Endodontic Chelators Induce Nitric Oxide Expression by Murine-cultured Macrophages

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Abstract

Introduction: Endodontic chelators may extrude to apical tissues during instrumentation activating cellular events on periapical tissues. This study assessed *in vitro* the expression of nitric oxide (NO) concentrations by murine peritoneal macrophages after contact with MTAD (Dentsply/Tulsa, Tulsa, OK), Tetraclean (Ogna Laboratori Farmaceutici, Muggio, Italy), Smear Clear (Sybron Endo, Orange, CA), and EDTA (Biodinâmica, Ibiporã, PR, Brazil). **Methods:** Macrophage cells were obtained from Swiss mice after peritoneal lavage. Chelators were diluted in distilled water obtaining 12 concentrations, and MTT assay identified the concentrations, per group, displaying the highest cell viability (analysis of variance, $p < 0.01$). Selected concentrations were tested for NO expression using Griess reaction. Culture medium and lipopolysaccharide (LPS) were used as controls. **Results:** Analysis of variance and Tukey tests showed that all chelators displayed elevated NO concentrations compared with the negative control ($p < 0.01$). MTAD induced the lowest NO expression, followed by Tetraclean, EDTA, and Smear Clear. No difference was observed between MTAD and Tetraclean ($p > 0.01$), Tetraclean and EDTA ($p > 0.01$), and EDTA and Smear Clear ($p > 0.01$). LPS ranked similar to both EDTA and Smear Clear ($p > 0.01$). **Conclusion:** The tested endodontic chelators displayed severe proinflammatory effects on murine-cultured macrophages. Citric acid–based solutions induce lower NO release than EDTA-based irrigants. *(J Endod 2009;35:824–828)*

Key Words

Chelators, cytotoxicity, irrigation solutions, macrophages, nitric oxide

In endodontics, instrumentation and irrigation intend to achieve root canal enlargement and disinfection. However, independently of the foraminal diameter (1), depth of irrigation needle penetration (2), or instrumentation technique used (3, 4), extrusion of debris and irrigants occurs, which may result in periapical inflammation and postoperative flare-ups (5).

Once extruded, endodontic irrigants could permeate into tissue and initiate cellular mechanisms including vascular alterations, activation of inflammatory cells, expression of chemical mediators, and reduction in cellular repair (6, 7). Besides, extruded debris and endodontic irrigants activate host defense in an attempt to eliminate the irritant (7).

Macrophages play a role in host defense mechanisms as they release reactive oxygen/nitrogen species such as nitric oxide (NO) and proinflammatory cytokines essential in intercellular communication (8). The proinflammatory role of NO in the development and maintenance of periapical lesions (8–12) through various biochemical mechanisms such as the promotion of osteoblast and macrophages apoptosis (8, 11) and the differentiation of osteoclasts (12) has been well reported. Additionally, the activation of NO synthase (NOS) positively correlates with prostaglandin E2 generation, an inflammatory mediator resultant from cyclooxygenase conversion of arachidonic acid. Both NO and prostaglandin E2 are capable of interacting with their own respective biosynthetic pathways retromodulating NOS and cyclooxygenase (13).

NOS can be trigged upon stimulation by virulence factors present in microorganisms, such as bacterial lipopolysaccharide (LPS) from gram negative (14) and lipoteichoic acid from gram positive (15) as well as by other inflammatory cytokines (14). Hence, if endodontic irrigants could also stimulate macrophage-induced NO expression *in vitro*, its determination would help to indicate the *in vivo* risk of a proinflammatory effect in case of apical extrusion.

During root canal instrumentation, chelating agents, such as EDTA, are widely used to remove inorganic debris and to facilitate root canal disinfection (16). The effect of EDTA on macrophage activity (17, 18) and viability (19) has been reported. However, it remains unknown whether EDTA and other currently used chelators induce the expression of NO by cultured macrophages.

