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ORIGINAL ARTICLE

In vitro inflammatory modulation of bioceramic endodontic sealer in macrophages stimulated by bacterial lipopolysaccharide

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Abstract

Aim: To evaluate the effects of AH Plus (Dentsply), Sealer 26 (Dentsply), and Sealer Plus BC (Produtos Médicos e Odontológicos) on cytotoxicity and inflammation in macrophage cultures exposed to bacterial lipopolysaccharide (LPS).

Methodology: After initial setting, the sealers were conditioned with serum-free culture medium for 24 h (1 ml/cm^2). Macrophages from the RAW 264.7 strain were exposed to sealer extracts in a 1:16 ratio in a culture medium with or without LPS. Cell morphology, viability, mitochondrial activity, oxidative stress and gene expression of interleukin 1β (IL-1β) and tumour necrosis factor-alpha (TNF-α) were evaluated. Data on mitochondrial activity, oxidative stress and TNF-α were analysed using a two-way analysis of variance (ANOVA) test, followed by the Student–Newman–Keuls post-test. IL-1β data were analysed using one-way ANOVA, followed by SNK, and the t-test was used for intragroup comparison. The significance level was set at 5%.

Results: In the absence of LPS, only AH Plus and Sealer 26 showed a reduction in cell density, while in the presence of LPS, Sealer 26 had the lowest density compared to the other groups. In terms of mitochondrial activity, at 24 and 48 h, Sealer Plus BC had significantly higher mean values than Sealer 26 and AH Plus ($p < .05$). Sealer 26 exhibited the lowest levels of oxidative stress and IL-1β and TNF-α expression, regardless of the presence of LPS ($p < .05$).

Conclusions: Although all sealers interfere with the response of macrophages to LPS, contact with epoxy resin-based sealers can impair cell activity *in vitro*, while bioceramic sealer seems to favour the inflammatory functions of these cells.

KEYWORDS

biomaterials, cytotoxicity, inflammation, lipopolysaccharides, macrophages

INTRODUCTION

Periapical pathologies are diseases with worldwide prevalence (Tibúrcio-Machado et al., 2021). Periapical pathologies are variations of tissue responses, which can be acute or chronic, and are directly related to the intensity and duration of the noxious stimuli toward the tissues involved, as well as to the host immune response (Machado et al., 2020). Macrophages account for 6% of the cells involved in the immune response, and their transmigration and multiplication at the inflammation site depend on chemotactic factors represented by the soluble components of microorganisms and factors derived from injured leukocytes (Cotti et al., 2021). They are responsible for the production of prostaglandins and proinflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), interleukin-1 (IL-1, IL-α, IL-1β and IL-1Ra), interleukin-6 (IL6), interleukin-12 (IL-12), interleukin-18 (IL-18), interleukin-23 (IL-23) and interleukin-27 (IL-27) (Braz-Silva et al., 2019). Prostaglandins cause vasodilation, while proinflammatory cytokines recruit polymorphonuclear cells (PMNs) to the injured tissues and cause systemic reactions (Cotti et al., 2021).

This chain of biochemical events is often triggered in response to the presence of microbial metabolic products or even components of the bacterial cell wall, such as lipopolysaccharides (LPS) (Hessle et al., 2005; Paula-Silva et al., 2020). Gram-negative bacteria, the most prevalent micro-organisms in primary endodontic infections, can release LPS from their cell walls during multiplication or after the death of micro-organisms (Machado et al., 2020). LPS binds to monocyte cell membrane receptors and induces the production of proinflammatory cytokines, such as TNF-α, IL-1, IL-5 and IL-8, which act as potent stimulators of bone resorption and prostaglandin release, which also influence osteoclast activation (Hessle et al., 2005; Machado et al., 2020; Paula-Silva et al., 2020).

Therefore, when a periradicular lesion is a consequence of a root canal infection, therapeutic measures are necessary to eliminate the source of infection and eliminate or neutralize LPS, ensuring that the periapical tissues can conduct the repair process (Machado et al., 2020).

Sealers containing calcium hydroxide, such as Sealer 26 (Dentsply-Sirona), have been shown to increase the local pH, causing superficial necrosis in the tissues involved. After the inflammation has resolved, dental pulp stem cells migrate, followed by the formation of osteoid matrix and deposition of dentine-like tissue. Ca^{2+} released by these sealers participates in the formation of calcium carbonate, causing cellular events that stimulate the expression of genes related to odontoblast differentiation and biomineralization (Cintra et al., 2013). Furthermore, Ca^{2+} neutralizes the LPS present in root canals by breaking the ester-type bonds of the union of fatty acids and hydroxyl (Bedran et al., 2020). AH Plus (Dentsply-Sirona) is an epoxy resin-based sealer that has significant evidence supporting its use for root canal filling. However, its cytotoxicity is related to formaldehyde release and amine degradation because of its long setting time (until it achieves complete polymerization) (Teixeira et al., 2017). This inhibits the initial healing capacity of the tissues in contact with this material.

In the last decade, bioceramic sealers have shown promise as root canal fillers (Candeiro et al., 2016; López-García et al., 2020). In a recent study conducted with EndoSequence BC sealer (Brasseler) and MTA Fillapex (Angelus) sealers in the presence of LPS in osteoblast cultures, a reduction in the levels of inflammatory mediators induced by LPS was observed, as well as an increase in osteogenic potential (Lee et al., 2019). Sealer Plus BC (MK Life Medical and Dental Products) is a bioceramic sealer manufactured in Brazil, which has been evaluated for its physicochemical properties (Mendes et al., 2018; Torres et al., 2020) and cytotoxicity (Benetti et al., 2019; Silva et al., 2020).

However, considering the possible impact of bacterial LPS on periapical pathology, there is no data on the immunomodulatory effects of endodontic sealers on macrophages exposed to LPS, which could help to understand the formation and persistence of periapical lesions after endodontic treatment. Therefore, this study aimed to evaluate the effects of sealers with different compositions and physicochemical properties on morphology, viability, oxidative stress and expression of proinflammatory cytokines in macrophages exposed to LPS. The null hypothesis was that the different compositions of the sealers would not interfere with the proposed parameters and consequently would not play a significant role in the inflammatory response.

MATERIALS AND METHODS

The manuscript of this laboratory study has been written in accordance with the Preferred Reporting Items for Laboratory Studies in Endodontology (PRILE) 2021 guidelines (Figure 1).

