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Palmitate induces apoptosis and inhibits osteogenic differentiation of human periodontal ligament stem cells

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ABSTRACT

Objective: The aim of the present study was to investigate the effect of palmitate on human periodontal ligament stem cells (PDLSCs).
Design: PDLSCs were isolated from the third molars of healthy adult donors, and cultured in normal or osteogenic medium supplemented with palmitate (0, 100, or 250 μM) for 21 days. Cell proliferation was evaluated by measuring the amount of formazan at 6, 24, 48, and 72 h. Apoptosis was detected by ELISA and terminal deoxynucleotidyl transferase dUTP nick end labeling assay at days 3 and 7. Osteogenic differentiation was evaluated by measuring the alkaline phosphatase (ALP) activity, production of procollagen type I C-peptide and osteocalcin, mineralization, and mRNA expression of Runx2 at days 3, 7, 14, and 21. In addition, mRNA expression of IL-6 and IL-8 was measured at day 3.
Results: Palmitate inhibited the proliferation, ALP activity, production of procollagen type I C-peptide and osteocalcin, mineralization, and mRNA expression of Runx2 in the cultured PDLSCs. Palmitate also induced apoptosis and mRNA expression of IL-6 and IL-8 in the PDLSCs.
Conclusions: The results of the present study demonstrate that palmitate induces apoptosis and inhibits osteogenic differentiation of PDLSCs. These findings may help clarify the relationship between palmitate and periodontal tissue regeneration.

1. Introduction

Palmitate is the most abundant saturated free fatty acid in plasma (Yi, He, Liang, Yuan, & Chau, 2006), and has toxic effects on various types of cells. For instance, palmitate induces apoptosis and proinflammatory responses in human osteoblasts and bone marrow-derived mesenchymal stem cells (Gillet et al., 2015; Kim et al., 2008; Lu et al., 2012), and inhibits the differentiation of human osteoblasts as well as their bone formation function (Gunaratnam, Vidal, Gimble, & Duque, 2014; Yeh, Ford, Lee, & Adamo, 2014). Moreover, palmitate is associated with the pathogenesis of metabolic syndrome and type 2 diabetes mellitus. High dietary intake of palmitate exacerbates the risk of metabolic syndrome, which can lead to the onset of type 2 diabetes mellitus (Cascio, Schiera, & Liegro, 2012; Phillips et al., 2012). Plasma concentrations of palmitate are significantly increased in patients with type 2 diabetes mellitus (Yi et al., 2006).

Recent studies have shown that palmitate may affect periodontal tissues and exacerbate periodontitis. High-fat diet, composed mainly of palmitic acid, exacerbates periodontal tissue inflammation and alveolar bone loss in an animal model (Li et al., 2015). Furthermore, palmitate promotes the expression of proinflammatory cytokines such as IL-6 and IL-8 in human gingival fibroblasts (Li et al., 2019; Shikama, Kudo, Ishimaru, & Funaki, 2015). However, whether palmitate has any effects on periodontal tissue regeneration remains unknown.

Periodontal ligament stem cells (PDLSCs) exist in periodontal tissues and have tissue regeneration capacity (Liu et al., 2008; Nagatomo et al., 2006; Sonoyama et al., 2006). PDLSCs have the potential to differentiate into osteoblasts and exhibit greater potential in the regeneration of periodontal tissues than other mesenchymal stem cells (Gronthos, Mrozik, Shi, & Bartold, 2006; Park et al., 2011). Therefore, PDLSCs play a particularly important role in periodontal tissue regeneration. The aim of the present study was to elucidate the effect of palmitate on the viability and osteogenic differentiation of PDLSCs.

