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Prostaglandin E_2 Induces Expression of Mineralization Genes by Undifferentiated Dental Pulp Cells

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Prostaglandin E_2 (PGE_2) is a lipid mediator usually released during inflammation. This study aimed to investigate the potential of soluble or microsphere-loaded PGE_2 on inducing differentiation of dental pulp stem cells. PGE_2-loaded microspheres (MS) were prepared using an oil-in-water emulsion solvent extraction-evaporation process and were characterized. Mouse dental pulp stem cells (OD-21) were stimulated with soluble or PGE2-loaded MS (0.01 and 0.1 μM). Cell viability was determined by MTT colorimetric assay. *Ibsp*, *Bmp2* and *Runx2* expression was measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) after 3, 6, and 24 h. The results showed that the soluble PGE_2 reduced dental pulp stem cells viability after 24 h of stimulation whereas PGE_2-loaded MS did not. Soluble PGE_2 up-regulated *Ibsp* and *Bmp2* at 3 h, differently from PGE_2-loaded MS. On the other hand, PGE_2-MS induced *Bmp2* and *Runx2* at 6 h and *Ibsp* at 24 h. In conclusion, our in vitro results show that PGE_2, soluble or loaded in MS are not cytotoxic and modulate *Ibsp*, *Bmp2*, and *Runx2* gene expression in cultured OD-21 cells.

Key Words: microspheres, prostaglandin E_2, dental pulp stem cell, differentiation

Introduction

Dental pulp present high regeneration capacity because of the presence of progenitor stem cells within the tissue. Acidic dissolution of carious dentin release repair signals that regulate the synthesis of reparative dentin, angiogenesis, and innervation (1). Thus, in response to caries, dental pulp responds dynamically by inflammation, expression of mineralization proteins and/or reparative dentin formation by odontoblast-like cells derived from dental pulp mesenchymal stem cells (2).

Successful handling of regeneration in this scenario involves the regulation of a complex molecular signalling network that shares similarities to the immune response to infection. Within that response, eicosanoid signalling are crucial players (3). Eicosanoids are oxidation products of C20 polyunsaturated fatty acids (e.g., arachidonic acid [AA]) that include prostaglandins (PGs) and leukotrienes (LTs) (4). During pulp inflammation, AA metabolism generates prostaglandin E_2 (PGE_2) (5) and this lipid mediator produced by stem cells shows such a potent immunomodulator with anti-inflammatory effects through regulation of cell maturation and activation (6). PGE_2 have demonstrate a dual role in proliferation and cell differentiation, because inhibit osteogenic differentiation of mesenchymal stem cells (7), but also has anabolic effects on osteogenic and odontogenic markers in vitro, enhances tertiary dentin formation in vivo (8). These findings shed light on this mediator as a potential therapeutical agent to accelerate pulp repair.

One shortcoming is that PGE_2 shows a half-life relatively short. Therefore to preserve its biological activities and protect the mediator from degradation, loading of lipid mediators in microspheres has been used as a pharmacological strategy (9,10). However, the role of lipid mediator, loaded in microspheres or are not, in dental pulp cell differentiation is not clear. For this reason, in this study we investigated the effects of the PGE_2 on the regulation of genes that encodes proteins as an indicator of odontoblastic cell differentiation. The null hypothesis of this study was that PGE_2 did not impact cell differentiation.

Material and Methods

Preparation of Microspheres

Microspheres (MS) were prepared as a pharmacological strategy using an oil-in-water emulsion solvent extraction-evaporation process (11). Briefly, PGE_2 (CAYM-14010; Cayman Chemical Company, Ann Arbor, MI, USA) was dissolved in absolute ethanol (5 mg/mL). Then, 0.3 mL of the organic phase, equivalent to 7 x 10-3 M of the PGE_2 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.

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. In addition, a sterility test was performed. Briefly, 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.

