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INTERLEUKIN-1 BETA AND INTERLEUKIN-8 IN HEALTHY AND INFLAMED DENTAL PULPS

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ABSTRACT

After aggression to the dental pulp, some cells produce cytokines in order to start and control the inflammatory process. Among these cytokines, interleukin-1 beta (IL-1ß) and interleukin-8 (IL-8) emerge as important ones. Objective: The purpose of this study was to analyze the location, distribution and concentration of these cytokines in healthy and inflamed dental pulps. Material and methods: Twenty pulps, obtained from healthy third molars (n=10) and from pulpectomies (n=10) were used for the study, with half of each group used for immunohistochemistry and half for protein extraction and ELISA assays. Fibroblasts obtained from healthy dental pulps, stimulated or not by *Escherichia coli* lipopolysaccharide (LPS), in order to simulate aggression on the cell cultures, were also used and analyzed by ELISA for IL-1ß and IL-8 as complementary information. Data obtained from immunohistochemistry were qualitatively analyzed. Data obtained from ELISA assays (tissue and cells) were statistically treated by the t-test ($p<0.05$). Results: Immunohistochemically, it was observed that inflamed pulps were strongly stained for both cytokines in inflammatory cells, while healthy pulps were not immunolabeled. ELISA from tissues quantitatively confirmed the higher presence of both cytokines. Additionally, cultured pulp fibroblasts stimulated by LPS also produce more cytokines than the control cells. Conclusions: It may be concluded that inflamed pulps present higher amounts of IL-1ß and IL-8 than healthy pulps and that pulp fibroblasts stimulated by bacterial LPS produce higher levels of IL-1ß and IL-8 than the control group.

Key words: Dental pulp. Inflammation. Cytokines.

INTRODUCTION

Dental pulp, as any other connective tissue, responds to aggression with the inflammation process, in order to eliminate pathogens and allow repair. However, due to its particular features as the confinement in a hard chamber and its unique blood irrigation and lymphatic circulation, a pulp inflammation process becomes hard to control and dissipate[3]. Thus, the understanding of pulp inflammation is essential in order to provide treatment strategies and better management of the harmed tissue, avoiding pulpectomy and subsequent loss of tooth sensibility and resistance.

Generally, an increase in vascular permeability and the migration of leukocytes from blood vessels can be observed during the first moments of the inflammation process, due to the presence of inflammatory substances in the inflamed site[9]. Most of the time, these substances are cytokines, produced and released by a range of cells in the dental pulp, playing an important role in the activation and control of the inflammatory process. Some studies have reported the presence of these substances in the dental pulp tissue or cells[2,4,6,11], and the interleukins 1 beta (IL-1ß) and 8 (IL-8) have importance in the study of inflammation in this tissue.

The IL-1ß is responsible for a wide range of actions, in order to mediate the inflammatory responses in the host. It can be produced and released by many different cell types and it is reported to be active in the dental pulp tissues or cell cultures[4,5,10,12], making it one of the most important interleukins in the study of pulp inflammatory process. Its main role in low concentrations is to mediate local inflammation. However, in high concentrations, it has endocrine effects.[1] The IL-8 is a potent inflammatory cytokine, since it recruits neutrophils to the inflamed site, being generally induced by bacterial antigens[8,13]. Additionally, it has been suggested and used as one of the main markers for acute pulp inflammation[7,14,18].

On this basis, the purpose of this study was to analyze the presence, location, distribution and concentration of these

