Leukotriene B₄ loaded in microspheres regulate the expression of genes related to odontoblastic differentiation and biomineralization by dental pulp stem cells

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Abstract

Background: Leukotriene B₄ (LTB₄) is a potent lipid mediator that stimulate the immune response. Because dental pulp inflammation and dentin repair are intrinsically related responses, the aim of this research was to investigate the potential of LTB₄ in inducing differentiation of dental pulp stem cells.

Methods: Microspheres (MS) loaded with LTB₄ were prepared using an oil emulsion solvent extraction evaporation process and sterility, characterization, efficiency of LTB₄ encapsulation and in vitro LTB₄ release assay were investigated. Mouse dental pulp stem cells (OD-21) were stimulated with soluble LTB₄ or MS loaded with LTB₄ (0.01 and 0.1 μM). Cytotoxicity and cell viability was determined by lactate dehydrogenase and methylthiazol tetrazolium assays. Gene expression were measured by quantitative reverse transcription polymerase chain reaction after 3, 6, 24, 48 and 72 h. Mineralized nodule formation was assessed after 28 days of OD-21 cell stimulation with LTB₄ in mineralized media or not. Groups were compared using one-way ANOVA test followed by Dunnett’s post-test (α = 0.05).

Results: Treatment with LTB₄ or MS loaded with LTB₄ (0.01 and 0.1 μM-μM) were not cytotoxic to OD-21 cells. Treatment with LTB₄ modulated the expression of the Ibsp (integrin binding sialoprotein) and Runx2 (runt-related transcription factor 2) genes differently depending on the experimental period analyzed. Interestingly LTB₄ loaded in microspheres (0.1 μM) allowed long term dental pulp cell differentiation and biomineralization.

Conclusion: LTB₄, soluble or loaded in MS, were not cytotoxic and modulated the expression of the Ibsp and Runx2 genes in cultured OD-21 cells. When LTB₄ was incorporated into MS, odontoblast differentiation and mineralization was induced in long term culture.

Keywords: Dental pulp stem cells, Leukotriene, Microspheres, Odontoblast, Differentiation

Introduction

Pulp and dentin are closely related tissues, being assembled as a single unit, the dentin-pulp complex, which is a strategic and dynamic barrier in face of injuries suffered by teeth, being caries the most common cause of injury to this complex [1, 2]. Odontoblasts, located around the pulp, are the first to have contact with
pathogens, producing dentine matrix in order to protect the pulp [3, 4]. However, deep cavity preparations or dental pulp exposure can disrupt the integrity of the dentin-pulp complex and may cause odontoblast cell death [5]. Thus, the regeneration of these tissues occurs through stimulation and proliferation of mesenchymal progenitor cells, which are attracted to the injury site to differentiate into odontoblast-like cells and produce reparative dentin [6, 7].

Response to infection that occurs in the dental pulp is a complex molecular reaction that aims to eliminate the foreign pathogen. Cells and tissues at the injury site express receptors that recognize pathogenic signals, such as lipopolysaccharides, lipoteichoic acids and bacterial DNA [8]. In response to that, several inflammatory mediators are produced locally to orchestrate the immune response. Among those are the eicosanoids, a class of lipid mediators that are synthesized from arachidonic acid through the action of cyclooxygenases or lipoxigenases to produce prostaglandins and thromboxanes or leukotrienes (LT) and lipoxins, respectively [9, 10]. In the presence of FLAP (5-lipoxygenase activating protein), a nuclear protein associated with the membrane, the enzyme 5-LO is activated and oxidizes arachidonic acid, converting it to 5S-hydroxyeicosatetraenoic acid (5S-HpETE), which is further reduced by the enzyme peroxidase to 5S acid-hydroxy-icosatetraenoic acid (5S-HETE) or is converted into LTA4, which, by the action of LTA4 hydrolase, results in LTB4 production [11].

