









Effects of Papain-Based Gel Used For Caries Removal on Macrophages and Dental Pulp Cells

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Papain-based gel is used for chemical-mechanical caries removal and present antimicrobial and anti-inflammatory activities. However, its effects on dental pulp cells and on macrophages remains largely unknown. Therefore, the aim of this study was to investigate whether the papain-based gel Papacárie Duo[®] acts as an immunomodulator in lipopolysaccharide (LPS)-activated macrophages and its effects on dental pulp cells. J774.1 macrophage and OD-21 dental pulp cells were stimulated with 0.5% and 5% of Papacárie Duo[®], following pre-treatment or not with LPS. After 24 h, a lactate dehydrogenase assay was used to measure cytotoxicity, a tetrazolium-based colorimetric assay (MTT) was used to measure cell viability, and qRT-PCR was used to analyze relative gene expression of Ptg2, Il10, Tnf, Mmp9, Runx2, Ibsp and Spp1. Papacárie Duo[®] was cytotoxic and reduced cell viability at 5% but not at 0.5% in both cultures. In macrophages, Papacárie Duo[®] increased the expression Il10 and LPS-induced Ptg2, but it did not affect Tnf or Mmp9. In OD-21 cells, Papacárie Duo[®] inhibited Runx2 and Ibsp expression, but stimulated Spp1 expression. Papain-based gel presented a concentration dependent cytotoxicity, without affecting cell viability, for dental pulp cells and macrophages. Interestingly, the gel presented an inhibitory effect on pulp cell differentiation but modulated the activation of macrophages stimulated with LPS. We speculate that in dental pulp tissue, Papacárie Duo[®] would impair reparative dentinogenesis but could activate macrophages to perform their role in defense and inflammation.

Key Words: papain-based gel, macrophages, dental pulp cells, chemo-mechanical caries removal.

Introduction

The success of chemo-mechanical caries removal relies on the selectivity of removing the carious tissue, which reduces the bacterial load in the interior of the cavity without removing the tissue that is capable of remineralization (1,2). This method can minimize the unpleasant perception that occur during caries removal by the conventional method (3). Some methods are becoming more popular and being improved to minimize the number of interventions and to maximize the preservation of dental tissues during removal of carious tissues (4).

The dentin is permeated by tubules that converge as they approach more internal areas. Therefore, the tubule diameter varies in relation to the distance towards the surface of the coronary chamber (5). The fact that dentin and the pulp tissue are intimately connected requires dental materials to be biocompatible and to ideally present anti-inflammatory properties. Some materials are biocompatible with the dentin-pulp complex when applied to superficial and moderate cavities, but when applied to deeper cavities may be toxic to the pulp tissue or may adversely affect the repair process (6). Papacárie Duo[®] is a material developed for chemo-mechanical caries removal and consists mainly

of papain (7), which has antimicrobial properties (3,8,9). This papain-based gel is basically composed of a proteolytic enzyme that interacts with the partially degraded collagen present in the necrotic carious tissue (7). Some studies have addressed the effects of chemo-mechanical caries removal agents, in terms of clinical efficacy, acceptability, levels of pain, and success in the long term (3,10-14). The effects of Papacárie Duo[®] on macrophage behaviour is not known, and its effects in cytotoxicity, viability and differentiation of undifferentiated dental pulp cells is scarce.

Therefore, the aim of this study was to determine whether Papacárie Duo[®] acts as an immunomodulator of macrophages activated or not by lipopolysaccharide (LPS) and evaluate its effects on cytotoxicity, viability and differentiation of dental pulp cells. Our null hypothesis is that Papacárie Duo[®] is neither cytotoxic nor impair cell function.