Sample size calculation revealed that for the analysis of mitochondrial activity and oxidative stress, five samples per group had 0.95 test power, considering a 5% type α error and 0.89 effect size. For gene expression analysis, three samples per group had 0.81 test power, considering a 5% type α error and 1.3 effect size.

Cell cultures

Mouse cells of the macrophage lineage RAW 264.7, American Type Culture Collection, were cultured in 75-cm^3 bottles (Corning) with 10 ml of DMEM

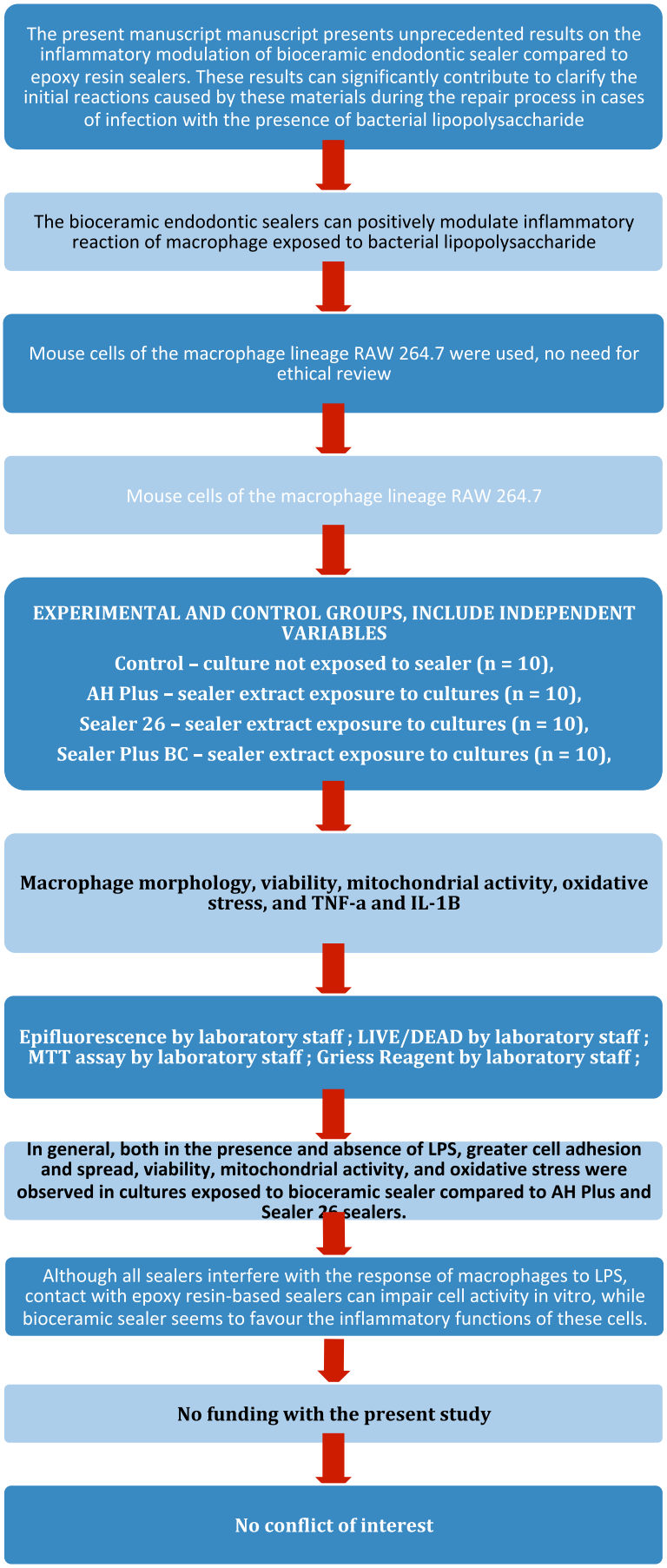


FIGURE 1 Flowchart according to preferred reporting items for laboratory studies in endodontology (PRILE) 2021 guidelines.

culture medium (Invitrogen), 10% foetal bovine serum (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% atmospheric air, and the medium was changed every 2 days until subconfluent. Cells were scraped from the substrate using a cell scraper, aspirated, plated in 96-well plates at a density of 1×104 cells/cm^2, allowed to adhere for 24 h and exposed to sealer extracts in the presence or absence of LPS (Sigma–Aldrich Chemicals) at 1 μg/ml for periods of up to 3 days. For morphological analysis, cells were plated on Thermanox coverslips (Nunc Inc.) and grown in 24-well plates.

Sealer extracts and exposure to cultures

The following endodontic sealers were used: AH Plus (Dentsply, Konstanz, BW, and DE), Sealer 26 (Dentsply) and Sealer Plus BC (Medical and Dental Products). The sealers were handled according to the manufacturers' instructions in a laminar flow cabinet under sterile conditions. A sterile insulin syringe was used to insert 0.2 cm^3 of each sealer and fill the bottom of the wells of 96-well plates (adapted from Pedano et al., 2018) and, after initial setting, the sealers were conditioned for 24 h with 1.9 ml of serum-free culture medium (1 ml/cm^2). For the exposure of cell cultures, macrophages of the RAW 264.7 strain were exposed to sealer extracts with a 1:16 ratio in a culture medium containing or not containing LPS. The sealer compositions are listed in Table 1. Cultures not exposed to sealers were used as controls.

Cell morphology

After 24 h, cells were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate-buffered solution (PB, pH 7.2) for 10 min at 28°C, washed in PB and permeabilized with Triton X-100 at 0.5% in CP for 10 min at room temperature (RT). Cells were then incubated with Alexa Fluor 488-conjugated phalloidin (green fluorescence) (1:200, Molecular Probes; Invitrogen) for actin cytoskeleton labelling for 60 min. Cultures were washed with deionized water, and cell nuclei were stained with 300 nM 4′,6-diamidino-2-phenylindole dihydrochloride (Molecular Probes) for 5 min. Thermanox coverslips were mounted with a mounting medium (AntifadeProlong, Molecular Probes), and cells were examined under epifluorescence using an AxioImager M2 Zeiss light microscope (Carl Zeiss Inc.) equipped with an AxioCam MRM digital camera (Carl Zeiss Inc.).

Cell viability

After 24 h, cell viability was qualitatively evaluated using the LIVE/DEAD™ Viability/Cytotoxicity Kit (viability/cytotoxicity for mammalian cells, Molecular Probes), following the manufacturer's instructions. Viable cells were stained with green fluorescence (488 nm) due to the reaction of calcein ($C_{30}H_{26}N_2O_{13}$) with intracellular esterase, and dead cells were stained with red fluorescence (561 nm) due to the binding of ethidium homodimer-1 (EthD-1) to nucleic acids. Images were obtained using an inverted trilocular microscope with direct fluorescence (Led B-g-u-uvTrilocular; Digilab) attached to a Nikon digital camera.