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2. Materials and methods

2.1. Isolation and primary culture of PDLSCs

PDLSCs were isolated and cultured from two females and a male (aged 21–29 years) as described in our previous study (Kato et al., 2013). After extraction, the teeth were rinsed in normal culture medium (Dulbecco's Modified Eagle's Medium with 500 U/mL penicillin, 500 μg/mL streptomycin, and 1.25 μg/mL amphotericin B (all from Nacalai Tesque, Kyoto, Japan)). After rinsing, the periodontal ligament tissues were separated from the middle one-third of the root surface and minced into 1-mm^3 pieces. The minced tissues were digested for 1 h at 37 °C in a solution of 3 mg/mL collagenase type I (Wako Pure Chemical Industrials Ltd, Tokyo, Japan) and 4 mg/mL dispase (Gibco, Thermo Fisher Scientific, Grand Island, NY, USA). The digested tissue samples were pooled, and single-cell suspensions were created by passing the pooled tissues through a strainer with 70 μm pore size (Falcon BD, Franklin Lakes, NJ, USA). The cells were resuspended in normal culture medium containing 10 % fetal bovine serum (Biowest, Nuaillé, France). The cells were then seeded onto 75 cm^2 culture dishes (AGC Techno Glass, Shizuoka, Japan), and incubated at 37 °C in 5% CO_2. The presence of single-cell colonies was confirmed after 5–10 days. PDLSCs at passage zero P0 were seeded, and cells at P3 to P5 were used for the experiments in the present study. Immnunocytochemistry for mesenchymal stem cell markers such as STRO-1 and SSEA-4 was used to confirm the phenotype of the cells. PDLSCs were obtained in accordance with the medical ethics guidelines of Osaka Dental University, and all the experiments were approved by the Osaka Dental University Medical Ethics Committee approval No. 110897. All participants provided written informed consent to participate in the present study, and the study design was approved by the appropriate ethics review board.

2.2. Culture medium and palmitate preparation

For cell proliferation assay and apoptosis detection, PDLSCs were incubated in normal culture medium containing 10 % fetal bovine serum (Biowest, Nuaillé, France). For osteogenic differentiation assays, PDLSCs were incubated in osteogenic medium containing 10 % fetal bovine serum (Biowest, Nuaillé, France), 50 μM L-ascorbic acid 2-phosphate (Nacalai Tesque), 10 mM β-glycerophosphate (Wako), and 10 nM dexamethasone (MP Biomedicals, LLC, Santa Ana, CA, USA).

Palmitate stock solution was prepared as previously described, with some modifications (Cousin et al., 2001). Briefly, a 100 mM solution of palmitic acid was prepared via dissolution in 0.1 M NaOH at 70 °C. The 100 mM solution of palmitate was mixed with 10 % bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) solution to obtain a 5 mM stock solution. The stock solution was then sterile-filtered through a membrane filter with 20 μm pore size (Toyo Roshi, Tokyo, Japan).

2.3. Cell proliferation assay

PDLSCs were plated onto 96-well microplates at a density of 2 × 10^4 cells/mL in normal culture medium (100 μL/well) containing palmitate (0, 100, or 250 μM), and the cells were cultured for 6, 24, 48, and 72 h. The number of viable cells at each time point was determined by measuring the amount of formazan using a formazan detection kit (Nacalai Tesque). The absorbance of formazan was measured at a wavelength of 450 nm, and the data were analyzed using the Soft Max® Pro Microplate Data Acquisition and Analysis software (Molecular Devices, Sunnyvale, CA, USA).

2.4. Apoptosis detection

PDLSCs were plated onto 24-well microplates at a density of 4 × 10^4 cells/mL and cultured until confluence in normal culture medium. The medium was replaced with normal medium containing palmitate (0, 100, or 250 μM), and the cells were cultured for 3 and 7 days. The cell pellets were collected, and the amount of fragmented DNA in apoptotic cells was measured using a cell death detection kit (Roche, Indianapolis, IN, USA). In addition, fragmented DNA was detected immunocytochemically by terminal deoxynucleotidyl transferase dUTP nick end labeling assay, using an *in situ* apoptosis detection kit (Takara Bio, Kusatsu, Japan). Pictures of apoptotic cells were processed using a microscope (Keyence, Osaka, Japan).

2.5. Measurement of alkaline phosphatase (ALP) activity

PDLSCs were plated onto 24-well microplates at a density of 4 × 10^4 cells/mL and cultured until confluence in normal culture medium. The medium was replaced with osteogenic medium containing palmitate (0, 100, or 250 μM), and the cells were cultured for 7 and 14 days. The cells were washed with phosphate-buffered saline and lysed with 0.2 % Triton X-100 (Sigma-Aldrich). The cell lysates were treated with a 1-Step PNPP substrate (Pierce Biotechnology, Inc., Pockford, IL, USA), and the absorbance was measured at a wavelength of 405 nm. The DNA content was measured using the PicoGreen dsDNA Assay Kit (Invitrogen, Paisley, UK). To normalize the ALP activity, the amount of ALP was normalized to the amount of DNA in the cell lysate. The data were analyzed using the Soft Max® Pro software (Molecular Devices).