Material and Methods

Macrophage Culture (J774.1)

The J774.1 murine macrophage cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1%

Penicilin/Streptomycin (Gibco, Grand Island, NY, USA) (DMEM). After the formation of a monolayer, cells were harvested with plastic cell scrapers and centrifuged at 1,500 rpm for 10 min at 10 °C. Next, supernatants were discarded and 10 mL of DMEM was added to each tube of cells. Cell viability and total cell numbers were determined by counting live and dead cells in a Neubauer chamber (BOECO Germany, Hamburg, Germany) after staining with Trypan blue (Gibco). Cells were plated in 96-well culture plates (Cell Wells – Corning Glass Workers, Corning, NY, USA) at a density of 1×10^5 cells / well and incubated overnight in DMEM in an incubator with a moist atmosphere of 5% CO₂ at 37 °C.

Pulp cell culture (OD-21)

Undifferentiated mouse pulp cells (OD-21) (15) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% Penicillin/Streptomycin (Gibco) in an incubator at 37 °C and 5% CO₂. For the experiments, 1×10^5 cells/well were plated in 96-well cell culture plates (Corning Glass Workers) using DMEM without FBS.

Preparation of Papacárie Duo® Solutions

Papacárie Duo® gel (1 ml syringe) (Formulation & Action, São Paulo, SP, Brazil) was prepared by serial dilution in DMEM without FBS to obtain concentrations ranging from 0.005% up to 50%. In pilot studies, we found that concentrations lower than 0.5% had no effect on cell viability and higher than 5% cell death was high (> 80% of the cells). Thus, we chose to investigate 5% and 0.5% Papacárie Duo® solutions.

J774.1 cells were pre-stimulated with LPS (0.5 mg/mL; Escherichia coli LPS, Sigma-Aldrich, St. Louis, MO, USA) for 2 h. After the pre-stimulation, the culture medium was removed and the cells were washed with 1x Phosphate Saline Buffer (PBS). Then, 200 µL of 0.5% and 5% Papacárie Duo® solutions, containing LPS or not, were added to each well. Cells exposed to LPS only were used as a positive control and cells maintained in DMEM without serum were used as a negative control. OD-21 had culture medium removed and the wells washed with PBS. Then, 200 µL of 0.5% and 5% Papacárie Duo® solution were added to each well. Following incubation, the stimuli were removed and the plates were either assayed or stored at -80 °C. The experiments were performed in triplicates and replicated twice. Experimentation and data collection was performed in an open label format. Data were then codified and analysis were performed blindly.

Cytotoxicity – Lactate Dehydrogenase (LDH) Assay

The cytotoxicity of 0.5 and 5% Papacárie Duo® solutions was evaluated on J774.1 macrophage cells and OD-21 cells by measuring the level of LDH released in the supernatant after cell lysis using the CytoTox96® non-radioactive cytotoxicity assay (Promega Corporation, Madison, WI, USA). The absorbance was measured at 490 nm with a spectrophotometer (mQuanti, Bio-Tek Instruments, Inc., Winooski, VT, USA). As positive control, 10x 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 (%) = $100 \times \text{Experimental LDH Release absorbance} / \text{Maximum LDH Release absorbance (positive control)}$.

Cell Viability – MTT Colorimetric Assay

Cell viability was evaluated using an MTT assay according to the manufacturer's instructions. J774.1 cells (1×10^5 /well), with or without LPS, and OD-21 cells (1×10^5 /well) were plated in 96-well plates and stimulated for 24 h with Papacárie Duo® solutions at different concentrations. Dimethyl sulfoxide (DMSO) was used as positive control.

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 Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco) was added to the plates. After 3h incubation at 37 °C, 40 µL of sodium dodecyl sulphate (SDS) 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 (%) = $100 \times \text{Experimental absorbance} / \text{Negative control absorbance (medium alone)}$.

