

Effects of tacrolimus on morphology, proliferation and differentiation of mesenchymal stem cells derived from gingiva tissue

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Abstract. Tacrolimus is a 23-membered macrolide lactone with potent immunosuppressive activity that is effective in the prophylaxis of organ rejection following kidney, heart and liver transplantation. Tacrolimus also exerts a variety of actions on bone metabolism. The aim of the present study was to evaluate the effects of different concentrations of tacrolimus on the morphology and viability of human stem cells derived from the gingiva. Gingival-derived stem cells were grown in the presence of tacrolimus at final concentrations ranging from 0.001 to 100 μ g/ml. The morphology of the cells was viewed under an inverted microscope and the cell viability was analyzed using Cell Counting kit-8 (CCK-8) on days 1, 3, 5 and 7. Alizarin Red S staining was used to assess mineralization of treated cells. The control group showed spindle-shaped, fibroblast-like morphology and the shapes of the cells in 0.001, 0.01, 0.1, 1 and 10 μ g/ml tacrolimus were similar to those of the control group. All groups except the 100 μ g/ml group showed increased cell proliferation over time. Cultures grown in the presence of tacrolimus at 0.001, 0.01, 0.1, 1 and 10 μ g/ml were not identified to be significantly different compared with the control at days 1, 3 and 5 using the CCK-8 assays. Increased mineralized deposits were noted with increased incubation time. Treatment with tacrolimus from 0.001 to 1 μ g/ml led to an increase in mineralization compared with the control group. Within the limits of this study, tacrolimus at the tested concentrations (ranging from 0.001 to 10 μ g/ml) did not result

in differences in the viability of stem cells derived from gingiva; however it did enhance osteogenic differentiation of the stem cells.

Introduction

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that have the capacity for self-renewal and differentiation into osteocytes, adipocytes and other cells (1). MSCs have been used in preclinical models for tissue engineering of bone, cartilage, muscle, marrow stroma, tendon, fat and other connective tissues (2-4). In addition, MSCs secrete a broad spectrum of bioactive macromolecules that are immunomodulatory and serve to structure regenerative microenvironments in fields of tissue injury (5). Human mesenchymal stem cells have previously been isolated and characterized from the gingiva, and gingiva-derived stem cells have been applied for tissue engineering purposes (6). Moreover, stem cells may be obtained intraorally, and gingiva is a readily accessible tissue source with a relatively high quantity of obtainable tissue (7).

Tacrolimus is a 23-member macrolide lactone (molecular weight, 803.5 Da) with potent immunosuppressive activity that is effective in the prophylaxis of organ rejection following liver, heart, kidney and small bowel transplantation (8). Tacrolimus has a narrow therapeutic window (9) and it is important to choose the right dose (10). Clinically, the doses of tacrolimus administered following kidney, heart and liver transplantation are 0.15-0.3, 0.05-0.075 and 0.10-0.2 mg/kg/day, respectively (11-14). In addition, whole blood tacrolimus concentrations were observed to be 10-15 ng/ml during months 1-3 and 5-12 ng/ml during months 4-12 with the initial tacrolimus dose of 0.15-0.2 mg/kg/day (14,15).

Tacrolimus has been shown to exert a variety of actions on bone metabolism (16). It was reported that tacrolimus causes bone loss when administered systemically (17,18). However, other studies have shown that tacrolimus promotes osteogenic differentiation (19,20). Conversely, another study demonstrated that tacrolimus was not associated with osteogenic differentiation of human heart-derived MSCs (21).

Limited information is currently available regarding the effects of tacrolimus on dental tissue, and none is available