Leukotriene B4 (LTB4) is a potent inflammatory mediator that also stimulates the immune response, induces the recruitment of phagocytes and potentiates the ingestion and death of pathogens, being one of the most recognized neutrophil activators, modulating the release of cytokines and increasing vascular permeability [12–14]. LTB4 binds either to high affinity receptor (BLT1), mainly in leukocytes, or to low affinity receptor (BLT2) [15]. However, soluble LTB4 present a short half-life and is rapidly degraded [16]. As a therapeutic strategy, the use of microspheres could preserve the biological activity and stability of the mediator for prolonged periods [13, 17, 18]. However, studies are lacking to investigate the role of these lipid mediators in dental pulp cell behavior, especially through the synthesis and deposition of dentinal matrix in undifferentiated cells. Therefore, the objective of this study was to investigate if LTB4 loaded in microspheres would induce odontoblastic cell differentiation and biomineralization. The null hypothesis of this study was that LTB4 did not impact odontoblast cell differentiation and function.

Material and methods
Preparation of microspheres
Microspheres (MS) were prepared as a pharmacological strategy using an oil-in-water emulsion solvent extraction-evaporation process [13, 19]. Briefly, LTB4 (CAYM-14010; Cayman Chemical Company, Michigan, USA) was dissolved in absolute ethanol (100 µg/mL). Then, 0.3 mL of the organic phase, equivalent to 3 × 10^{-5} M of the LTB4 solution was added to 10 mL of methylene chloride supplemented with 30 mg of 50:50 poly (lactic-co-glycolic acid) (PLGA) (Boehringer Ingelheim, Germany). Next, 40 mL of 3% polyvinyl alcohol (3% w/v PVA) (Sigma-Aldrich CO., St. Louis, MO, USA) were added and the mixture was mechanically stirred at 600 rpm for 4 h (RW-20; Ika®-Werke GmbH & CO. KG, Staufen, Germany). Microspheres were washed (3x) with deionized water (Milli-Q®, Merck Millipore, Darmstadt, Germany), lyophilized, and stored at –20 °C until use.

LPS contamination tests
For sterility test small microsphere aliquots were diluted in 500 µL of 1 × PBS (phosphate buffered saline) and 100 µL of solution was spread on Brain Heart Infusion (BHI)-Agar medium and kept in an incubator at 37 °C for 24 h to detect microbial contamination.

Microspheres were tested for LPS contamination using the Limulus Amebocyte Lysate (LAL) QCL-1000™ kit (Lonza Walkersville, Inc., Olten, Switzerland) according to the manufacturer’s instructions. To obtain the standard curve, the serial dilution regime was performed, starting from 1.0 EU/mL of E. coli endotoxin 0111: B4 (E50-640). Optical density was analyzed using a µQuant™ photometer at a wavelength of 405 µm (BioTek® Instruments Inc.), in order to determine the concentration of endotoxin units/mL of solution containing microspheres (EU/ml).

Characterization of microspheres
Size distribution of MS was determined using a LS 13 320 Laser Diffraction Particle Size Analyzer (Beckman Coulter, USA). Samples (1 mg) of either unloaded-MS or LTB4-loaded MS was dispersed in 0.4 mL of purified sterile water and then analyzed at 25 °C. Zeta potential of MS was determined using a Zetasizer Nano (Malvern Instruments, England). Each sample was prepared dispersing 1 mg of unloaded-MS or LTB4-loaded MS in 0.4 mL of purified water containing 10 mM NaCl and then analyzed at 25 °C. Morphology of MS samples was assessed by scanning electron microscopy (SEM) using a FEIInspect S 50 scanning microscope (FEI; Oregon, USA).
Efficiency of LTB₄ encapsulation in MS

For calculation of encapsulation efficiency, samples of LTB₄-loaded MS (4 mg) were dissolved in 1 mL of acetonitrile/ethanol (7:3 v/v), to disrupt the MS structure. The solvent was then evaporated off in a vacuum concentrator centrifuge for 4 h, and the residue was reconstituted in 100 µL of methanol. Then, the supernatants were transferred to appropriate vials for determination of the concentration of LTB₄ by a competition enzyme immunoassay, according to manufacturer’s instructions (EIA, Amersham Biosciences, Piscataway, NJ, USA). Quantification in µM was accomplished using calibration curve containing LTB₄ synthetic standards (Cayman Chemical, Ann Arbor, MI, USA).

