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ORIGINAL ARTICLE

In vitro treatment of *Enterococcus faecalis* with calcium hydroxide impairs phagocytosis by human macrophages

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ABSTRACT

Objective: Monocyte-derived macrophages (MDMs) ability to phagocytize and produce nitric oxide (NO) was tested against root-canal strains of *Enterococcus faecalis* submitted to alkaline stress. Root-canal strains were also compared with urine Enterococci.

Materials and Methods: *Enterococcus faecalis* were stressed with alkaline-BHI broth and incubated *in vitro* at a cell/bacteria ratio of 1:5. Phagocytosis was analyzed by fluorescence microscopy using acridine orange stain, and NO concentration was measured in supernatants.

Results and Conclusions: Alkaline-stress significantly impaired MDMs phagocytosis of *E. faecalis* strains analyzed, except in ATCC4083 isolated from a pulpless tooth, but NO production was unchanged. Comparison of different strains showed the urine isolate had higher NO levels than root canal strains. Alterations in the bacterial cell wall structures after alkaline-stress possibly made bacteria less recognizable and phagocytized by MDMs but did not affect their ability to activate NO production. Furthermore, root canal strains elicited different responses by immune cells compared with strains from urine. Clinically, impaired phagocytosis of *E. faecalis* could contribute to their persistence in root canal systems previously treated with calcium hydroxide.

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Introduction

In apical periodontitis, the predominant genera are *Staphylococcus*, *Propionibacterium*, *Prevotella*, *Streptococcus*, *Pseudomonas* and *Fusobacterium*; showing a combination of strict and facultative anaerobic microbiota [1]. Whereas, in endodontically treated teeth presenting persistent apical periodontitis, *Enterococcus faecalis* (*E. faecalis*) is frequently associated with unsatisfactory filling [2,3], and even in a recent publication *E. faecalis* infection was identified at the root ends and in surfaces surrounding periradicular lesions, together with other bacteria strains [4]. Although *E. faecalis* is a gram-positive bacterium normally residing in the gastrointestinal tract of mammals as part of the commensal flora [5], some studies have shown that viable food-borne *E. faecalis* could leak into the pulp chamber space from cheese and meat, as example, and persist in periapical lesions [6–8].

Calcium hydroxide (CH), the most popular antimicrobial root canal medication used to inhibit bacterial growth during endodontic treatment, has an alkaline pH in which most bacteria cannot survive [9], due to its lethal effect on bacterial cells related to protein denaturation, and damage to DNA and cytoplasmic membranes. However, CH is less effective against *E. faecalis* and *Candida albicans* [10]. CH can also inactivate lipoteichoic acid and lipopolysaccharides in bacteria, reducing their ability to induce the production of inflammatory mediators such as tumor necrosis factor (TNF)-α and nitric oxide (NO) in macrophages, and possibly promoting progression of apical periodontitis progression [11].

Studies have shown that *E. faecalis* can survive in harsh environments with high alkalinity levels, and is resistant to direct exposure to alkaline dressings in endodontic treatments using CH, probably because of an effective proton pump mechanism, which maintains optimal cytoplasmic pH levels [2,9,11–13].

This could determine its high prevalence in persistent periapical lesions, since *E. faecalis* is the most commonly identified microorganism in these endodontic infections [14]. Enterococci virulence factors, which may enable the pathogen to remain inside root canal and cause damage to periradicular tissues, include cytolysin and proteolytic enzymes, adhesins, as well as capsular and cellular wall polysaccharides [2,14].

Macrophages are prevalent in endodontic infections and represent the main immune system cells against microbial pathogens, directly killing the microorganisms through

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phagocytosis and activating inflammatory signaling pathways [15–17]. Intracellular killing of bacteria is accomplished through the production of reactive species within the phagolysosome, such as NO, a reactive nitrogen intermediate synthesized by means of NO synthase activity [18]. Substances generated from NO molecule reactions by themselves or with other molecules, such as reactive oxygen species, act effectively on several microorganisms, including *E. faecalis* [9,19,20]. As a mediator of inflammatory responses, NO may modulate vascular permeability and vasodilatation, leukocyte adhesion and transmigration, cytokine and chemokine synthesis, exerting direct influence on assembling of the immune response [21,22].