Based on the essential role of macrophages in biochemical reactions in which cellular products are released, this study intend to determine *in vitro* the effect of MTAD (Dentsply/Tulsa, Tulsa, OK), Tetraclean (Ogna Laboratori Farmaceutici, Muggio, Italy), Smear Clear (Sybron Endo, Orange, CA), and EDTA (Biodinâmica, Ibiporã, PR,

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TABLE 1. Endodontic Chelators, Their Compositions, and Manufactures

Chelator	Composition	Manufacturer
MTAD	3% Doxycycline hyclate 4.25% Citric Acid 0.5% Tween 80	Dentsply/Tulsa, Tulsa, OK
Tetraclean	1% Doxycycline hyclate 10% Citric Acid 2% Cetrimide Polypropylene Glycol	Ogna Laboratori Farmaceutici, Muggio, Italy
SmearClear	17% ethylenediaminetetraacetic acid Cetrimide Polyoxyethylene (10)iso-octylcyclohexyl ether Water	Sybron Endo, Orange, CA
EDTA	17% ethylenediaminetetraacetic acid	Biodinâmia, Ibiporã, PR, Brazil

Brazil) on NO concentrations released by murine peritoneal macrophages. The null hypothesis tested is that none of the chelators induce proinflammatory effects on murine peritoneal macrophages.

Materials and Methods

Experimental Design

This laboratory-based study quantified NO concentrations expressed by cultured murine peritoneal macrophages after contact with MTAD, Tetraclean, Smear Clear, and EDTA (Table 1). Twelve subsequent dilutions of endodontic chelators were first evaluated for cell viability using the MTT-tetrazolium method (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide). The dilutions displaying significantly higher levels of cell viability on the MTT test were selected for NO evaluation after an enzymatic reaction. All measurements for MTT and NO tests were performed in triplicate. The NO results of the selected different concentrations of the same solution were grouped and a mean obtained for statistical analysis.

Materials

Each chelating solution was diluted in distilled water to obtain 12 different concentrations ranging from 1.9 μg/mL to 3,900 μg/mL.

Peritoneal Macrophages

Three Swiss mice, 6- to 8-weeks old, weighing 18 to 25 g, provided by the Animal House of the Faculty of Pharmaceutical Sciences of Araraquara, São Paulo, Brazil, were used to provide macrophages. Resident and thioglycollate-elicited peritoneal exudate cells were obtained after an intraperitoneal injection of 3 mL of thioglycollate medium (3 g/100mL) and lavage of the peritoneal cavity with 5 mL of 10 mmol/L of phosphate-buffered saline (pH = 7.2) 3 to 4 days later. The number of macrophages in the peritoneal exudate was determined by cell staining using May-Grünwald-Giemsa stain (Sigma-Aldrich, São Paulo, SP, Brazil). Cell preparations contained more than 95% macrophages. The cells were washed twice with PBS and resuspended in the appropriate medium for each test.

Macrophages (2×10^6 cells/mL) were suspended in RPMI-1640 containing 5% heat-inactivated fetal bovine serum, 100 IU/mL of penicillin, 10 μg/mL of streptomycin, and 50 mmol/L of 2-mercaptoethanol. One hundred microliters of the suspension was added to each well of a 96-well tissue culture plate, and the cells were incubated at 37°C in a moist environment containing 5% CO_2. After 24 hours, the adhering cells were exposed to the 12 different concentrations of tested solutions. *Escherichia coli*'s LPS and culture medium were used as controls, positive and negative, respectively.

Cytotoxicity Assay (MTT Test)

The MTT assay (20) was performed to identify the concentrations reaching the highest cell viability. Because NO expression is influenced by cell mortality, a solution concentration leading to significant low amount of viable cells should be not included on the NO assay. MTT assay reflects cellular process in mitochondrial metabolism. After 24 hours of cell growth in both controls and experimental solutions diluted in culture medium, the medium was replaced with 10 mL/well of 5 mg/mL of MTT solution (Sigma-Aldrich) in fresh medium and reincubated for 3 hours. Then, the cultures were removed from the incubator and the resulting formazan crystals dissolved by adding 100 mL of MTT solubilizer solution. The absorbance was measured in a spectrophotometer at 540-nm wavelength (Labsystems Mulstiscan Ascent, Thermo Labsystems, Finland). Background absorbance was measured at 620 nm and subtracted from the 540-nm measurement. Tests were performed in triplicate for each solution concentration.

