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Enterococcus faecalis and *Staphylococcus aureus* stimulate nitric oxide production in macrophages and fibroblasts *in vitro*

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Aim: Nitric oxide (NO) is an important mediator related to damage of the pulp tissue and at the same time to regenerative pulp processes. However, it is not clear how common endodontic microorganisms can regulate this mediator. This study aimed to investigate NO production by macrophages and fibroblasts against *Enterococcus faecalis*- and *Staphylococcus aureus*-antigens. **Methods:** RAW 264.7 macrophages and L929 fibroblast cell lines were stimulated with different heat-killed (HK) antigen concentrations (10^5-10^8 colony forming units - CFU) from *E. faecalis* and *S. aureus* with or without interferon-gamma (IFN-γ). Cell viability by MTT colorimetric assay and NO production from the culture supernatants were evaluated after 72 h. **Results:** Data here reported demonstrated that none of the antigen concentrations decreased cell viability in macrophages and fibroblasts. The presence of HK-*S. aureus* and HK-*E. faecalis* antigen- stimulated NO production with or without IFN-γ on RAW 264.7. The HK-*S. aureus* antigen stimulated NO production in L929 fibroblasts with or without IFN-γ, and the highest concentration of HK-*E. faecalis* with IFN-γ also stimulated NO production by these cells. **Conclusion:** The amount of NO produced by macrophages and fibroblasts may be involved in the concentration and type of prevalent endodontic microorganisms, generating new answers for the understanding of pulpal revascularization/regeneration processes.

Keywords: *Enterococcus faecalis*. *Staphylococcus aureus*. Fibroblasts. Macrophages. Nitric oxide.

Introduction

Regenerative endodontic therapies have become widespread, especially in immature teeth with open apex[1]. Difficulties during root canal instrumentation and disadvantages of the conventional apexification technique motivate pulp revascularization therapy. Briefly, this process consists of accessing the root canal system (RCS), irrigating it and subsequently using an intracanal medication aiming to remove the largest number of microorganisms in the RCS. This aseptic environment for blood clot formation is essential for the new tissue formation[1,2]. The success of regenerative therapies will depend on three important factors, namely the presence of stem cells, growth factors and scaffold[3].

Several mesenchymal stem cells, originating from the apical papilla (SCAP), dental pulp stem cells (DPSCs) or from exfoliated deciduous teeth (SHED) can contribute to pulp tissue regeneration[4,5]. However, the biomarkers needed for tissue reconstruction are still unknown. In addition, many clinical studies have reported the formation of fibroblast-rich scar tissue, without free nerve endings[6]. Until now, the role of other cells such as macrophages and fibroblasts in this process is unclear[7]. Moreover, the presence of some Gram-positive bacterial species such as *Enterococcus faecalis* has been found in revascularized tissues, triggering the production of mediators and pro-inflammatory cytokines, which may hamper tissue repair[8].

Among all the mediators produced by macrophages and fibroblasts, nitric oxide (NO) can act both in the elimination of invading agents and in the formation or destruction of tissues[9]. In this way, in a previous study, NO was upregulated in the presence of *E. faecalis in vitro*[10]. In relation to the formation of new tissues, low concentrations of NO may stimulate new vessel formation during pulpal regeneration / revascularization processes[11]. However, on the other hand, high concentrations can cause pulp tissue damage and particularly hinder new tissue formation[9]. This happens because NO may be associated with odontoblast differentiation and the production of enzymes and proteins related with bone and dentin formation, including alkaline phosphatase and calcitonin[12,13].

Therefore, considering the difficulty of promoting an aseptic environment, the purpose of this study was to evaluate *in vitro* cell viability and the production of NO in two cell lines. These cells were stimulated with different concentrations of heat-killed antigens from prevalent endodontic bacterial *E. faecalis* and *S. aureus*, mimicking the environment related to pulp revascularization.