Mitochondrial activity

At 24 and 48 h, mitochondrial activity was evaluated using the colourimetric mean transit time (MTT) assay {[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]} (Sigma–Aldrich Chemicals). Aliquots of MTT (5 mg/ml in phosphate-buffered saline) were prepared briefly, followed by incubation of the primary cultures with this 10% solution in culture medium for 4 h at 37°C in a humidified

TABLE 1 Composition of the endodontic sealers

Sealer	Composition	Batch
AH Plus	Paste A: Bisphenol-a epoxy resin; bisphenol-f epoxy resin; calcium tungstate; zirconium oxide; silica and iron oxide. Paste B: adamantized amine; N,N′-dibenzyl-5-oxanonane-diamine-1,9; TCD -diamine; calcium tungstate; zirconium oxide; silica and silicone oil.	L:350598 K
Sealer 26	Powder: Bismuth trioxide; calcium hydroxide; urotropine and titanium dioxide. Resin: epoxy.	L:338639 J
Sealer PlusBC	Paste: Zirconium oxide, tricalcium silicate, dicalcium silicate, calcium hydroxide, propylene glycol.	L:WR770100

Source: Leaflet provided by the respective manufacturers.

atmosphere containing 5% CO_2 and 95% atmospheric air. After this period, acidic isopropanol solution (isopropanol and HCl) was added to each well under moderate agitation for 5 min to completely solubilize the formed precipitate. Aliquots (150 µl) were collected from the wells and transferred to a 96-well plate for colourimetric measurements using a spectrophotometer (570 nm, Epoch 2; BioTek Instruments Inc.).

Oxidative stress

After 24 h, oxidative stress was evaluated by measuring the production of nitric oxide (NO), using the method described by Griess (1879). For this assay, the cells were exposed to sealer extracts diluted in serum-free foetal bovine culture medium. After the exposure period, 100 µl of the cell culture medium from each group was transferred to a new 96-well plate, mixed with an equal volume of Griess reagent (Sigma–Aldrich Chemicals) and incubated for 10 min at RT. After this period, the concentration of NO was determined by colorimetric measurement using a spectrophotometer (530 nm; Epoch 2, BioTek Instruments Inc.), with reference to a standard curve of NO (500–0.5 µg/ml $NaNO_2$ in culture medium). Wells containing culture medium with sealer extracts were used as control.

Gene expression

After 3 days, the culture medium was removed from the wells, and Trizol LS reagent (Invitrogen) was added at RT for 5 min under agitation by pipetting. Total RNA extraction was performed using the SV Total RNA Isolation System kit (Promega), according to the manufacturer's specifications. Total RNA was then quantified, and cDNA strands were prepared from 1 µg of the total RNA. This procedure was performed in a MasterCycler Gradient thermocycler (Eppendorf) by reaction with the reverse transcriptase enzyme using the GoScript Reverse Transcriptase kit (Promega), following the manufacturer's instructions. For real-time PCR, the GoTaq® qPCR Master Mix reagent (Promega) and StepOnePlus device (Thermo Fisher Scientific) were used. The reactions were performed in triplicate with a final volume of 10 µl containing 12.5 ng of cDNA. The amplification reactions consisted of 2 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. The results were analysed based on the Ct value (cell threshold or threshold cycle), which allows for quantitative analysis of the expression of the factors evaluated. The expression of TNF-α and IL-1β was also evaluated. The expression of the constitutive gene β-actin (ACTB) was evaluated as an endogenous control. Primer sequences used in this study are listed in Table 2.

Analysis of the results

The quantitative data obtained were subjected to the Shapiro–Wilk test for adherence to the normal curve and Levene's test for homogeneity of variance. When necessary, data transformation was performed using a logarithmic reduction and the normality rechecked by Shapiro–Wilk test. The MTT, oxidative stress and TNF-α expression data were analysed using a two-way analysis of variance (ANOVA) test, followed by the Student–Newman–Keuls (SNK) post-test. SNK post-test was used to compare groups under the same conditions of LPS exposure. To compare intragroup LPS exposure on IL-1β data, a *t*-test was performed. The significance level was set at 5%. Statistical analysis was performed using the SigmaStat 3.5 software (Systat Software). The statistical analyses were performed blinded to the materials tested. The images obtained for cell morphology and viability were analysed qualitatively.

RESULTS

Means and standard deviation values of mitochondrial activity, oxidative stress and gene expression are provided in Tables S1–S3.

Cell morphology

After 24 h, it was observed that in the presence and absence of LPS, exposure to AH Plus sealer extract promoted a reduction in cell density and less spreading compared to the control. The group exposed to Sealer 26 also presented a significant reduction in cell volume and the presence of pyknotic nuclei. Higher cell density was observed in the control and Sealer Plus BC groups, both in the presence and absence of LPS (Figure 2).

Cell viability

From the images obtained by epifluorescence of cultures not exposed to LPS, a predominance of viable cells was observed in the control and all sealers, with a reduction in density in the groups exposed to AH Plus and Sealer 26 (Figure 3). In cultures exposed to LPS, there was a predominance of viable cells with small visible portions of dead

cells and a slight reduction in density in the group exposed to Sealer 26. In the comparison between groups, there was a higher cell density in the Sealer Plus BC cultures, with a slight decrease in the Sealer 26 group (Figure 3).

Mitochondrial activity

ANOVA showed a statistically significant difference in mitochondrial activity after 24 h between the sealers and in the presence of LPS ($p < .001$). No interaction was observed between these factors ($p = .945$).

TABLE 2 Sequence of primers used for real-time PCR reactions

IL-1β	Foward	GCT ACC TGT GTC TTT CCC GT
IL-1β	Reverse	CAT CTC GGA GCC TGT AGT GC
TNF-α	Foward	AGG CCT TGT GTT GTG TTT CCA
TNF-α	Reverse	ATG GGG GAC AGC TTC CTT
ACTB	Foward	CTC TGG CTC CTA GCA CCA TGA AGA
ACTB	Reverse	GTA AAA CGC AGC TCA GTA ACA GTC CG

Abbreviations: ACTB, β-actin; IL-1β, interleukin 1β; TNF-α, tumour necrosis factor-alpha.