Characterization of Microspheres

Size distribution of MS was determined using a LS 13 320 Laser Diffraction Particle Size Analyzer (Beckman Coulter, Inc., Atlanta, GA, USA). Samples (1 mg) of either unloaded-MS or PGE_2-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 PGE_2-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 PGE_2 Encapsulation in MS

For calculation of encapsulation efficiency, samples of PGE_2-loaded MS (2 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 PGE_2 by high-performance liquid chromatography-tandem mass spectrometry using an Acquity UPLC-MS system coupled to a Xevo TQ-S mass spectrometer (Waters Corporation, Milford, MA, USA). Chromatographic separation was conducted using a Supelco Ascentis EXPRESS C_{18} HPLC column (Sigma-Aldrich Co.) with dimensions of 100 mm×3.0 mm, 2.7 µm. A binary gradient system was employed in which phases A and B consisted of water/acetonitrile/acetic acid (70:30:0.02, v/v) and acetonitrile/isopropanol (70:30, v/v), respectively. Samples (10 µL) were eluted with a flow rate of 0.6 mL/min, with a linear gradient starting with 0% B, which was increased to 15% B at 2 min, 20% B at 5 min, 35% B at 8 min, 40% B at 11 min, 100% B at 15 min, 100% B at 18 min, and 0% B at 19 min and held there for 30 min. Analyses were performed using multiple reaction monitoring (MRM) scan mode employing negative ionization (transitions 351.2 → 171 for PGE_2). Quantification was accomplished using calibration lines constructed with PGE_2 synthetic standards (Cayman Chemical Co.). Data were collected and analyzed using the software Mass Lynx 4.0 (Waters Corporation).

Cell Culture

Murine immortalized undifferentiated pulp cells line (OD-21) was used. The cells were kept in cryopreservation and stored in a freezing solution composed of 90% Bovine Fetal Serum (Gibbs, Carlsbad, CA, USA) and 10% Dimethylsulfoxide (DMSO) (Mallinckodt Chemicals , Mallinckrodt Baker Inc., Phillipsburg, USA). For cell culture, cells were transferred to a conical tube (Falcon ™, Corning Inc., NY, USA) containing 10 mL of DMEM with 10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin (Gibco, USA), supplemented with 10% FBS (DMEM-c) and centrifuged at 1200 rpm for 10 min at 10 ° C. The supernatant was discarded and cells resuspended in 30 mL of DMEM-c. After that, the cells were transferred to 150 cm^2 culture bottles (FalconTM, Corning Inc., NY, USA) at 37 °C in humidified incubator with 5% CO_2/95% air, according Ferreira et al. (12) The medium was changed every 3 days, and the bottle was washed with 30 mL Phosphate Buffered Saline (PBS) in order to remove debris and dead cells present in the culture.

After the culture reached confluence, the culture medium was removed, and the bottle was washed with 30 mL of PBS. Then, 15 mL of DMEM-c were placed in the bottle, and with a sterile scraper (Cell Scraper, Corning Glass Workers, NY, USA) cells were detached. After that, the supernatant was transferred to a conical tube and centrifuged at 1200 rpm for 10 min at 10 °C. The supernatant was discarded and the pellet resuspended in 10 mL of DMEM-c. The viable cells number was counted in Newbauer Chamber (BOECO Germany, Hamburg, Germany) using 10 µL of the solution containing the cells were added to 90 µL of Trypan blue solution (Gibco, NY, USA) (1:10).

For the experiments, 1×10^5 cells/well were plated into 48-well cell culture plates (Cell Wells; Corning Glass Workers, NY, USA) using DMEM without FBS (DMEMi) and cells were left to attach overnight.

Next, the culture medium was removed; wells were washed with 1x PBS and 300 µL PGE_2-loaded MS or soluble PGE_2 were added to each well. The experiments were done in duplicate for 3, 6, 24 or 36 h. Following incubation, the stimuli were removed and plates were either assayed or stored at -80 °C.

Cell Viability - MTT Colorimetric Assay

Cell viability was evaluated using MTT assay according

manufacturer instructions. Briefly, 1 x 10^5 OD-21 cells/well were plated into 96-well cell culture plates and stimulated with PGE_2-loaded MS or soluble PGE_2 (Cayman Chemical Co.) for 24 h.