2.6. Measurement of procollagen type I C-peptide

PDLSCs were plated onto 24-well microplates at a density of 4 × 10^4 cells/mL and cultured until confluence in normal culture medium. The medium was replaced with osteogenic medium containing palmitate (0, 100, or 250 μM), and the cells were cultured for 7 days. The culture supernatant was collected, and the levels of procollagen type I C-peptide were measured using a procollagen type I C-peptide detection kit (Takara Bio).

2.7. Measurement of osteocalcin

PDLSCs were plated onto 24-well microplates at a density of 4 × 10^4 cells/mL and cultured until confluence in normal culture medium. The medium was replaced with osteogenic medium containing palmitate (0,100, or 250 μM), and the cells were cultured for 21 days. The culture supernatant was collected, and the osteocalcin levels were measured using an osteocalcin detection kit (Takara Bio).

2.8. Measurement of calcium deposition

PDLSCs were plated onto 24-well microplates at a density of 4 × 10^4 cells/mL and cultured to confluence in normal culture medium. The medium was replaced with osteogenic medium containing palmitate (0, 100, or 250 μM), and the cells were cultured for 21 days. Extracellular calcium deposition was measured after dissolution in 10 % formic acid. The amount of calcium was quantified using a Calcium E-test Kit (Wako), according to the manufacturer's protocol. The absorbance was measured at a wavelength of 610 nm, and the data were analyzed using the Soft Max® Pro software (Molecular Devices).

2.9. Alizarin red staining

PDLSCs were plated onto 24-well microplates at a density of 4 × 10^4 cells/mL and cultured until confluence in normal culture medium. The medium was replaced with osteogenic medium containing palmitate (0, 100, or 250 μM), and the cells were cultured for 21 days. The cells were washed with phosphate-buffered saline and fixed in 70 % ethanol for 10 min at −20 °C. PDLSCs were stained with a solution of 1% alizarin red S (Wako) for 3 min at room temperature and washed 3 times with distilled water. Pictures of the calcified nodules were processed using a microscope (Olympus, Tokyo, Japan).

2.10. Quantitative real-time polymerase chain reaction (PCR)

The mRNA expression levels of Runx2, IL-6, and IL-8 were determined by quantitative real-time PCR analysis. PDLSCs were plated onto 24-well microplates at a density of 4×10^4 cells/mL and cultured until confluence in normal culture medium. The medium was replaced with osteogenic medium containing palmitate (0, 100, or 250 μM), and the cells were cultured for 3 and 7 days. The total cellular RNA was extracted using a commercial kit (Qiagen, Venlo, the Netherlands), and 10 μl RNA from each sample was reverse-transcribed into complementary DNA using another commercial kit (Takara Bio). All real-time PCR assays were performed according to the manufacturer's protocol. Gene expression levels were estimated using a StepOnePlus Real-time PCR System (Thermo Fisher Scientific Inc.) and normalized to GAPDH expression.

2.11. Statistical analysis

In the present study, all experiments were performed in triplicate ($n = 3$). Data are presented as mean ± SD, and were analyzed using SPSS Statistics Ver. 17 (IBM, Chicago, IL, USA). One-way analysis of variance followed by Tukey's post hoc test was used to determine significance. P values < 0.05 were considered significant.

3. Results

3.1. Cell proliferation

The proliferation of PDLSCs in the normal culture medium containing 100 and 250 μM palmitate was significantly decreased compared with the cells cultured without palmitate at 24, 48, and 72 h (Fig. 1).

3.2. Apoptosis detection

The amount of fragmented DNA in apoptotic cells was significantly increased in the normal culture medium containing 100 and 250 μM palmitate at day 3 (Fig. 2A) and day 7 (Fig. 2B). The number of apoptotic cells was increased in the normal culture medium containing 100 and 250 μM palmitate at day 3 and 7 (Fig. 2C).

3.3. ALP activity

The ALP activity of PDLSCs was significantly decreased in the osteogenic medium containing 100 μM palmitate at day 14 (Fig. 3B), and 250 μM palmitate at day 7 (Fig. 3A) and day 14 (Fig. 3B).