After 24 h of exposure, in the comparisons between the groups, similarities were observed between the means of AH Plus and Sealer 26 ($p > .05$) and between the control and Sealer Plus BC ($p > .05$), both in the presence and absence of LPS. Regardless of LPS exposure, the mitochondrial activity of AH Plus and Sealer 26 was significantly lower than that of the control and Sealer Plus BC ($p < .001$). A statistically significant difference was observed in the presence or absence of LPS in all groups ($p < .001$), with higher mean values observed in the presence of endotoxins (Figure 4).

ANOVA showed a statistically significant difference in mitochondrial activity after 48 h in sealers ($p < .001$) and in the presence of LPS ($p < .001$). An interaction between these factors was observed, that is, the effects of sealers on this parameter varied in the presence of LPS ($p < .001$).

After 48 h, the mean values of mitochondrial activity for AH Plus and Sealer 26 were similar, both in the presence ($p = .871$) and absence of LPS ($p = .128$), and lower than those of control and Sealer Plus BC ($p < .001$) under the same conditions of exposure to endotoxins. In addition, regardless of LPS, the control group showed higher mean values than the Sealer Plus BC group ($p < .001$).

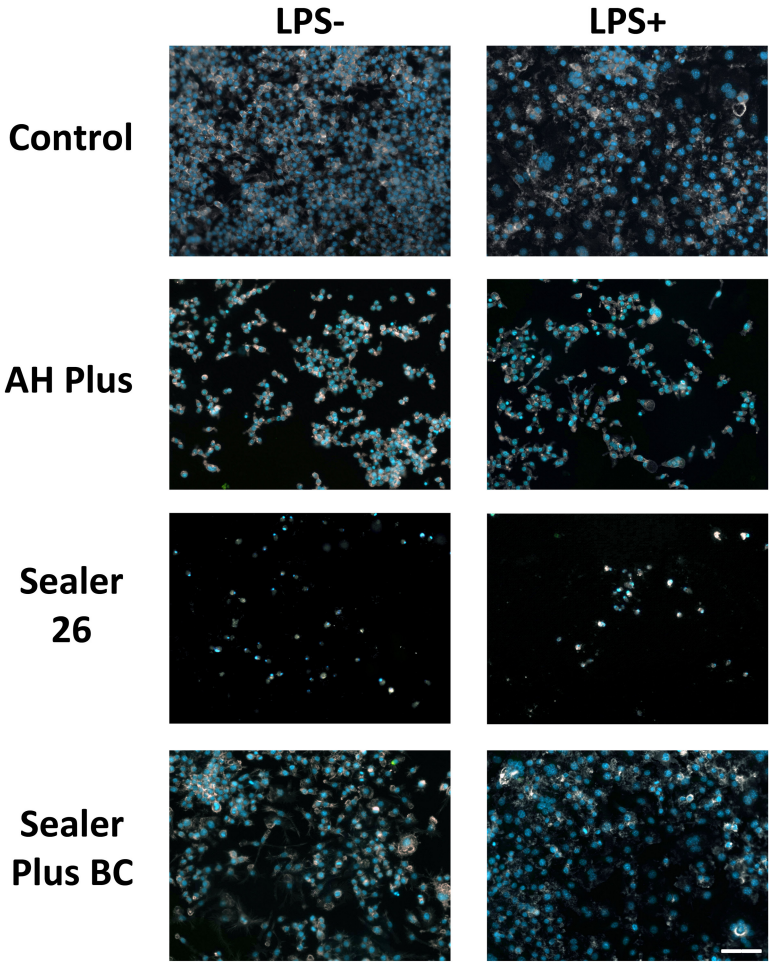


FIGURE 2 Morphological aspects of macrophage cultures of the RAW 264-7 strain cultured for 24 h with sealer extracts, exposed or not to lipopolysaccharide. Blue fluorescence indicates cell nuclei, in pale white, the Actin cytoskeleton. Magnification = 200×.

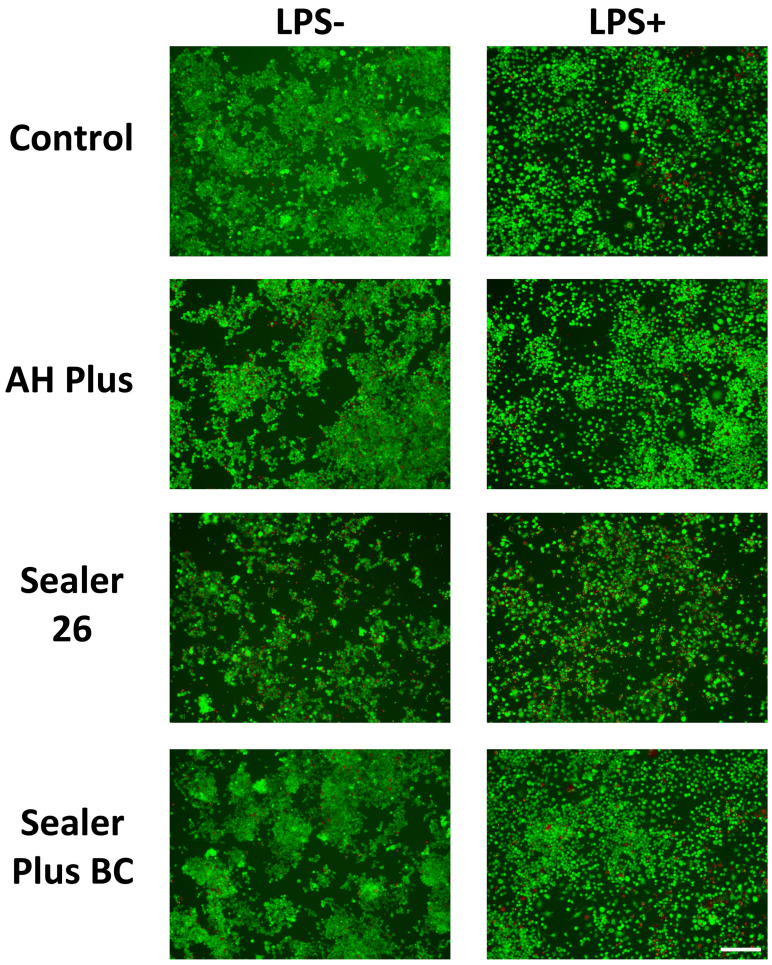


FIGURE 3 Microscopic aspects of macrophage cultures of the RAW 264-7 strain cultured for 24 h with sealer extracts, exposed or not to lipopolysaccharide (LPS), after staining using the live/dead kit. In green, viable cells and in red, dead cells. Magnification = 100×.