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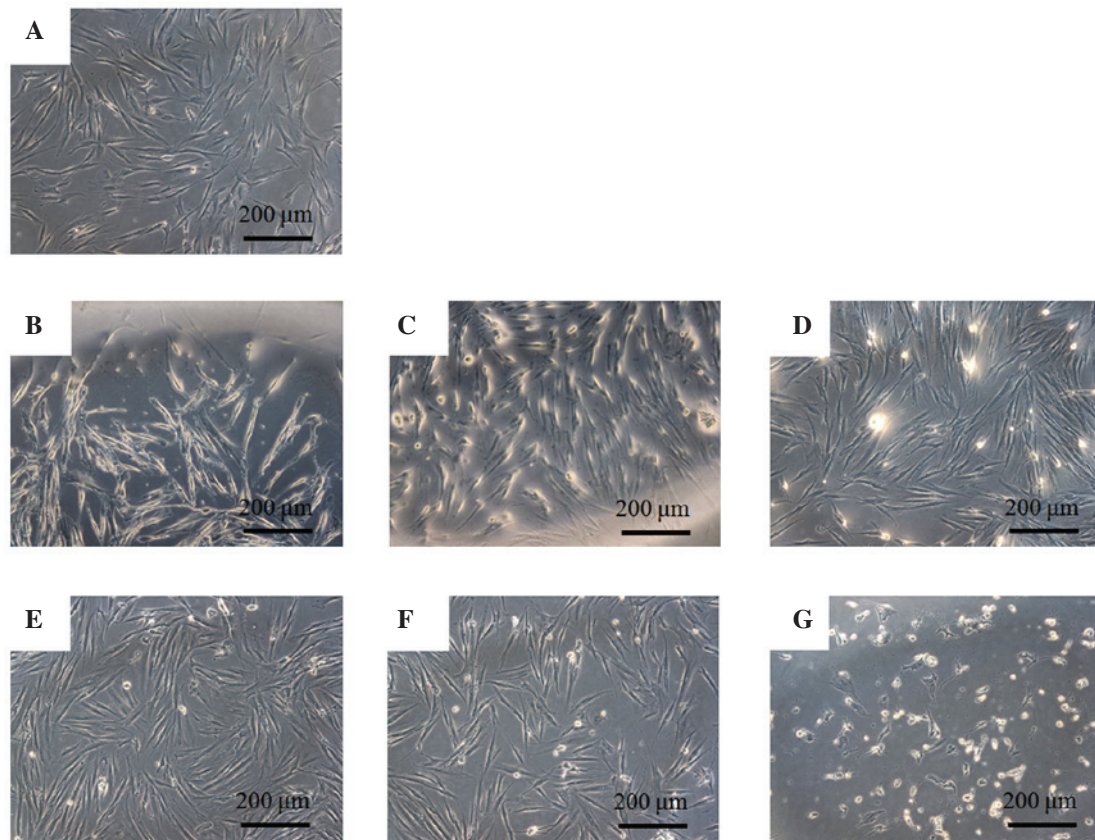


Figure 1. Evaluation of cell morphology on day 1 using inverted microscopy following treatment with tacrolimus of different concentrations. (A) Control, (B) 0.001 $\mu\text{g/ml}$ group, (C) 0.01 $\mu\text{g/ml}$ group, (D) 0.1 $\mu\text{g/ml}$ group, (E) 1 $\mu\text{g/ml}$ group, (F) 10 $\mu\text{g/ml}$ group and (G) 100 $\mu\text{g/ml}$ group.

on its effects on human mesenchymal stem cells derived from the gingiva. The aim of the present study was to evaluate the effects of a broad range of concentrations of tacrolimus on the morphology and viability of human stem cells derived from the gingiva.

Materials and methods

Isolation and culture of stem cells derived from the gingiva. Healthy gingival tissue samples were collected from healthy patients (mean age, 51.8 ± 18.1 years; 2 male and 2 female) and this study was reviewed and approved by the Institutional Review Board of Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea (Seoul, Korea; approval no. KC11SISI0348); informed consent was obtained from all patients. The resected gingival tissues were immediately placed in sterile phosphate-buffered saline (PBS; Welgene Inc., Daegu, Korea) with 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 4°C . The gingival tissue was de-epithelialized, minced into 1-2 mm^2 fragments, and digested in 0.2 μm filtered α -Minimum Essential Medium (MEM; HyClone; GE Healthcare Life Sciences, Chalfont, UK) with collagenase IV (Sigma-Aldrich). The cells were incubated at 37°C in a humidified incubator with 5% CO_2 and 95% O_2 . After 24 h, the non-adherent cells were washed with PBS, and adherent cell were administered fresh medium and replaced every 2-3 days. A previous report demonstrated that these cells showed colony-forming abilities, plastic adherence, and multilineage differentiation (osteogenic,

adipogenic, chondrogenic) potency (22). The cells expressed CD44, CD73, CD90, and CD105, but did not express CD14, CD45, CD34, and CD19 in flow cytometry (22).

Evaluation of cellular morphology. The cells were plated at a density of 2.0×10^3 cells/well in 96-well plates. The cells were incubated in α -MEM containing 15% fetal bovine serum (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin in the presence of tacrolimus at final concentrations ranging of 0 (control), 0.001, 0.01, 0.1, 1, 10 and 100 $\mu\text{g/ml}$. Tacrolimus was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and filter-sterilized. Equal quantities of DMSO were added to each culture sample to offset the influence of this dissolving vehicle. The morphology of the cells was viewed under an inverted microscope (Leica DM IRM, Leica Microsystems, Wetzlar, Germany) on days 1, 3, 5 and 7. The images were saved as JPEGs.

Determination of cell viability. The cell viability analysis was performed on days 1, 3, 5 and 7. 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H tetrazolium, monosodium salt (WST-8 from Cell Counting kit-8; Dojindo, Tokyo, Japan) was added to the culture and the cells were incubated for 3 h at 37°C . Viable cells were identified using the CCK-8 assay, which relies on the ability of mitochondrial dehydrogenases to oxidize WST-8 to a formazan product. The spectrophotometric absorbance at 450 nm was measured using a microplate reader (BioTek Instruments Inc., Winooski, VT, USA). The tests were performed in triplicate.

