Brazilian Dental Journal (2021) 32(3): 10-20
http://dx.doi.org/10.1590/0103-6440202104503

ISSN 0103-6440

Profile of host cell responses to exposure to stressed bacteria in planktonic; dislodged, and intact biofilm mode

Rafaela Fernandes Zancan [1], José Burgos Ponce [2], Thiago José Dionisio [3], Rodrigo Cardoso de Oliveira [4], Rafaela Alves da Silva [4], Jussaro Alves Duque [1] and Marco Antonio Hungaro Duarte [1]

1 Bauru Dental School, University of São Paulo, Bauru, São Paulo, Brazil - Department of Restorative Dentistry, Dental Materials and Endodontics

2 Bauru Dental School, University of São Paulo, Bauru, São Paulo, Brazil - Department of Surgery, Stomatology, Pathology and Radiology

3 Bauru Dental School, University of São Paulo, Bauru, São Paulo, Brazil - Department of Biological Sciences,

4 Bauru Dental School, University of São Paulo, Bauru, São Paulo, Brazil - Department of Pediatric Dentistry, Orthodontics and Public Health

Correspondence: Rafaela Fernandes Zancan Al. Octávio Pinheiro Brisola no. 9-75 17012-901 Bauru, São Paulo, Brazil Telephone +55-14-32358344 Fax +55-14-32242788
E-mail: rafaelazancan@yahoo.com.br

The host defense response to microbial challenge emerging from the root canal system leads to apical periodontitis. The aim of this study was to evaluate the expression of inflammatory cytokines and Nitric Oxide (NO) by macrophages after interaction with *Enterococcus faecalis* in the: plankton and dislodged biofilm mode; intact biofilm mode stimulated by calcium hydroxide (CH), CH and chlorhexidine (CHX) or Triple Antibiotic Paste (TAP). For this purpose, culture of macrophages from monocytes in human peripheral blood (N=8) were exposed to the different modes of bacteria for 24 hours. Subsequently, the cytokines, such as, Tumor Necrotic Factor- alfa (TNF-α), interleukin (IL)-1β, IL-6, IL-10; and NO were quantified by Luminex xMAP and Greiss reaction, respectively. In addition to the potential therapeutic effects of the intracanal medication, their antimicrobial activity against *Enterococcus faecalis* biofilm were also tested in vitro by confocal microscopy. The experiments` data were analyzed by the Kruskal–Wallis test with the Dunn post hoc test ($\alpha < 0.05$). Bacteria in dislodged biofilm mode were shown to be more aggressive to the immune system than bacteria in plankton mode and negative control, inducing greater expression of NO and TNF-α. Relative to bacteria in intact biofilm mode, the weakest antimicrobial activity occurred in Group CH. In Groups CH/CHX and TAP the percentage of dead bacteria was significantly increased to the same extent. Interestingly, the biofilm itself did not induce the release of pro-inflammatory cytokines – except for NO – while the biofilm treated with TAP and CH based pastes enhanced the levels of IL-6 and TNF-α; and IL-1 β, respectively. In contrast, the levels of a potent anti-inflammatory (IL-10) were increased in Group TAP.

Key Words: Enterococcus faecalis; interleukin; intracanal medication; nitric oxide.

Introduction

Enterococcus faecalis (E. faecalis), a Gram-positive facultative anaerobic bacterium, is recognized as the leading cause of re-treatment and persistent endodontic infections. E. faecalis is capable of resisting unfavorable conditions within dentinal tubules - such as nutritional limitation for up to 12 months, as they go into "starvation phase/mode"(1). In addition they can easily organize themselves in biofilms that have high levels of antibiotic resistance and virulence (2). Biofilms are high-density bacterial communities, adherent to a surface and embedded in an exopolymer matrix (3). Earlier studies with *Staphylococcus epidermidis* have found evidence that owing the complexity of biofilm, bacteria within them, expressing different degrees of pathogenicity when compared with their planktonic counterparts, may be recognized differently by the host immune system (4, 5).

The choice of an intracanal medication is essentially based on the knowledge of its physicochemical characteristics and antimicrobial action, and it is an important adjunct therapy for control of root canal infection (6). An imbalance between the microbiological factors and the host defenses favoring the former, can stimulate macrophages to release proinflammatory cytokines inductors of bone resorption, such as, Interleukin 1 beta (IL-1β), tumor necrosis factor (TNF-α) and Interleukin 6 (IL-6). Therefore, inactivation of these cytokines also needs to be considered when choosing an intracanal medication, as it influences the progress and maintenance of apical periodontitis (7-9), and manifestations such as periapical granulomas, radicular cysts and periapical abscesses (10-12).

Calcium hydroxide (CH) powder in a vehicle has been widely used by many clinicians, due to ts antimicrobial and biological action. CH plays an important role in hard-tissue formation, as its alkalinity activates alkaline phosphatase (13). Its capacity to modulate inflammation by inhibiting the function of macrophages may also be of physiological significance in promoting the healing of periradicular tissues

(14). However, as CH is less effective against E. faecalis (6, 15), chlorhexidine (CHX) has been proposed as an additive to CH, due to its broad spectrum of activity (16) against Gram-positive and Gram-negative microorganisms (17). Ciprofloxacin, a fluoroquinolone; metronidazole, a nitroimidazole, and minocycline, a tetracycline antibiotics, are used in combination as an intracanal medication named Triple Antibiotic Paste (TAP). Its wide spectrum of antimicrobial activity has made it the medication of first choice against persistent endodontic infections, but it also used in regenerative endodontics as a substitute for CH. Apart from disinfection, these antibiotics are known to down-regulate lipopolysaccharide (LPS)-mediated TNF-α (18-20), encouraging the expression of ECM genes related to periodontal healing (21). However, few studies have described the immunomodulatory effect of TAP on macrophages associated with a gram-positive bacteria (22).