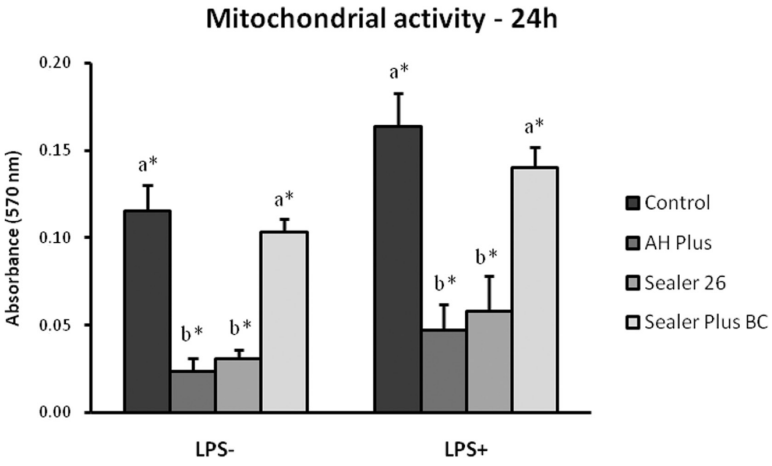


FIGURE 4 Mitochondrial activity in cultures of RAW 264-7 macrophages exposed for 24 h to sealer extracts in the presence or absence of lipopolysaccharide (LPS). Values presented as mean ± standard deviation. Different lowercase letters indicate a statistical difference between the sealers in the same condition of exposure to LPS, while the asterisk indicates a statistical difference of the same sealer in the presence or absence of LPS ($p < .001$).

When comparing the mean values in the presence and absence of LPS, no differences were observed between the sealers ($p > .05$), except for the control ($p < .001$) (Figure 5).

Oxidative stress

ANOVA revealed a statistically significant difference in NO production at 24 h, both in sealers and in the presence of LPS ($p < .001$). An interaction between these factors was observed, that is, the effects of sealers on this parameter varied in the presence of LPS ($p < .001$).

In the absence of LPS, the mean values of NO production were similar between all sealers and the control ($p > .05$). In the presence of LPS, there was interference in oxidative stress, with a difference in mean values between all groups ($p \leq .027$), with the highest values obtained in the control, followed by Sealer Plus BC, AH Plus and Sealer 26, which presented the lowest NO values. For all sealer extracts evaluated, significantly higher levels of NO were observed in cultures exposed to LPS ($p < .001$) (Figure 6).

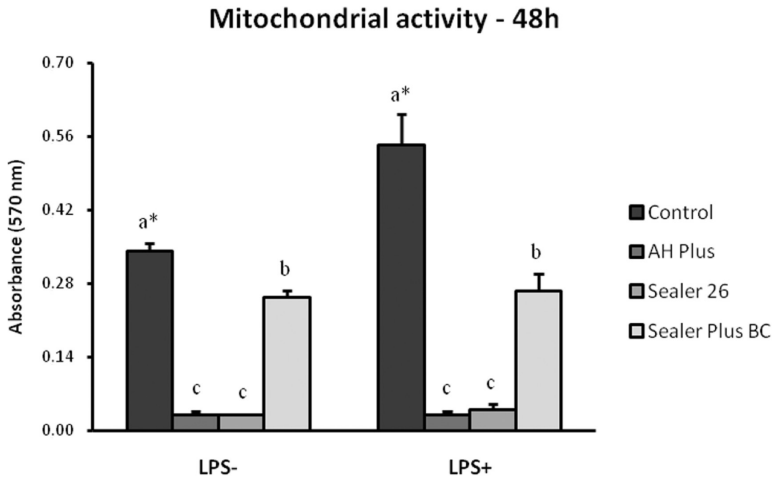


FIGURE 5 Mitochondrial activity in macrophage cultures of the RAW 264-7 strain exposed for 48 h to sealer extracts in the presence or absence of lipopolysaccharide (LPS). Values presented as mean ± standard deviation. Distinct lowercase letters indicate a statistical difference between sealers in the same condition of exposure to LPS.

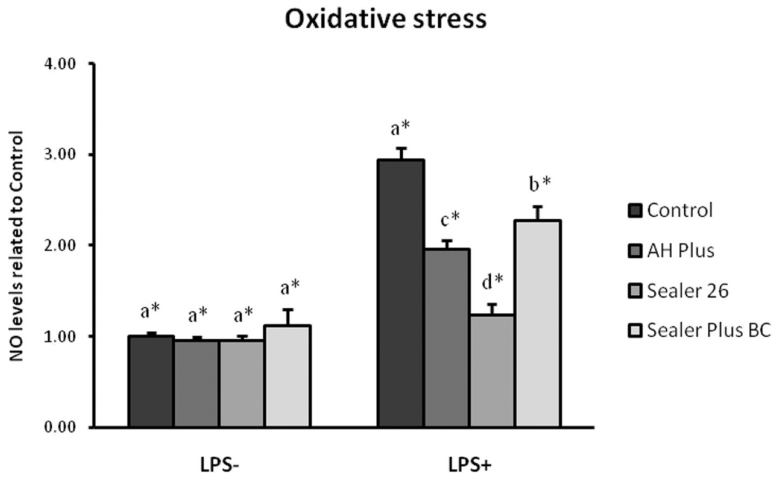


FIGURE 6 NO concentration related to oxidative stress in cultures of macrophages of the RAW 264-7 strain exposed to sealer extracts in the presence or absence of lipopolysaccharide (LPS). Values presented as mean ± standard deviation. Different lowercase letters indicate a statistical difference between the sealers in the same condition of exposure to LPS, while the asterisk indicates a statistical difference of the same sealer in the presence or absence of LPS ($p < .001$).

Gene expression

ANOVA showed a statistically significant difference in TNF-α expression between the sealers and the presence of LPS ($p < .001$). Interactions were observed between the factors, that is, the effects of sealers on this parameter varied in the presence of LPS ($p < .001$).

In the absence of LPS, the highest TNF-α expression values were observed for AH Plus ($p < .001$), followed by control and Sealer Plus BC, which were similar ($p = .328$), and Sealer 26, which showed the lowest gene expression values ($p < .001$). In the presence of LPS, the lowest mean values of TNF-α expression were observed for Sealer 26 and control ($p \leq .002$) compared to AH Plus and Sealer Plus BC, which in turn were similar ($p = .15$). Interference was observed in the expression of TNF-α in the presence of LPS in all groups ($p < .001$), in which significantly lower values were obtained in cultures exposed to endotoxins (Figure 7).