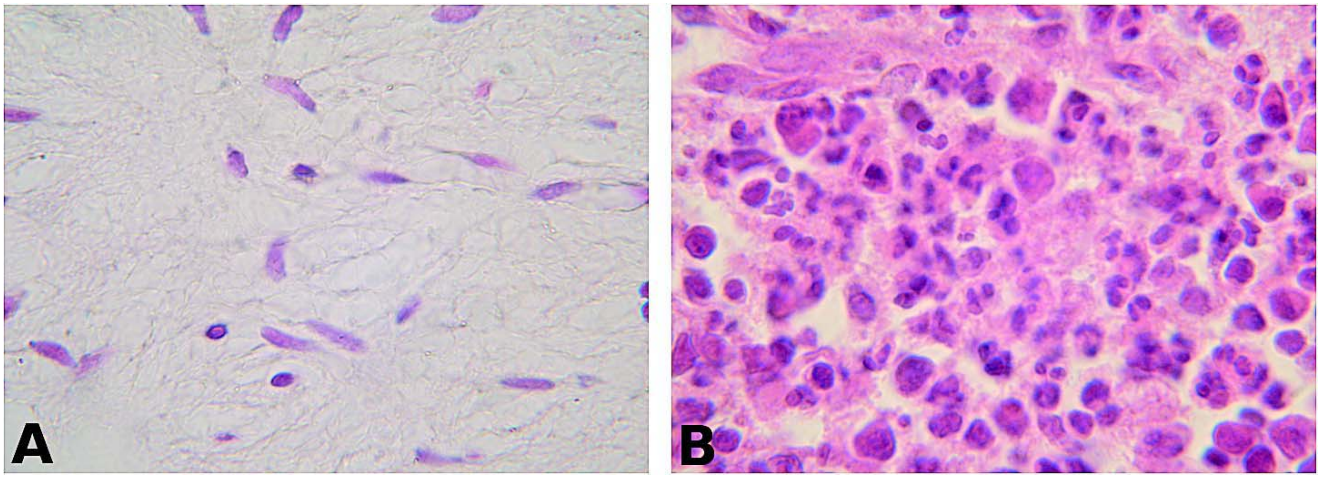

FIGURE 1- Representative histological sections stained by hematoxylin-eosin for healthy pulps (A) and inflamed pulps (B). Hematoxilin-eosin, 100x

cytokines in healthy and inflamed dental pulp by immunohistochemistry and ELISA. Additionally, an in vitro experiment with dental pulp fibroblasts was performed in order to provide complementary information on pulp response.

MATERIALS AND METHODS

This study was conducted under the approval of a Research Ethics Committee (document #93/05). All participating subjects provided written informed consent. Twenty pulp tissues (10 healthy pulps and 10 inflamed pulps) were obtained from patients who had teeth indicated for extraction (for the healthy pulps) or pulpectomy (for the inflamed pulps).

After the extraction, healthy third molars were placed in 4% paraformaldehyde solution and immediately prepared with a longitudinal 1 mm depth sulcus, made with a diamond disc in low speed hand piece (Kavo, Joinville, SC, Brazil). After this preparation, a chisel was inserted in the sulcus, and the teeth were cleaved, in order to allow the collection of the unharmed pulp tissue. Tissues were placed into a new 4% paraformaldehyde solution, in 0.1 M phosphate buffer at 4º C, for 24 h.

The inflamed pulps were obtained in the urgency services of our dental school. Teeth with pulp exposures, or which received intrapulpal anesthesia were discarded. After a complete clinical examination, the following inclusion criteria were used: localized pain, stimulated by cold; absence of pain in vertical or horizontal percussion; absence of radiographic lesion on the root apex; presence of abundant and red bleeding. After the collection of these pulps, the tissues were also placed in 4% paraformaldehyde solution, in 0.1 M phosphate buffer at 4º C, for 24 h.

Both conditions (health or inflammation) were histologically confirmed by hematoxylin-eosin staining (Figure 1).

Immunohistochemistry

For immunohistochemical reactions, 3 mm sections from 5 healthy and 5 inflamed pulps were obtained from formalin-fixed, paraffin-embedded tissue. These tissues were mounted on silanized slides, deparaffinized and re-hydrated. Sections were submitted to a water bath antigen retrieval step, immersed in EDTA (pH 8.0) for 30 min at 95ºC, and then incubated on a 6% hydrogen peroxide and methanol solution v/v to quench endogenous peroxidase activity (30 min at room temperature). Subsequently, sections were submitted to the primary monoclonal antibodies anti-IL-1b (C-20, sc1250, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-IL-8 (C-19, sc1269, Santa Cruz Biotechnology) at 1:75 concentration for 30 min, followed by incubation with biotinylated swine-anti-mouse, rabbit, goat antibody (LSAB+ System-HRP K0690; DakoCytomation, Carpinteria, CA, USA) and with streptavidin-biotin peroxidase conjugate (LSAB+ System-HRP K0690; DakoCytomation), both for 30 min. Antibody complexes were developed after the addition of a buffered diaminobenzidine substrate (Liquid DAB+ Substrate chromogen system K3468; DakoCytomation), for 10 min, and counter-stained by Mayer's hematoxylin. The immunohistochemical staining, from the primary antibody to the counterstaining, was performed on an autostainer (DakoCytomation).

For both antibodies, mucoceles were used as positive control (Figure 2A). These tissues were obtained from the archives of the Oral Pathology Department, and were chosen due to its great concentration of inflammatory cells, which are the main source of interleukins. As negative controls, the mucoceles were incubated with non-immune goat serum and primary antibodies, diluted at the same antibody concentration (1:75) (Figure 2B). Qualitative immunohistochemical evaluation was performed in a blinded analysis.