In vitro LTB₄ release assay

The release kinetics of LTB₄ from LTB₄-MS were monitored in vitro. LTB₄ (4 mg) was suspended in 1 mL of PBS/ethanol (50:50, v/v), pH 7.4, and incubated at 37 °C on a rotating incubator. At each time point 6, 12, 18, 24, 30, 36, 42, 48 and 54 h of rotation, the suspension was centrifuged and the supernatant was collected for assay of LTB₄ concentration, then 1 mL of fresh PBS/ethanol was added to the flask containing the LTB₄-MS and the experiment was continued.

The supernatants were transferred to appropriate vials for determination of the concentration of LTB₄ by a competition enzyme immunoassay, according to manufacturer’s instructions (EIA, Amersham Biosciences, Piscataway, NJ, USA). Quantification was accomplished using calibration curve containing LTB₄ synthetic standards (Cayman Chemical, Ann Arbor, MI, USA).

OD-21 cell culture

Murine immortalized undifferentiated dental pulp cells (OD-21) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% Penicillin/Streptomycin (Gibco) in an incubator at 37 °C and 5% CO₂. For the experiments, 1 x 10⁵ OD-21 cells/well were plated into 96-well cell culture plates and stimulated with LTB₄-loaded MS or soluble LTB₄ (Cayman Chemical Company) for 24 h.

The stimuli were removed and 10 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich CO., Catalog number M2128) supplemented with 150 µL RPMI (Roswell Park Memorial Institute) medium 1640 (Gibco) was added to the plates. After 3 h incubation, 40 µL of SDS (sodium dodecyl sulphate) buffer was added and cell viability was determined using a SpectraMax® Paradigm® spectrophotometer (Molecular Devices, LLC, Sunnyvale CA, USA). Data obtained was analyzed using a standard curve containing a known number of cells.

RNA extraction, reverse transcription, and polymerase chain reaction in real time (qRT-PCR)

For evaluation of cell differentiation and biomineralization signaling, integrin binding sialoprotein (Ibsp), runt-related transcription factor 2 (Runx2), dentin sialophosphoprotein (Dspp) and dentin matrix protein-1 (Dmp1) mRNA levels were assayed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). mRNA levels were measured by quantitative reverse transcriptase-polymerase chain reactions (qRT-PCR). To this end, total RNA was extracted using the RNeasy® Mini kit (Qiagen Inc., Valencia, USA) and quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA). A total of 1 µg of RNA were used for cDNA synthesis with the

Cytotoxicity: lactate dehydrogenase (LDH) assay

For cytotoxicity assessment, cells were plated in serum-free medium, at a concentration of 1 x 10⁵ cells per well, kept in an incubator at 37 °C and 5% CO₂ for 12 h (overnight). After this period, cultures were stimulated with different concentrations of soluble LTB₄ or microspheres with or without LTB₄ at 0.01 µM e 0.1 µM, for 24 h. Next, 50 µL of the supernatant was collected and transferred to a new 96-well plate with a transparent, flat bottom and 50 µL of the CytoTox 96® Reagent was added to each sample. The plate was then covered with foil to protect against light and the samples incubated at 25 °C for 30 min. After this period, 50 µL of the Stop Solution was added to each well. The absorbance was measured at 490 nm with a spectrophotometer (mQuanti, BioTek Instruments, Inc., Winooski, VT, USA). As positive control, 10 x Lysis Solution was added to the cells, 45 min prior to adding CytoTox 96® Reagent. LDH levels were expressed as percentages, according to the formula: cytotoxicity (%) = 100 x Experimental LDH Release absorbance/Maximum LDH Release absorbance (positive control).

Cell viability: MTT colorimetric assay

Cell viability was evaluated using methylthiazol tetrazolium (MTT) assay according manufacturer instructions. Briefly, 1 x 10⁵ OD-21 cells/well were plated into 96-well cell culture plates and stimulated with LTB₄-loaded MS or soluble LTB₄ (Cayman Chemical Company) for 24 h.

The stimuli were removed and 10 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich CO., Catalog number M2128) supplemented with 150 µL RPMI (Roswell Park Memorial Institute) medium 1640 (Gibco) was added to the plates. After 3 h incubation, 40 µL of SDS (sodium dodecyl sulphate) buffer was added and cell viability was determined using a SpectraMax® Paradigm® spectrophotometer (Molecular Devices, LLC, Sunnyvale CA, USA). Data obtained was analyzed using a standard curve containing a known number of cells.
were less than 0.1 EU/μg (Fig. 1B).