RNA Extraction, Reverse Transcription, and Polymerase Chain Reaction In Real Time (qRT-PCR)

To evaluate J774.1 cell activation and OD-21 cell differentiation, the mRNA levels of Ptg2s, Il10, Tnf, and Mmp9, and Ibsp, Runx2, and Spp1, respectively, were measured by quantitative reverse transcriptase-polymerase chain reactions (qRT-PCR) 24 h after cell stimulation. 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 800 ng of total 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. All protocols were performed according to the manufacturers' instructions. Primer-probe pairs were obtained commercially, thus their sequences are not available (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and beta-actin (Actb) were used as reference genes for normalization purposes. The results were analyzed based on cycle threshold (Ct) values. Relative expression was calculated by the $\Delta\Delta C_t$ method.

Statistical Analysis

For each experiment, data were compared using analysis

of variance (ANOVA) followed by the Tukey's test ($\alpha=0.05$) using Graph Pad Prism Software version 6.0.

Results

Cytotoxicity and Cell Viability

The 5% Papacárie Duo® solution was cytotoxic to macrophages ($p<0.05$). However, at 0.5% concentration, no cytotoxicity was observed ($p>0.05$) when compared to positive and negative controls (Fig. 1A). The same pattern was observed for cell viability (Fig. 1B). In the presence of LPS, no cytotoxicity was observed ($p>0.05$) (Fig. 2A), but cellular viability was reduced, regardless of the presence

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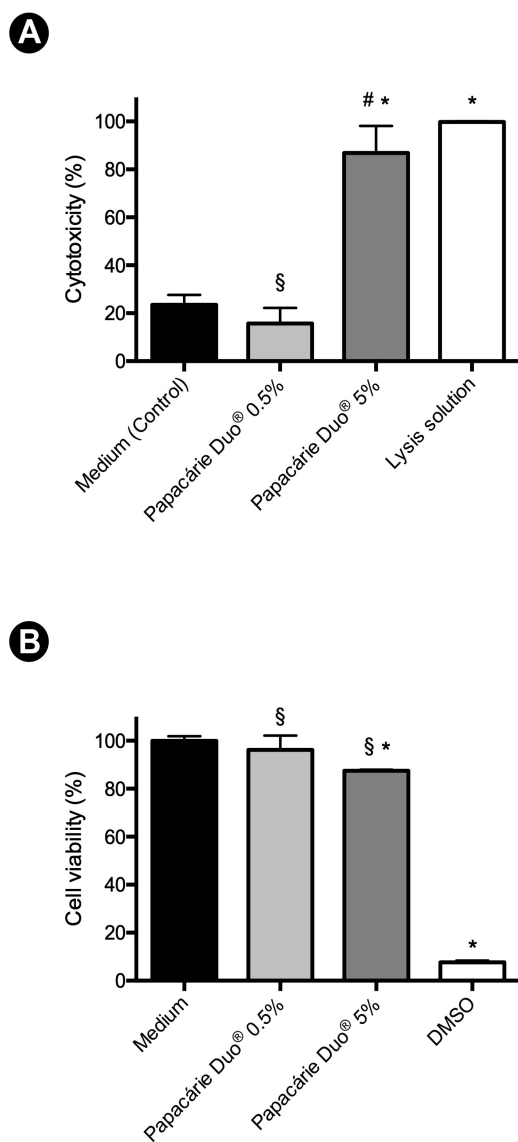


Figure 1. Papacárie Duo® cytotoxicity in macrophages (J774.1) by LDH assay (A) and cell viability by MTT assay after 24 h (B). Lysis solution or DMSO were used as positive control. Untreated cells (culture medium) were used as a negative control * $p<0.05$ compared to culture medium alone; § $p<0.05$ compared to lysis buffer or DMSO; # $p<0.05$ compared to Papacárie Duo® 0.5%.