Materials and methods

RAW 256.7 and L929 fibroblast cultures

RAW 264.7 osteoclast precursor monocyte cells (CR108, Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil) were cultured at $1x10^5$ cells per well in 96-well culture plates (TPP, USA). L929 fibroblasts (ATCC 929) were cultivated at $1x10^5$ per well in 96-well culture plates (TPP, USA). Both cells were cultured in Dulbecco modified Eagle medium (Gibco, USA) supplemented with 10 % fetal bovine serum (Gibco, USA), 1 %

penicillin / streptomycin (1000 U.mL^{-1}) (Invitrogen, Grand Island, NY), 1 % nonessential amino acids (Invitrogen), 1% L-glutamine and 0.1% gentamicin (Invitrogen)[14,15]. These cell cultures were stimulated *in vitro* with different concentrations of heat-killed antigens (HK) from *E. faecalis* (ATCC 19433) and *S. aureus* (ATCC 25923) with or without recombinant (r) IFN-γ (10 U per well, Peprotech, USA), mimetizing the endodontic environment in the necrosis of incomplete rhizogenesis processes. As a control, both cells were also stimulated with lipopolysaccharide (LPS) (3 µg.ml^{-1}, Sigma-Aldrich, USA)[16]. Cell viability assay and NO production were assessed after 72 h of incubation.

HK antigen preparations

Experimental groups determined for cytotoxicity and NO production analyses were stimulated with HK-*E. faecalis* and -*S. aureus*. Heat-killed antigens were prepared, as previously described[10]. Briefly, colonies were grown in Luria Bertani agar (LB; Kasvi, pH 7.3; USA) and subsequently resuspended in sterile phosphate-buffered saline solution, followed by their quantification by optical density. Then, they were heated at 121 °C, for 50 min[10]. Different concentrations of antigens from both microorganisms (10^5-10^8 colony-forming units/well) were tested. Death of microorganisms was confirmed by the absence of colonies, after 24 hours of incubation in Luria Bertani agar (LB; Kasvi, pH 7.3; USA). Optical microscopy images (inverted microscope Axiovert 40 CFL, USA; objective 20x) were obtained after 72h of incubation, from the experimental groups stimulated with rIFN-γ, LPS, and rIFN-γ, HK-*S. aureus* (10^6 CFUs) with or without rIFN-γ and HK-*E. faecalis* (10^6 CFUs) with or without rIFN-γ.

Viability assay

Cell viability assays were performed on both cells with antigen stimulus after a period of 72 h incubation at 37 °C, 5 % CO_2 and 95 % humidity, with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylte-trazolium (MTT) bromide (0.25 mg.mL^{-1}). The absorbance was measured at 595 nm in a microplate spectrophotometer (Bio-Tek, Winooski, VT). The results were compared to a positive control (unstimulated cells) and negative controls (cell culture in lysis buffer solution - 10 mmol.L^{-1} Tris, pH 7.4, 1 mmol.L^{-1} EDTA and 0.1% Triton X-100) and plotted as mean±standard error of absorbance[16].

Nitric oxide production

NO levels in culture supernatant from both stimulated cell lines were determined by Griess reaction. After 72h of incubation, the supernatant was mixed with an equal volume of Griess reagent (1 % sulfanilamide and 0.1 % naphtylethylene in 2.5 % ortho-phosphoric acid; Sigma-Aldrich, GB, Brazil). The absorbance was measured by a microplate spectrophotometer (Bio-Tek, Winooski, VT) at 490 nm. The nitrite concentration was determined according to a standard curve (0–200 mmol.L^{-1} sodium nitrite)[17].

Statistical Analysis

All experiments were carried out in technical and biological triplicates. Statistical analyses were performed by Kolmogorov-Smirnov test followed by one-way analysis of

variance (ANOVA) and Bonferroni *post hoc* by using GraphPad Prism 6 (GraphPad Software, San Diego, CA); $p<0.05$ was considered statistically significant.