4.53

TaqMan® PCR reactions were performed in duplicate using the TaqMan® system in a StepOne Plus® real-time PCR system (StepOne Plus® Real-time PCR System, Applied Biosystems) and the following cycle program: 95 °C for 20 s, 40 cycles at 95 °C for 1 s, and 60 °C for 20 s. Primer-probe pairs were not available (TaqMan® Gene Expression Assay, Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as reference genes for normalization purposes. The results were analyzed based on cycle threshold (Ct) values. Relative expression was calculated by the ΔΔCt method.

Biomineralization assay
Mineralized nodule formation was assessed by culturing confluent OD-21 cells in biomineralization media for 28 days with changes of media every third-day. Biomineralization media consisted of DMEM culture media supplemented with 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and 1% FBS. OD-21 cells were treated with LTB4-MS or mineralizing media alone and with the combination of both. Mineralized monolayer cell cultures were stained for matrix biomineralization as described previously [21]. Briefly, cultures were fixed with 70% ethanol for 10 min and stained with 2% Alizarin Red solution (Sigma) for 5 min at room temperature. To quantify the degree of calcium accumulation in the mineralized extracellular matrix, Alizarin Red-stained cultures were incubated with 100 mM cetylpyridinium chloride (Sigma) for 1 h to release calcium-bound dye into solution. The absorbance of the released dye was measured at 570 nm using a spectrophotometer, and normalized by the total protein concentration in the culture.

Statistical analysis
Statistical analysis was performed using GraphPad Prism 6 software (GraphPad software Inc., La Jolla, USA). Groups were compared using the one-way ANOVA test followed by Dunnett’s post-test (α = 0.05).

Results
PLGA microspheres (loaded with LTB4 or empty) exhibited no bacterial growth after 24 h incubation in BHI-agar at 37 °C (Fig. 1A). Also, the endotoxin levels in all samples (encapsulated LTB4 or in empty microspheres) were less than 0.1 EU/μg (Fig. 1B).

Microspheres presented similar diameter with average diameter of 5.01 ± 4.4 μm for LTB4 loaded MS and 4.53 ± 2.23 μm for unloaded-MS (p > 0.05). The zeta potential was −12.3 ± 3.49 mV for LTB4 loaded MS and −20.6 ± 4.8 mV for unloaded-MS. In the scanning electron microscopy (SEM) was observed spherical, nonporous and non-aggregated microspheres.

The encapsulation efficiency of LTB4 was 39 ± 3.13% (Fig. 1C). Analysis of LTB4 release showed a burst release from MS at 6 h, when approximately 20% of the mediator was detected in the medium. After 48 h, 48% of LTB4 was released. These results indicate that PLGA biodegradation allows for a progressive release of LTB4 up to 54 h (Fig. 1C).

Treatment with empty microspheres or with LTB4 0.01 μM and 0.1 μM showed low cytotoxicity, which was similar to the control (p > 0.05) (Fig. 2A). The number of viable cells treated with LTB4 encapsulated in microspheres compared to the empty microspheres and LTB4 soluble were not statistically significant (p > 0.05) (Fig. 2B).

Runx2 expression increased after a 3 h stimulation period with LTB4 in both concentrations (p < 0.05). Within 6 h, the non stimulated group and groups of cells stimulated with LTB4 microspheres in both molarities had increased Runx2 expression (p < 0.05). At 24 h only the 0.01 μM LTB4 microspheres group increased Runx2 expression (p < 0.05). After a stimulation period of 48 and 72 h, the group that received treatment with microspheres with 0.01 μM LTB4 showed an increased Runx2 expression (p < 0.05) (Fig. 3).

Regarding Ibsp gene expression in the early period of time (3 h), the LTB4 0.1 μM showed higher expression of this gene (p < 0.05). On the other hand, in the periods of 6, 48 and 72 h, gene expression was higher in group with 0.1 μM LTB4 microsphere (p < 0.05) (Fig. 4). Dmp1 and Dspp gene expression was not detected in short term culture.