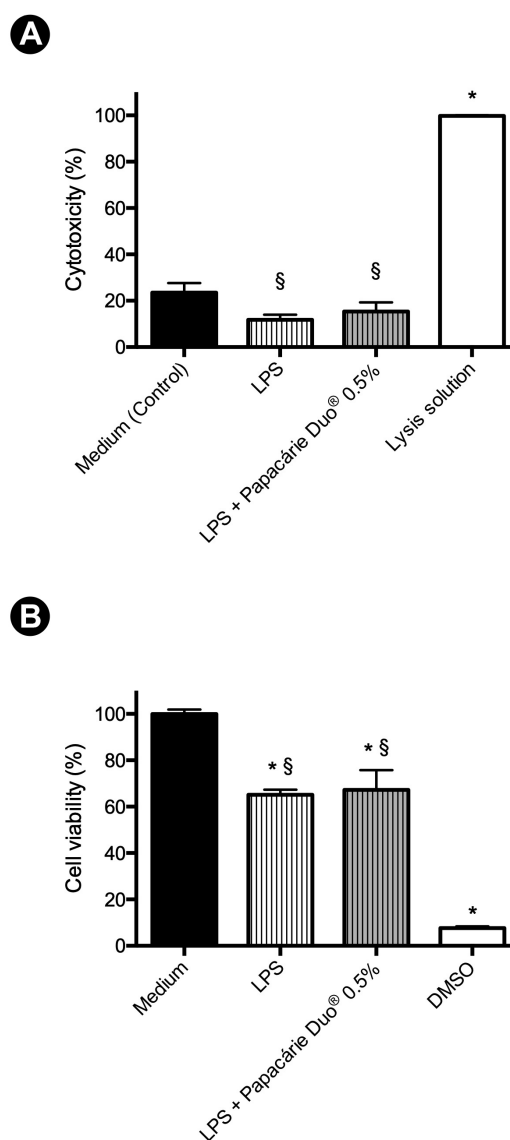


Figure 2. Papacárie Duo® cytotoxicity in undifferentiated dental pulp cells (OD-21) by LDH assay (A) and cell viability by MTT assay after 24 h (B). Lysis solution or DMSO were used as positive control. Untreated cells (culture medium) were used as a negative control. * $p<0.05$ compared to culture medium alone; § $p<0.05$ compared to lysis buffer or DMSO; # $p<0.05$ compared to 0.5% Papacárie.

of Papacárie Duo® ($p < 0.05$) (Fig. 2B).

In dental pulp cells, Papacárie Duo® was cytotoxic when applied at 5% ($p < 0.05$). However, it was not cytotoxic when applied at 0.5% ($p > 0.05$), compared to positive and negative controls (Fig. 3A). Papacárie Duo® reduced cell viability in a dose-dependent fashion, compared to the culture medium alone ($p > 0.05$) (Fig. 3B).

Cell Differentiation

In undifferentiated dental pulp cells, Papacárie Duo® inhibited the expression of Runx2 ($p < 0.05$) and Ibsp ($p < 0.05$), while stimulated Spp1 expression ($p < 0.05$) (Fig. 4).

Papacárie Duo® activated macrophages with or without LPS. LPS stimulated Tnf expression compared to negative

control ($p < 0.05$), while the 0.5% Papacárie Duo® at solution did not change the LPS-induced Tnf expression ($p > 0.05$). Mmp9 expression was not altered by LPS or by Papacárie Duo®, or by the combination of both ($p > 0.05$). LPS induced expression of Ptgs2 ($p < 0.05$) and 0.5% Papacárie Duo® increased LPS-induced Ptgs2 expression ($p < 0.05$). On the