Results

Cellular viability related to microbial antigen and controls

First, the cell viability of both chosen cell lines was evaluated, in the presence of HK-*E. faecalis* or HK-*S. aureus*. In this context, antigen concentration may be determinant for cell viability. Thus, RAW 264.7 stimulated with different concentrations of *S. aureus* did not diminish cell viability and the LPS-stimulated group induced cell proliferation (Fig. 1A). Otherwise, the addition of rIFN-γ and 10^8 CFU.mL^{-1} of *S. aureus* upregulated cell proliferation (Fig. 1C). Regarding *E. faecalis,* this antigen did not reduce cell viability and, at 10^8 CFU.mL^{-1}, it was also able to increase cell viability (Fig. 1B). Furthermore, the LPS-control group induced cell proliferation, while the presence of rIFN-γ did not represent an additional stimulus to alter cell viability (Fig. 1C and 1D). In order to relate our viability results with the morphological characteristics presented by these cells, the optical microscopy images (Fig. 1A-L) were observed, and these showed that after three days' incubation, the groups containing HK-*S. aureus* (Fig. 1I and 1J) and HK-*E. faecalis* (Fig. 1K and 1L) antigens with or without rIFN-γ significantly altered the cellular morphology of RAW 264.7, when compared to the control group (Fig. 1E).

Regarding L929 fibroblasts cell viability, none of the concentrations studied was cytotoxic. Therefore, neither L929 fibroblasts stimulated with different concentrations of *S. aureus*, nor LPS, diminished cell viability (Fig. 2A). Besides, the highest concentration of this HK upregulated cell proliferation (Fig. 2C). A similar relationship was observed when the rIFN-γ was added to HK-*S. aureus.* These stimuli did not reduce cell viability and, at 10^6 CFU.mL^{-1}, they also stimulated cell proliferation (Fig. 2C). HK-*E. faecalis* did not reduce cell viability, and at 10^6, 10^7 and 10^8 CFU.mL^{-1}, it again increased cell viability (Fig. 2B). The rIFN-γ stimulus was not able to alter cell viability in any of the tested groups (Fig. 2D). Concerning the morphological alterations by optical microscopy, the images demonstrated the structural differences under stress that both cells may present in the presence of both tested antigens. Thus, the optical microscopy images (Fig. 1A-L) showed that after three days' incubation, groups containing LPS (Fig. 1G), HK-*S. aureus* (Fig. 1I and 1J) and HK-*E. faecalis* (Fig. 1K and 1L) antigens with or without rIFN-γ significantly altered cellular morphology of L929, when compared to the control group (Fig. 1E).

NO production related to microbial antigen and controls

The evaluation of NO production was performed after 72h in both cells studied. Assembling a system involving both the different antigens and the presence of the rIFN-γ recombinant, it was possible to mimic an *in vitro* infection. In this way, in RAW 264.7 cultures, the presence of LPS and different concentrations of HK-*S. aureus* was able to upregulate the production of sodium nitrite, after 72 hours of incubation, except for the group containing 10^8 CFU.mL^{-1} (Fig. 3A). Therefore, the presence of IFN-γ increased nitrite production by these cells in different concentra-