ANOVA showed a statistically significant difference in IL-1β expression between sealers not exposed and those exposed to LPS ($p < .001$). The *t*-test showed a difference in gene expression for all groups ($p < .001$), with an increase observed in cultures exposed to endotoxins.

In the absence of LPS, the highest values of IL-1β expression were observed for AH Plus ($p < .001$), followed by Sealer Plus BC, control and Sealer 26, which showed the lowest gene expression ($p < .001$) (Figure 8).

In the presence of LPS, the highest values of IL-1β expression were observed for AH Plus ($p < .001$), followed by Sealer Plus BC, control and Sealer 26, which presented the lowest gene expression ($p < .001$) (Figure 9).

DISCUSSION

Despite multiple studies evaluating the cytotoxicity and associated inflammatory response of endodontic sealers

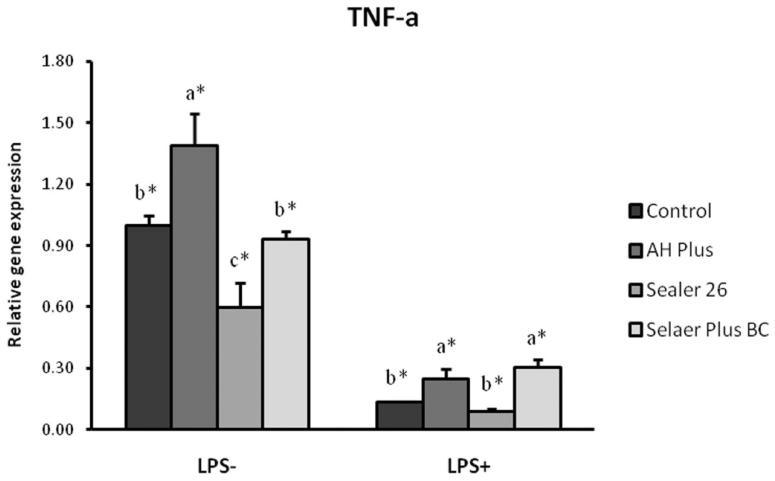


FIGURE 7 Relative expression of tumour necrosis factor-alpha in cultures of RAW 264-7 macrophages exposed to sealer extracts in the presence or absence of lipopolysaccharide (LPS). Values presented as mean ± standard deviation. Different lowercase letters indicate a statistical difference between the sealers in the same condition of exposure to LPS, while the asterisk indicates a statistical difference of the same sealer in the presence or absence of LPS ($p < .001$).

FIGURE 8 Relative expression of interleukin 1β in cultures of RAW 264-7 macrophages exposed to sealer extracts without lipopolysaccharide. Values presented as mean ± standard deviation. Different lowercase letters indicate a statistical difference between the sealers ($p < .001$).

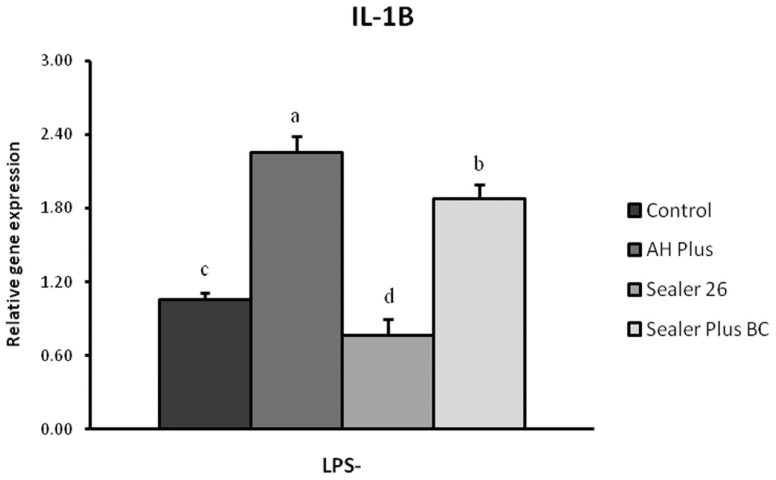
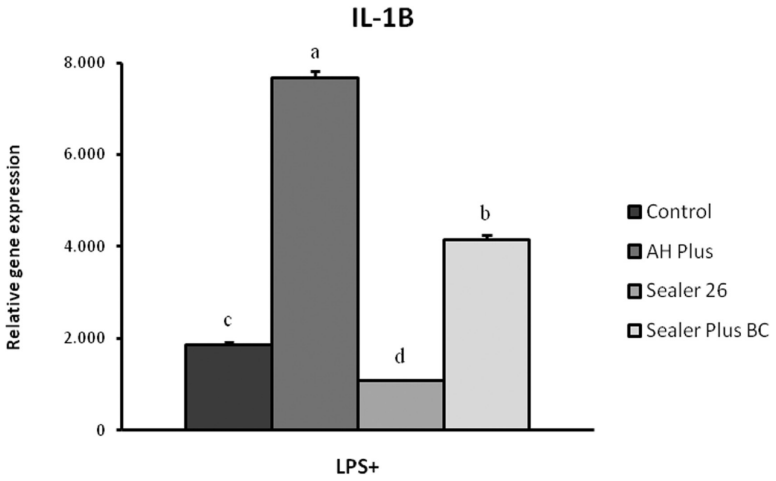


FIGURE 9 Relative expression of interleukin 1β in cultures of RAW 264-7 macrophages exposed to sealer extracts in the presence of lipopolysaccharide. Values presented as mean ± standard deviation. Different lowercase letters indicate a statistical difference between the sealers ($p < .001$).



in fibroblasts and odontoblasts, there are still no data on the immunomodulatory effect of these materials on macrophages exposed to bacterial LPS. In this study, *in vitro* biocompatibility and inflammatory modulation of the bioceramic sealer Plus BC was compared with calcium hydroxide-based sealers (Sealer 26) and epoxy resin

(AH Plus) on exposed macrophage cultures with or without LPS. The null hypothesis was rejected, as both in the presence and absence of LPS, greater cell adhesion and spread, viability, mitochondrial activity and oxidative stress were observed in the cultures exposed to the bioceramic sealer (Sealer Plus BC) compared to resin-based (AH Plus) and calcium hydroxide-based (Sealer 26) sealers. Proinflammatory markers for the AH Plus sealer were observed, while Sealer 26 exhibited the lowest levels, regardless of the presence of LPS.