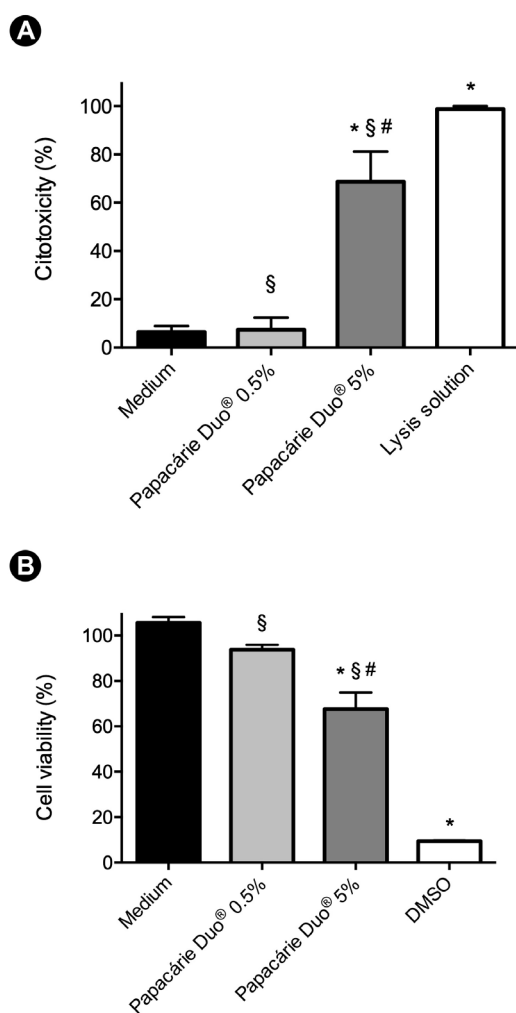


Figure 3. Papacárie Duo® cytotoxicity in undifferentiated dental pulp cells (OD-21) by LDH assay (A) and cell viability by MTT assay after 24 h (B). Lysis solution or DMSO were used as positive control. Untreated cells (culture medium) were used as a negative control. * $p < 0.05$ compared to culture medium alone; § $p < 0.05$ compared to lysis buffer or DMSO; # $p < 0.05$ compared to 0.5% Papacárie.

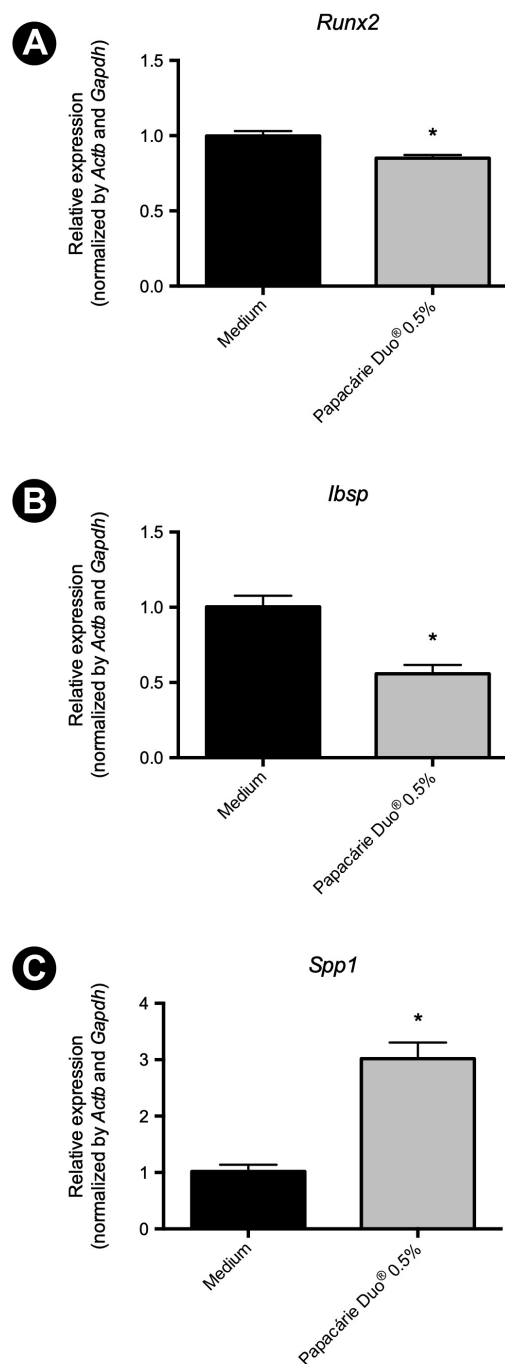


Figure 4. Effect of 0.5% Papacárie Duo® on the expression of Runx2 (A), Ibsp (B) and Spp1 (C) by undifferentiated dental pulp cells (OD-21) after 24 h. Expression was analysed by qRT-PCR. * $p < 0.05$ compared to culture medium alone.

other hand, 0.5% Papacárie Duo® alone inhibited Ptgs2 expression ($p < 0.05$). Papacárie Duo® at 0.5%, in the presence or not of LPS, stimulated Il10 expression ($p < 0.05$). However LPS alone did not modulate Il10 expression ($p > 0.05$) (Fig. 5).