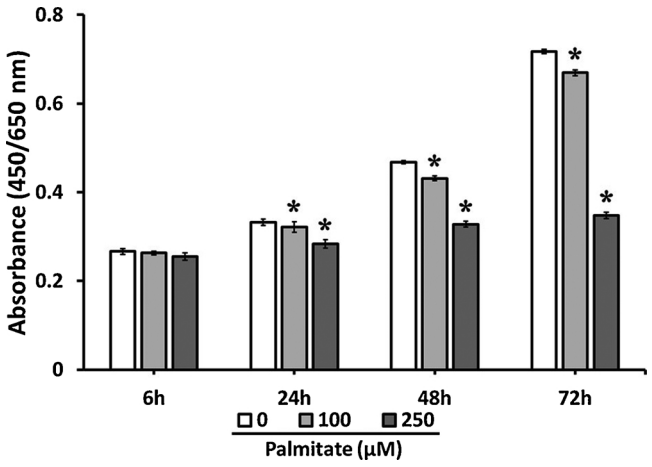

Fig. 1. Effect of palmitate on the proliferation of PDLSCs. The proliferation of the PDLSCs treated with 100 and 250 μM palmitate was significantly suppressed compared with the cells cultured without palmitate (*$p < 0.05$ at 24, 48, and 72 h).

3.4. Production of procollagen type I C-peptide and osteocalcin

The production of procollagen type I C-peptide was significantly lower in the osteogenic medium containing 100 and 250 μM palmitate than in the control at day 7 (Fig. 4A). The osteocalcin production was significantly lower in the osteogenic medium containing 250 μM palmitate than in the control at day 21 (Fig. 4B).

3.5. Calcium deposition and alizarin red staining

The calcium deposition was significantly higher in the osteogenic medium containing 100 μM palmitate than in the control, and was significantly lower in the osteogenic medium containing 250 μM palmitate at day 21 (Fig. 5A). The number of calcified nodules stained with alizarin red was lower in the osteogenic medium containing 250 μM palmitate than in the control at day 21 (Fig. 5B).

3.6. Expression of Runx2 mRNA

The expression of Runx2 mRNA was significantly decreased in the osteogenic medium containing 250 μM palmitate at day 3 (Fig. 6A) and day 7 (Fig. 6B).

3.7. Expression of IL-6 and IL-8 mRNA

Expression of IL-6 and IL-8 mRNA were measured at day 3. The expression of IL-6 mRNA was significantly increased in the osteogenic medium containing 250 μM palmitate (Fig. 6C). In addition, the expression of IL-8 mRNA was significantly increased in the osteogenic medium containing 250 μM palmitate (Fig. 6D).

4. Discussion

The results of the present study indicate that palmitate inhibits proliferation, osteogenic differentiation, and mineralization of PDLSCs. Moreover, palmitate induced apoptosis and proinflammatory cytokine expression in PDLSCs.

Palmitate inhibits cell survival and induces apoptosis in human osteoblasts (Kim et al., 2008). Palmitate also induces apoptosis in human bone marrow-derived mesenchymal stem cells (Lu et al., 2012). In the present study, palmitate suppressed the proliferation of PDLSCs, and induced apoptosis in PDLSCs. Thus, it is suggested that palmitate has a lipotoxic effect and inhibits the survival of PDLSCs, resulting in the inhibition of periodontal tissue regeneration and alveolar bone formation.

ALP is a marker of osteoblastic differentiation which is secreted during the mid-stage of differentiation and is closely related to initiation of mineralization (Aubin, Liu, Malaval, & Gupta, 1995; Weinreb, Shinar, & Rodan, 1990). Palmitate suppresses ALP activity in human osteoblasts (Gunaratnam et al., 2014; Yeh et al., 2014). In the present study, palmitate inhibited ALP activity in PDLSCs, suggesting that palmitate inhibits the mid-stage of osteoblastic differentiation of PDLSCs.

The synthesis of type I collagen is required for the formation of new bone (Blair, Zaidi, & Schlesinger, 2002). Procollagen type I C-peptide is a precursor of type I collagen and produced in the early stages of osteoblastic differentiation. Palmitate decreases mRNA expression of type I collagen in cardiac fibroblasts (Sokolova et al., 2017). In the present study, palmitate inhibited the production of procollagen type I C-peptide in PDLSCs, suggesting that palmitate inhibits osteogenic differentiation of PDLSCs at early stages of differentiation.