To evaluate the biocompatibility and immune modulatory response of sealers, macrophages from the RAW 264.7 cell line were chosen. These cells participate in the tissue defence mechanism mediated by the presentation of antigens to T lymphocytes, phagocytosis of foreign bodies and release of proinflammatory and anti-inflammatory cytokines upon cessation of the cytotoxic stimulus. It is known that the degradation of endodontic sealers causes the release of chemical products or particles that alter the function of macrophages, which may activate or suppress their function. Therefore, understanding the effect of sealers on the function of these cells is relevant for predicting endodontic prognosis (Braz-Silva et al., 2019). Therefore, in the present study, the analysed sealers were chosen based on their composition, that is, their effects on biocompatibility and inflammatory modulation in the presence or absence of LPS.

Among the resin sealers available for endodontic use, the AH Plus sealer was selected because significant evidence supports its use for filling the root canal system owing to its high flow rate and low solubility after the final setting (Torres et al., 2020). However, its cytotoxicity is attributed to the release of formaldehyde and degradation of amines, which accelerate the polymerization process of the epoxy resin-based sealer (Lee et al., 2019; Teixeira et al., 2017). Sealer 26 is also an epoxy resin-based sealer, but was chosen because it contains calcium hydroxide, as it is observed that this component can interfere with the inflammatory process (Queiroz et al., 2005; Souza et al., 2019; Tanomaru-Filho et al., 2009). Finally, Sealer Plus BC was selected because it is a bioceramic sealer with calcium hydroxide, di-calcium and tri-calcium silicate in its composition and is free of resin; therefore, when in contact with tissue fluid, it leads to the formation of compounds with biological properties and biomineralization potential (Benetti et al., 2019). However, there is a lack of data on the effects of all evaluated sealers on macrophages exposed to bacterial LPS. Therefore, this is a relevant type of assessment that warrants further exploration.

Morphological evaluation by epifluorescence was performed for the initial verification of *in vitro* biocompatibility. Moreover, viability/cytotoxicity effects were evaluated using the LIVE/DEAD™ Viability/Cytotoxicity Kit. Previous studies have proven and corroborated the stability, specificity and sensitivity of these assessment methods (Kargarpour et al., 2020; Tawakoli et al., 2013), which have been improved through the application of fluorogenic probes whose conversion into fluorescent molecules is related to the maintenance and/or loss of cell integrity (Pfeffer & Fliesler, 2017). The two fluorophores used in this study were noncytotoxic, stable and did not degrade rapidly. In addition, the MTT assay was chosen as the quantitative analysis of the cell viability of the present manuscript as is considered a sensitive method among viability assays (Dias Corpa Tardelli et al., 2021; Kumar et al., 2018). The yellow salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] used for MTT assay, undergoes degradation and reduction by mitochondrial proteinases, active only in viable cells, forming, as a by-product of this reaction is the precipitation of purple-coloured formazan crystals (Kumar et al., 2018).

Another parameter widely used to assess the cytotoxicity and genotoxicity of endodontic sealers is the oxidative stress caused by the contact of these materials with periapical tissue cells (Sato-Suzuki et al., 2020;). The Griess method was used to verify the existence of an imbalance between oxidizing and antioxidant compounds due to the excessive generation of free radicals, such as NO, or to the detriment of their removal speed. This process leads to the oxidation of biomolecules, and consequently, there is a loss of their biological functions and/or homeostatic imbalance, which manifests as potential oxidative damage to cells and tissues. NO is considered as one of the mediators of tissue inflammation, as the third isomer (iNOS) identified in the immune system cells intervenes not only in proinflammatory activity but also in the regulation of cell growth and differentiation (Teixeira et al., 2005). Furthermore, NO is involved in the periapical bone resorption process since cells, such as macrophages, neutrophils and fibroblasts, have already been reported to produce NO in periapical cysts; it can be understood that this free radical is significantly associated with the destruction of mineralized tissue mediated by a chronic inflammatory process (Queiroz et al., 2005).

In addition, the expression of inflammatory markers was evaluated to identify endodontic sealers that favour tissue repair and resolution of the inflammation when placed in contact with biological tissues. According to the composition of dental materials, direct contact or ionic diffusion of endodontic sealers with the periapical tissues can stimulate the production of proinflammatory cytokines, such as IL-1β and TNF-α (Barkhordar et al., 1999), which are related to the development, maintenance and repair of periapical lesions (Souza et al., 2019).

Regarding the results obtained, it was verified that regardless of the presence of LPS, higher cell density in cultures exposed to control and Sealer Plus BC were observed, whereas for AH Plus and Sealer 26, lower density, spreading and cell viability were observed. The nature of the initial interaction between cells and biomaterials can influence cell adhesion and function and is a good predictor of their biocompatibility (Benetti et al., 2019; Candeiro et al., 2016; da Silva et al., 2017). However, the viability of low dilutions of Sealer Plus BC was higher than that of AH Plus (Benetti et al., 2019) and an abundant cell adhesion to the sealer surface (López-García et al., 2020) was noted. The highest number of viable cells in contact with Sealer Plus BC may be related to the release of Ca^{2+} into the medium, which favours cell migration and adhesion (Benetti et al., 2019; Candeiro et al., 2016; da Silva et al., 2017). Considering the results of macrophage viability and morphology in this study, a favourable response can be expected from Sealer Plus BC when in contact with inflammatory tissue, as macrophage adhesion and dissemination are the first steps in the phagocytic process that favour the immune system response (Braga et al., 2015).

The viability and morphology of macrophages exposed to AH Plus extracts can be explained by the long setting time of this sealer, which can lead to the release of formaldehyde and amines with significant initial cytotoxic potential (Benetti et al., 2019; Silva et al., 2020). For Sealer 26, in addition to the release of bisphenol-A formaldehyde diglycidyl ether from hexamethylenetetramine during the hardening process, the continuous release of Ca^{2+} can lead to an overload of this ion in the intracellular medium and cell death by necrosis and the induction of apoptosis (Souza et al., 2019), justifying the drastic reduction in cell density and spreading.

Corroborating previous data on morphology and cell viability, considering the ISO norms and the dilution presented in the quantitative evaluation of MTT, in both analysed periods independent of the presence of LPS, the macrophages exposed to the control and extracts of Sealer Plus BC showed significantly higher values of mitochondrial activity than the Sealer 26 and AH Plus groups.