To further understand the role of LTB4-MS in OD-21 cell differentiation, the ability of cells to produce mineralized nodules was investigated. On day 28, LTB4-MS (0.1 μM) induced mineralized nodule formation more than cells maintained in biomineralization media alone (p < 0.05). Ibsp, Runx2, Dspp and Dmp1 gene expression at 28 days were higher in cells treated with LTB4-MS (0.1 μM) compared to biomineralization media alone (p < 0.05) (Fig. 5).

Discussion
Here we found that LTB4 induced an odontoblastic phenotype in dental pulp cells and production of mineralized nodules. LTB4 is a proinflammatory mediator derivate from the enzymatic oxidation of arachidonic acid involved in dental pulp inflammatory reactions [9, 10, 14, 22, 23], but none of them evaluated your effect in the osteogenic
Fig. 1 Characterization of PLGA-microspheres. A Culture of microspheres containing LTB4 on BHI-agar after 24 h incubation. B Data from LPS contamination of microspheres (MS) with or without LTB4. Endotoxins (below 0.1 EU/1 µg of polymer). C MEV image, size distribution, zeta potential distribution and in vitro LTB4 release assay.

Fig. 2 A Cytotoxicity using LDH assay in undifferentiated dental pulp cells (OD-21) added to microspheres (MS) with or without LTB4 after 24 h. B Cell viability of undifferentiated dental pulp cells (OD-21) added to microspheres (MS) with or without LTB4 using MTT assay after 24 h.
Fig. 3  Runx2 gene expression after stimulation or not with microspheres associated or not with LTB4 on the experimental times of 3, 6, 24, 48 and 72 h. *p < 0.05 compared to control (non-stimulated cells), ‡p < 0.05 compared to empty microspheres, §p < 0.05 comparison between MS-LTB4 0.01 µM and 0.1 µM, †p < 0.05 comparison between LTB4 0.01 µM and 0.1 µM, and ‡‡p < 0.05 comparison between soluble and MS at the same concentration.
and odontogenic differentiation of dental pulp stem cells. Therefore, the null hypothesis was rejected once LTB₄ loaded in microspheres regulated the expression of genes related to odontoblastic differentiation and biomineralization in mouse dental pulp stem cells.

As LTB₄ shows a half-life relatively short, in this study the use of microspheres had the aim to preserve its biological activities a longer time and protect the mediator from degradation [24]. LTB₄ showed no cytotoxic to dental pulp cells, measured by the percentage of cell death of less than 30% and in accordance to the International Organization for Standardization guidelines [25]. Other studies that used the PLGA microspheres demonstrated that it is biocompatible and act as particulate adjuvants [17, 24–29]. All these studies showed that microspheres are a viable way to deliver mediators for prolonged time.

The expression of Runx2 was upregulated by LTB₄ soluble after 3 h and after 6, 24, 48 and 72 h by LTB₄—loaded MS in different concentrations (0.01 and 0.1 μM), indicating the involvement of this mediator in Runx2 expression [30]. Runx2 is a transcription factor highly expressed in mesenchymal cells and dental papilla, which is essential for osteoblast and odontoblast differentiation and regulates these cell proliferations [31–33]. High doses of LTB₄ can stimulate the osteoblastic cell proliferation while low doses exhibited an inhibitory effect [34]. In this study, the use of microspheres prolonged the action of LTB₄ and it may have corroborated to this effect by increasing the expression of Runx2.

Integrin binding sialoprotein belongs to a family of proteins, exclusively located in mineralized tissues and crucial for the homeostasis of bone remodeling. The role of this protein involves the initiation of mineral deposition (hydroxyapatite) and increasing of osteoclastogenesis (bone resorption) [35]. In bacterial-induced apical periodontitis, the LTB₄ is involved in the signaling for osteoclastogenesis by the action of leukotriene B₄ type 1 receptor (BLT1) [10].

In this study Ibsp presented high relative expression after 3 h of stimulation with LTB₄ soluble, however it decreases in the other times analyzed, 6, 24, 48 and 72 h. While LTB₄—loaded MS upregulated the expression of Ibsp at 48 and 72 h. This upregulation can be associated to high expressions of Runx2 as some in-vitro studies demonstrated that the expression of bone matrix protein genes, as integrin binding sialoprotein (Ibsp) can be upregulated by Runx2 [33, 36].