ELISA

The pulp tissues (n=5 for each group) were homogenized

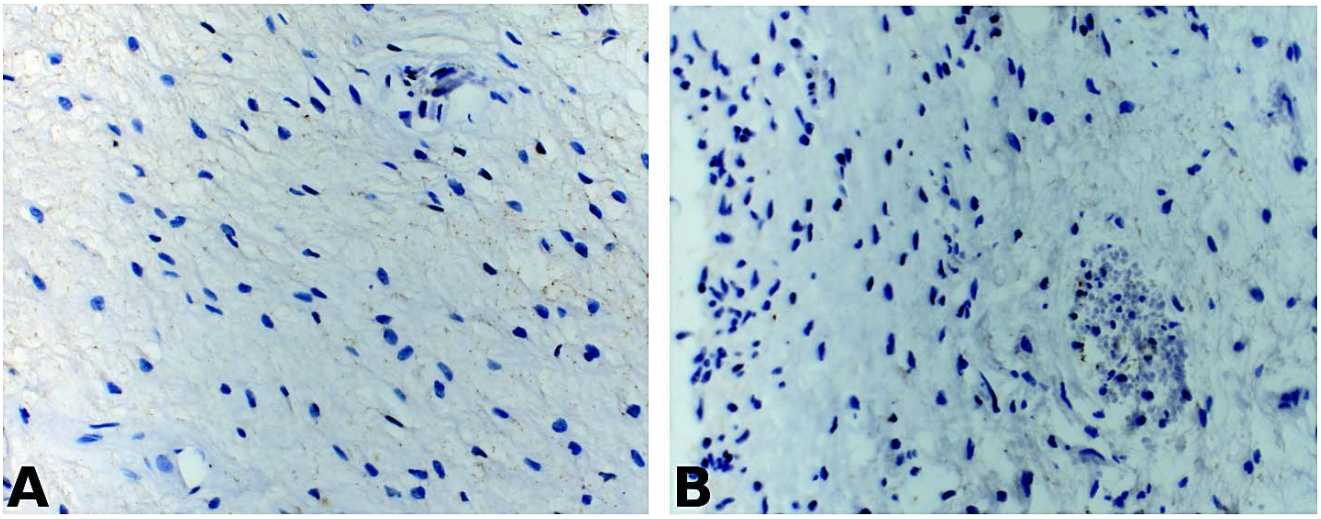

FIGURE 2- Immunohistochemistry reactions made in mucoceles, used as controls for both cytokines (here the staining for IL-8). Note the intense staining in the positive control (A) and the absence of staining in the negative control (B). Mayer's hematoxilin, 400x

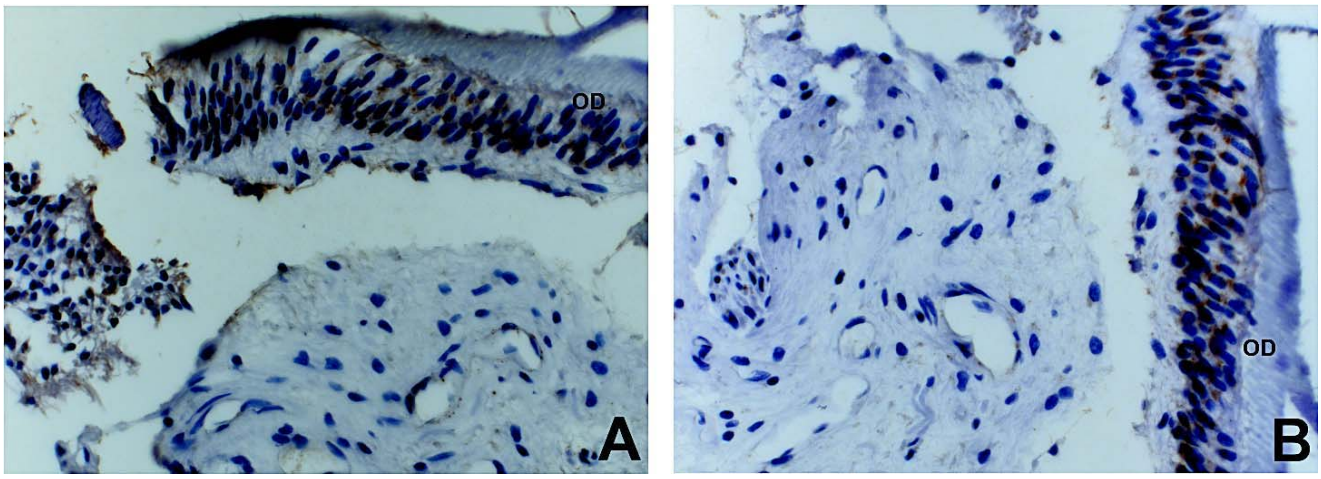

FIGURE 3- Immunohistochemical staining for healthy pulps. Note the odontoblast layer (OD) staining for IL-1ß (A) and IL-8 (B). Mayer's hematoxilin, 400x