The stimuli were removed and 10 µL of MTT (3-(4,5-dymethylthiazol-2-yl)-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). Cell viability was expressed as percentages, according to the formula: cell viability (%)=100× Experimental absorbance / Negative control absorbance (medium alone).

RNA Extraction, Reverse Transcription, and qRT-PCR

For evaluation of cell differentiation and mineralization signalling, *Ibsp*, *Bmp2*, *Runx2*, *Alpl*, *Msx1*, and *Bglap* mRNA levels were assayed by qRT-PCR. For that, total RNA was extracted using the RNeasy® Mini kit (Qiagen Inc., Valencia, USA) and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, MA, USA). Next, cDNA synthesis was done from 800 ng of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) in a thermal cycler (Veriti® Thermal Cycler, Applied Biosystems). Quantitative reverse transcriptase-polymerase chain reactions (qRT-PCR) were done in duplicate using the TaqMan® system in a StepOne Plus® real-time PCR system (StepOne Plus® Real-Time PCR System, Applied Biosystems) using the following cycle program: 95 °C for 20 s, followed by 40 cycles at 95 °C for 1 s, and 60 °C for 20 s. All protocols were performed according to the manufacturers' instructions. Primer-probe pairs were obtained commercially, and thus their sequences are not available. The quantifications were normalized using glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and beta-actin (Actb) as reference genes. The results were analyzed based on cycle threshold (Ct) values. For each gene, relative expression was calculated by the ΔΔCt method.

Statistical Analysis

The groups were compared using analysis of variance (ANOVA) followed by the Tukey's test (α=0.05).

Results

Endotoxin levels in PLGA MS were less than 0.1 EU / µg, as detected by the LAL assay and no bacterial growth was detected after 24 h of incubation in BHI-agar at 37 °C.

MS prepared with PLGA co-polymers presented monomodal population with similar diameter. For PGE_2-loaded MS average diameter was 5.332±3.480 µm and for unloaded-MS was 4.153±2.723 µm ($p>0.05$). Zeta potential of all MS was near the neutrality (-25±8.78 mV for PGE_2- loaded MS and -21.7±5.78 mV for unloaded-MS; $p>0.05$). Regarding morphology, MS prepared with PLGA co-polymers were nonporous, spherical and non-aggregated (Fig. 1).

The efficiency of encapsulation was determined using mass spectrometry to quantify PGE_2. The concentration of PGE_2 in 2 mg of PGE_2- loaded MS was calculated to be 853.9 ng/mL, which corresponds to an encapsulation efficiency of 85.4%.

Soluble PGE_2 at 0.01 and 0.1 µM reduced dental pulp cell viability ($p<0.05$) while PGE_2-loaded microspheres did not ($p>0.05$). Reduced cell viability was also detected in unloaded-MS (Fig. 2).

Soluble PGE_2 up-regulated gene expression at 3 h, differently from PGE_2-loaded MS, that sustained delivery for longer periods. *Ibsp* expression was stimulated by soluble PGE_2 (0.01 µM) compared to control (DMEM) cells after 3 h, differently from PGE_2-loaded MS (0.01 µM) or soluble