Discussion

The null hypothesis of this study was rejected because we found a concentration dependent effect of cytotoxicity, cell viability and cell function, i.e., activation of macrophages and differentiation of dental pulp cells. Papacárie Duo® at 0.5% solution was not cytotoxic to macrophages or dental pulp cells, but when used at 5% concentration reduced cell viability and was highly cytotoxic for both cultures. A previous study demonstrated that Papacárie Duo® cytotoxicity may also vary depending on the cell type on which it is applied. Major effects were found in periodontal ligament fibroblasts, epithelial cells and tumor cells of the oral mucosa. Minor cytotoxic effect

was found in cells from human pulp and human gingival fibroblasts (16). These divergent effects might be due to cell type specific effects. OD-21 cells are from a mesenchymal lineage whereas J774.1 are from an hematopoietic lineage. The cytotoxicity of Papacárie Duo® was also tested on fibroblasts isolated from the central region of the pulp tissue and no cytotoxicity was observed (2).

It is noteworthy that the cytotoxicity of a material may also depend on the thickness of the remaining dentin and on dentin permeability (6,17). Thus, considering that Papacárie Duo® is possibly cytotoxic to dental pulp cells (as indicated by the results at 5% concentration), the thickness of the remaining dentin after cavity preparation is a highly relevant factor. The thickness and morphological characteristics of the dentin may favor transdental diffusion of chemical components from the materials. Indeed, severe pulp reactions occur when the remaining dentin is low (18). Therefore, considering clinical parameters, deeper cavities require greater care and attention in choosing the dental

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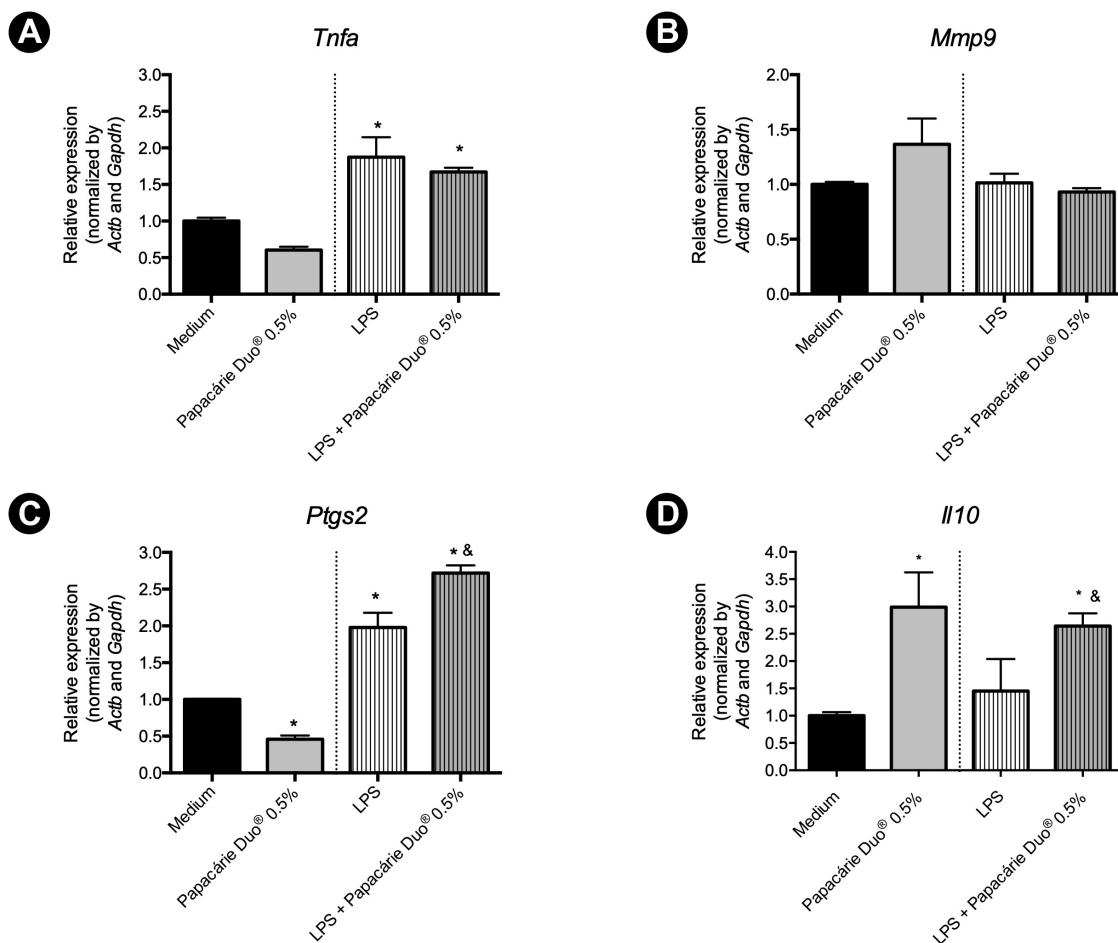


