (the change of median fluorescence intensity). We compared the ΔMFIs of CD44, CD105, and CD146 in hDDPF treated with NTAPP.

2.6. Statistical Analysis

Data were expressed as means ± standard error of the mean (SE). Comparison among groups was performed with one-way ANOVA, followed by the Student-Newman–Keuls test. For comparisons between two groups with normally distributed data, two-tailed unpaired Student's t tests were used to determine statistical significance. Significance was set at a *p*-value of <0.05 (KaleidaGraph 4.00; SynergySoftware, Reading, PA, USA).

3. Results

3.1. Cell Proliferation Assay

3.1.1. Investigation of NTAPP Irradiation Conditions

We observed cell proliferation in hDDPFs treated 1–4 times with NATPP for 20 s compared with that in the non-treated group. When hDDPFs were irradiated with NTAPP 4 times, we observed cell detachment and irreversible disturbance of the proliferative capacity (Figure 2). Optimum NTAPP protocol (3 times with NTAPP for 20 s) was followed for all the following experiments.

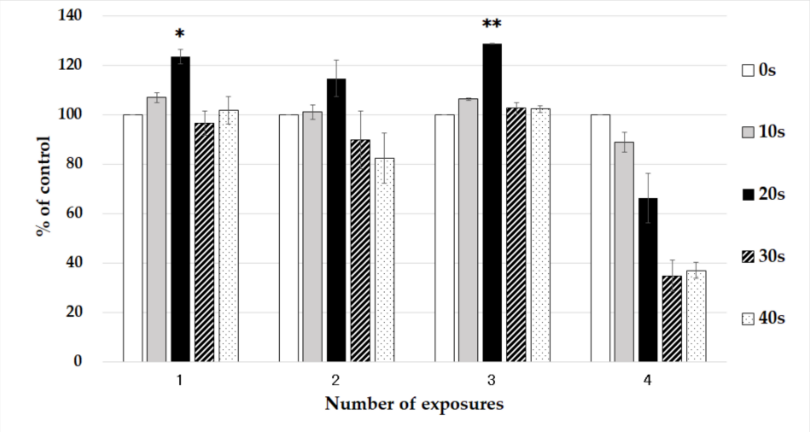

Figure 2. Direct effects of non-thermal atmospheric pressure plasma (NTAPP) on the proliferation of human deciduous dental pulp fibroblast-like cells (hDDPFs). These hDDPF (1×10^4 cells per well) were incubated for 96 h at 37 °C and 5% CO_2 in 24-well plates and then treated 1, 2, 3, or 4 times with NTAPP for 10, 20, 30, or 40 s per h. NTAPP-treated cells were incubated for 72 h at 37 °C and 5% CO_2. Cell proliferation was measured using the MTS assay. Data are presented as means ± SE ($n = 4$). Markers * and ** indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively.

3.1.2. Effects on the Culture Medium

As shown in Figure 3, compared with the control (Group 1), in which neither cells nor culture medium were irradiated, a significant increase in the proliferative capacity of cells in Group 4 was confirmed, in which both cells and culture medium were irradiated.

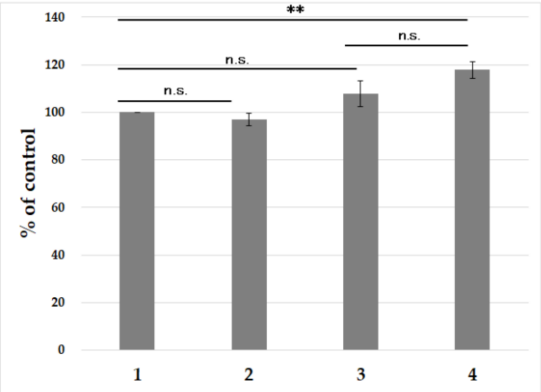

Figure 3. Effects of NTAPP on the culture medium. Group 4 (in which both cells and culture medium were NTAPP-irradiated) showed a significant increase in the proliferative capacity compared with the control (group 1) (in which neither cells nor culture medium was irradiated). Data are presented as means ± SE (n = 4). ** indicates significant differences at $p < 0.01$.

3.2. RT-PCR

The results showed NTAPP upregulated *Oct3/4*, *Sox2* and *Nanog* in hDDPFs (Figure 4).