Based on the foregoing, the aims of this study were to evaluate the release of TNF- α, IL-1β, IL-6, IL-10 and NO after bacteria-macrophage interactions by using: bacteria in planktonic and dislodged biofilm mode; biofilm stimulated by different intracanal medications in order to compare the potential therapeutical effects of these compounds and their antimicrobial activity. The null hypotheses tested were that:

The stimulus of pro- and anti-inflammatory cytokines and NO released by macrophages would be similar in: 1. Bacteria in planktonic and dislodged biofilm mode; 2. Biofilm treated and not treated with different intracanal medications.

Material and methods

Antimicrobial Test and Microscopy Analysis

Microbiological procedures and manipulation of pastes were conducted under aseptic conditions in a laminar flow chamber (VecoFlow Ltda, Campinas, SP, Brazil).

Biofilm Growth

For E. faecalis biofilm, 15 µL standard strain (American Type Culture Collection [ATCC] 29212) were put into 3 ml sterile brain–heart infusion (BHI; Oxoid, Basingstoke, UK) at 37°C in air for growth overnight. After this period, the bacterial density was adjusted to 108 cells/ml with a spectrophotometer (UV-VISIBLI, Shimadzu, Japan) at an optical density of 1 at 600 nm according to the 0.5 MacFarland standard.

Subsequently, we proceeded with contamination of the dentin blocks. These were obtained from bovine central incisors with fully developed roots, with the use of trephine drills 4.0 mm in diameter under abundant irrigation. The size of blocks obtained was adjusted to standardize their measurements to 4 mm x 1.2 mm (diameter x thickness), by means of polishing the pulp surface with 500 and 800 grit SiC papers, and subsequently sterilizing these samples in an autoclave. For their contamination, one dentin block +100 µl of E. faecalis + 900 µl of BHI were put into each well of a 24-well multiwell plate that was kept in an oven at 37°C, under aerobic conditions, for a period of 21 days. To prevent nutrient deficiency, the BHI was renewed every 2 days. After biofilm maturation, the infected samples were washed with 1 mL of phosphate-buffered-saline (PBS) to remove loosely adherent and planktonic bacteria.

Direct Contact Test

The contaminated dentin blocks were randomly divided into 4 groups according to the intracanal medication, of which, one received no treatment (control).

Three intracanal medications were evaluated:

- Calcium Hydroxide / Poliethylene glycol (CH; Calen, SS White Artigos Dentários Ltd, Rio de Janeiro, Brazil);
- Calcium Hydroxide (Merck & Co, Kenilworth, NJ)/ 2% Chlorhexidine gluconate (FGM, Joinville, Brazil) / Propylene glycol (CH/CHX; 1 g/0.5 mL/0.3 mL);
- Triple Antibiotic Paste: Metronidazole, Ciprofloxacin, Minocycline (Brainfarma Indústria Química e Farmacêutica S.A., São Paulo, Brazil) / Propylene glycol (TAP; 500 mg of each antibiotic/1 mL)

Dentin blocks were immersed in the test medications and remained in an oven at 37°C for a period of 7 days.

Microscopy Analysis

After the direct contact test, the samples were washed with PBS and stained with 15 µL of SYTO9 and propidium iodide solution (Live/Dead BacLight Viability Kit; Molecular Probes, Eugene, USA) for 15 minutes, for microscopy analysis. After this, the samples were washed again and directly observed by inverted confocal laser scanning microscopy (CLSM; Leica TCS-SPE; Leica Biosystems CMS, Mannheim, Germany) with a 40x oil lens. Four confocal "stacks" of random areas were obtained per dentin block totaling 20 images per group. The percentage of dead bacteria cells were obtained by means of Bioimage v2-1 software (www.bioImageL.com).

Expression of Cytokines and Nitric Oxide by Host Cells Exposed to Bacteria in Biofilm in Intact, Dislodged and Planktonic Mode

This study was approved by the Research Ethics Committee of the Bauru School of Dentistry of the University of São Paulo (#CAAE 66228717.4.0000.5417). After signing the term of free and informed consent, in compliance with resolution 196/96 of the National Health Council, blood collection was performed in healthy volunteer patients.

Culture of macrophages from monocytes of human peripheral blood

A skilled laboratory technician trained to obtain data from a previous calibration record collected 30 mL of blood from the brachial veins of 8 healthy individuals with the aid of vacuum glass tube (BD Vacutainer, United States), containing sodic heparin as anticoagulant. By means of the Histopaque gradient 1083 (Sigma, Stoinheim, Germany), the mononuclear cells were differentiated followed by RPMI 1640 (Invitrogen, United States) washing, and their concentration was adjusted to 1x106. Then, they were inserted into 24 wells culture plates (Orange Scientific, Belgium) and incubated at 37 °C in a humidified atmosphere (5 % CO2/air) for 2 hours so that monocytes could adhere to the plate. After this, the plates were again washed twice with RPMI 1640 to remove non-adherent cells, including lymphocytes. Then, 1 mL of RPMI 1640 containing 10% fetal calf serum (FCS) and 1% penicillin were added to each well and the plates were incubated for 7 days (time required for monocytes to differentiate into macrophages) at 37 °C in a humidified atmosphere (5 % CO2/air). Every 48 hours, 500uL of culture medium of each well were renewed.