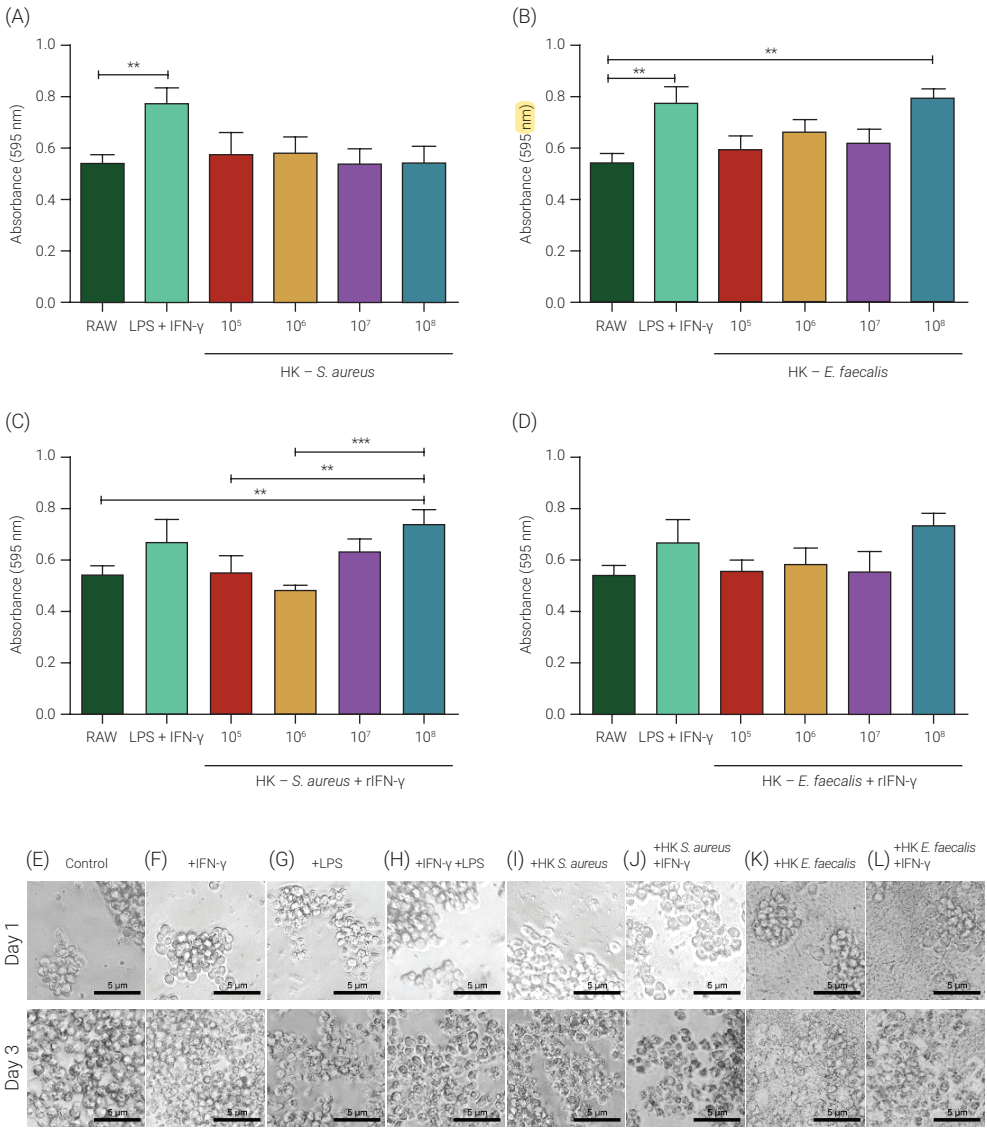

Figure 1. RAW 264.7 cell viability. Graphs represent cell cultures with different concentrations (10^5-10^8 CFU.mL^{-1}) of HK-*S. aureus* antigen (A) with rIFN-γ (C) or HK-*E. faecalis* antigen (B) with rIFN-γ (D), after 72h of cell incubation. Bars represent mean and standard error of cellular absorbance (595 nm) carried out in technical and biological triplicates. Statistical differences by one-way ANOVA test and Bonferroni *post hoc* were represented by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p< 0.001$). Optical microscopy (20x) shows the initial (day 1) and final (day 3) cell morphology aspects (E-K) of RAW 264.7 stimulated with rIFN-γ (F), LPS (G), LPS and rIFN-γ (H), HK-*S. aureus* 10^6 CFUs without (I) or with rIFN-γ (J), HK-*E. faecalis* 10^6 CFUs without (K) or with rIFN-γ (L) compared to the cell control group (E).

tions of *S. aureus* or LPS, except for the group stimulated by 10^8 CFU.mL^{-1} (Fig. 3C). Only the 10^8 CFU.mL^{-1} of *E. faecalis* stimulus was able to induce the production of sodium nitrite (Fig. 3B). The addition of rIFN-γ led to an increase in sodium nitrite production, except for the group containing 10^8 CFU.mL^{-1} of *E. faecalis* (Fig. 3D). Regarding the L929 cultures, these cells may represent the most abundant cells on the pulp tissue: fibroblasts. Then, the presence of LPS or different HK-*S. aureus*