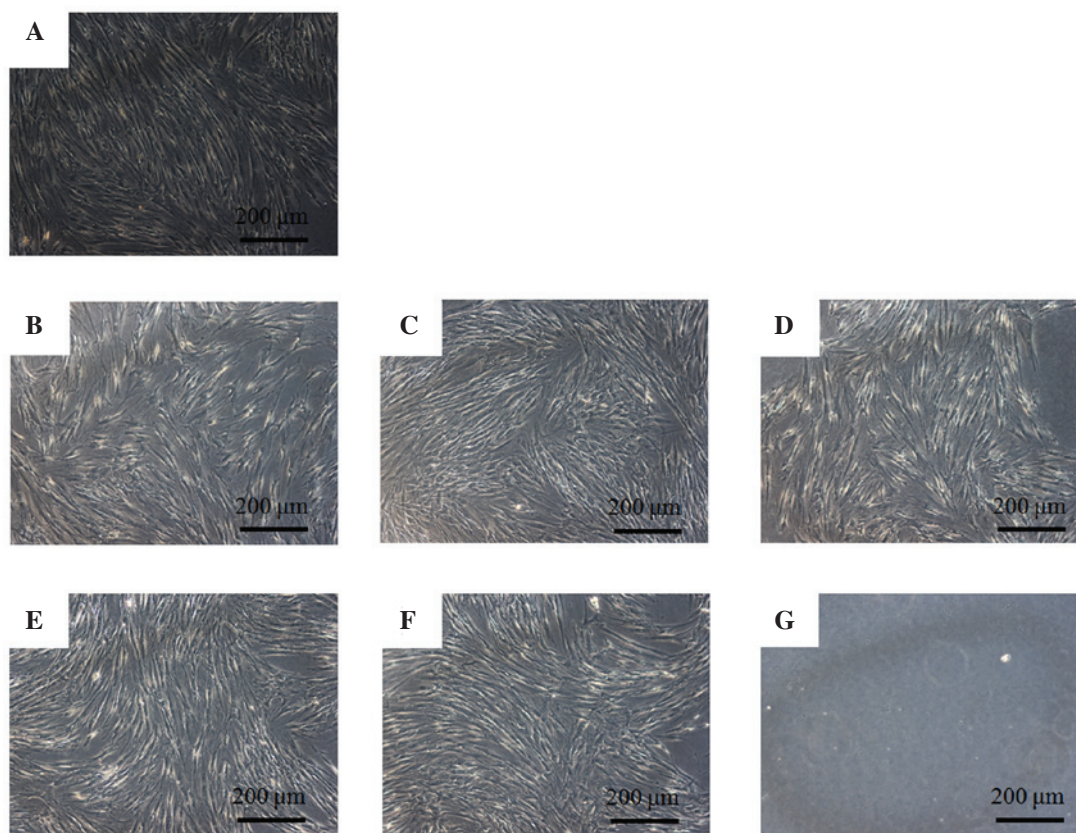


Figure 2. Cell morphology on day 3 using inverted microscopy following treatment with tacrolimus of different concentrations. (A) Control, (B) 0.001 $\mu\text{g/ml}$ group, (C) 0.01 $\mu\text{g/ml}$ group, (D) 0.1 $\mu\text{g/ml}$ group, (E) 1 $\mu\text{g/ml}$ group, (F) 10 $\mu\text{g/ml}$ group and (G) 100 $\mu\text{g/ml}$ group.