Expression of cytokines and NO by host cells exposed to bacteria in planktonic and dislodged biofilm mode

After differentiation of blood monocytes into macrophages (section 2.1), human macrophages were exposed to bacteria in planktonic and dislodged biofilm mode in the ratio of 1:5 in 1 mL of broth. After biofilm maturation (section 1.1), the adhered bacterial cells were dislodged gently but firmly by scraping the dentin with a cell scraper, resuspended in appropriate buffer, and vortexed vigorously to disrupt any clumps in order to produce the dislodged biofilm mode cells. Microscopic observation was also performed to confirm disruption of aggregates. For planktonic mode cells, aliquots of E. faecalis strain were taken from storage at -80°C and incubated in 3 mL BHI at 37°C overnight. Subsequently, bacteria in both modes were inoculated for 4 hours into test tubes containing neutral BHI (positive control) and alkaline-BHI broth, buffered at pH 9.5 with 5M NaOH, adjusted with a pH meter (model 371; Micronal, São Paulo, SP, Brazil). Cells were then washed twice with PBS to remove remaining BHI. Bacterial density of both bacterial modes was then adjusted to 5x106 with a spectrophotometer (UV-VISIBLI, Shimadzu, Japan), at an optical density of 1 at 600 nm according to the 0.5 MacFarland standard.

Subsequently, these suspensions were added to the culture plate wells containing adhered macrophages at a concentration of 1x106. After 24 hours of bacteria-macrophage interaction at 37 °C in a humidified atmosphere (5 % CO2/air), the supernatants of the wells were collected and stored in a freezer at -80°C for subsequent cytokine and NO dosage.

Expression of cytokines and NO by host cells exposed to intact biofilm mode bacteria

For this experiment the macrophage cells adhering to the wells (section 2.1) were dislodged gently but firmly by scraping with a cell scraper and resuspended in RPMI Then, the macrophages in suspension at the concentration of 1x106 were added into the culture plate wells containing bacterial biofilm without BHI.

The maturation of the biofilm occurred as described in section 1.1, with the difference that the process was developed on glass coverslips that perfectly adjusted to the wells of the 24-well plate, thus ensuring effective contact between the bacterial biofilm and macrophages. The biofilms treated, or not

(positive control), with the different intracanal medications tested in this study (section 1.2) for 7 days, were abundantly irrigated with PBS to remove all of the intracanal medication, before exposure to macrophages. All plates were incubated at 37 °C in a humidified atmosphere (5 % CO2/air) for 24 hours. The supernatants of the wells were collected and stored in a freezer at -80°C for subsequent cytokine and NO dosage.

Quantification of TNF- α, IL-1β, IL-6 and IL-10

In order to estimate the cytokine synthesis after exposure to E. faecalis strains (section 2.2 and 2.3), the culture supernatants of macrophages were quantified using MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel (Merck, Cat. No: HCYTOMAG-60K-03) in the Luminex xMAP (MAP: Multiple Analyte Profiling) according to the manufacturer's instructions. Macrophages exposed to non-treated bacteria or on their own, served as positive and negative control, respectively. The sensitivity levels for detection of each cytokine were TNF- α =1.9 ng/uL, IL-1β = 2.8 ng/uL, IL-6 = 30.7 ng/uL and IL-10 = 2.7 ng/uL. The samples were evaluated using the Analyst software and fitting model cubic log standard curve.

Quantification of Nitric Oxide

Analyses of nitric oxide (NO) were performed by using the Greiss reaction.Thus, 100 µL of supernatant culture of each well was mixed to 1% sulfanilamide in 2.5% phosphoric acid solution (100 µL) for 10 minutes. After this, 100 µL of 1% N-(1-Naphthyl) ethylenediamine in 2.5% phosphoric acid was added for the same length of time. To evaluate the production of NO, this was measured by absorbance at 490 nm in a microplate reader (Synergy 2, BioTek, VT, USA), using the sodium nitrite standard curve with RPMI 1640 as a control.

Statistical analysis

The Shapiro-Wilk test was used to verify the normality of data of all analyses, and absence of normality was observed. Therefore, statistical comparisons between the groups were made by the Kruskal-Wallis and Dunn tests. The significance level was established at 5%.

Results

Table 1 presents the percentages of live cells of E. faecalis biofilm after 7 days of contact with the experimental pastes CH, CH/CHX or TAP. Representative confocal laser scanning microscope images of the groups described in Table 1 are shown in Figure 1, where live cells are indicated in green, and dead cells are indicated in red. The weakest antimicrobial activity occurred in Group CH, showing no statistical differences when compared with the control group (P > .05). In Groups CH/CHX and TAP, significant increase in the percentage of dead bacteria was found, when compared with the other groups (P = .0001) This occurred to the same extent in E. faecalis biofilm (P > .05).

Table 1. Median (Med) and Minimum and Maximum (Min–Max) Values of the Percentage of Live Cells of Different Biofilms after Contact with the Experimental Medicaments for one Week

	CH Med (Min – Max)	*CH/CHX* Med (Min – Max)	*TAP* Med (Min – Max)	*Control* Med (Min – Max)
% live cells	84.88^{A} (41.71 – 95.54)	57.95^{B} (25.17 – 89.98)	16.78^{B} (0.02 – 70.8)	95.50^{A} (76.97 – 99.99)