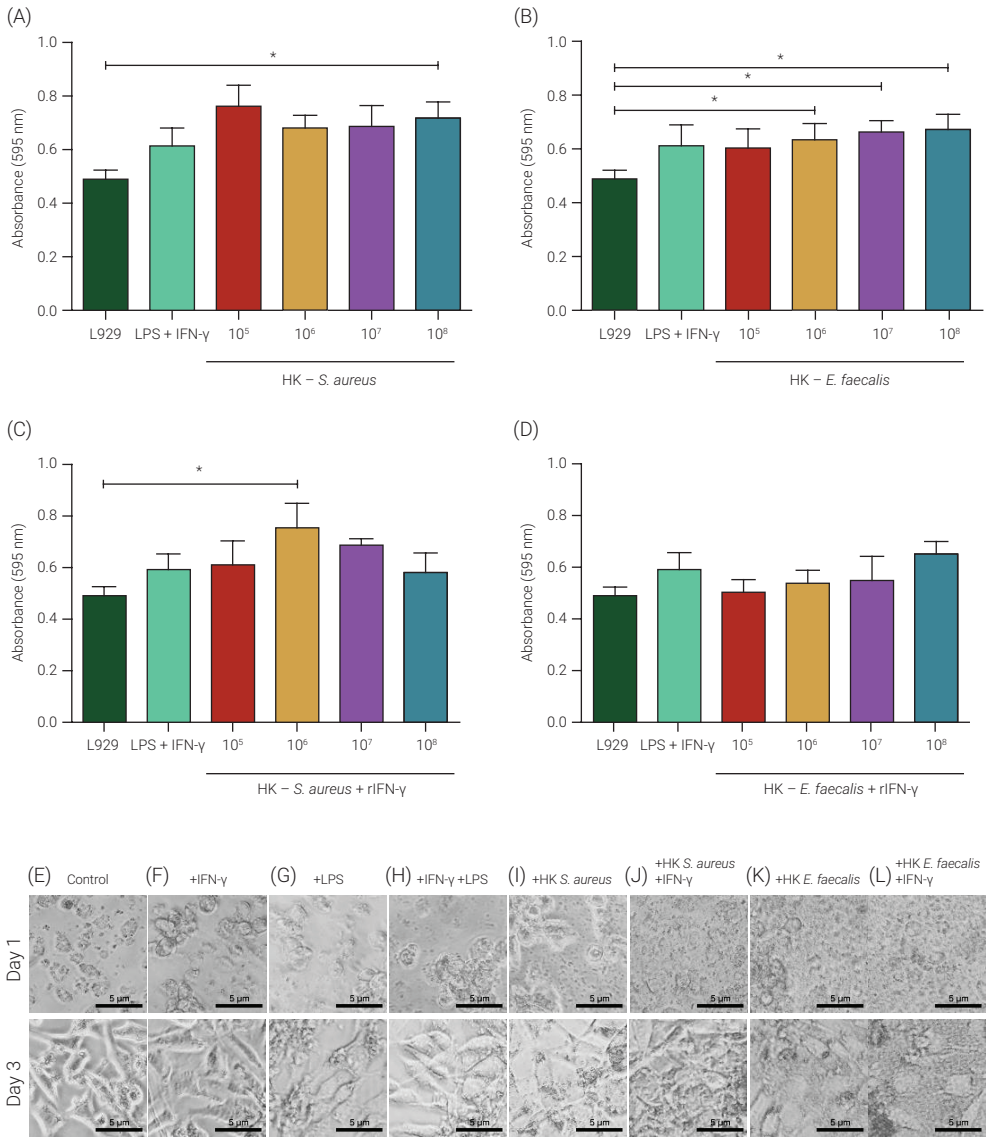

Figure 2. L929 cell viability. Graphs represent cell cultures with different concentrations (10^5-10^8 CFU. mL^{-1}) of HK-*S. aureus* antigen (A) with rIFN-γ (C) or HK-*E. faecalis* antigen (B) with rIFN-γ (D), after 72h of cell incubation. Bars represent mean and standard error of cellular absorbance (595 nm) carried out in technical and biological triplicates. Statistical differences by one-way ANOVA test and Bonferroni *post hoc* were represented by * ($p < 0.05$). Optical microscopy (20x) shows the initial (day 1) and final (day 3) cell morphology aspects (E-K) of L929 stimulated with rIFN-γ (F), LPS (G), LPS and rIFN-γ (H), HK-*S. aureus* 10^6 CFUs without (I) or with rIFN-γ (J), HK-*E. faecalis* 10^6 CFUs without (K) or with rIFN-γ (L) compared to the cell control group (E).

concentrations were capable of stimulating the production of sodium nitrite, with or without rIFN-γ (Fig. 4A and 4C). Only the highest concentration of HK-*E. faecalis* was able to stimulate the production of sodium nitrite without rIFN-γ (Fig. 4B). Nevertheless, when the rIFN-γ was added to all groups, sodium nitrite production was upregulated (Fig. 4D).