and had their proteins extracted by buffer solution (RIPA; Pierce Biotechnology, Rockford, IL, USA) in micro centrifuge tubes prepared for this purpose (Sample Grinding Kit, Ref. 80-6483-37; GE Healthcare, UK), at 10,000 rpm for 5 min in a refrigerated centrifuge (Sorvall Biofuge, Thermo Scientific, Waltham, MA, USA). Supernatants were collected and quantified by ELISA kits for IL-1b (RPN-5969, Biotrak, Amersham Pharmacia Biotech, UK) and IL-8 (RPN-5694, Biotrak, Amersham Pharmacia Biotech), following manufacturer's instructions, in a triplicate for each specimen. Equally processed mucoceles were used as positive controls and the standards provided by the kit were used as negative controls. Three independent assays were performed to confirm the results.

Data were calibrated, in order to avoid different concentration ranges due to the size of the tissues. For this calibration, samples obtained as described above were also used for a total protein assay (BCA Protein Assay Kit, Pierce Biotechnology), following manufacturer's instructions. With this assay, the total protein ratio obtained was 1:1.236 for the control (mucocele): healthy pulps and 1:0.674 for the control: inflamed pulps. These ratios were expected, since the inflamed pulps were smaller than the healthy pulps. The calibrated data were analyzed by the t-test ($p<0.05$).

Cell Cultures

In order to compliment the *in vivo* data obtained from pulp tissues, human pulp fibroblasts were obtained from the cell bank of our school. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Cultilab, Campinas, SP, Brazil), supplemented by 10% fetal bovine serum (Cultilab) and 1% antimycotic-antibiotic solution (10,000 units of penicillin, 10 mg of streptomycin and 25 ìg of amphotericin B per mL in 0.9% sodium chloride; Sigma, St. Louis, MO, USA). The cells were kept in an incubator at 37ºC and a humidified 5% CO_2 atmosphere. Cultures were supplied with

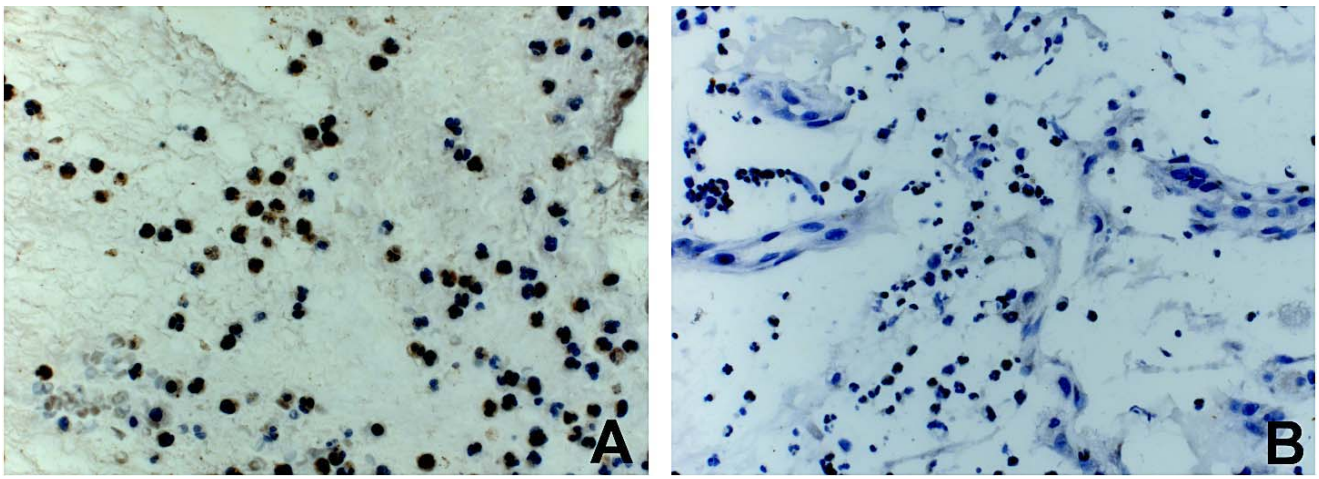

FIGURE 4- Immunohistochemical staining for healthy pulps. Note the absence of staining for IL-1ß (A) and IL-8 (B). Mayer's hematoxilin, 400x