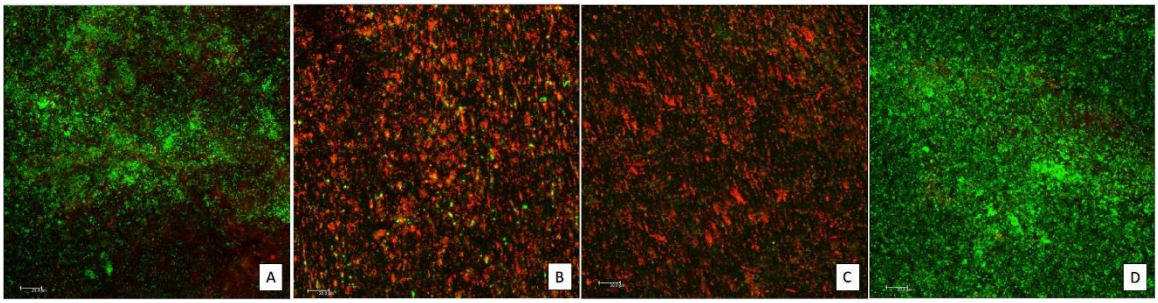

Figure 1. Confocal laser scanning microscopy of biofilms treated with (A) calcium hydroxide, (B) calcium hydroxide + chlorhexidine, (C) triple antibiotic paste, and (D) control group. Live cells are indicated in green, and dead cells are indicated in red. Each picture represents an area of 275 x 275 mm.

Table 2 presents the expression of cytokines (TNF- α, IL-1β, IL-6 and IL-10) and NO by host cells exposed to planktonic and dislodged biofilm mode bacteria, stressed or not (positive control) for 4 hours, in test tubes containing alkaline-BHI broth. Both modes of bacteria, whether stressed or not in alkaline media, did not elicit significant IL-10, IL-1 and IL-6 production by macrophages, showing no differences when compared with negative control ($P > .05$). E. faecalis in dislodged biofilm mode induced the highest excretion of NO ($P = .0001$) and TNF-α ($P = .02$), with significant differences when compared with planktonic bacteria and negative control ($P < .05$).

Table 2. Median (Med). minimum (Min) and maximum (Max) values of protein levels (pg/mg) released by macrophages after 24 hours of contact with biofilm or planktonic form of *Enterococcus faecalis* stressed in an alkaline environment for 4 hours

	Planktonic bacteria (Alkaline stress)	Planktonic bacteria (Control)	Dislodged biofilm (Alkaline stress)	Dislodged biofilm (Control)	Negative Control
IL-10	1.16^{A} (1.01 – 1.39)	1.19^{A} (1.13 – 1.26)	1.07^{A} (0.72 – 5.07)	2.09^{A} (1.39 – 2.87)	1.66^{A} (1.13 – 2.39)
IL-1 β	0.37^{A} (0.21 – 1.05)	0.91^{A} (0.25 – 2.09)	0.24^{A} (0.15 – 1.66)	1.03^{A} (0.72 – 3.76)	0.23^{A} (0.19 – 0.26)
IL-6	0.64^{A} (0.43 – 0.91)	0.53^{A} (0.36- 43.20)	0.69^{A} (0.43 – 3.20)	6.03^{A} (2.73 – 21.37)	0.69^{A} (0.36 – 1.00)
TNF-α	43.91^{AB} (11.24 – 85.00)	19.73^{B} (6.85 – 48.38)	32.35^{AB} (29.26 – 108.2)	253.0^{A} (119.6 – 540.9)	14.81^{AB} (7.26 – 42.66)
Nitric Oxide	42.46^{AB} (16.09 – 89.27)	20.19^{B} (13.36 – 65.18)	55.19^{AB} (27.91 – 170.6)	115.8^{A} (101.7 – 128.1)	12.41^{B} (9.91 – 48.55)

Table 3 presents the expression of cytokines (TNF- α, IL-1β, IL-6 and IL-10) and NO by host cells exposed to intact biofilm mode bacteria treated or not with CH, CH/CHX or TAP for a period of 7 days. Non-treated biofilm did not elicit significant production of cytokines by macrophages, showing no differences when compared with negative control ($P > .05$), but their NO production was triggered ($P = .0001$). NO was produced in groups containing CH/CHX to the same extent as in positive control ($P > .05$). CH alone or with CHX enhanced IL-1β production when compared with negative control ($P = .0004$) showing no differences when compared with the other groups ($P > .05$).TAP significantly increased Il-10 ($P = .0006$), Il-6 ($P = .001$); and TNF-α ($P = .001$) production when compared with the control groups, but had decreased NO production when compared with positive control ($P = .0001$).

Table 3. Median (Med), minimum (Min) and maximum (Max) values of protein levels (pg/mg) released by macrophages after 24 hours of contact with *Enterococcus faecalis* biofilm stressed with intracanal dressing treatment for 7 days

	CH Med (Min – Max)	CH/CHX Med (Min – Max)	TAP Med (Min – Max)	Positive Control Med (Min – Max)	Negative Control Med (Min – Max)
IL-10	8.91^{AB} (1.26 – 183.4)	8.06^{AB} (3.29 – 170.1)	34.46^{A} (4.09 – 47.18)	1.94^{B} (1.39 – 2.87)	2.09^{B} (1.13 – 5.07)
IL-1 β	4.40^{A} (0.31 – 97.83)	58.86^{A} (0.38 – 940.6)	1.62^{AB} (0.35 – 10.92)	0.50^{AB} (0.19 – 3.76)	0.23^{B} (0.19 – 2.82)
IL-6	13.83^{AB} (0.64 – 270.9)	17.30^{AB} (0.86 – 311.1)	192.0^{A} (6.03 – 1372)	1.66^{B} (0.43 – 21.37)	0.69^{B} (0.36 – 27.82)
TNF-α	569.5^{AB} (31.95 – 2615)	586.2^{AB} (118.1 – 2848)	1125^{A} (132.3 – 5528)	63.24^{B} (1.21 – 540.9)	28.74^{B} (1.59 – 41.07)
Nitric Oxide	36.27^{ABC} (10.36 – 177.6)	46.28^{AB} (29.91 – 172.6)	12.64^{BC} (11.27 – 32.18)	112.4^{A} (68.55 – 133.1)	11.73^{C} (9.91 – 13.08)

Discussion

The null hypothesis was rejected since differences in pro- and anti-inflammatory cytokines and NO released by macrophages were found for: 1. planktonic and dislodged biofilm mode bacteria; 2. intact biofilm mode treated or non-treated with different intracanal medications. Differences in the antimicrobial activity of the latter against E. faecalis biofilm were also found.