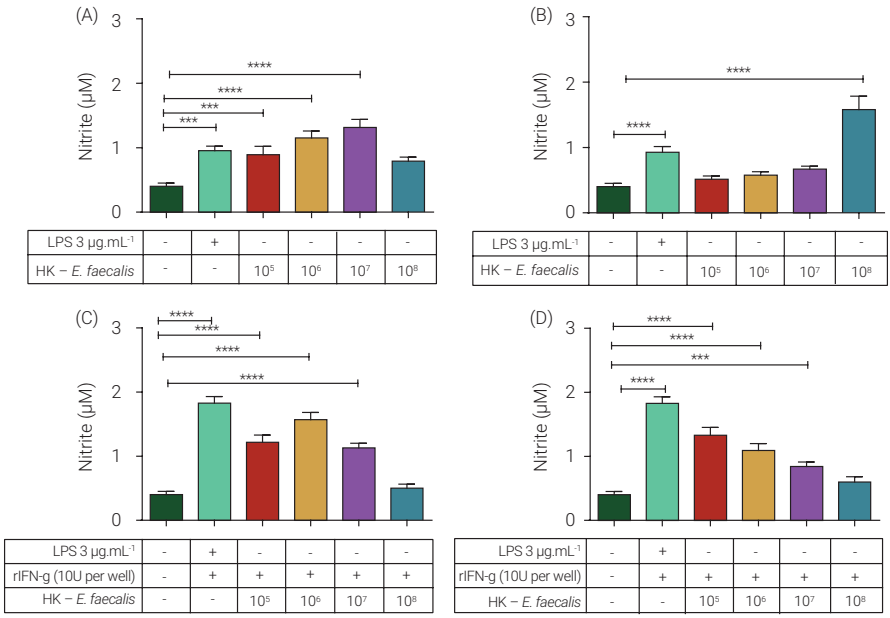

Figure 3. NO production by RAW 264.7 cells. Graphs represent values of sodium nitrite with different concentrations (10^5-10^8 CFU.mL^{-1}) of HK-*S. aureus* antigen (A) with rIFN-γ (C) or HK-*E. faecalis* antigen (B) with rIFN-γ (D), after 72h of cell incubation. Bars represent mean and standard error of sodium nitrite production in µM carried out in technical and biologic triplicates. Statistical differences by one-way ANOVA test and Bonferroni *post hoc* were represented by *** ($p < 0.001$) and **** ($p < 0.0001$).