Figure 5. Effect of 0.5% Papacárie Duo®, alone or in combination with LPS, on the expression of Tnf (A), Mmp9 (B) Ptgs2 (C) and Il10 (D) by macrophages (J774.1) after 24 h. Expression was analysed by qRT-PCR. * $p < 0.05$ compared to culture medium alone; and & $p < 0.05$ compared to LPS.

materials to be used, as improper handling or choice can result in significant damage to dental tissue and pulp. Also, the dilution effect of the dentinary fluid found in dentin tubules should be considered. This fluid can dilute the material, depending on its solubility, and therefore modify the intensity of the inflammatory response in the subjacent dental pulp tissue. The *in vivo* effect of Papacárie Duo® on dental pulp, when applied to dentin, is not known and deserves further investigation.

In our study, it was found increased expression of Ptg2 and Il10 mRNA in the presence of Papacárie Duo®. Increased levels of the pro-inflammatory lipid mediator prostaglandin E2 (PGE2) was induced by Papacárie Duo® in gingival fibroblasts that were synergistically increased in presence of IL-1 β (16). The Ptg2 gene encodes the COX-2 enzyme, which is responsible for the synthesis of prostaglandins involved in inflammatory response (19). IL-10 is an anti-inflammatory cytokine and a modulator of macrophage activation during infection (20). Until now, nothing was known about the Papacárie Duo® immunomodulatory capacity in the presence or absence of LPS. Increased expression of Ptg2 by Papacárie Duo® in the presence of LPS or induction of Il10 expression in the presence or absence of LPS demonstrated that this material modulate macrophage activation and indicate that it could modulate the inflammatory response.

In contrast, undifferentiated dental pulp cells had the expression of Runx2 and Ibsp inhibited by Papacárie Duo®. Runx2 regulates several signaling pathways related with odontoblast differentiation (21). Runx2 is expressed in odontoblast-like cells and in dental pulp stem cells located in the region of deposition of reparative dentin. This transcription factor promotes differentiation of pulp stem cells, enabling them to form repairing dentin (22,23). Ibsp codes for a phosphorylated glycoprotein and is mainly expressed in mineralized connective tissues. It has several roles during development, regulating the volume and mineralization of bone and dentin tissues. Induction of this gene coincides with the initial formation of the mineralized matrix and maximum induction is achieved during bone formation (24,25). Considering the roles of Runx2 and Ibsp in cell differentiation, it is expected that their inhibition results in suppression of differentiation of OD-21 cells. However, the expression of Spp1, a gene that codes for osteopontin (OPN) was induced. OPN is a highly phosphorylated glycoprotein that composes the bone extracellular mineralized matrix. It is important for inflammatory and mineralization events because it is capable of regulating several physiological and pathological processes, including wound healing, bone remodeling, tumorigenesis, inflammation and immunological responses (26). The protein is then deposited in the dentin-pre-dentin