The Luminex xMAP (multi-analyte profiling) technology was chosen for the immunological analyses, as it provides simultaneous measurement of multiple cytokines in small sample volumes, as it is a validated technique with good correlation coefficients with the "gold standard" ELISA (enzyme-linked immunosorbent assays) in evaluating cytokine profiling in vitro (23). Bacteria-macrophage interactions were assessed using bacteria from both planktonic inoculums and disrupted biofilm, as individual bacteria at the same concentration. Bacteria have been shown to survive in nature by forming biofilms on surfaces, and to expand with the dispersal of physiologically differentiated free-living cells from biofilm to colonize other niches (24), which enhances the clinical relevance of the present study. The results showed greater activity by macrophages to produce inflammatory mediators - such as TNF- α and NO - in contact with E. faecalis cells from dislodged biofilm when compared with the planktonic mode. Macrophages use NO, a diatomic free radical gas, to kill the internalized microorganisms such as, fungi and bacteria (25-28). Bacteria that grow as biofilm retain some distinctive features and mechanisms to avoid phagocytosis (29), which leads to a continues stimulus for eliminating these microorganisms, therefore, higher levels of NO are released by macrophages (Table 2) (30). This mediator is also related to the early stages of periapical lesion progression via inducing macrophage and osteoblast apoptosis (31). TNF-α contributes to further tissue damage, with the capacity to stimulate osteoclast development and thereby the process of bone resorption (32). As a pro-inflammatory protein in the acute phase, it is expected to have the highest release in an early phase of inflammation (33). This explains why it is the only cytokine that showed differences from the negative control.

In the second part of the experiments, the pro- and anti-inflammatory effects of different intracanal medications on human macrophages stimulated by biofilm were analyzed in order to compare the potential therapeutical effects of these compounds.

The resistance of biofilms to antibiotics is directly related to their developmental stage (34). The E. faecalis biofilm is poorly structured during the first days of it development (35); according to the literature (6) biofilm growth occurred for an extended period of time in this study (21 days). E. faecalis biofilm survived the highly alkaline environment resulting from the ionic dissociation of CH (6, 15), probably because of its proton pump that maintains optimal cytoplasmic pH levels (Figure 1) (36). CHX is believed to be capable of inhibiting adenosine triphosphatase activity, which is vital to proton pump functioning (6). The benefits of adding CHX to enhance the antiseptic action of CH paste against E. faecalis were proved in this and other studies (6, 37).

Interestingly, the biofilm itself did not induce the release of cytokines – except for NO – while the treated biofilm did (Table 3). Macrophages might have expressed more NO to kill internalized

microorganisms in the positive control group, due to the higher number of live bacteria . As expected, TAP achieved a greater reduction in biofilm levels after 7 days (38), leading to levels of NO similar to those of the negative control. CH inhibits the adhesion of macrophages, and consequently, their phagocytic process (14). Adding CHX to CH appears to have interfered with this reaction, as the levels of NO were higher than those of the negative control. It is not known whether the reactive by-products such as reactive oxygen from CHX decomposition into alkaline pHs (39), could have influenced the results.

Lipoteichoic acids (LTA), a cell wall component of E. faecalis, is a key virulence factor in the release of major proinflammatory mediators such as NO, TNF-α and IL-6 (40, 41). Increases in IL-6 stimulate major osteoclastic bone destruction, and the synthesis of acute phase proteins (42), important characteristics in the evolution of symptomatic dental abscesses (43). Biofilm treated with CH and additives released lower levels TNF-α and IL-6 due its capacity to inactivate LTA (44), or TNF-α itself (45, 46). LTA can accumulate and persistently reside within dentinal tubules even after bacterial death, due its high affinity for hydroxyapatite (47). CH would prevent the continuous stimulation of the surrounding tissues by LTA, and therefore, chronic inflammation (7-9).

Although the combination of ciprofloxacin and metronidazole causes a significant decrease in TNF-α, suggesting a synergistic anti-inflammatory effect (21, 48), TNF-α expression was increased when they were used in combination with minocycline. The release of LTA could be triggered by TAP resulting in the highest values of TNF-α and IL-6 for this paste when compared with the Control. TNF-α potentiates the effects of IL-1β (49). TAP showed no differences in the release of this pro-inflammatory cytokine, when compared with Groups CH. However, only biofilms treated with CH-based pastes exacerbated IL-1β expression by macrophages when compared with negative control. This cytokine is known to be one of the most active stimulators of osteoclastic bone reabsorption (50) - 500-fold more potent than TNF-α (51) - being related to persistent apical periodontitis (52).