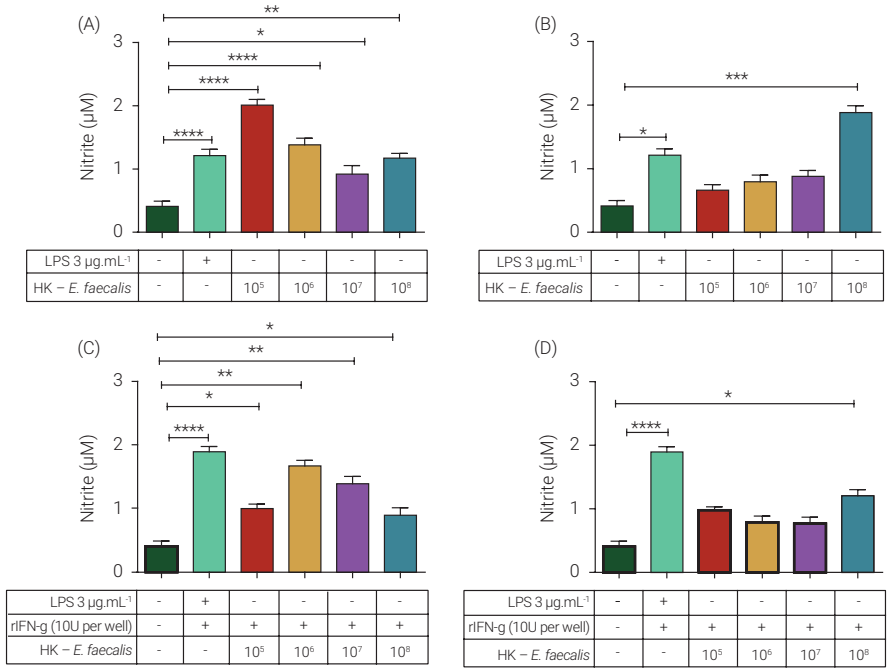

Figure 4. NO production by L929 fibroblasts. Graphs represent values of sodium nitrite with different concentrations (10^5-10^8 CFU.mL^{-1}) of HK-*S. aureus* antigen (A) with rIFN-γ (C) or HK-*E. faecalis* antigen (B) with rIFN-γ (D), after 72h of cell incubation. Bars represent mean and standard error of sodium nitrite production in µM carried out in technical and biologic triplicates. Statistical differences by one-way ANOVA test and Bonferroni *post hoc* ($p<0.05$) were represented by * ($p < 0.05$), ** ($p < 0.01$) *** ($p< 0.001$) and **** ($p < 0.0001$).

Discussion

Regenerative therapies may contribute to endodontic treatment in immature teeth with open-apex[3,18]. However, both the absence of microorganisms and the presence of mediators and growth factors are essential for the construction of new pulp tissue[18]. Thus, the polymicrobial infected root canal system is composed of Gram-positive and Gram-negative bacteria. Among the microorganisms, *E. faecalis* is prevalent in infected immature permanent teeth[19]. Moreover, this microorganism is associated with different forms of periradicular disease, including primary endodontic infections as well as persistent periapical lesions[20]. In the category of primary endodontic infections, *E. faecalis* is present in 40% of them[20]. *S. aureus* might be another bacterium found in pulpitis and may have quorum-sensing as its main mechanism of virulence[21]. This factor contributes to the control of the pathogenesis of this microorganism, which is involved with the density that occurs through cellular communications[22].

The presence of microorganisms may inhibit the development of new tissue, modifying the normal function of these cells[23]. In an *in vitro* study, LPS from *Pseudomonas aeruginosa* did not reduce cell viability, but reduced the ability of periodontal ligament stem cells to differentiate into osteoblasts; in addition, it upregulated the production of proinflammatory cytokines such as IL-1β, IL-6, and IL-8 by these cells[23]. In the same way, the activation of immune system cells, metalloproteinase, reactive oxygen species (ROS) and bacterial endotoxins, for instance LPS and lipoteichoic acid (LTA), may compromise the development of loose connective tissue[24]. It has been described that the presence of *S. aureus* antigens downregulated the bone marrow stem cell and human fibroblast adhesion factors, by blocking TLR2[25]. In addition, LTA from *S. aureus* walls was related with the production of NO in RAW 264.7 macrophages, via TLR2[26].

The antigen-fighting process of resistant microorganisms is mostly associated with the first line of immune response, represented by cytokines and lysosomal enzyme-macrophage producers, related to tissue destruction[27]. However, fibroblasts are the main cells present in connective tissue, deploying a structural and repair role, including the release of tissue repair mediators[28]. Thus, for this study, RAW 264.7 macrophages and L929 fibroblasts were chosen. In this context, IFN-γ may be responsible for upregulating the class I and II major histocompatibility complexes and activating reduced nicotinamide adenine dinucleotide phosphate–dependent phagocyte oxidase and NO production in macrophages, besides exacerbating the response to the production of NO in fibroblasts[29,30].

In this study, the *S. aureus* and *E. faecalis* stimuli in RAW 264.7 macrophages were not able to decrease cell viability, even at higher tested concentrations. However, the HK antigens altered cell morphology at all tested concentrations. These results were also observed in a previous study, in which RAW 264.7 cells remained viable even at higher *S. aureus* antigen concentrations, after 48 h of incubation[31].