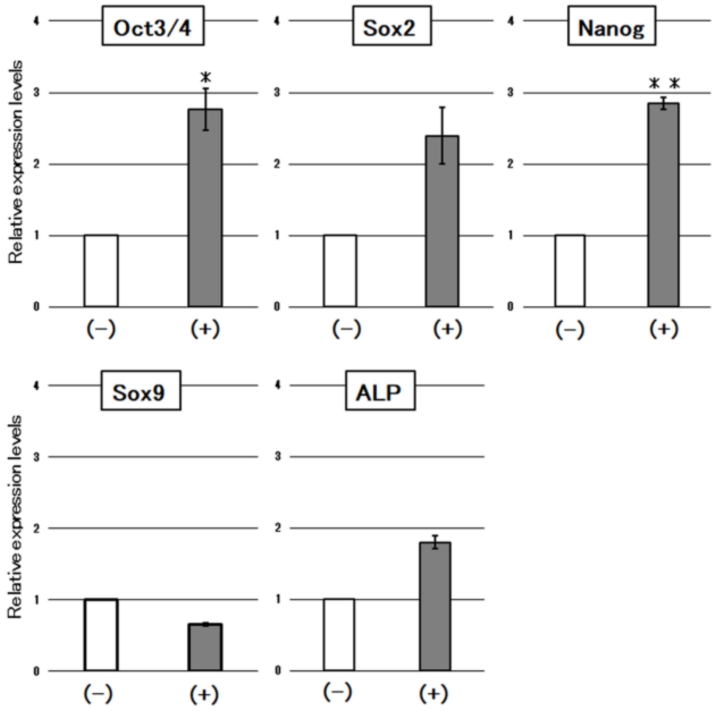

Figure 4. Effect of non-thermal atmospheric pressure plasma (NTAPP) on the expression of pluripotency markers (*Oct3/4*, *Sox2*, *Nanog*, *Sox9*, and *ALP*) in human deciduous dental pulp fibroblast-like cells (hDDPFs). (−) indicates the control group (non-NTAPP treated cells), and (+) indicates NTAPP-treated cells. Values were normalized using GAPDH as the internal control. Data are presented as means ± SE (n = 5). Data from five different experiments are shown. * and ** indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively.

3.3. Flow Cytometry

The increased protein expression of stemness markers in NTAPP-treated hDDPFs was further confirmed using flow cytometry. We found that the ΔMFI of CD105 was significantly increased post-incubation for 72 h compared with that of non-treated cells, whereas no change was observed in CD44 and CD146 (Figure 5).

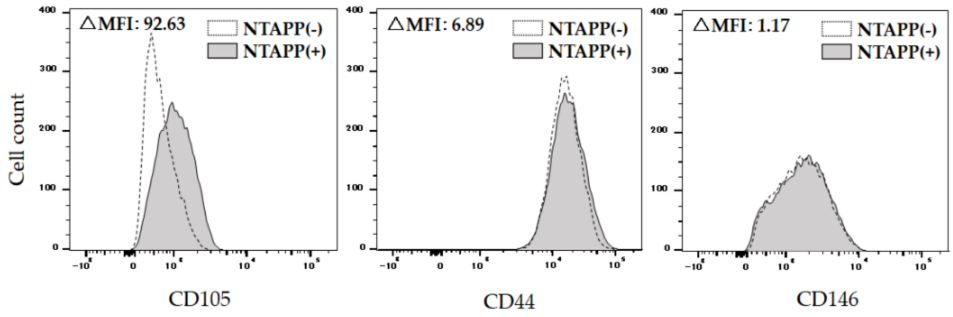

Figure 5. Effect of non-thermal atmospheric pressure plasma (NTAPP) on the expression of stem cell-specific markers (CD44, CD105, and CD146) in human deciduous dental pulp fibroblast-like cells (hDDPFs). ΔMFI (%), change in median fluorescence intensity.

4. Discussion

NTAPP is a partially ionized gas containing electrically charged particles and radicals at atmospheric pressure [18]. Previous studies reported that NTAPP exhibits various physiological effects beneficial for applications in regenerative medicine. It activates the proliferation of various mesoderm-derived adult stem cells, including human adipose tissue-derived stem cells, bone marrow-derived (BM)-MSCs, and hematopoietic stem cells, in vitro without affecting their stem-like properties [15,19].