Percentages of cell viability were calculated comparing the absorbance values with the absorbance of the negative control (100%). The percentage values for each solution dilution as well as for control groups were compared by using one-way analysis of variance. Concentrations grouped as highest percentages of viable cells were selected for the NO assay (Fig. 1). Differences were considered significant at $p < 0.01$.

NO Assay

The chosen concentrations (Fig. 1) were checked for NO synthesis by the quantification of nitrite (NO_2^-), a stable metabolite of NO, using the Griess reaction (21). After 24 hours of incubation of macrophages with the different concentrations, 50-μL aliquots of culture supernatant were mixed with 50 μL of Griess reagent (1% w/v sulfanilamide, 0.1% w/v naphthyl ethylenediamine, and 3% phosphoric acid) (Sigma-Aldrich) and incubated for 5 minutes at room temperature. A spectrophotometer at 540 nm recorded the absorbance. The NO_2^- concentration (μmol/L) was calculated from a calibration curve generated with $NaNO_2$. For each solution concentration, the experiment was performed in triplicate. All manipulations were performed under a laminar flow hood to avoid contamination from outside organisms.

Statistical Analysis

Because the data from NO assay did not reach a normal distribution (Kolmogorov-Smirnov test) and the sample size was too low for a nonparametric test, data were transformed using the $\log_{10}$ function (GraphPad Prism 5.0, GraphPad Software, Inc., San Diego, CA). One-way analysis of variance and Tukey post hoc tests verified differences in NO expression among endodontic irrigants. The results from LPS and culture medium were included in statistical analysis to verify