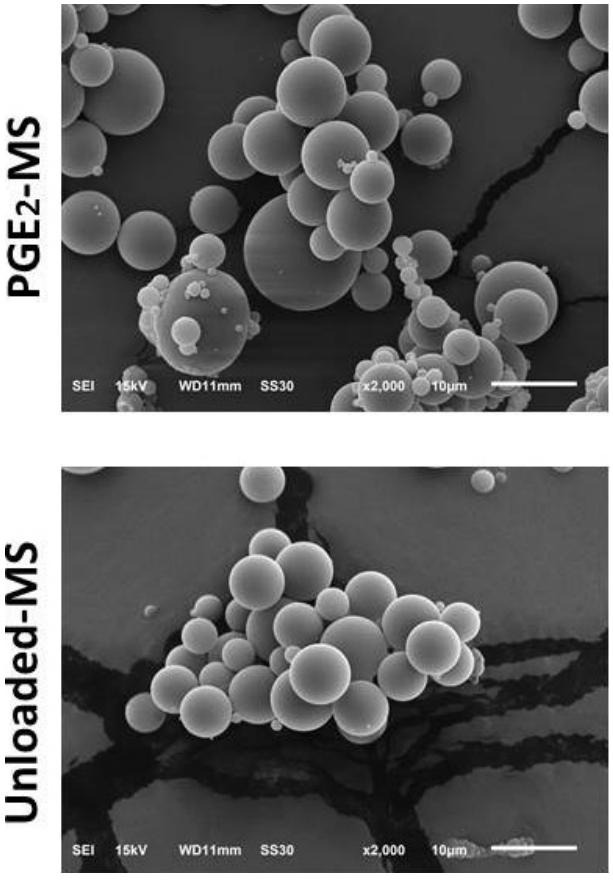


Figure 1. PGE_2-MS and unloaded-MS morphology assessed by scanning electronic microscopy (SEM)

PGE_2 in a higher concentration (0.1 µM) ($p<0.05$). After 6 h, no difference was found between treatments ($p>0.05$) whereas *Ibsp* expression at 24 h was inhibited by soluble PGE_2 (0.01 and 0.1 µM) ($p<0.05$). Treatment with PGE_2-loaded MS (0.01 µM) induced *Ibsp* expression compared to unloaded-MS ($p<0.05$) (Fig. 3).

Bmp2 expression was stimulated by soluble PGE_2 (0.1 µM) compared to control (DMEM) cells after 3 h ($p<0.05$) whereas PGE_2-loaded MS exerted no effect ($p>0.05$). After 6 h, there was an invertion in this pattern, because PGE_2-loaded MS (0.1 µM) induced *Bmp2* expression higher than PGE_2-loaded MS (0.01 µM) or controls (DMEM and unloaded-MS). At 24 h, PGE_2-loaded MS (0.01 or 0.1 µM) sustained *Bmp2* expression, differently from soluble PGE_2 that inhibited gene expression ($p<0.05$; Fig. 4).

Runx2 expression was not modulated after 3 h of treatment ($p<0.05$) but was upregulated by PGE_2-loaded MS (0.1 µM) after 6 h, differently from controls (DMEM and unloaded) or soluble PGE_2 (0.1 µM) ($p<0.05$). At 24 h, PGE_2-loaded MS (0.01 and 0.1 µM) exerted no effect on gene expression while soluble PGE_2 inhibited (0.1 µM) ($p<0.05$) (Fig. 5).

Bglap, *Alpl*, and *Msx1* gene expression were not detected in dental pulp stem cells with or without PGE_2 stimulation at any concentration or time.

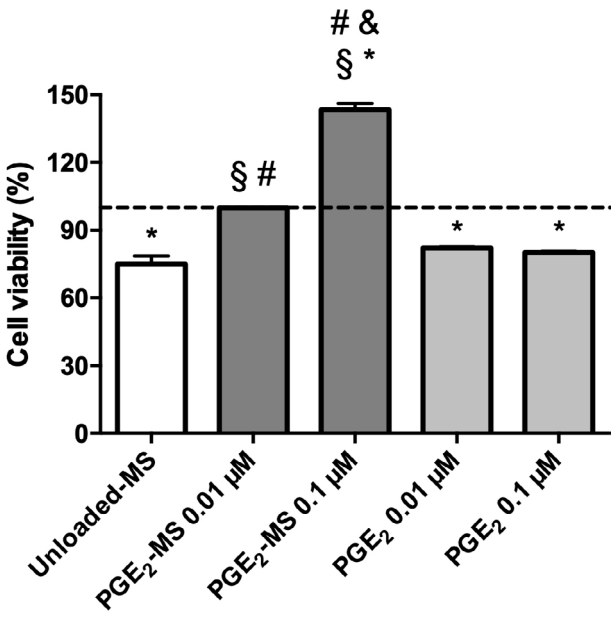


Figure 2. Effect of soluble or microsphere-loaded PGE_2 on undifferentiated dental pulp cells (OD-21) viability by the MTT assay after 24 h. *$p<0.05$ compared to medium alone; #$p<0.05$ compared to unloaded-MS; §$p<0.05$ comparison between PGE_2-loaded-MS and corresponding soluble PGE_2 at the same concentration; ¤$p<0.05$ comparison between different concentration of soluble or PGE_2-loaded-MS