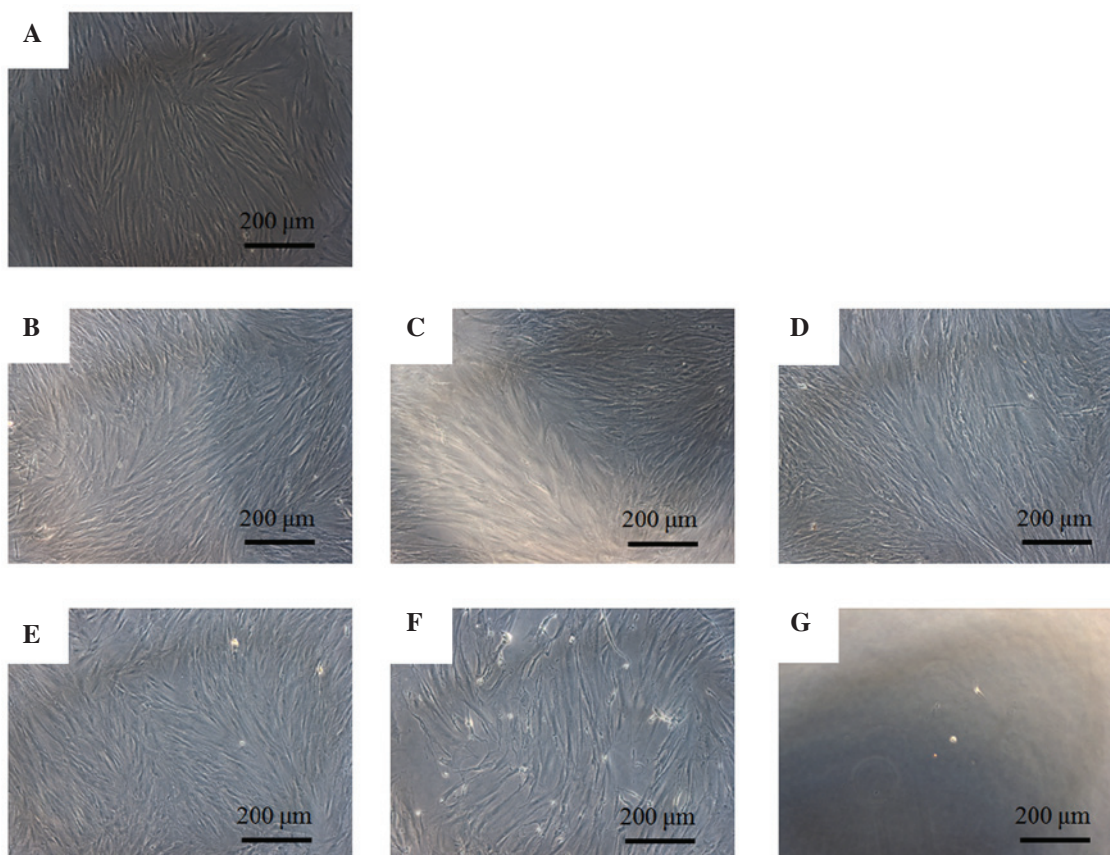


Figure 3. Cell morphology on day 5 using inverted microscopy following treatment with tacrolimus of different concentrations. (A) Control, (B) 0.001 $\mu\text{g/ml}$ group, (C) 0.01 $\mu\text{g/ml}$ group, (D) 0.1 $\mu\text{g/ml}$ group, (E) 1 $\mu\text{g/ml}$ group, (F) 10 $\mu\text{g/ml}$ group, (G) 100 $\mu\text{g/ml}$ group.