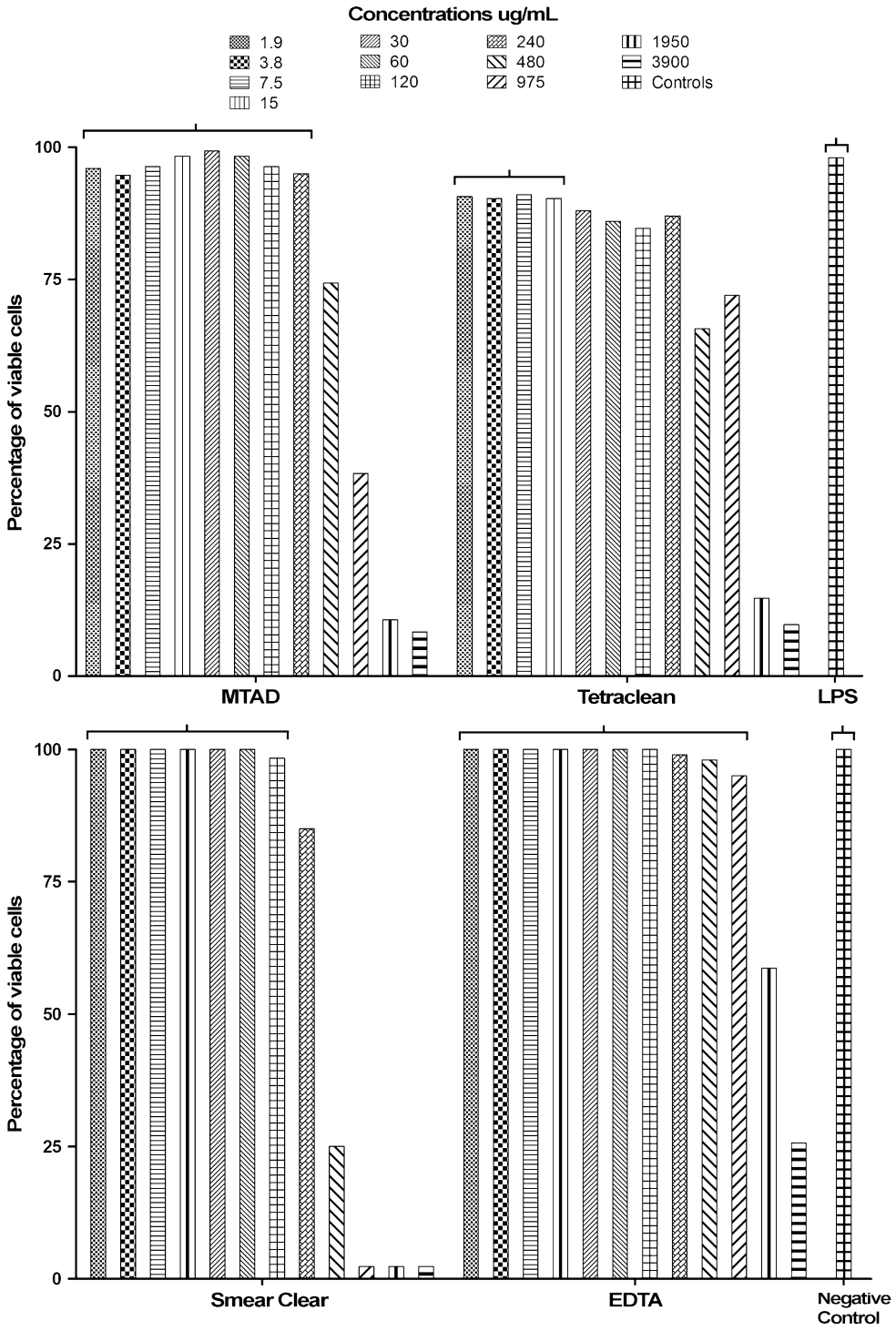

Figure 1. Cell viability observed on the MTT test. Claves indicate the solution concentration displaying similar and highest cell viability (analysis of variance, $p < 0.01$). Those concentrations were selected for NO assay.

similarities on NO concentrations between experimental and control groups. Differences were considered significant at a $p < 0.01$.

Results

On the MTT assay, one-way analysis of variance showed that 29 solution concentrations (dilutions) (8 from MTAD [1.9 μg/mL-240 μg/mL], 4 from Tetraclean [1.9 μg/mL-15 μg/mL], 7 from Smear Clear [1.9 μg/mL-120 μg/mL] and 10 from EDTA [1.9 μg/mL-975 μg/mL]), and the two controls were not significantly different ($p > 0.01$), displaying the highest means of cell viability (ranging from 90.03% to 100%). Those solutions were, therefore, selected for the NO assay (Fig. 1).

Data from NO quantification are expressed in Figure 2. All chelators presented a significantly higher NO release compared with the negative control ($p < 0.01$). A significant difference among chelators was observed ($p < 0.01$). MTAD displayed the lowest mean NO concentration (79.7 μmol/L) followed by Tetraclean (79.8 μmol/L), EDTA

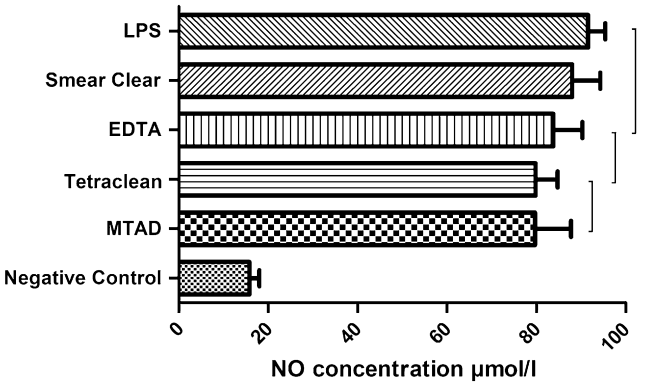

Figure 2. Representation of NO concentrations expressed by viable cells after contact with different concentrations of endodontic irrigants, negative and positive (LPS) control. Claves indicate groups with statistically similar results at a $p < 0.01$ (one-way analysis of variance and Tukey test).

(83.8 μmol/L), and Smear Clear (88.0 μmol/L). MTAD presented significantly lower NO release compared with EDTA and Smear Clear ($p < 0.01$) and Tetraclean significantly lower than Smear Clear ($p < 0.01$). EDTA and Smear Clear displayed similar NO concentrations compared with LPS ($p > 0.01$), whereas MTAD and Tetraclean did not ($p < 0.01$).