Osteocalcin is a non-collagenous protein component of bone matrix and a marker of late osteoblast differentiation (Ikeda, Nomura,

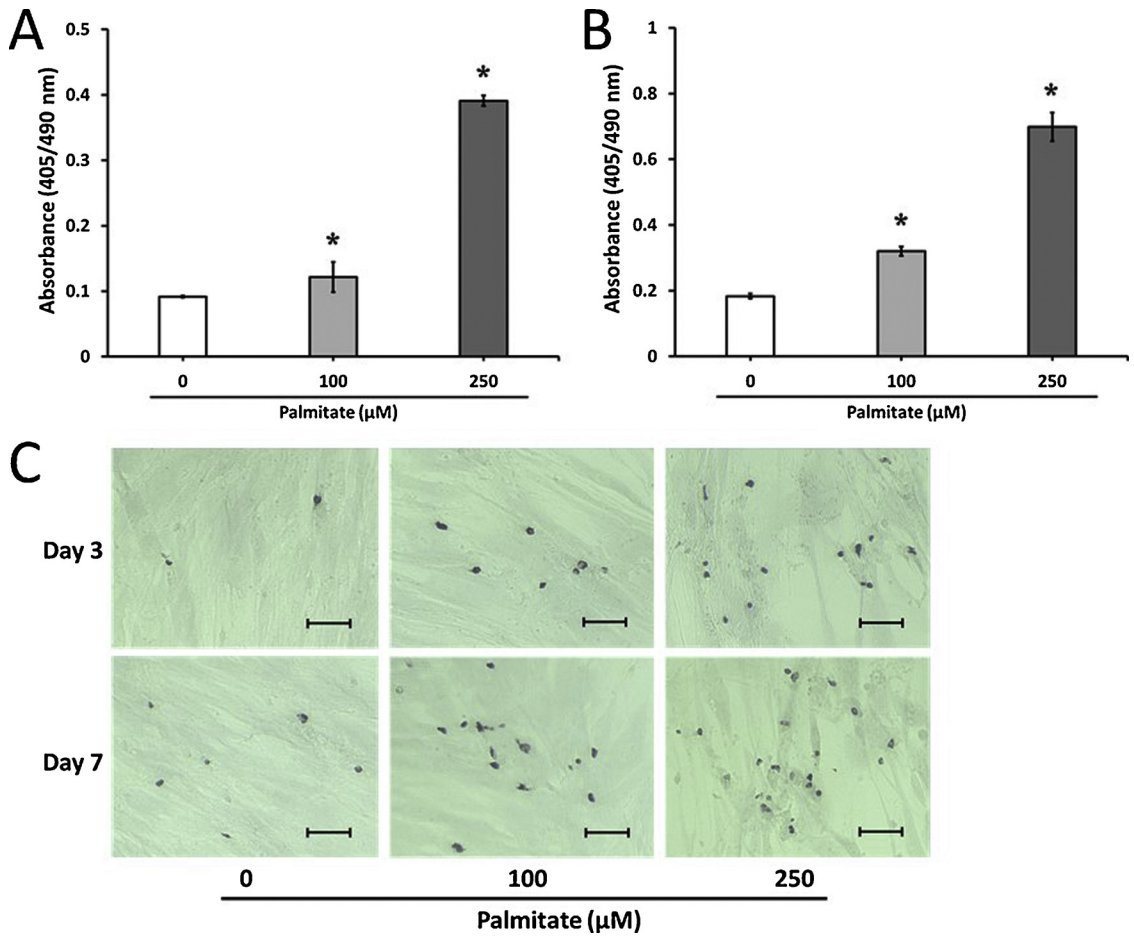

Fig. 2. Effect of palmitate on apoptosis in the cultured PDLSCs. (A) The fragmented DNA in the apoptotic cells was quantified by ELISA. The amount of fragmented DNA was significantly increased in the medium containing 100 and 250 μM palmitate (*$p < 0.05$ at day 3 and 7). (B) The fragmented DNA in the apoptotic cells were stained black with diaminobenzidine in terminal deoxynucleotidyl transferase dUTP nick end labeling assay. The number of apoptotic cells was increased in the medium containing 100 and 250 μM palmitate (day 3 and 7). Scale bar = 50 μm.