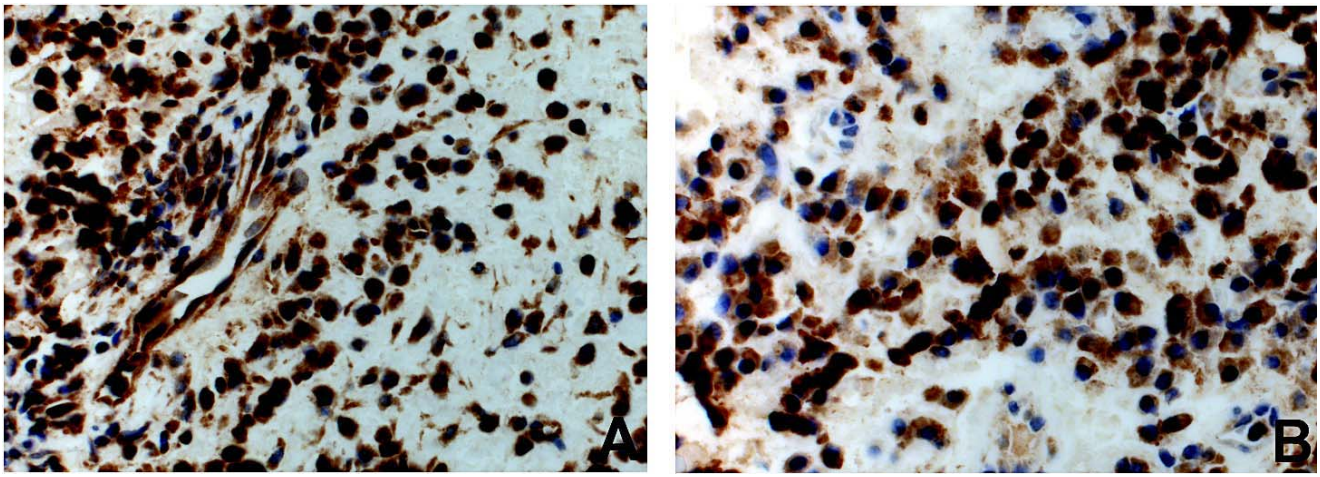

FIGURE 5- Immunohistochemical staining for inflamed pulps. Note the intense staining of the cytoplasm of inflammatory cells, for IL-1ß (A) and IL-8 (B). Mayer's hematoxilin, 400x

fresh medium every other day. Cells between the fifth and 10th passages were used in all experimental procedures.

After cell culture, the cells were counted and plated on 6 well plates (1 x10^6 cells per well), in a total of 3 wells per group. After 24 h, the DMEM was replaced by the treated culture media, being only fresh DMEM for control group and DMEM with 10 ng/ml of *Escherichia coli* lipopolysaccharide (LPS, L4391, Sigma) for the treated group. Control group (fresh DMEM) simulates healthy pulps, while treated (LPS) group represents inflammatory state. All experiments were made in triplicate, in order to confirm data and reduce variation among samples.

After 24 h-incubation, these media were removed and cells washed with 4ºC phosphate buffered solution (PBS, Sigma). During the extraction of proteins, the plates were always kept on ice, in order to avoid the denaturation of cytokines. Each well was filled with 100 µL of buffer (RIPA, Pierce Biotechnology), supplemented by 1 µl of protease inhibitor (Sigma), kept in contact with the cells for 15 s. After that, cells were vigorously scrapped from the bottom of wells and transferred for a micro centrifuge tube. The tubes were centrifuged in a refrigerated centrifuge (4ºC) at 20,000 g for 15 min. After centrifugation, the supernatant was collected and diluted to a 1:4 ratio for the ELISA assay. Again, data were statistically analyzed by the t-test.

RESULTS

Cytokine Expression in Dental Pulp Tissues

As a histological finding, it was observed that in the slices from healthy tissues where odontoblasts were present, they were positively stained for both antibodies (Figure 3). No sections from the inflamed tissues had the odontoblast layer preserved.