The levels of a potent anti-inflammatory cytokine (IL-10) were increased by TAP, as it occurred with double antibiotic paste (21). This cytokine avoids exaggerated inflammatory response, due its suppression of proinflammatory mediator production that could culminate in the development of inflammatory diseases (53) and inhibits the development and activation of the bone-resorbing cells, fundamental feature of periapical lesion progression (54). This is not only important for healing the periradicular tissues but also for the success of regenerative endodontic procedures such as the blood-clot- interaction with the immature infected root structure.

In conclusion, E. faecalis cells from dislodged biofilm triggered a pro-inflammatory response from macrophages by releasing TNF- α and NO when compared to it planktonic mode. Although the intracanal medications were primarily selected because of their antimicrobial activity, their ability to possibly influence the degree of the systemic and local immune response to infection, which is also related to the progress and maintenance of apical periodontitis, should be more appreciated. The release of NO by macrophages seems to be directly related to the amount of live bacteria, with higher values for non-treated biofilm and lower values for TAP. In contrast, the biofilm it self did not induce the release of pro-inflammatory cytokines, while the biofilm treated with TAP and CH- based pastes triggered the release of IL-6 and TNF-α; and IL-1 β, respectively. The levels of the anti-inflammatory cytokine IL-10 increased in Group TAP. Given the importance of E. faecalis in endodontic infections, further studies on how the biofilm mode of this bacteria affects the host defense mechanisms, and the possible influence of the degree to which an inter appointment medication and its antimicrobial action could have on this response should be conducted.

Acknowlegments

Supported by the State of São Paulo Research Foundation (FAPESP 2016/25133-1).

Resumo

A resposta de defesa do hospedeiro ao desafio microbiano que emerge do sistema de canais radiculares leva à periodontite apical. Os objetivos deste estudo foram avaliar a expressão de citocinas pró e anti-inflamatórias e Óxido Nítrico (NO) por macrófagos após interação com *Enterococcus faecalis* no modo: planctônio e de biofilme desalojado; biofilme intacto estimulado por hidróxido de cálcio (CH), CH e clorexidina ou Pasta Tri Antibiótica (TAP). Para isto, a cultura de macrófagos originados de monócitos do sangue periférico de humanos (N=8) foi exposta aos diferentes tipos de bactéria por 24 horas. Então, a quantificação da produção de of Fator de Necrose Tumoral- alfa (TNF-α), interleucina

(IL)-1β, IL-6, IL-10 e NO por macrófagos se deu por meio do Luminex xMAP e reação de Greiss, respectivamente. Além dos potenciais efeitos terapêuticos desses compostos, sua atividade antimicrobiana contra E. faecalis também foi testada através microscopia confocal. Os dados dos experimentos foram analisados através do teste de Kruskal-Wallis com Dunn`s post hoc ($\alpha < 0.05$). Bactéria em modo de biofilme desalojado se mostrou mais agressivo ao sistema imune que as bactérias planctônicas e controle negativo induzindo a maior excreção de NO e TNF-α. Em relação ao biofilme intacto, a atividade antimicrobiana mais fraca ocorreu no grupo de CH. Os grupos CHX e TAP aumentaram significativamente a porcentagem de bactérias mortas na mesma extensão. Interessantemente, o biofilme por ele mesmo não induziu a liberação de citocinas pro-inflamatórias – exceto por NO – enquanto que o biofilme tratado com TAP ou pastas a base de CH aumentaram os níveis de IL-6; e TNF-α e IL-1 β respectivamente. Em contraste, os níveis da potente citocina anti-inflamatória (IL-10) foram aumentados pelo grupo TAP.

References

1. Sedgley CM, Lennan SL, Appelbe OK. Survival of *Enterococcus faecalis* in root canals ex vivo. International endodontic journal. 2005;38(10):735-42.

2. Portenier I, Waltimo T, Ørstavik D, Haapasalo M. The susceptibility of starved, stationary phase, and growing cells of *Enterococcus faecalis* to endodontic medicaments. Journal of endodontics. 2005;31(5):380-6.

3. Yoo A, Rossi-Fedele G, Kidd SP, Rogers AH, Zilm PS. Association between Extracellular Material and Biofilm Formation in Response to Sodium Hypochlorite by Clinical Isolates of *Enterococcus faecalis*. Journal of endodontics. 2018;44(2):269-73.

4. Johnson GM, Lee D, Regelmann W, Gray E, Peters G, Quie P. Interference with granulocyte function by *Staphylococcus epidermidis* slime. Infection and immunity. 1986;54(1):13-20.

5. Riber U, Espersen F, Kharazmi A. Comparison of adherent and non-adherent staphylococci in the induction of polymorphonuclear leukocyte activation in vitro. APMIS : acta pathologica, microbiologica, et immunologica Scandinavica. 1995;103(6):439-46.

6. Zancan RF, Vivan RR, Milanda Lopes MR, Weckwerth PH, de Andrade FB, Ponce JB, et al. Antimicrobial Activity and Physicochemical Properties of Calcium Hydroxide Pastes Used as Intracanal Medication. Journal of endodontics. 2016;42(12):1822-8.

7. Zhao L, Chen J, Cheng L, Wang X, Du J, Wang F, et al. Effects of E nterococcus faecalis lipoteichoic acid on receptor activator of nuclear factor-κ B ligand and osteoprotegerin expression in periodontal ligament fibroblasts. International endodontic journal. 2014;47(2):163-72.

8. Costa Junior ED, Souza-Filho FJd, Barbosa SV. Tissue reactions to a component of root canal system bacteria: lipoteichoic acid. Brazilian dental journal. 2003;14(2):95-8.

9. Wang JE, Dahle MK, McDonald M, Foster SJ, Aasen AO, Thiemermann C. Peptidoglycan and lipoteichoic acid in gram-positive bacterial sepsis: receptors, signal transduction, biological effects, and synergism. Shock. 2003;20(5):402-14.