However, LPS promoted an increase in mitochondrial activity in all groups after 24 h. Although LPS can inhibit macrophage proliferation *in vitro* (Bastos et al., 2019), there is no evidence suggesting cytotoxic effects to endotoxins. Thus, the higher mitochondrial activity of macrophages observed for these sealers when in contact with LPS could be related to the polarization and activation of endotoxin-induced cell functions (Shapouri-Moghaddam et al., 2018), evident in the process of phagocytosis and neutralization of toxic components released by sealers.

In the evaluation of oxidative stress, an increase in NO production was observed in all cultures exposed to endotoxins. These results were expected as previous studies have shown that LPS can increase the levels of reactive oxygen species and is directly related to the production of proinflammatory cytokines (Shapouri-Moghaddam et al., 2018). Sealer 26 had the lowest concentration of NO compared to the other sealers and the control. In addition to the differences observed in the cell densities of these groups, the intense release of $Ca(OH)_2$ in the first 24 h observed in Sealer 26 (Tanomaru-Filho et al., 2009), may have been responsible for the hydrolysis of LPS (Guo et al., 2014), which could explain the obtained results. The difference in the results of oxidative stress in the presence of LPS between Sealer 26 and Sealer Plus BC may be related to lower ionic release and formation of $Ca(OH)_2$ from bioceramic sealers. In fact, in previous studies, Sealer 26 released 0.730 mg/ml of Ca^{2+} in the first 24 h (Tanomaru-Filho et al., 2009), whereas Sealer Plus BC released only 0.379 mg/ml of Ca^{2+} in the same period (Mendes et al., 2018).

Cytokines are protein molecules that can be synthesized by cells after stimulation with endodontic material degradation by-products or bacterial aggression (Shapouri-Moghaddam et al., 2018). In the present study, the expression of TNF-α genes, which have inflammatory and autoimmune activities (de la Haba et al., 2016; dos Santos Costa et al., 2020), and IL-1β, which stimulates the proliferation of lymphocytes, neutrophils and macrophages, thereby increasing chemotactic and phagocytic activities (Rossol et al., 2011; Shapouri-Moghaddam et al., 2018) was evaluated. From the results, in the absence of LPS, the highest expression values of TNF-α were observed for AH Plus and the lowest values were observed for Sealer 26. The highest production of TNF-α observed for the AH Plus sealer corroborates the results of morphology, viability and mitochondrial activity in the present study, as degradation of the plasma membrane caused by cell death may have stimulated cytokine expression in the remaining cells (de la Haba et al., 2016). For Sealer 26, the lower expression of TNF-α may be related to its high release of $Ca(OH)_2$ (Tanomaru-Filho et al., 2009), which could have decreased the activation of the NF-κB protein complex, which functions as a transcription factor and neutralizes macrophage signalling for cytokine expression (Guo et al., 2014).

In the presence of LPS, regardless of the sealer evaluated, the mean values of TNF-α expression were significantly lower than those in cultures that were not exposed to endotoxins. These results may be related to the high NO levels observed in the cultures exposed to LPS. The production of TNF-α is related to the fluidization of the plasma membrane of macrophages. However, under oxidative stress, hardening of its membrane and inhibition of the expression of this cytokine is observed (de la Haba

et al., 2016), which was also confirmed in this study by the results of the control group. Moreover, because of the conditions observed in the expression of TNF-α under the effect of LPS, macrophage function can be compromised for the resolution of the inflammatory process, regardless of the sealer used.

Regarding the expression of IL-1β, in both the presence and absence of LPS, the highest values of gene expression were observed for AH Plus, followed by Sealer Plus BC and Sealer 26, which showed the lowest values of gene expression. IL-1β expression observed in macrophages exposed to Sealer 26 under the effect of LPS may be related to the high release of OH^-, promotion of endotoxin hydrolysis and minimization of cytokine expression (Guo et al., 2014). The intermediate values of IL-1β expression for Sealer Plus BC could be explained by the lower release of $Ca(OH)_2$ compared with Sealer 26 (Mendes et al., 2018; Tanomaru-Filho et al., 2009). In general, cultures exposed to LPS showed higher expression of IL-1β than those not exposed to endotoxins. Consistent with these results, previous studies have shown that LPS can increase the expression of proinflammatory cytokines in different cell types, including macrophages (Shapouri-Moghaddam et al., 2018).

The evaluation of dental materials through *in vitro* cell culture has some limitations, such as the requirement for progressive dilution of the material owing to the renewal of the culture medium, short evaluation periods to avoid cell culture deterioration and evaluation after initial setting, which represents a reduction in solubility and consequent lower release of material components. However, emphasizing the importance of each evaluative stage is necessary because *in vitro* methodologies allow one to obtain results at different periods of cell development. These methods are highly reproducible, and the response of an isolated cell line is pertinent to understanding the reactions observed in biological tissues (Queiroz et al., 2005).

Considering the results and limitations of the present study, epoxy resin-based sealers—Sealer 26 and AH Plus—have a cytotoxic potential on macrophages, which is not observed for the bioceramic sealer, Sealer Plus BC. In contrast, sealers that release $Ca(OH)_2$ (Sealer 26 and Sealer Plus BC) negatively modulate the expression of inflammatory markers compared to AH Plus, especially in the presence of LPS. Together, these findings suggest that bioceramic sealers exhibit characteristics that make it favourable for the repair of periapical tissues when in contact with tissues that present LPS.

CONCLUSIONS

The null hypothesis was rejected, as independent of the presence of LPS, Sealer Plus BC supports the adhesion, spreading, viability and mitochondrial activity of macrophages, whereas AH Plus and Sealer 26 promoted a reduction in these parameters. Furthermore, Sealer 26 exhibited the lowest inflammatory potential, with lower levels of TNF-α and IL-1β expression in macrophages, independent of LPS.

AUTHOR CONTRIBUTIONS

Nadyne Saab Messias was a graduate student at our institution; actively participating in all phases of this work that represents the dissertation of her graduation's degree. Antônio Secco Martorano and Rayana Longo Bighetti-Trevisan contributed significantly with regard to the study design, implementation of the experiment and data processing. Paulo Tambasco de Oliveira and Larissa Moreira Spinola de Castro Raucci contributed with regard to the study design and statistical analyses. The corresponding author Walter Raucci Neto is a professor of this institution that was involved with all phases of this work, from the study design to its conclusion.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICS STATEMENT

The present study used macrophage lineage RAW 264.7, American Type Culture Collection (Manassas, VA, USA), thefore, the ethics committe of authors'intitution did not require the study evaluation.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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