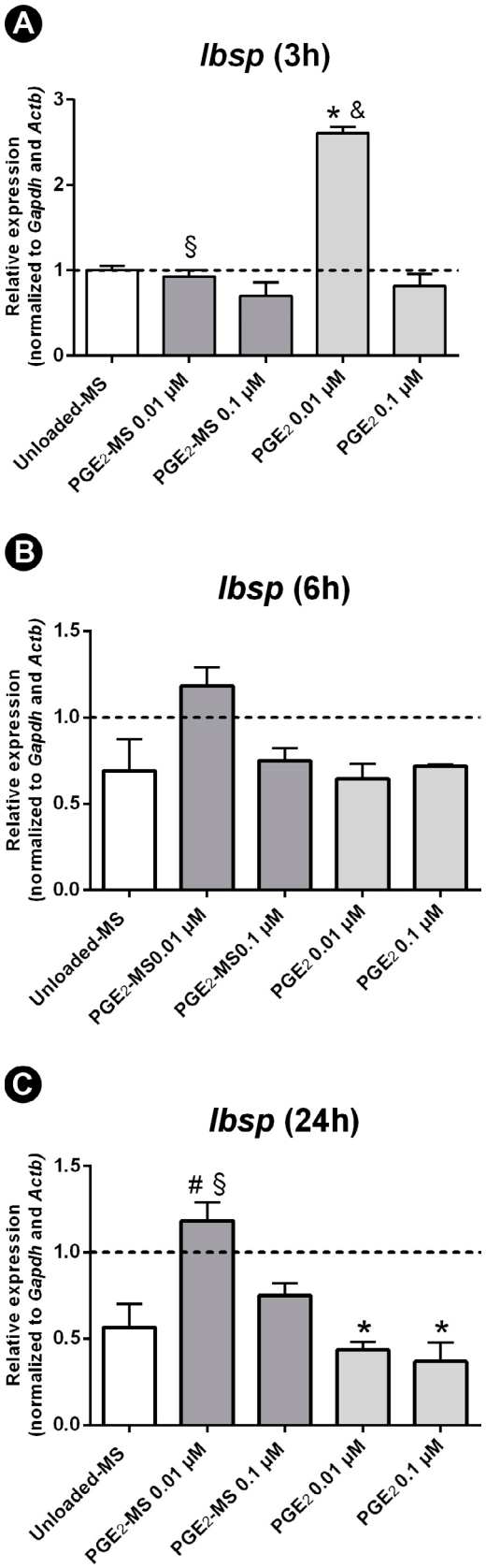
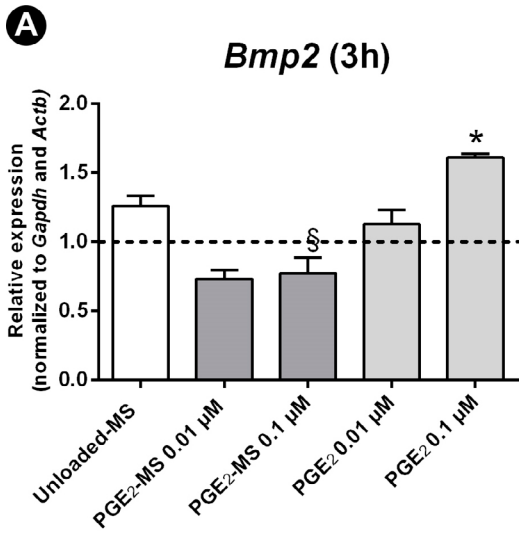
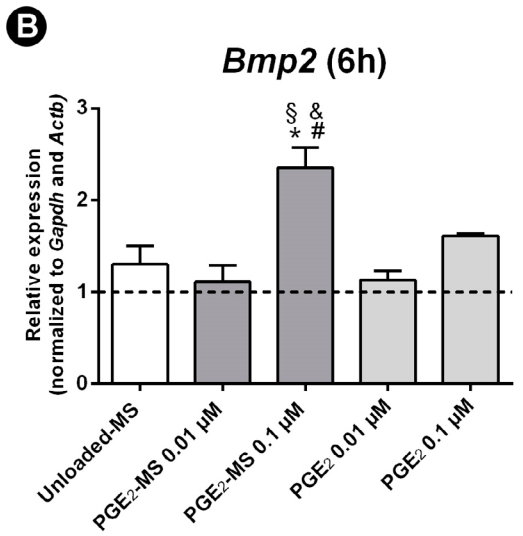