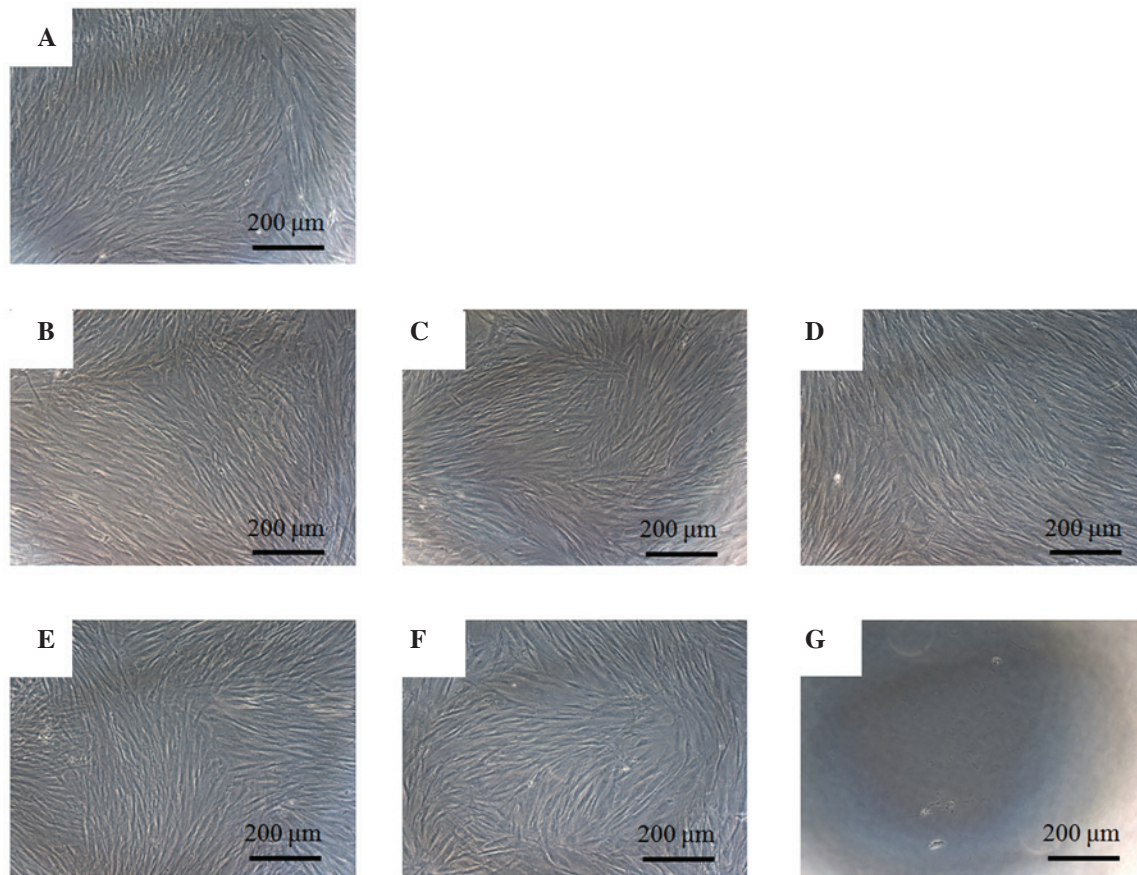


Figure 4. Cell morphology on day 7 using inverted microscopy following treatment with tacrolimus of different concentrations. (A) Control, (B) 0.001 $\mu\text{g/ml}$ group, (C) 0.01 $\mu\text{g/ml}$ group, (D) 0.1 $\mu\text{g/ml}$ group, (E) 1 $\mu\text{g/ml}$ group, (F) 10 $\mu\text{g/ml}$ group and (G) 100 $\mu\text{g/ml}$ group.

Alizarin Red S staining. Cell cultures obtained on days 5 and 7 were washed twice with PBS, fixed with 70% ethanol and rinsed twice with deionized water. Cultures were stained with Alizarin Red S (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and evaluated with a microscope (Leica DM IRB; Leica Microsystems). To remove non-specifically bound stain, cultures were washed three times with deionized water and once with PBS for 15 min at ambient temperature. Bound dye was solubilized in 10 mM sodium phosphate containing 10% cetylpyridinium chloride (Sigma-Aldrich) and quantified spectrophotometrically at 560 nm (PowerWave XS2; BioTek Instruments, Inc., Winooski, VT, USA).

Statistical analysis. The data are presented as the mean \pm standard deviation of the experiments. A one-way analysis of variance with post-hoc test was performed to determine the differences between the groups using a commercially available program (SPSS 12 for Windows, SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Evaluation of cell morphology. The control group showed normal fibroblast morphology on day 1 (Fig. 1). The shapes of the cells treated with 0.001, 0.01, 0.1, 1 and 10 $\mu\text{g/ml}$ tacrolimus were similar to those of the control group. However, the 100 $\mu\text{g/ml}$ group was markedly different when compared

with the control group. The shapes of the cells in the 100 $\mu\text{g/ml}$ group were rounder, and fewer cells were present. The morphology of the cells on day 3 is shown in Fig. 2. An increased number of cells were observed in each group and the shapes of the cells in the tested groups were similar to those in the control group. The results for cells on days 5 and 7 are shown in Figs. 3 and 4, respectively. Noticeable differences were observed in the 100 $\mu\text{g/ml}$ group.

Cellular viability. The CCK-8 results demonstrated cellular viability on days 1, 3, 5 and 7 are shown in Fig. 5. All groups except the 10 and 100 $\mu\text{g/ml}$ group showed relatively increased cell proliferation over time. The cultures grown in the presence of tacrolimus at 0.001, 0.01, 0.1, 1 and 10 $\mu\text{g/ml}$ did not show any statistically significant differences compared with the control at days 1, 3 and 5 using the CCK-8 assays ($P > 0.05$). However, growth in the presence of tacrolimus at a concentration of 100 $\mu\text{g/ml}$ resulted in decreases in the CCK-8 values at days 3, 5 and 7 ($P < 0.05$).

Mineralization assay. Mineralized extracellular deposits were minimally observed after Alizarin Red S staining on day 5 (Fig. 6). Increased mineralized deposits were noted on day 7 (Fig. 7). The quantitative results regarding bound dye on days 5 and 7 are shown in Fig. 8. The cultures grown in the presence of 0.001, 0.01, 0.1 and 1 $\mu\text{g/ml}$ tacrolimus exhibited increased mineralized deposits compared with the control on day 5. The relative values of the mineralization at 0.001, 0.01,