10. Ramachandran Nair PN, Pajarola G, Schroeder HE. Types and incidence of human periapical lesions obtained with extracted teeth. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics. 1996;81(1):93-102.

11. Gbadebo SO, Akinyamoju AO, Sulaiman AO. PERIAPICAL PATHOLOGY: COMPARISON OF CLINICAL DIAGNOSIS AND HISTOPATHOLOGICAL FINDINGS. Journal of the West African College of Surgeons. 2014;4(3):74-88.

12. Saraf PA, Kamat S, Puranik RS, Puranik S, Saraf SP, Singh BP. Comparative evaluation of immunohistochemistry, histopathology and conventional radiography in differentiating periapical lesions. Journal of conservative dentistry : JCD. 2014;17(2):164-8.

13. Estrela C, Sydney GB, Bammann LL, Felippe Junior O. Mechanism of the action of calcium and hydroxy ions of calcium hydroxide on tissue and bacteria. 1995.

14. Segura JJ, Llamas R, Rubio-Manzanares AJ, Jimenez-Planas A, Guerrero JM, Calvo JR. Calcium hydroxide inhibits substrate adherence capacity of macrophages. Journal of endodontics. 1997;23(7):444-7.

15. Portenier I, Waltimo TM, Haapasalo M. *Enterococcus faecalis*–the root canal survivor and 'star'in post-treatment disease. Endodontic topics. 2003;6(1):135-59.

16. Gomes BP, Souza SF, Ferraz CC, Teixeira FB, Zaia AA, Valdrighi L, et al. Effectiveness of 2% chlorhexidine gel and calcium hydroxide against *Enterococcus faecalis* in bovine root dentine in vitro. International endodontic journal. 2003;36(4):267-75.

17. Vahdaty A, Pitt Ford TR, Wilson RF. Efficacy of chlorhexidine in disinfecting dentinal tubules in vitro. Endodontics & dental traumatology. 1993;9(6):243-8.

18. Krehmeier U, Bardenheuer M, Voggenreiter G, Obertacke U, Schade FU, Majetschak M. Effects of antimicrobial agents on spontaneous and endotoxin-induced cytokine release of human peripheral blood mononuclear cells. Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy. 2002;8(2):194-7.

19. Bode C, Diedrich B, Muenster S, Hentschel V, Weisheit C, Rommelsheim K, et al. Antibiotics regulate the immune response in both presence and absence of lipopolysaccharide through modulation of Toll-like receptors, cytokine production and phagocytosis in vitro. International immunopharmacology. 2014;18(1):27-34.

20. Liu D, Yang PS. Minocycline hydrochloride nanoliposomes inhibit the production of TNF-α in LPS-stimulated macrophages. International journal of nanomedicine. 2012;7:4769-75.

21. Tan EE, Quah SY, Bergenholtz G, Rosa V, Hoon Yu VS, Tan KS. Antibiotics Used in Regenerative Endodontics Modify Immune Response of Macrophages to Bacterial Infection. Journal of endodontics. 2019;45(11):1349-56.

22. Sousa MGC, Xavier PD, Cantuária APdC, Porcino RA, Almeida JA, Franco OL, et al. Host defense peptide IDR-1002 associated with ciprofloxacin as a new antimicrobial and immunomodulatory strategy for dental pulp revascularization therapy. Microbial Pathogenesis. 2021;152:104634.

23. Codorean E, Nichita C, Albulescu L, Răducan E, Popescu ID, Lonită AC, et al. Correlation of XMAP and ELISA cytokine profiles; development and validation for immunotoxicological studies in vitro. Roumanian archives of microbiology and immunology. 2010;69(1):13-9.

24. Webb JS, Givskov M, Kjelleberg S. Bacterial biofilms: prokaryotic adventures in multicellularity. Current Opinion in Microbiology. 2003;6(6):578-85.

25. Nathan CF, Hibbs JB. Role of nitric oxide synthesis in macrophage antimicrobial activity. Current Opinion in Immunology. 1991;3(1):65-70.

26. Malawista SE, Montgomery RR, Van Blaricom G. Evidence for reactive nitrogen intermediates in killing of staphylococci by human neutrophil cytoplasts. A new microbicidal pathway for polymorphonuclear leukocytes. The Journal of clinical investigation. 1992;90(2):631-6.

27. Privett BJ, Deupree SM, Backlund CJ, Rao KS, Johnson CB, Coneski PN, et al. Synergy of nitric oxide and silver sulfadiazine against gram-negative, gram-positive, and antibiotic-resistant pathogens. Molecular pharmaceutics. 2010;7(6):2289-96.

28. Hetrick EM, Shin JH, Stasko NA, Johnson CB, Wespe DA, Holmuhamedov E, et al. Bactericidal efficacy of nitric oxide-releasing silica nanoparticles. ACS nano. 2008;2(2):235-46.

29. del Fresno C, García-Rio F, Gómez-Pina V, Soares-Schanoski A, Fernández-Ruíz I, Jurado T, et al. Potent phagocytic activity with impaired antigen presentation identifying lipopolysaccharide-tolerant human monocytes: demonstration in isolated monocytes from cystic fibrosis patients. The Journal of Immunology. 2009;182(10):6494-507.

30. Hernández-Jiménez E, del Campo R, Toledano V, Vallejo-Cremades MT, Muñoz A, Largo C, et al. Biofilm vs. planktonic bacterial mode of growth: Which do human macrophages prefer? Biochemical and Biophysical Research Communications. 2013;441(4):947-52.