The presence of different concentrations of *S. aureus* and *E. faecalis* antigens also did not diminish the L929 fibroblast viability. This is the first study, according to our knowledge, that has evaluated the effects of Gram-positive bacteria on the L929 cell line.

The presence of heat-killed *Porphyromonas gingivalis* in fibroblasts from periodontal ligament was not cytotoxic, after 48 h of incubation, even at the highest cells: bacteria proportion (1:100)[32].

The main events reported with the presence of antigens in the root canal systems are related to changes in the pattern of response and production of mediators by these cells[10]. Among these mediators, NO is a gaseous free radical produced by NO-synthase, by converting L-arginine to L-citrulline[33]. The action of inducible nitric oxide (NO_2) on pulp tissue can contribute to the destruction of microorganisms, but at the same time, high concentrations (500 μM) were able to cause apoptosis of macrophages and osteoblasts in an *in vitro* periapical lesion model[34]. The beneficial or malignant action of NO may be related to the levels of NO produced. Low concentrations of NO in pulp space can contribute to tissue formation and regeneration processes, since the formation of new vessels may be essential for the construction of new tissue[11]. And because it is lipophilic, NO can easily be permeable to biological membranes, causing vasodilation[35]. In addition, NO (100 μM) may upregulate the vascular endothelial growth factor (VEGF), which is essential for angiogenesis[36]. As the pulp tissue have a higher concentration of blood vessels, the synthesis of this free radical becomes essential in the support and establishment of their physiology[11]. An *in vitro* study demonstrated an increase in NO synthase expression in pulp cells derived from immature permanent teeth when compared to third molar pulp cells[37].

This study showed that both stimuli (*S. aureus* and *E. faecalis*) were able to induce the production of NO in RAW macrophage 264.7, based on a standard curve of sodium nitrite. NO production in macrophages in the presence of *S. aureus* stimuli seems to be dose dependent. However, at the highest concentration the abundance of this specific mediator was not improved. In this context, macrophages are the first defense line and, when in contact with antigens, are specialized in producing inducible NO[38]. Macrophage polarization (M1) may perpetuate an inflammatory response, whereas a macrophage response (M2) may contribute to the formation of new tissues. An *in vitro* study associated the autocrine action of NO on LPS-stimulated macrophages with the polarization in profile M1[39].

Virulence factors such as LTA from Gram-positive bacteria, peptide glycol and adhesion factors are generally associated with the induction of NO synthase in macrophages[32,40]. Here, the higher antigen concentrations, in the presence of IFN-γ, did not stimulate NO production in RAW 264.7 cells. In situations of high antigen concentrations, the immune cells can lose their response pattern and may act unresponsively due to the mechanisms of immune regulation mediated by regulatory T lymphocytes[39,41].

The presence of *S. aureus* antigens stimulated NO production at all tested concentrations, with or without IFN-γ in L929 fibroblasts. In the presence of *E. faecalis*, the highest concentration of antigens was significantly important in inducing NO production. Fibroblasts are known to have an important role in tissue repair; however, they also respond to the antigen by producing IL-6, MCS-F, TGF-β, and NO[42,43]. Until then, the classical stimuli studied for the evaluation of NO production in L929 are IFN-γ, LPS or both[30]. In this way, NO production in human pulp fibroblasts in response to heat-killed

antigens from *E. faecalis* may increase alkaline phosphatase production in fibroblasts and, consequently, pulp calcification[44]. In addition, the production of NO and other proinflammatory cytokines in fibroblasts may favor the expression of OPG in these cells and consequently the formation of calcified pulp tissue[45]. Besides, fibroblasts may be susceptible to NO. An *in vitro* study demonstrated that 3 mmol.L^{-1} of NO were responsible for the apoptosis of gingival fibroblasts. This action was associated with the c-Jun N-terminal kinase signaling pathway[46].

In conclusion, NO production by RAW 264.7 monocytes and L929 fibroblasts against the pathogens presented in this study may contribute to the understanding of how microorganisms prevalent in the root canal system lead to a pro-inflammatory response, increasing NO. This is an initial study and in view of the real role of this mediator, new studies with human cells must be carried out to establish its action both in the elimination of microorganisms and in the formation of new tissues during pulp revascularization/regeneration processes.

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