Leukotriene B 4 Loaded in Microspheres Regulate the Expression of Genes Related to Odontoblastic Differentiation and Biomineralization by Dental Pulp Stem Cells

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Research Article

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Abstract

Background: Leukotriene B$_4$ (LTB$_4$) 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 LTB$_4$ in inducing differentiation of dental pulp stem cells.

Methods: Microspheres (MS) loaded with LTB$_4$ were prepared using an oil emulsion solvent extraction evaporation process and sterility, characterization, efficiency of LTB$_4$ encapsulation and in vitro LTB$_4$ release assay were investigated. Mouse dental pulp stem cells (OD-21) were stimulated with soluble LTB$_4$ or MS loaded with LTB$_4$ (0.01 and 0.1 μM). Cytotoxicity and cell viability was determined by lactate dehydrogenase (LDH) and MTT (methylthiazol tetrazolium) assays. Gene expression were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) after 3, 6, 24, 48 and 72 h.

Results: Mineralized nodule formation was assessed after 28 days of OD-21 cell stimulation with LTB$_4$. Groups were compared using the one-way ANOVA test followed by Dunnett's post-test ($\alpha = 0.05$). Treatment with LTB$_4$ or MS loaded with LTB$_4$ (0.01 and 0.1 micrometer-μM) were not cytotoxic to OD-21 cells. Treatment with LTB$_4$ 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 LTB$_4$ loaded in microspheres (0.1 μM) allowed long term dental pulp cell differentiation and biomineralization. LTB$_4$ loaded in MS was not cytotoxic and induced an odontoblastic cell phenotype differentiation.

Conclusion: These findings shed light on a novel pharmacological strategy to induce dental pulp cell 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 lipoxygenases 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-hydroxyperoecosaetraenoic acid (5S-HpETE), which is further reduced by the enzyme peroxidase to 5S acid - hydroxyieicositetaenoic (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 therapeutical 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 1x 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 µQuantTM spectrophotometer at a wavelength of 405 nm (BioTek® Instruments Inc., Winooski, USA), with KC4™ Data Analysis Software (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 (1mg) of either unloaded-MS or LTB₄-loaded MS was dispersed in 0.4mL 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 1mg 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 FEI Inspect 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, Amershan 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
Quantification was accomplished using calibration curve containing LTB₄ synthetic standards (Cayman Chemical, Ann Arbor, MI, USA).

**OD-21 cell culture**

Undifferentiated mice dental pulp stem cells (OD-21 cell line) 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 × 10⁵ cells/well were plated into 48-well cell culture plates (Cell Wells, Corning Glass Workers, NY, USA) using DMEM without FBS and cells were left overnight for attachment.

Next, the culture medium was removed; wells were washed with phosphate buffered saline (PBS) and 300 µL LTB₄-loaded MS or soluble LTB₄ were added to each well. The experiments were done in duplicate and the stimuli were maintained for 3, 6, 24, 48 and 72 h for short term experiments or 28 days for long term biomineralization assay.

**Cytotoxicity – Lactate dehydrogenase (LDH) assay**

For cytotoxicity assessment, cells were plated in serum-free medium, at a concentration of 1 × 10⁵ cells per well, kept in an incubator at 37°C and 5% CO₂ for 12 hours (*overnight*). After this period, cultures were stimulated with different concentrations of soluble LTB₄ or microspheres with or without LTB₄ at 0.01 µM and 0.1 µM, for 24 hours. 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 minutes. 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, Bio-Tek Instruments, Inc., Winooski, VT, USA). As positive control, 10× Lysis Solution was added to the cells, 45 minutes prior to adding CytoTox 96® Reagent. LDH levels were expressed as percentages, according to the formula: cytotoxicity (%) = 100 × Experimental LDH Release absorbance / Maximum LDH Release absorbance (positive control).

**Cell viability – MTT colorimetric assay**

Cell viability was evaluated using 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-dymethylthiazol-2-yi)-2,5-diphenyltetrazoluim 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 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 High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, USA) in a thermal cycler (Veriti® Thermal Cycler, Applied Biosystems, USA). qRT-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 obtained commercially, and thus their sequences are 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 [20]. Briefly, cultures were fixed with 70% ethanol for 10 minutes and stained with 2% Alizarin Red solution (Sigma) for 5 minutes 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 hour 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 ($\alpha = 0.05$).

## Results

PLGA microspheres (loaded with LTB$_4$ or empty) exhibited no bacterial growth after 24h incubation in BHI-agar at 37° C (Figure 1A). Also, the endotoxin levels in all samples (encapsulated LTB$_4$ or in empty microspheres) were less than 0.1 EU/μg (Figure 1B).

Microspheres presented similar diameter with average diameter of 5.01±4.4 μm for LTB$_4$ loaded MS and 4.53±2.23 μm for unloaded-MS (p>0.05). The zeta potential was -12.3±3.49 mV for LTB$_4$ loaded MS and -20.6±4.8 mV for unloaded-MS. In the scanning electron microscopy (MES) was observed spherical, nonporous and non-aggregated microspheres.

The encapsulation efficiency of LTB$_4$ was 39±3.13% (Figure 1C). Analysis of LTB$_4$ 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 LTB$_4$ was released. These results indicate that PLGA biodegradation allows for a progressive release of LTB$_4$ up to 54 h (Figure 1C).