Figure 3. *Ibsp* expression after stimulation with soluble and PGE_2-MS after 3 (A), 6 (B), and 24 h (C). *$p<0.05$ compared to medium alone; #$p<0.05$ compared to unloaded-MS; §$p<0.05$ comparison between PGE_2-loaded-MS and corresponding soluble PGE_2 at the same concentration; ¤$p<0.05$ comparison between different concentration of soluble or PGE_2-loaded-MS





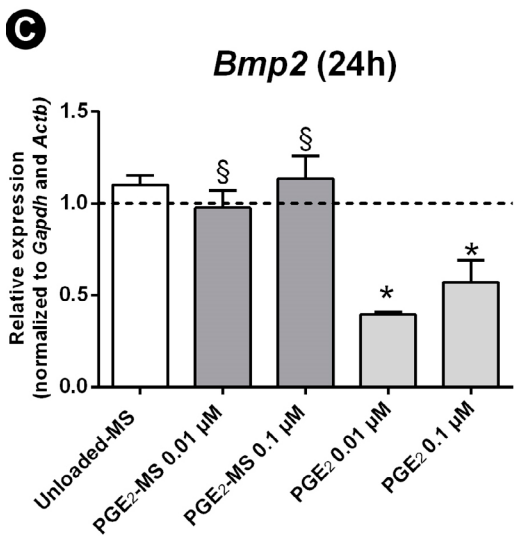
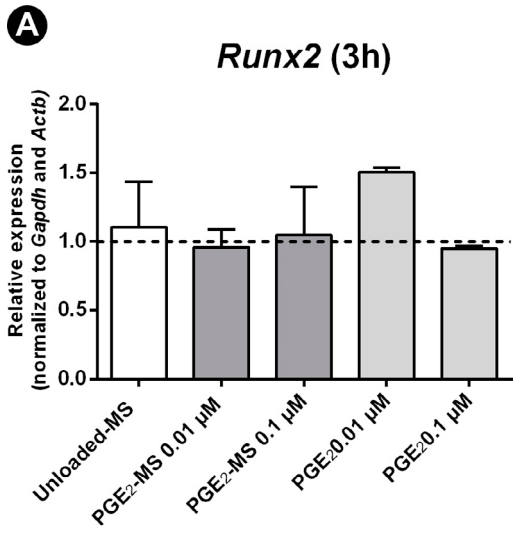
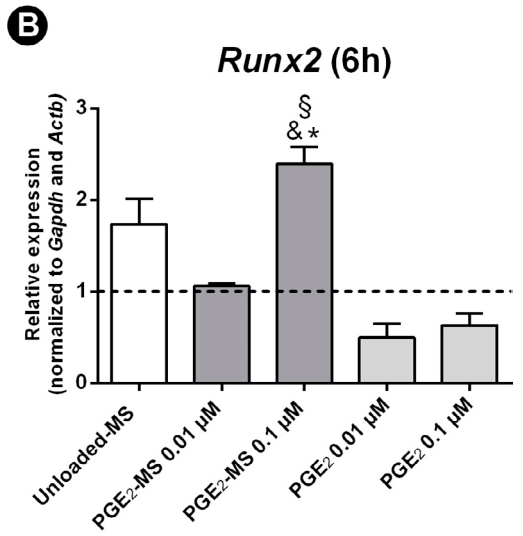