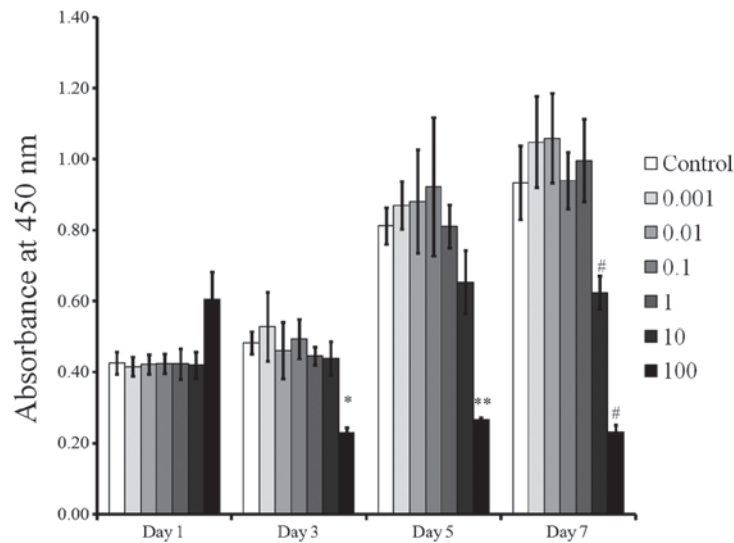


Figure 5. Cellular viability using cell counting kit-8 assay on days 1, 3, 5 and 7 following treatment with different concentrations of tacrolimus. * $P < 0.05$ compared with the control group at day 3. ** $P < 0.05$, compared with the control group at day 5. # $P < 0.05$ compared with the control group at day 7.

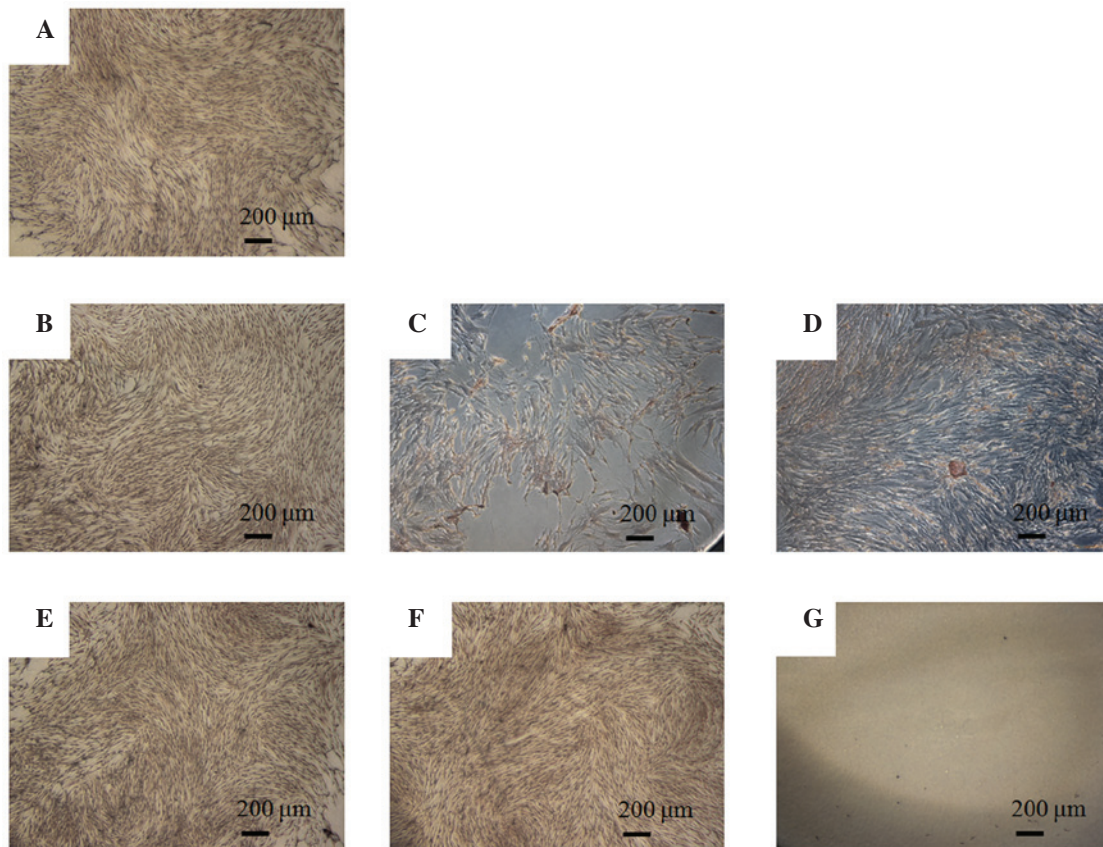


Figure 6. Results of Alizarin Red S staining on day 5 following treatment with different concentrations of tacrolimus. (A) Control, (B) 0.001 $\mu\text{g/ml}$ group, (C) 0.01 $\mu\text{g/ml}$ group, (D) 0.1 $\mu\text{g/ml}$ group, (E) 1 $\mu\text{g/ml}$ group, (F) 10 $\mu\text{g/ml}$ group, (G) 100 $\mu\text{g/ml}$ group.