interface, prior to the differentiation of odontoblast-like cells. Therefore, OPN may have a role in the differentiation of these cells (27). Once deposited in the pulp-dentin border, OPN induces the formation of tertiary dentin and of type I collagen, which is essential for the development of a new mineralized matrix (28). Future studies are needed to clarify the role of Spp1, a gene related not only with mineralization but also with inflammatory events (29,30) and to investigate the effect of Papacárie Duo® in other types of inflammatory cells, as well as *in vivo*.

In macrophages and undifferentiated dental pulp cells, Papacárie Duo® was cytotoxic and reduced cell viability at 5% but not at 0.5%. In macrophages, Papacárie Duo® at 0.5% induced the expression Il10 and LPS-induced Ptg2, but it did not affect Tnf or Mmp9 gene expression. In undifferentiated dental pulp cells, Papacárie Duo® at 0.5% inhibited Runx2 and Ibsp, while stimulated Spp1 expression. We speculate that in dental pulp tissue, Papacárie Duo® would impair reparative dentinogenesis but could activate macrophages to perform their role in defense and inflammation. Further studies should be conducted *in vivo* to confirm that.

Resumo

O gel à base de papaína é utilizado para remoção químico-mecânica do tecido cariado e apresenta propriedades antimicrobianas e anti-inflamatórias. Entretanto, seu efeito sobre as células da polpa dentária e macrófagos é desconhecido. Portanto, o objetivo deste estudo foi investigar o efeito de um gel de papaína (Papacárie Duo®) em células indiferenciadas da polpa dentária e a capacidade de induzir a ativação e síntese de mediadores inflamatórios por macrófagos estimulados com lipopolissacarídeo bacteriano (LPS). O gel de papaína foi diluído nas concentrações de 0,5 e 5%. Células indiferenciadas da polpa dentária OD-21 e macrófagos J774.1 foram mantidos em cultura com os diferentes estímulos por um período de estimulação de 24 h para realização do teste de citotoxicidade (Ensaio LDH) e para avaliação da viabilidade celular (Ensaio Colorimétrico MTT). A seguir foi realizada avaliação da expressão gênica relativa dos genes Ibsp, Runx2 e Spp1 em células OD-21; e dos genes Il10, Mmp9, Ptg2 e Tnf em células J774.1, pelo método de transcrição reversa e reação em cadeia de polimerase em tempo real (qRT-PCR), após estimulação pelo período de 24 h. O extrato do gel diluído a 5% foi citotóxico às células da polpa dental, reduziu a viabilidade celular, inibiu a expressão de Runx2 e Ibsp e estimulou a expressão de Spp1. Em macrófagos, o extrato do gel foi citotóxico e reduziu a viabilidade celular na concentração de 5%. O LPS inibiu a viabilidade celular na presença ou não do extrato do gel, sem apresentar citotoxicidade. O extrato do gel induziu a expressão de Ptg2 e Il10, sem alterar Tnf e Mmp9. O extrato do gel de papaína foi citotóxico, dependente da concentração, tanto em células da polpa dentária como em macrófagos, sem alterar a viabilidade celular. Interessantemente, apresentou efeito inibitório na diferenciação de células da polpa dentária e modulou a ativação de macrófagos estimulados com LPS. No tecido pulpar, o Papacárie Duo® poderia impedir a dentinogênese de reparação, porém ativar macrófagos para desempenhar seu papel na inflamação e defesa.

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