Although enterococci are clinically important in endodontic failure, considering that they are capable of surviving at alkaline levels produced by CH, little is known about the host immune system behavior in response to persistent enterococcal infections within the root canal system and periapical microenvironment after endodontic dressings with CH. In addition, many researches in the endodontic field have performed assays using urine-isolated *E. faecalis* despite the possible influence of environmental stress on bacteria biology [23]. In this sense, application of non-endodontic strains of enterococci for these purposes might lead to misconceptions, since these bacteria also adapt their physiology to environmental conditions set by endodontic procedures [24]. Therefore, studies using bacteria specifically from the pulp or periapical environment should provide more reliable results in the endodontic research field, since they represent microorganisms with differential expression of virulence factors in comparison with those colonizing other sites of the host [25,26].

Considering the foregoing approach, this study evaluated phagocytosis and NO production from monocyte-derived macrophages (MDMs) cultured with alkaline-stressed *E. faecalis* from root-canals. The results were also compared with a strain originally isolated from urine, widely used in endodontic-related researches.

Material and methods

This study was approved by Ethics Committee from Bauru School of Dentistry (Process #CAAE 46839215.0.0000.5417). All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study. All results were obtained from the average of blood samples from 15 young healthy volunteers aged 25–45 years.

Bacterial strains

The ATCC 4083 (CANAL 1) isolated from a pulpless tooth was obtained from American Type Culture Collection (ATCC®). Another root canal strain from a pulpless tooth presenting primary infection was obtained from a patient at the authors' University Endodontic Clinic (CANAL 2). As a reference for comparison, *E. faecalis* isolated from urine, ATCC 29212 (URINE), was used. All strains were stored at −80 °C until use. For the CANAL 2 strain, the isolation protocol was performed as previously described [26,27]. To confirm their enterococcal identification, polymerase chain reaction (PCR) with 16s rRNA specific for this species was performed with the sequence of primers according to Sedgley *et al.* [28]: Forward: CCGAGTGCTTGCACTCAATTGG; Reverse: CTCTTATGCCATGCG GCATAAAC. The PCR reaction resulted in amplification of a fragment of 138 base pairs. Previously, the DNA was purified with QIAamp DNA Mini kit (Qiagen, Valencia, CA) according to the manufacturer's guidelines. Enterococci strains were cultured in brain-heart infusion (BHI) broth, stored at −80 °C.

For all assays, before MDMs were challenged with the different strains of *E. faecalis*, these strains were previously exposed to an alkaline stress protocol. Briefly, aliquots of each *E. faecalis* strain were taken from storage at −80 °C and incubated overnight at 37 °C in 4 mL of BHI broth. Subsequently, these bacterial strains were inoculated into test tubes containing alkaline-BHI broth, buffered with 5M NaOH at pH 9.5 (checked with a pH-meter). The strains were incubated at 37 °C for 4 hours; growth was assessed by spectrophotometry with the McFarland 0.5 standard, immediately before the assay (ALKALINE-STRESS group). As controls, bacteria inoculated in neutral BHI broth were used.

Isolation and culture of human *MDMs*

Peripheral blood monocytes were separated from heparinized whole blood (30 mL) by using Histopaque 1083 gradients (Sigma-Aldrich Brazil Ltda., São Paulo, Brazil) as previously described [29]. The cell suspension was adjusted to contain 1×10^6 monocytes/mL by using a hemocytometer and neutral red stain, and 1 mL per well was distributed in triplicate in a 24-well culture plate containing sterile spherical glass coverslips (13 mm in diameter). After 2 hours in 5% CO_2 at 37 °C, non-adherent cells were removed by aspiration. The monocytes were incubated in 1 mL of complete medium (RPMI 1640 + 10% heat-inactivated fetal-calf-serum +1% penicillin/streptomycin) for 7 days to obtain MDMs. Cellular viability was assessed in triplicate by trypan blue exclusion (>94%) in samples specifically designed for this purpose. About 100% of adhered cells on coverslips were characterized as MDMs by the detection of the surface molecule CD14. After trypsinization and incubation with phosphate-buffered saline (PBS) plus 2% of bovine serum albumin (BSA) for 30 minutes, the cells (1×10^6) were labeled with FITC-conjugated mouse anti-human CD14 antibodies (BD Bioscience, cat number 555397) at 4 °C for 1 hour. Subsequently, labeled-cells were fixed in 4% paraformaldehyde and visualized with a confocal scanning laser microscope (Leica TCS-SPE; Leica Microsystems GmbH, Mannheim, Germany).