31. Lin S-K, Kok S-H, Lin L-D, Wang C-C, Kuo MYP, Lin C-T, et al. Nitric oxide promotes the progression of periapical lesion via inducing macrophage and osteoblast apoptosis. Oral Microbiology and Immunology. 2007;22(1):24-9.

32. Manolagas SC. Role of cytokines in bone resorption. Bone. 1995;17(2 Suppl):63s-7s.

33. Bannerman D. Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows. Journal of animal science. 2009;87(suppl_13):10-25.

34. Sedlacek M, Walker C. Antibiotic resistance in an in vitro subgingival biofilm model. Oral microbiology and immunology. 2007;22(5):333-9.

35. Sun J, Song X. Assessment of antimicrobial susceptibility of *Enterococcus faecalis* isolated from chronic periodontitis in biofilm versus planktonic phase. Journal of periodontology. 2011;82(4):626-31.

36. Evans M, Davies JK, Sundqvist G, Figdor D. Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. International endodontic journal. 2002;35(3):221-8.

37. Delgado RJ, Gasparoto TH, Sipert CR, Pinheiro CR, Moraes IG, Garcia RB, et al. Antimicrobial effects of calcium hydroxide and chlorhexidine on *Enterococcus faecalis*. Journal of endodontics. 2010;36(8):1389-93.

38. Arruda MEF, Neves MAS, Diogenes A, Mdala I, Guilherme BPS, Siqueira JF, Jr., et al. Infection Control in Teeth with Apical Periodontitis Using a Triple Antibiotic Solution or Calcium Hydroxide with Chlorhexidine: A Randomized Clinical Trial. Journal of endodontics. 2018;44(10):1474-9.

39. Siren EK, Haapasalo MP, Ranta K, Salmi P, Kerosuo EN. Microbiological findings and clinical treatment procedures in endodontic cases selected for microbiological investigation. International endodontic journal. 1997;30(2):91-5.

40. Baik JE, Ryu YH, Han JY, Im J, Kum K-Y, Yun C-H, et al. Lipoteichoic Acid Partially Contributes to the Inflammatory Responses to *Enterococcus faecalis*. Journal of endodontics. 2008;34(8):975-82.

41. Koch L, Frommhold D, Buschmann K, Kuss N, Poeschl J, Ruef P. LPS- and LTA-induced expression of IL-6 and TNF-α in neonatal and adult blood: role of MAPKs and NF-κB. Mediators of inflammation. 2014;2014:283126.

42. Crowl RM, Stoller TJ, Conroy RR, Stoner CR. Induction of phospholipase A2 gene expression in human hepatoma cells by mediators of the acute phase response. The Journal of biological chemistry. 1991;266(4):2647-51.

43. Kurihara N, Bertolini D, Suda T, Akiyama Y, Roodman GD. IL-6 stimulates osteoclast-like multinucleated cell formation in long term human marrow cultures by inducing IL-1 release. The Journal of Immunology. 1990;144(11):4226-30.

44. BARTHEL CR, LEVIN LG, REISNER HM, TROPE M. TNF-α release in monocytes after exposure to calcium hydroxide treated Escherichia coli LPS. International endodontic journal. 1997;30(3):155-9.

45. Barthel C, Levin L, Reisner H, Trope M. TNF-α release in monocytes after exposure to calcium hydroxide treated Escherichia coli LPS. International endodontic journal. 1997;30(3):155-9.

46. Pinar Karapinar S, Ulum YZA, Ozcelik B, Dogan Buzoglu H, Ceyhan D, Balci Peynircioglu B, et al. The effect of N-acetylcysteine and calcium hydroxide on TNF-α and TGF-β1 in lipopolysaccharide-activated macrophages. Archives of Oral Biology. 2016;68:48-54.

47. Ciardi J, Rölla G, Bowen W, Reilly J. Adsorption of Streptococcus mutans lipoteichoic acid to hydroxyapatite. European journal of oral sciences. 1977;85(6):387-91.

48. Bamias G, Marini M, Moskaluk CA, Odashima M, Ross WG, Rivera-Nieves J, et al. Down-Regulation of Intestinal Lymphocyte Activation and Th1 Cytokine Production by Antibiotic Therapy in a Murine Model of Crohn's Disease. The Journal of Immunology. 2002;169(9):5308-14.

49. Fried R, Voelkel E, Rice R, Levine L, Gaffney E, Tashjian Jr A. Two squamous cell carcinomas not associated with humoral hypercalcemia produce a potent bone resorption-stimulating factor which is interleukin-1α. Endocrinology. 1989;125(2):742-51.

50. Lang NP, Tonetti MS, Suter J, Sorrell J, Duff GW, Kornman KS. Effect of interleukin-1 gene polymorphisms on gingival inflammation assessed by bleeding on probing in a periodontal maintenance population. Journal of periodontal research. 2000;35(2):102-7.

51. Stashenko P, Dewhirst FE, Peros WJ, Kent RL, Ago JM. Synergistic interactions between interleukin 1, tumor necrosis factor, and lymphotoxin in bone resorption. The Journal of Immunology. 1987;138(5):1464-8.

52. Morsani JM, Aminoshariae A, Han YW, Montagnese TA, Mickel A. Genetic predisposition to persistent apical periodontitis. Journal of endodontics. 2011;37(4):455-9.

53. Iyer SS, Cheng G. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. Critical Reviews™ in Immunology. 2012;32(1).

54. Arron JR, Choi Y. Bone versus immune system. Nature. 2000;408(6812):535-6.

Received: 17/05/2021
Accepted: 22/06/2021