All healthy pulps presented negative immunolabeling for IL-1ß and IL-8 antibodies (Figure 4). On the other hand, the inflamed tissues presented intense staining for both antibodies, also specific and restricted to inflammatory cells (Figure 5). Data were complemented by the ELISA assay

TABLE 1- Concentration (pg/mL) of IL-1ß and IL-8 obtained from tissue processing (± standard deviation)

	Control +	Healthy	Inflamed	Control -*
IL-1ß	31.22 ± 8.491	3.72 ± 0.788	28.02 ± 4.219**	0.72 ± 0.351
IL-8	39.37 ± 5.849	2.05 ± 0.834	45.77 ± 4.663**	0.85 ± 0.437

* Readings in this case can be related to the accuracy of the microplate reader. ** statistically significant difference as compared to healthy group

TABLE 2- Concentration (pg/mL) of IL-1ß and IL-8 obtained from cell processing (± standard deviation)

	Control	LPS
IL-1ß	1.19 ± 0.36	10.60 ± 4.99*
IL-8	3.61 ± 4.06	137.46 ± 42.45*

* statistically significant difference as compared to control

(Table 1). Statistically significant differences were found between healthy and inflamed pulps ($p<0.001$), for both cytokines (IL-1ß and IL-8).

Cytokine Production from Pulp Fibroblasts

The ELISA assays made with cell cultures complemented data observed in tissue processing (Table 2). According to these results, treated (LPS) group (representing an inflammatory state) presented higher expression of both analyzed cytokines than the control group. Statistically significant differences were found between the control and treated (LPS) groups, both for IL-1ß and for IL-8 ($p<0.001$).

DISCUSSION

The purpose of this study was to analyze the presence, localization and quantity of IL-1ß and IL-8 in healthy and inflamed dental pulps. Additionally, this study also co-related data obtained from pulp tissues and cultured pulp fibroblasts treated or not with LPS, since it is accepted that LPS is able to induce an inflammation[5,8,11]. The cited cytokines were chosen due to their importance in the acute inflammation process[4,5,6,8,11,13,14,18]. Additionally, these cytokines have an important relationship: chemokines, and among them the IL-8 are generally induced by bacteria and other inflammatory cytokines, mainly IL-1[1]. It is also known that the interaction between IL-8 and IL-1ß leads to the migration and recruiting of neutrophils, the most prevalent cells in the acute inflammation[15].

In the present study, higher levels of IL-1ß were detected in inflamed pulps. This data was expected, since it is known that increases in IL-1ß levels are also able to stimulate inflammation.[5] This is in accordance with many other studies, independently of the methods used.[2,4,5,10] Here, using three different methods for the same purpose, it was possible to validate all methods and confirm all data presented before.

The IL-8 is one of the most potent chemokines, with great chemo attractive activity for neutrophils[1,6]. For this reason, it must be studied in order to provide more information of its action. It can be produced by leukocytes, endothelial cells and fibroblasts, due to the contact of an antigen with these cells[1,13], starting its effects on neutrophils[13,14,17]. Considering this relation between inflammation and IL-8, the results presented here are also in agreement with the literature[6,7,8,11,13,14,18].

The positive staining in inflamed dental pulp was only observed in inflammatory cells, and not in fibroblasts as observed with the cell culture ELISA detection. However, it can be explained by the sensitivity of both assays, from which ELISA is much more sensible. Additionally, cell cultures provide a much more controlled environment, from where the LPS induction can be achieved in a easier way, differently from tissues, where the presence of the extra-cellular matrix, blood flow and lymph vessels can reduce this signal.

From data presented here, a finding can be highlighted: the staining of the odontoblast layer for both cytokines, even in healthy pulps. This is already described for IL-8,[6] but without any good explanation. However, it is reported that these cells can also respond with expression of IL-1ß and IL-8 genes, when stimulated by bacterial LPS, showing their capacity to respond to external aggression[16]. Thus, a further study with fresh healthy odontoblasts can be suggested, analyzing the production and expression of these cytokines both in the protein and molecular levels. Additionally, despite the simple comparison made in this study, it was possible to corroborate, with three different methods, data described in the literature, emphasizing the importance of the complete understanding of these cytokines, regarding pulp inflammation. This will be further extended in the future with the study, under the same conditions, of the membrane receptors and signaling pathways involved in the inflammatory process.

CONCLUSIONS

Based on data presented here, it may be concluded that inflamed pulps present higher amounts of IL-1ß and IL-8 than healthy pulps and that pulp fibroblasts stimulated by bacterial LPS to produce higher levels of IL-1ß and IL-8 than the control group.

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