0.1 and 1 $\mu\text{g/ml}$ of tacrolimus were $110.9 \pm 10.7\%$, $102.1 \pm 8.4\%$, $106.5 \pm 8.0\%$, and $111.8 \pm 6.7\%$, respectively when the result of the control group on day 5 was considered to be 100% ($100.0 \pm 9.1\%$). A significant decrease in mineralization was observed in the 100 $\mu\text{g/ml}$ group on day 5 in comparison with the control group ($P < 0.05$). The results for day 7 showed that treatment with tacrolimus at concentrations 0.001-1 $\mu\text{g/ml}$ led

to increase mineralized deposits compared with the control group. The relative value of the mineralization at 0.001, 0.01, 0.1 and 1 $\mu\text{g/ml}$ of tacrolimus were 118.0 ± 11.2 , 123.6 ± 12.3 , 118.3 ± 4.5 and $104.4 \pm 5.9\%$, respectively, when the result of the control group on day 7 was considered 100% ($100.0 \pm 5.4\%$). A statistically significant decrease was observed in the 100 $\mu\text{g/ml}$ group on day 7 in comparison with the control group ($P < 0.05$).

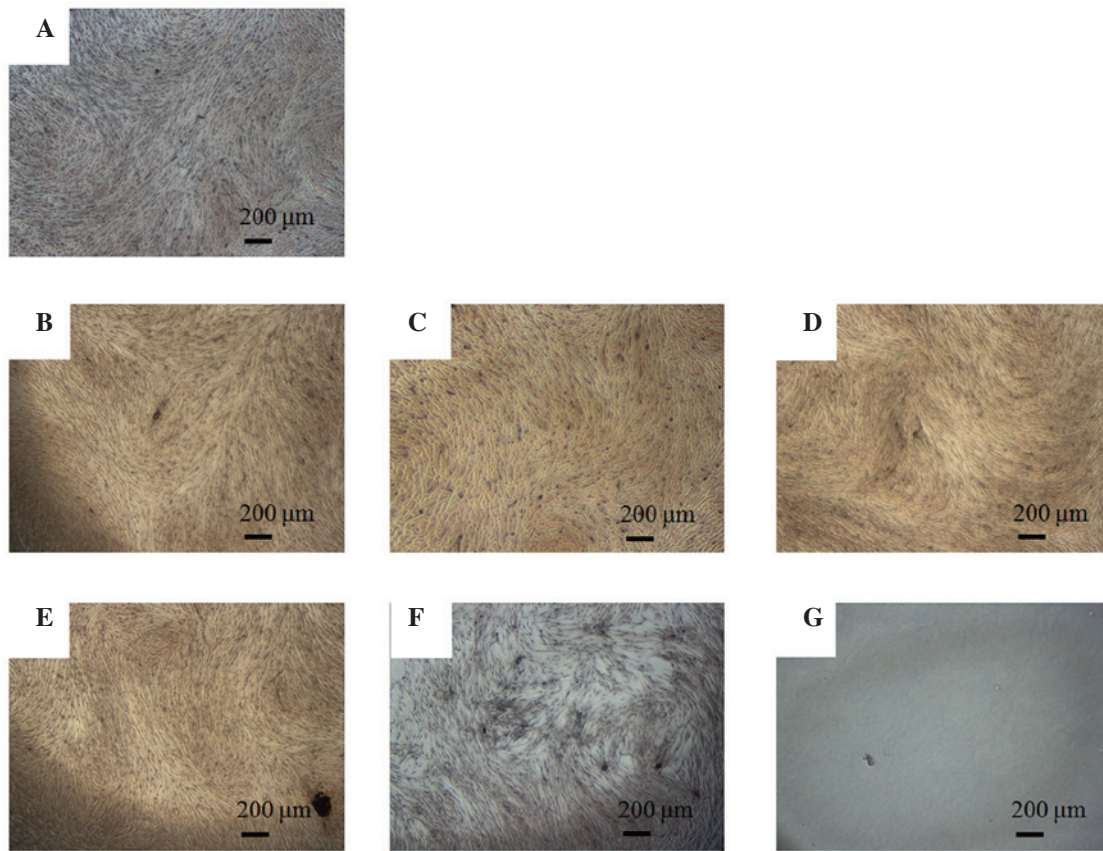


Figure 7. Results of Alizarin Red S staining on day 7 following treatment with different concentrations of tacrolimus. (A) Control, (B) 0.001 $\mu\text{g/ml}$ group, (C) 0.01 $\mu\text{g/ml}$ group, (D) 0.1 $\mu\text{g/ml}$ group, (E) 1 $\mu\text{g/ml}$ group, (F) 10 $\mu\text{g/ml}$ group, (G) 100 $\mu\text{g/ml}$ group.