Figure 4. *Bmp2* expression after stimulation with soluble and PGE_2-MS after 3 (A), 6 (B), and 24 h (C). *p<0.05 compared to medium alone; #p<0.05 compared to unloaded-MS; §p < 0.05 comparison between PGE_2-loaded-MS and corresponding soluble PGE_2 at the same concentration; &p<0.05 comparison between different concentration of soluble or PGE_2-loaded-MS.





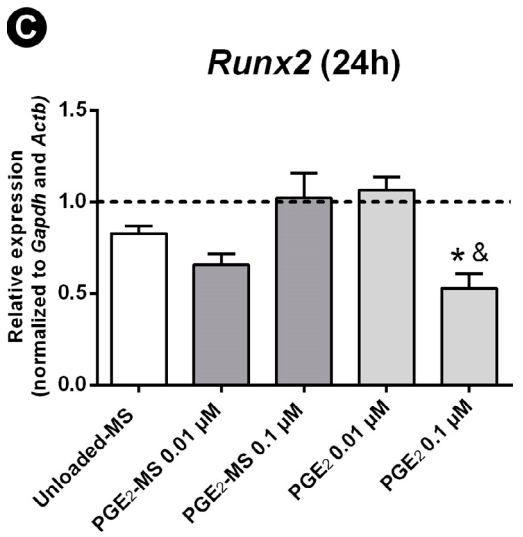


Figure 5. *Runx2* expression after stimulation with soluble and PGE_2-MS after 3 (A), 6 (B), and 24 (C) h. *p<0.05 compared to medium alone; #p<0.05 compared to unloaded-MS; §p<0.05 comparison between PGE_2-loaded-MS and corresponding soluble PGE_2 at the same concentration; &p<0.05 comparison between different concentration of soluble or PGE_2-loaded-MS.

Discussion

In dental pulp cells, little was known about the mechanisms involved in cell differentiation induced by prostaglandins. In this study, we showed that PGE_2 is not cytotoxic to dental pulp cells, although when loaded in microspheres, it reduced cell viability. Nonetheless, the percentage of cell death is not considered cytotoxic according to International Organization for Standardization (13). Therefore, PGE_2-loading strategy is suitable for further dental pre-clinical investigation. It should be noted that PLGA degradation products are biocompatible and consist of lactic acid and glycolic acid (14), but these products are important cell metabolites (15). A study that used another lipid mediator (Leukotriene B4 - LTB4) loaded microspheres to stimulate murine bone marrow-derived macrophages showed that cells were activated by unloaded microspheres and produced significant amounts of cytokines, confirming that PLGA microspheres act as particulate adjuvants (16,17). Conversely, in this study we showed that the polymer used had inhibitory on cell viability and that PGE_2- loaded MS is able to recover and even upregulate gene expression, demonstrating sustained delivery of the mediator overtime.

Bmp2 expression was upregulated by soluble PGE_2 (0.1 µM) after 3 h and by PGE_2- loaded MS (0.1 µM) after 6 h, indicating that this mediator is involved in early *Bmp2* expression. Interestingly, PGE_2-loaded MS sustained gene expression up to 24 h, differently from soluble PGE_2. The human bone morphogenetic protein 2 gene is essential for odontogenic differentiation of dental pulp cells (18). PGE_2 stimulation of human tendon stem cells (hTSC) in culture induces *BMP2* production and hTSC differentiation into osteoblast-like cells (19).