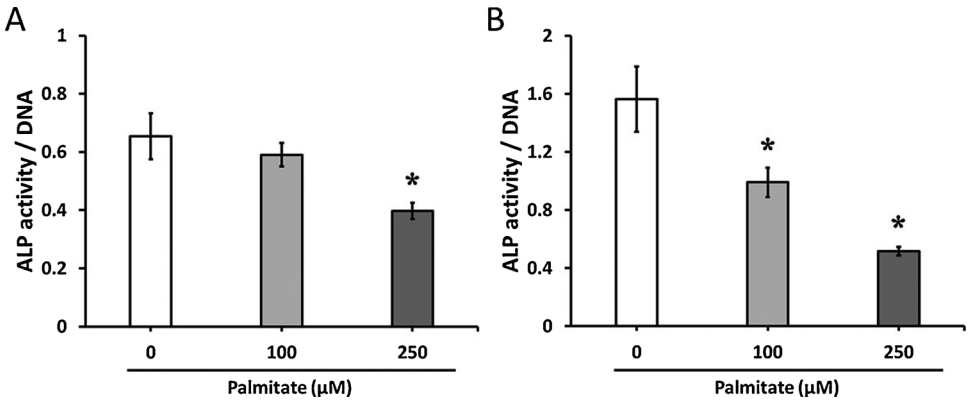

Fig. 3. Effect of palmitate on ALP activity in the cultured PDLSCs. ALP activity was measured at days 7 and 14. To normalize the ALP activity levels, the amount of ALP was normalized to that of the DNA. (A) At day 7, the ALP activity of the PDLSCs was significantly decreased in the medium containing 250 μM palmitate (*$p < 0.05$). (B) At day 14, the ALP activity was significantly decreased in the medium containing 100 and 250 μM palmitate (*$p < 0.05$).

Yamaguchi, Suda, & Yoshiki, 1992). Palmitate suppresses the osteocalcin mRNA expression in osteoblasts (Gunaratnam et al., 2014; Yeh et al., 2014). In the present study, 250 μM palmitate inhibited the osteocalcin production, suggesting that palmitate inhibits the differentiation of PDLSCs into mature osteoblasts.

Calcium deposition in cultured PDLSCs was quantified with a biochemical mineralization assay based on alizarin red staining and measurement of extracellular calcium deposition. Alizarin red staining is used to ascertain the presence of mineralized nodules formed by cells of osteogenic lineages; these nodules are indicative of bone matrix calcification. In previous studies, alizarin red staining was used to assess the mineralization of PDLSCs (Park et al., 2011; Seo et al., 2004). Palmitate inhibits the mineralization of extracellular matrix and bone nodule formation in osteoblasts (Gunaratnam et al., 2014; Yeh et al., 2014). In the present study, 250 μM palmitate reduced the number of mineralized nodules and extracellular calcium deposition in PDLSCs, indicating that high concentrations of palmitate inhibit the differentiation of PDLSCs into mature osteoblasts. On the other hand, 100 μM palmitate increased extracellular calcium deposition in PDLSCs. Palmitate may contribute to vascular calcification by inducing apoptosis and calcium deposition in vascular smooth muscle cells (Kageyama et al., 2013). Apoptotic bodies derived from vascular smooth muscle cells act as cores of

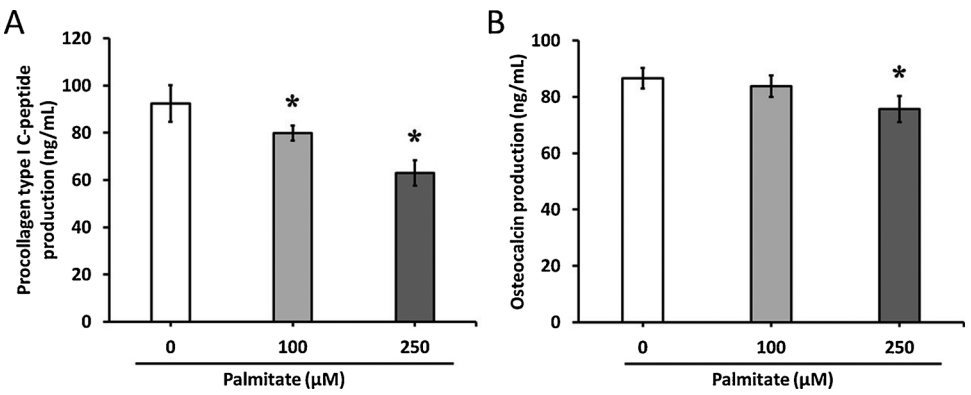

Fig. 4. Effect of palmitate on the production of procollagen type I C-peptide and osteocalcin in the cultured PDLSCs. (A) Production of procollagen type I C-peptide was measured at day 7. The production of procollagen type I C-peptide was significantly decreased in the medium containing 100 and 250 μM palmitate (*$p < 0.05$). (B) Osteocalcin production was measured at day 21. The osteocalcin production was significantly decreased in the medium containing 250 μM palmitate (*$p < 0.05$).