Two LTB₄ receptor have been cloned: BLT1 and BLT2. BLT1 is the high-affinity receptor predominantly expressed in leukocytes and acts as a potent chemotactic receptor for inflammatory cells [15, 37]. LTB₄ can stimulate the osteoclast differentiation and bone resorption [38] by the activation of LTB₄/BLT1 mechanism [39]. BLT2 is the low-affinity receptor and has been associated with reduction of pain and wound-healing acceleration by cell proliferation [40]. The prolonged effect of LTB₄ promoted by the microspheres could activate the LTB₄/BLT2 mechanism and promote cell proliferation and differentiation. The increase in the relative expression of Runx2 and Ibsp might be related to that as BLT2 plays an important role in the wound-healing by cell proliferation [18].

A recent study demonstrated that LTB₄ needs an incubation time of 24 h to assure an adequate ligation with the receptor and present the intended pharmacological effects, as accelerated wound-healing rate [40]. Therefore, the use of microspheres can be a strategy to preserve the biological activities of the mediator for prolonged times and activated this receptor. One should not expect a direct correlation between in vitro and in vivo concentration of mediators released from microspheres, specially because the environment might influence that, due to inflammation, edema, dilution, etc. In this preclinical in vitro study, cell differentiation under LTB₄ stimuli was investigated. Later on, in vivo investigation should be performed to optimize the delivery to in vivo preclinical and clinical studies.

There are several clinical procedures that the materials can be directly applied to dental pulp which includes direct pulp capping, partial pulpotomy or full pulpotomy. Our findings shed light on a novel pharmacological strategy to delivery stimuli capable of inducing differentiation of dental pulp cells. Because LTB₄—MS can efficiently drive OD-21 cells into an odontoblast phenotype, these findings opens the avenue for a future clinical application. One limitation of our study is that the results were obtained in an in vitro study, requiring further in vivo investigation.

**Conclusion**

LTB₄ soluble or loaded in MS, were not cytotoxic and modulated the expression of the Ibsp and Runx2 genes in cultured OD-21 cells. When LTB₄ was incorporated into MS, odontoblast differentiation and mineralization was induced in long term culture. Our findings shed light on a novel pharmacological strategy to delivery stimuli capable of inducing differentiation of dental pulp cells obtained from a mouse cell lineage.
Fig. 4 (See legend on previous page.)
Fig. 5 A Mineralized nodule formation after stimulation with microspheres associated or not with LTB4 for 28 days. B Ibsp, Runx2, Dsp and Dmp1 gene expression after stimulation or not with MS-LTB4 for 28 days in biomineralization media. *p < 0.05 compared to control (non-stimulated cells), †p < 0.05 compared to biomineralization media alone, ‡p < 0.05 comparison between soluble and MS at the same concentration.
Abbreviations

LTB₄: Leukotriene B₄; MS: Microspheres; LDH: Lactate dehydrogenase; MTT Assay: Methylthiazol tetrazolium (MTT) assay; μM: Micrometer; OD‑21: Dental pulp cells; Ibsp: Integrin binding sialoglycoprotein; LTA₄: Leukotriene A₄; BLT1: Leukotriene receptor 1; BLT2: Leukotriene receptor 2; PLGA: Lactic-co-glycolic acid; 5°C: Degrees Celsius; μL: Microliter; PBS: Phosphate Buffered Saline; BH4: Brain Heart Infusion; LAL: Limulus Amebocyte Lysate; EU/mL: Endotoxin units per milliliter; mg: Milligram; mL: Milliliter; SEM: Scanning electron microscopy; h: Hour; DMEM: Dulbecco’s Modified Eagle’s Medium; FBS: Fetal bovine serum; RPMI: Roswell Park Memorial Institute; SDS: Sodium dodecyl sulphate; Dpp: Dentin sialophosphoprotein; Omp: Dentin matrix protein-1; μg: Microgram; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Ct: Cycle threshold; nm: Nanometer.

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Authors’ contributions

All authors contributed to the study conception and design. Material preparation and data collection were performed by FLS, GCCL and FMMPCO. FWGPS, LHF, PNF, LABS and RABS contributed to design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. All authors read, revised and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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