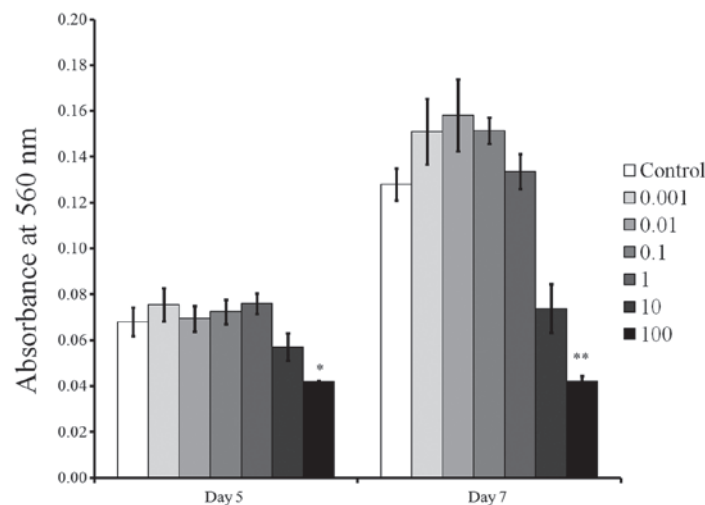


Figure 8. Quantitative results of mineralization assay on days 5 and 7. * $P < 0.05$ compared with the control group at day 5. ** $P < 0.05$ compared with the control group at day 7.

Discussion

Immunosuppressants have provided great improvement in organ transplantation by suppressing the rejection of allografts; this has increased the survival rate of organ transplant patients (23). Tacrolimus (FK506) is a widely-used, well-known immunosuppressant used following kidney or heart transplantation, and has been recognized as effective in

promoting the growth of bone grafts (24). The present study was performed in order to investigate the effects of tacrolimus on proliferation and osteoblastic differentiation of mesenchymal stem cells *in vitro*.

This study determined the effects of tacrolimus on the morphology, cell viability and mineralization following treatment with 0.001-100 $\mu\text{g/ml}$ tacrolimus. The cells exposed to tacrolimus at concentrations of 0.001-10 $\mu\text{g/ml}$ exhibited

a similar fibroblastic spindle shape. Short-term application of tacrolimus did not result in morphologic changes at final concentrations ranging from 0.001 to 10 $\mu\text{g/ml}$.

Cellular viability was determined using a CCK-8 assay, which is based on mitochondrial enzyme reduction of the WST-8 and spectrophotometric quantification of the water-soluble formazan dye generated (25). This study showed that tacrolimus at the tested concentrations was not identified to exhibit a significant effect on the viability of stem cells derived from the gingiva at final concentrations ranging from 0.001 to 10 $\mu\text{g/ml}$. However, tacrolimus at 100 $\mu\text{g/ml}$ decreased cell viability on days 3, 5 and 7.

Osteogenic differentiation can be evaluated by Alizarin red S staining (26). The presence of calcium in cellular deposits was confirmed by Alizarin Red S staining with cetylpyridinium chloride for quantification (27). Tacrolimus at 0.001, 0.01 and 1 $\mu\text{g/ml}$ resulted in the highest degree of mineralized nodule formation on day 7. A previous study showed that tacrolimus at 0.04 and 0.4 $\mu\text{g/ml}$ enhanced osteoblastic differentiation of rat mesenchymal stem cells (20). Co-stimulation with tacrolimus (1.0 $\mu\text{g/ml}$) and bone morphogenetic protein-9 (100 ng/ml) induced marked osteoblastic differentiation of dedifferentiated fat cells, which were isolated from mature adipocytes using the ceiling culture method and exhibit similar characteristics to mesenchymal stem cells (28). In the present study, the highest differentiation was achieved at 0.01 $\mu\text{g/ml}$; however, a previous study demonstrated that remarkable osteoblast differentiation was achieved at 1.0 $\mu\text{g/ml}$ (20). The differences may be explained by the different type and stage of the cells, the culturing time period and the culture system (29,30).

A previous study demonstrated that tacrolimus promoted the early stage of osteoinduction (24). The mechanism underlying osteogenic differentiation has not yet been fully elucidated; however, it has been suggested that the osteogenic effect of tacrolimus may involve bone morphogenetic protein signaling (16). A previous report suggested that tacrolimus promoted osteogenic differentiation by activating BMP receptors through interacting with FK506-binding protein 12 (16). Tacrolimus enhanced the positive effects of bone morphogenetic proteins on alkaline phosphatase activity and osteocalcin in mRNA (19). The effects of the combination treatment with bone morphogenetic protein and tacrolimus acted in a dose- and time-dependent manner (19).

In conclusion, the present study demonstrated that treatment with tacrolimus at the tested concentrations ranging from 0.001 to 10 $\mu\text{g/ml}$ did not result in significant changes in the viability of stem cells derived from gingiva, but increased osteogenic differentiation of the stem cells. Further studies related to this phenomenon in an *in vivo* model are necessary in order to ascertain greater understanding.

Acknowledgements

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