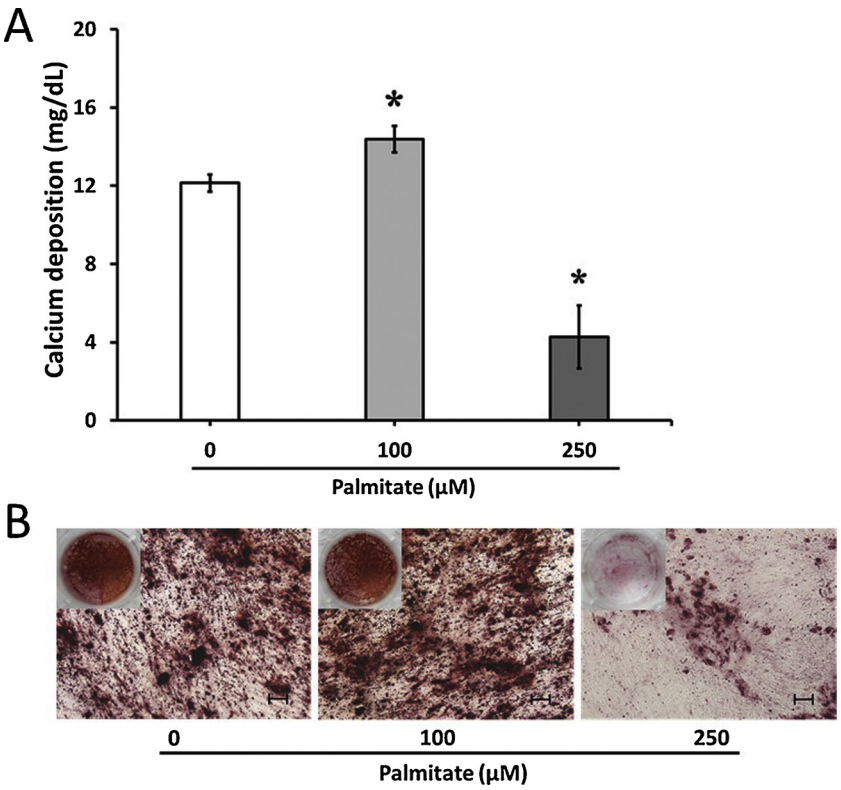

Fig. 5. Effect of palmitate on mineralization in the cultured PDLSCs. (A) The calcium deposition was significantly increased in the medium containing 100 μM palmitate (*$p < 0.05$), and significantly decreased in the medium containing 250 μM palmitate (*$p < 0.05$). (B) The number of calcified nodules stained with alizarin red was decreased in the medium containing 250 μM palmitate. Scale bar = 100 μm.

calcium deposition similar to matrix vesicles in osteoblasts and chondrocytes, and contribute to vascular calcification (Reynolds et al., 2004). The results of the present study suggest that low concentrations of palmitate induce apoptosis moderately in PDLSCs, and the apoptotic cells may act as cores of calcium deposition based on a mechanism similar to vascular calcification.

Runx2 is an essential transcription factor for osteoblastic differentiation and is expressed in immature osteoblasts (Komori, 2006). Palmitate inhibits Runx2 mRNA expression in osteoblasts (Gunaratnam et al., 2014; Yeh et al., 2014). In the present study, 250 μM palmitate suppressed the Runx2 mRNA expression in human PDLSCs, suggesting that palmitate inhibits the differentiation of PDLSCs into immature osteoblasts.

IL-6 and IL-8 are associated with the pathogenesis of periodontitis (Duarte et al., 2007; Javed, Al-Askar, & Al-Hezaimi, 2012). Palmitate induces the production of IL-6 and IL-8 in gingival fibroblasts and osteoblasts (Gillet et al., 2015; Li et al., 2019; Shikama et al., 2015). In the present study, 250 μM palmitate induced the expression of IL-6 and IL-8 in PDLSCs, indicating that palmitate causes inflammatory responses in periodontal tissues.