Phagocytosis assay

The phagocytosis assay and analysis of internalized bacteria was performed as previously described with some modifications [30,31]. A fluorescent-quenching technique was also

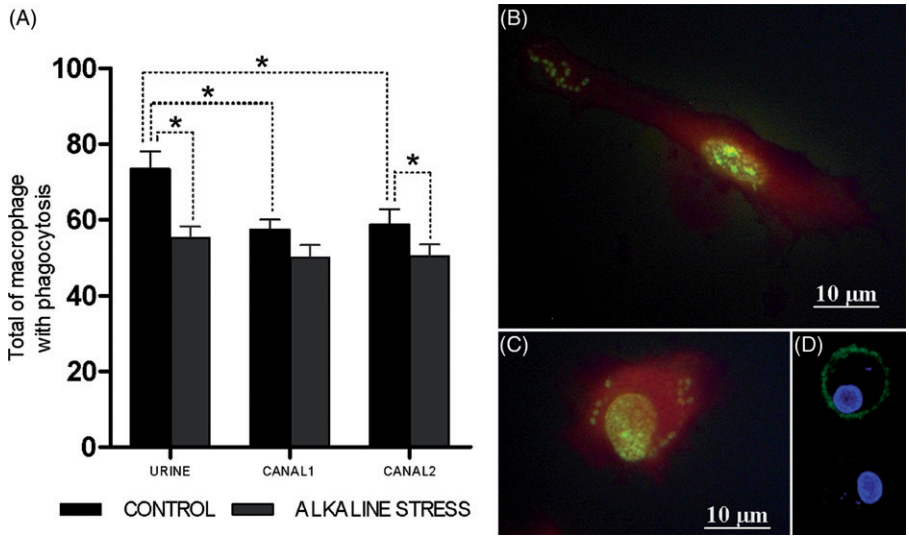

Figure 1. Number of MDMs with internalized bacteria after infection with different strains of E. faecalis, either stressed (ALKALINE STRESS) or not (CONTROL) with alkaline-BHI broth (A). *$p < .05$. E. faecalis internalized by MDMs after 30 minutes of infection (B and C). The stain of extracellular bacteria was quenched by crystal violet. MDMs labeled with DAPI (nucleus), expressing CD14-FITC (D). The results were obtained from the average of blood samples from 15 volunteers.

performed [32]. Briefly, the macrophage medium was washed away and fresh medium without antibiotics was added. The MDMs cultured in triplicate in 24-well plate were infected with *E. faecalis* (ALKALINE-STRESS or CONTROL) at a cell/bacteria ratio of 1:5, previously determined (MDMs viability ≥80%, after challenge). After 30 minutes, at 37 °C and in a 5% CO_2 atmosphere, the wells were washed three times with sterile PBS to remove the extracellular bacteria. After remaining at 37 °C for 30 minutes in a 5% CO_2 atmosphere, the wells were washed three times with sterile PBS to remove the extracellular bacteria. MDMs were fixed with 4% paraformaldehyde for 30 minutes and 0.05mg/mL acridine orange was used for 15 minutes to stain the bacteria and cells. Immediately, fluorescence of the remaining extracellular bacteria was quenched by crystal violet stain. The coverslips with adherent MDMs were carefully removed and placed on glass slides by using a mounting medium (VECTASHIELD®).

A qualitative analysis was performed by confocal laser-scanning microscopy (TCS model, SPE, Leica, Mannheim, Germany) to confirm that only internalized bacteria had been emitting fluorescence. The unidentified slides were randomly chosen for quantitative analysis by Axiostar HBO plus 50/AC Fluorescence Microscopy (Carl Zeiss, Göttingen, Germany). Phagocytosis by MDMs was quantified, considering the number of internalized bacteria per cell (<5 and ≥5), at 100× magnification in 20 randomly selected fields. In total, 15 independent experiments in triplicate were performed.

NO production

MDMs were infected as described above, and the supernatants recovered after 24 hours of bacterial stimulation (CONTROL and ALKALINE STRESS). NO production was assayed by Griess reaction to determine nitrite levels. Absorbance was measured in a spectrophotometer at 540 nm and the values expressed in micromolar (µM).