In addition to *Ibsp* and *Bmp2*, *Runx2* (runt-related transcription factor 2) expression was also upregulated by PGE_2- loaded MS in the first 6 h of stimulation. *Runx2* regulates stem cell differentiation and is the main gene controlling odontoblast differentiation. *Runx2* is expressed in odontoblast-like cells and dental pulp stem cells at sites of dentin deposition, thus promoting the differentiation of dental pulp stem cells to form reparative dentin (20,21). Because increased PGE_2 production has been shown in experimentally-induced inflamed pulp (5), it is important to determine if there is a correlation between *Runx2* and PGE_2. PGE_2 plays a dual role by promoting both bone resorption and formation (22). Similarly to what we observed for undifferentiated dental pulp cells in the current study, PGE_2 upregulated *Runx2* expression in human periodontal ligament cells (hPDLC) cultured in osteogenic medium at different molarities, indicating that PGE_2 modulates the osteogenic differentiation of hPDLC via regulation of *Runx2* expression (23).

However, differently to that observed in loaded microspheres, PGE_2 did not exert effects on OD-21 cells after 24 h or even inhibited gene expression. That might be explained by the fact that eicosanoid encapsulation preserves the biological activity of lipid mediators by preventing their degradation (9). In fact, PGE_2 have been effectively encapsulated in PLGA with no loss of biological activity, because lactic/glycolic acid polymers show high encapsulation efficiency and adequate stability (10). A similar method for loading of PGE_2 showed that peak levels were reached in the first 4 h, but it could also be released continuously over longer periods (11). Additionally, release of encapsulated products has been observed after 21 days and their effects on cells remained unaltered (24). 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. The encapsulation of bioactive molecules in microspheres has shown to be an interesting pharmacological strategy because besides modulate the immune response and protect biomolecules against fast degradation, this method prolong the delivery over longer periods of time and prevents toxic side effects (25). It has been already demonstrated that biomaterials in microspheres have the ability to induce stem cells differentiation and results in odontogenic differentiation and mineralized tissue regeneration (26).

Therefore, in accordance to satisfactory results in this study, PGE_2- loaded MS should be further investigated in direct contact with dental pulp tissue, aiming to promote the repair by mineralization gene expression regulation. Our in vitro results show that PGE_2, soluble or loaded in MS were not cytotoxic and modulate *Ibsp*, *Bmp2*, and *Runx2* gene expression in cultured OD-21 cells. Based on these results we speculate that PGE_2 may play an important role in dentin-pulp complex response to inflammatory stimuli and that delivery of PGE_2 in microspheres might be an interesting approach to induce dental pulp cell differentiation. Further studies should be conducted in primary human dental pulp cells that represent a mixed cell culture in short and long terms to investigate the effects of PGE_2 on both cell differentiation and mineralization.

Resumo

A Prostaglandina E_2 (PGE_2) é um mediador lipídico comumente liberado durante a inflamação. Este estudo teve como objetivo investigar o potencial da PGE_2, solúvel ou na forma de microesferas, na diferenciação de células-tronco de polpa dentária. Microesferas de PGE_2 (MS) foram preparadas por meio do processo de extração/evaporação de solvente em emulsão óleo-em-água e foram caracterizadas. Células-tronco de linhagem derivadas da polpa dentária de camundongos (OD-21) foram estimuladas com PGE_2 solúvel ou na forma de MS (0,01 e 0,1 µM). A citotoxicidade foi determinada por ensaio colorimétrico MTT. A expressão gênica de *Ibsp*, *Bmp2* e *Runx2* foi avaliada por meio de reação em cadeia da polimerase em tempo real (qRT-PCR) após 3, 6 e 24 h. Os resultados mostraram que as MS contendo PGE_2 não foram citotóxicas para células-tronco da polpa dentária, enquanto MS vazias ou PGE_2 solúvel reduziram a viabilidade

celular após 24 h de estimulação. PGE_2 solúvel aumentou a expressão de *Ibsp* e *Bmp2* após 3 h, diferentemente da PGE_2 em MS. Por outro lado, PGE_2-MS induziram a expressão de *Bmp2* e *Runx2* após 6h de estímulo e *Ibsp* após 24h. Em conclusão, nossos resultados in vitro demonstram que a PGE_2, solúvel ou em microesferas não são citotóxicas e modulam a expressão gênica de *Ibsp*, *Bmp2* e *Runx2* em células OD-21.

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