Previous studies have reported the mechanisms of palmitate-induced lipotoxic effects in various types of cells. Palmitate induces apoptosis in osteoblasts by impairing the activation of ERK (Kim et al., 2008). On the other hand, palmitate induces apoptosis in bone marrow-derived mesenchymal stem cells via the activation of p38 MAPK (Lu et al., 2012). Furthermore, palmitate induces inflammatory responses in human endothelial cells via the activation of Toll-like receptor 4 and NF-κB signaling pathway (Maloney et al., 2009). Palmitate also upregulates Toll-like receptor 4 expression as well as the increase of IL-6 and IL-8 expression in human bone marrow-derived mesenchymal stem cells and osteoblasts (Gillet et al., 2015). In the present study, the expression of IL-6 and IL-8 induced by palmitate suggests that the activation of Toll-like receptor 4 might contribute to the inflammatory responses in PDLSCs. However, whether these molecular mechanisms are associated with the effects of palmitate in PDLSCs is unclear, and the mechanisms need to be elucidated in future studies.

In conclusion, the present study showed that the saturated free fatty acid, palmitate induces apoptosis and inhibits osteogenic differentiation of periodontal ligament stem cells, which play an important role in periodontal tissue regeneration. The present study reports the first

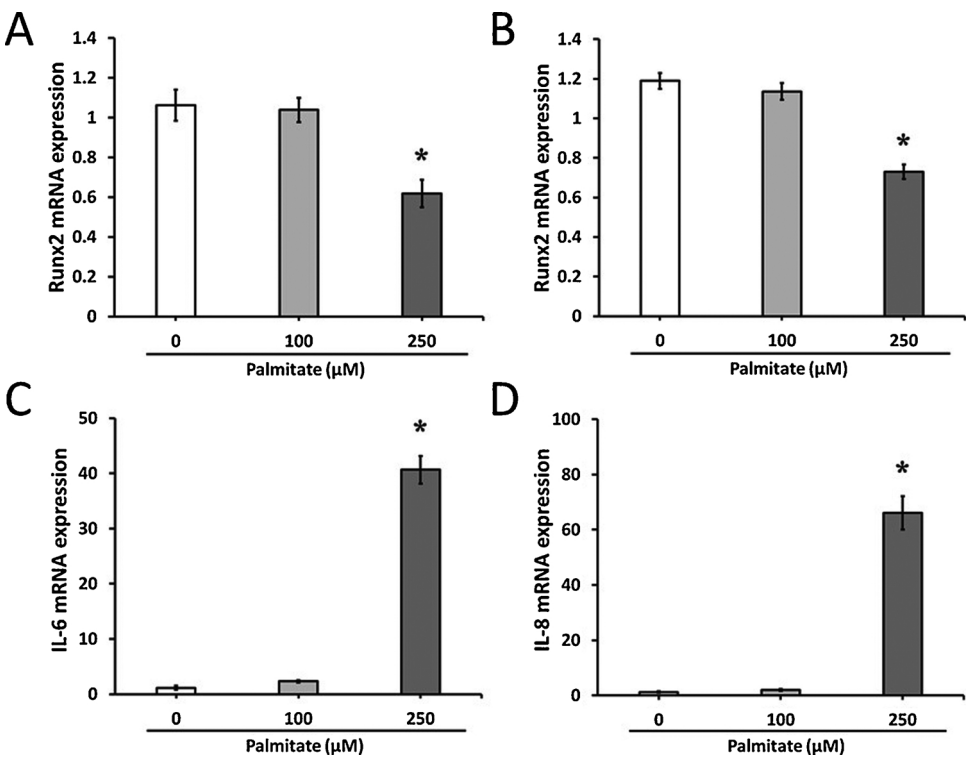

Fig. 6. Effect of palmitate on Runx2, IL-6, and IL-8 expression in the cultured PDLSCs. The expression of Runx2 mRNA was significantly decreased in the medium containing 250 μM palmitate (A: day 3, $*p < 0.05$; B: day 7, $*p < 0.05$). (C) The expression of IL-6 mRNA was significantly increased in the medium containing 250 μM palmitate ($*p < 0.05$). (D) The expression of IL-8 mRNA was significantly increased in the medium containing 250 μM palmitate ($*p < 0.05$).

findings concerning the effects of saturated free fatty acids on periodontal ligament stem cells, and may help clarify the relationship between palmitate and periodontal tissue regeneration.

Authors' contributions

1 The conception and design of the study, acquisition of data, analysis and interpretation of data.
2 Drafting the article and revising it critically for important intellectual content.
3 Final approval of the version to be submitted.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest related to this study.

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