Statistical analysis

Data were analyzed by GraphPad Prism version 5.0 program. One-Way ANOVA and *t* student tests were applied.

Results

Phagocytosis of E. faecalis

Alkaline stress affected the phagocytosis of *E. faecalis* strains by MDMs after 30 minutes. A significant reduction was observed in the number of MDMs presenting internalized CANAL 2 or URINE strains, which were previously stressed, when compared with their matched CONTROLS ($p = 0.0014$) (Figure 1(A)). Phagocytosis differed for each *E. faecalis* strain. In the absence of alkaline stress (CONTROL), there were statistically lower numbers of MDMs with internalized bacteria in root-canal strain assays than in those of URINE strains ($p < 0.05$). These differences were not observed in the ALKALINE STRESS group assays (Figure 1(A)).

Green stained-bacteria were easily observed inside MDMs cytoplasm after fluorescent acridine orange staining (Figure 1(B,C)). Irrespective of the strain or the presence of alkaline stress, most MDMs showed more than five bacteria internalized per cell. MDMs showed marked expression of the surface molecule CD14 (Figure 1(D)).

NO production

After 24 hours, MDMs infected with root-canal strains showed diminished NO production compared with those cultured with URINE strains ($p < 0.0001$). Alkaline-stressed bacteria showed no change in NO production when compared with their corresponding CONTROL groups (Figure 2).

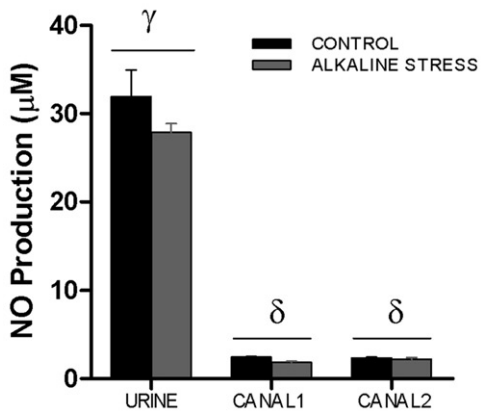

Figure 2. NO production by MDMs in response to *E. faecalis* infection for 24 hours. The three strains were either stressed (ALKALINE STRESS) or not (CONTROL) with alkaline-BHI broth. Nitrite accumulation was used as an indicator of NO production. Different symbols indicate $p < .05$.

Discussion

Several studies focusing on practical clinical applications in Endodontics have used the urine strain ATCC 29212 despite enterococcal cell pathogenicity differing among strains and being influenced by the microenvironment [9,10,12,33–35]. The presence of virulence factors is a strain-specific aspect and a clear difference has been observed relative to the type of virulence factors in strains of different origin, such as expression of enzymes, adherence and aggregation substances, Ace, Esp and gelatinase gelE [36–38]. Unrestricted use of enterococcus in endodontic studies may compromise the clinical application of their results since researchers have reported differences in virulence factors between bacteria from urine and root-canal, which are most likely due to their niche specialization and variable genetic traits [25,26]. Thus, it seems likely that different strains elicit equally different responses by immune cells recruited to the root canal to fight endodontic infections.

In fact, our in vitro results showed that different strains of *E. faecalis* (root-canal and urine), when maintained in normal pH (control), triggered different phagocytic profiles from macrophages, a useful model to study host-*E. faecalis* interactions [5]. After 30 minutes, about 75% of MDMs challenged with URINE enterococcus (ATCC 29212) presented internalized bacteria, while only 50 and 51% of MDMs from CANAL 1 and CANAL 2, respectively, had internalized root-canal enterococci.

According to these results of the phagocytosis assays, the lowest NO production was detected in supernatants from cultures of MDMs challenged with root-canal enterococci. These findings may be supported by the fact that the production of this gaseous molecule is inducible by several inflammatory stimuli such as cytokines and microbial products, and has a striking association with phagocytosis [21,39].

Taken together, the findings regarding the different *in vitro* macrophagic responses, both phagocytic and NO production, against strains of *E. faecalis* from different sources, strengthened the idea that researchers should be careful when using urine strains to study *E. faecalis* involvement in endodontic lesions, particularly those that propose therapeutic tools.