In the present study, we investigated the effectiveness of NTAPP in activating the proliferation of cells from the deciduous dental pulp for potential use in regenerative dentistry. Our results showed that the cell proliferative capacity of cells treated 3 times with NTAPP for 20 s each time was approximately 1.2-fold higher than that of non-treated cells. In this study, the increased rate of cell proliferation was detected by MTS which is an indirect indicator. To confirm that proliferation (and not only metabolic activity) was increased by the NTAPP treatment, work should be carried out to directly compare cell numbers between the control group and the group treated with the optimum NTAPP protocol in future study. The optimal NTAPP conditions vary depending on plasma generator devices, the gas type used for plasma generation, and cell type. For instance, it has been reported that the proliferative potential of MSCs and hematopoietic stem cells is increased when treated with helium-based NTAPP 10 times for 50 s each time, in contrast to that of human synovial cells when treated with argon-based NTAPP for 60 s [19,20]. Here, the proliferative potential of hDDPFs decreased when treated >3 times with NTAPP for >20 s each time. Thus, the appropriate treatment conditions for each cell line need to be established before any clinical application.

In plasma medicine, NTAPP acts directly on cells and indirectly through the plasma-treated medium. For instance, NTAPP-activated media have anti-cancer effects on various human cancer cells [16], and He-based low-temperature atmospheric plasma jet-activated media have anti-bacterial properties [17,21]. Here, we found that cells cultured in NTAPP-treated medium had a higher proliferative capacity than those cultured in non-treated medium. Therefore, NTAPP enhanced cell proliferation both directly and indirectly by modifying the culture medium. Many oral periapical tissues contain large amounts of water. For instance, the dental pulp is a soft gelatinous tissue and water comprises 75–80% of its volume [22]. Therefore, it is essential to consider the effects of NTAPP on the cells and the surrounding fluid. Our findings showed the effectiveness of NTAPP in modifying the cell culture media, possibly expanding the applications of NTAPP using dental pulp.

Previous studies reported that *Oct4*, *Sox2*, and *Nanog* play essential roles in maintaining stem cell pluripotency and promoting cell proliferation in various adult stem cells [23–26]. To determine whether NTAPP treatment (3 times, 20 s each) could promote the proliferation of hDDPFs without affecting pluripotency, we examined the expression of *Oct4*, *Sox2*, and *Nanog*. We also studied the expression of *Sox9*, a marker of pluripotency of undifferentiated neural crest cells, and *ALP*, a marker of pluripotency of undifferentiated osteoblasts since hDDPFs are known to differentiate into chondrocytes and osteoblasts [27–29]. Our results showed that mRNA expression of *Oct3/4*, *Sox2* and *Nanog* was upregulated in NTAPP-treated cells.

A study on multipotent myoblasts has reported that the expression of *Sox9* increases after 3 to 5 days of incubation with material trioxide aggregate (MTA), which promotes hard tissue formation [30]. Therefore, in our experiments, confirming the expression of Sox9 in NTAPP-irradiated cells cultured for a longer period was necessary.

It has been reported that the MSC markers CD44, CD73, and CD90, as well as the stem cell markers CD105 and CD146, are expressed in dental pulp cells. Of these, CD105 is highly expressed in deciduous teeth [31]. In the present study, we studied CD44, CD105, and CD146 and found that they are all expressed in non-treated cells; however, only the expression of CD105 was increased after NTAPP treatment. It is known that MSCs from various human tissues and organs are adherent to plastic, have a fibroblastoid morphology, and are positive for CD73, CD90, and CD105 [32,33], but they vary in potency and self-renewal potential. Therefore, NTAPP treatment (3 times, 20 s each) is an effective tool that activates cell proliferation while maintaining and increasing MSC and stem cell markers in hDDPFs.

The multi-gas plasma jet source can generate atmospheric plasma of various gas species, including argon, oxygen, helium, nitrogen, air, and carbon dioxide, at low gas temperatures (<57 °C) [34]. Nevertheless, a study has reported that the effect of plasma depends on the type of gas, and different gases change the active species produced, thus changing the effectiveness and speed of the treatment. [35] For example, NTAPP using nitrogen and carbon dioxide is effective for hemostasis [36].

Helium and argon have been used in many plasma studies, but we preferred the latter because of the lower temperature, according to the manufacturer's instructions. We found that NTAPP based on argon gas was effective for promoting proliferation in hDDPFs; however, further research is necessary to examine other gas species and elucidate the complex biological effects of NTAPP on human stem cells.

5. Conclusions

Our study showed that argon-generated NTAPP activates the proliferative potential of hDDPFs both directly and indirectly through the culture medium. Furthermore, we revealed the possibility of enhancing the undifferentiated and proliferative cell potential, suggesting that NTAPP could be an effective tool in regenerative dentistry.

Author Contributions: K.H. and K.A. conceived and designed the experiments. M.O., S.A. and S.K. performed the experiments. R.I. and Y.A. analyzed the data, and M.O. and K.H. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Osaka Dental University (No.111039 date of approval: 29 July 2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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