Treatment with empty microspheres or with LTB$_4$ 0.01 μM and 0.1 μM showed low cytotoxicity, which was similar to the control (p > 0.05) (Figure 2A). The number of viable cells treated with LBT$_4$ encapsulated in microspheres compared to the empty microspheres and LTB$_4$ soluble were not statistically significant (p > 0.05) (Figure 2B).

Runx2 expression increased after a 3-hour stimulation period with LTB$_4$ in both concentrations (p < 0.05). Within 6 hours, the non stimulated group and groups of cells stimulated with LTB$_4$ microspheres in both molarities had increased Runx2 expression (p < 0.05). At 24 hours only the 0.01 μM LTB$_4$ microspheres group increased Runx2 expression (p < 0.05). After a stimulation period of 48 and 72 hours, the group that received treatment with microspheres with 0.01 µM LTB$_4$ showed an increased Runx2 expression (p < 0.05) (Figure 3).

Regarding Ibsp gene expression in the early period of time (3h), the LTB$_4$ 0.1 μM showed higher expression of this gene (p < 0.05). On the other hand, in the periods of 6, 48 and 72 hours, gene expression was higher in group with 0.1 μM LTB$_4$ microsphere (p < 0.05) (Figure 4). Dmp1 and Dspp gene expression was not detected in short term culture.

To further understand the role of LTB$_4$-MS in OD-21 cell differentiation, the ability of cells to produce mineralized nodules was investigated. On day 28, LTB$_4$-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 LTB$_4$-MS (0.1 μM) compared to biomineralization media alone (p < 0.05) (Figure 5).
Discussion

Here we found that LTB$_4$ induced an odontoblastic phenotype in dental pulp cells and production of mineralized nodules. LTB$_4$ is a proinflammatory mediator derivative from the enzymatic oxidation of arachidonic acid involved in dental pulp inflammatory reactions [10, 14, 21-23], but none of them evaluated your effect in the osteogenic and odontogenic differentiation of dental pulp stem cells. Therefore, the null hypothesis was rejected once LTB$_4$ loaded in microspheres regulated the expression of genes related to odontoblastic differentiation and biomineralization in mouse dental pulp stem cells.

As LTB$_4$ 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]. LTB$_4$ 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 [17,24,26-29]. All these studies showed that microspheres are a viable way to delivery mediators for prolonged time.

The expression of Runx2 was upregulated by LTB$_4$soluble after 3 h and after 6, 24, 48 and 72h by LTB$_4$ - loaded MS in different concentrations (0.01 and 0.1 μM), indicating the involvement of this mediator in Runx2expression (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]. Hight doses of LTB$_4$ 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 LTB$_4$ 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 LTB$_4$ 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 hours of stimulation with LTB$_4$ soluble, however it decreases in the other times analyzed, 6, 24, 48 and 72 hours. While LTB$_4$ - loaded MS upregulated the expression of Ibsp at 48 and 72 hours. 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 LTB$_4$ 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]. LTB$_4$ can stimulate the osteoclast differentiation and bone resorption [38] by the activation of LTB$_4$/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 LTB$_4$ promoted by the
microspheres could activate the LTB$_4$/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 LTB$_4$ needs an incubation time of 24 hours 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 LTB$_4$ stimuli was investigated. Later on, *in vivo* investigation should be performed to optimize the deliver 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 findingsshed light on a novel pharmacological strategy to deliver stimuli capable of inducing differentiation of dental pulp cells. Because LTB$_4$-MS can efficiently drive OD-21 cells into an odontoblast phenotype, these findings opens the avenue for a future clinical application.

**Abbreviations**

LTB$_4$ = Leukotriene B$_4$

MS = Microspheres

LDH = lactate dehydrogenase

MTT Assay = methylthiazol tetrazolium (MTT) assay

μM = micrometer

OD-21 = dental pulp cells

*Ibsp* = integrin binding sialoprotein

*Runx2* = runt-related transcription factor 2

LT = leukotrienes

FLAP = 5-lipoxygenase activating protein

5-LO = 5-Lipoxygenase

5S-HpETE = 5S-hydroxyperoeicosatetraenoic acid
5S-HETE = 5S acid - hydroxyeicosatetraenoic
LTA4 = Leukotriene A4
BLT1 = leukotriene receptor 1
BLT2 = leukotriene receptor 2
PLGA = lactic-co-glycolic acid
°C = degrees Celsius
µL = microliter
PBS = Phosphate Buffered Saline
BHI = 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
PBS = phosphate buffered saline
RPMM = Roswell Park Memorial Institute
SDS = sodium dodecyl sulphate
Dspp = dentin sialophosphoprotein
Dmp1 = dentin matrix protein-1
µg = microgram
Gapdh = Glyceraldehyde-3-phosphate dehydrogenase
Ct = cycle threshold

nm = nanometer

Declarations

- Ethics approval and consent to participate
  Not applicable

- Consent for publication
  Not applicable

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

- Competing interests
  The authors declare that they have no competing interests

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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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Figures
Figure 1

Characterization of PLGA-microspheres. (A) Culture of microspheres containing LTB4 on BHI-agar after 24 hours 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.
Figure 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.
Figure 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 hours.
Figure 4

Ibsp gene expression after stimulation or not with microspheres associated or not with LTB4 on the experimental times of 3, 6, 24, 48 and 72 hours.
Figure 5

(A) Mineralized nodule formation after stimulation with microspheres associated or not with LTB4 for 28 days. (B) Ibsp, Runx2, Dspp and Dmp1 gene expression after stimulation or not with MS-LTB4 for 28 days in biomineralization media.