It is noteworthy that this divergence in macrophage functions associated with the origin of bacteria was not observed among *E. faecalis* strains that were previously stressed at alkaline pH, since all stressed strains, irrespective of their origin (root canal or urine), resulted in similar percentages of MDMs with internalized bacteria.

On the other hand, alkaline stress was determinant in influencing the phagocytic process by MDMs challenged with enterococcal bacteria originating from urine or root-canal from a pulpless tooth presenting primary infection, when compared with the phagocytosis of non-stressed bacteria. The percentage of MDMs presenting internalized enterococci (groups URINE and CANAL 2) was lower when these cells were challenged with bacteria previously incubated at pH 9.5, in comparison with matched-bacteria maintained in neutral pH. Possibly, this alkaline stress resulted in structural changes in the enterococci virulence factors, since researchers have demonstrated down or up-regulation of virulence genes in *E. faecalis* under alkaline stress conditions [40–42], as well as amplification of polypeptides [43] and inactivation of moieties present in virulence factors [11]. Alterations involving surface components of bacteria may impair the ability of macrophages to recognize and subsequently phagocytose them [44]. Cortes-Perez Dumoulin et al. (2015) revealed that overexpression of the protein ElrA (associated with the bacterial surface) of *E. faecalis* provides resistance to phagocytosis by macrophages in vitro.

In summary, our data indicated that the microenvironment may influence the interaction between some strains of enterococcal bacteria and macrophages, since the enterococci submitted to alkaline-stress appeared to have escaped from efficient phagocytosis by human macrophages, reflecting the capacity of *E. faecalis* to elude immune defenses. However, according to our findings, this impact caused by alkaline stress did not result in changes in NO production by human macrophages challenged in vitro with *E. faecalis*, irrespective of strain. Considering the importance of these two processes for bacterial clearance [45], the impaired phagocytosis of alkaline-stressed *E. faecalis* could contribute to their persistence in root canal systems that were previously treated with CH, irrespective of NO production, since large amounts of this reactive are generated within the phagosomes. Worth noting is that phagocytosis is a complex process whose success may involve other microbicidal molecules apart from NO. Thus, impaired phagocytosis, although accompanied by unchanged NO production, may be related to survival of the internalized microorganisms. In this sense, evidence have shown an association between the apically colonizing bacteria and the immune response present in the periapical granuloma, with macrophages being one of the main types of cells found in these lesions [46,47]. Moreover, macrophages challenged with root-canal strains did not produce amounts of NO as large as those generated in the presence of bacteria from urine. Possibly, NO poorly produced in response to root canal strains of *E. faecalis* could be crucial to the persistence of apical periodontitis.

The biological aspect surrounding host-stressed bacteria is of great interest in clinical practice in Endodontics, because

E. faecalis plays a pathogenic role in endodontic treatment failure. This bacterium has frequently been found in obturated root canals exhibiting signs of chronic apical lesions, by presenting the ability to tolerate harsh environmental conditions and to resist to intracanal medicaments such as CH that releases hydroxyl ions in an aqueous environment, thereby inducing an alkaline environment [2,10–12,48].

Baik et al. [11] observed that CH promoted damage to lipid moieties of *E. faecalis* virulence factors, decreasing TNF-α production by macrophages, and attenuating the host response. Therefore, it is possible that these structural alterations after alkaline stress occur in enterococci remnants when alkaline intracanal medications have failed to completely eliminate *E. faecalis* from the root canal, involving the impairment of the host immune response, as observed in the present study. We believe that this escape from immune defenses through impairment of the phagocytic process would contribute to enterococcal persistence in root canal system after endodontic treatment based on CH, along with other factors already known, such as the *E. faecalis* ability to invade dentinal tubules and remain viable within the tubule, and to compete with other bacterial cells [48].

Removal of bacterial biofilm from root canals is critical for the management of endodontic disease. Studies evaluating strains from root-canals and changes in the bacterial structures caused by alkaline stress (which prevent effective phagocytosis) would contribute to the design of treatments, including those focused on improving immune mechanisms.

Conclusion

The alkaline stress impairs the phagocytosis of *E. faecalis* by professional phagocytes but did not affect the NO production by these cells. Clinically, the impaired phagocytosis of *E. faecalis* could contribute to their persistence in root canal systems previously treated with calcium hydroxide. Moreover, root canal strains elicited different responses by immune cells when compared with strains from urine.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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