Discussion

Macrophage-induced NO is capable of eliciting a series of proinflammatory events on local environment (22). Increased NO production by macrophages contribute to pathogenic mechanisms of periapical periodontitis (8, 10, 11) through the induction of osteoblasts and macrophage apoptosis (8), the differentiation of osteoclasts (12), MMP-1 production by osteoclasts (11), and cytokine gene expression (23) resulting in cytotoxic effects on both microorganisms and host cells. When present in periapical granulomas, NO interacts with the receptor for advanced glycation end products (RAGE), a multiligand member of immunoglobulin superfamily of cell surface molecules, resulting in modulation of tissue injuries and bone destruction (24). In fact, elevated NO concentrations are usually observed in exudates from human periapical lesions (11, 25). Hence, NO expression is an important marker of periapical inflammation.

To prevent that NO concentrations could be the result of different degrees of cell mortality induced by chelators, a complete standardization was performed in the present study. First, chelators were subsequently diluted in an attempt to reduce solutions potential to kill cells. Furthermore, the 12 dilutions per solution were subjected to a cell viability assay (MTT test) to verify concentrations displaying comparable highest cell viability. Although there was a 10% variation in cell viability among selected dilutions, the statistical procedure for MTT test indicated no difference in cell viability among them. This guaranteed that concentrations selected for NO evaluation allowed similar cell death and that solution concentrations resulting in significantly lower cell viability were not included for NO assay. Because different chelators lead to different levels of cytotoxicity (26), multiple dilutions among the groups were used for the NO assay (Fig. 1). Following this approach, a higher reliability for NO assay was accomplished because the NO concentrations recorded were not influenced by cell mortality. Considering that cultured cells are more susceptible to drug toxic effects than the periapical tissue (26) because in the body, phagocytic cells, lymph, and blood channels help to dilute and carry away the drug (27), the dilutions and MTT assay performed in this study helped to mimic the *in vivo* situation in which solutions would induce the release of proinflammatory species rather than the cell death.

All endodontic chelators tested displayed an elevated proinflammatory effect because NO levels were significantly higher than those observed in the negative control group (Fig. 2). This way, the null hypothesis that none of the endodontic chelators induces proinflammatory effects on cultured macrophages should be rejected. This clearly indicates that the extrusion of chelators to periapical tissues would induce significant NO production by macrophages. Nonetheless, statistical differences among solutions were observed. Citric acid–containing solutions (MTAD and Tetraclean, Table 1) presented significantly lower levels of NO release compared with EDTA-based solutions (EDTA and Smear Clear, Table 1) (Fig. 2).

The EDTA and citric acid–negative effects on macrophage viability have been previously reported (19). Although cytotoxic, citric acid has been ranked as less irritating than EDTA on cell cultures (19, 26). Zhang et al (28) observed MTAD lower cytotoxicity compared with EDTA on L929 fibroblast cultured cells. The less irritating to the cell is the chemical the lower proinflammatory effect it might display. This assumption is supported by the results of the present study in which the citric acid–containing solutions displayed significantly lower NO concentrations compared with EDTA-based solutions (Fig. 2). Additionally, doxycycline, the active antibiotic present on both MTAD and Tetraclean (Table 1), is rather less cytotoxic than other tetracycline derivates (29).

Although citric acid–containing solutions induced lower NO concentrations compared with EDTA-based solutions, they still present a significant proinflammatory effect that could be explained by cell growth inhibition and cytotoxic response on cell cultures induced by Tween 80 (MTAD) and Cetrimide (Tetraclean) detergents (30, 31). It could also be inferred that Smear Clear adds the negative effects of EDTA and Cetrimide (Table 1) leading to its extreme proinflammatory effect.

Interestingly, both EDTA and Smear Clear presented similar NO expression to that of LPS. LPS is the major component of the outer membrane of gram-negative bacteria. It acts as endotoxin and is regarded as one of the most potent osteolytic factors (32), displaying a positive correlation with apical periodontitis severity (33, 34). The observation that EDTA-based solutions exhibit similar NO induction to LPS together with reports of EDTA interference on macrophage viability and activity (17–19, 35) indicate that care should be taken to avoid apical extrusion of EDTA-based solutions during root canal preparation.

Although extensively used in endodontics, there are no reports of complications after the extrusion of EDTA-based solutions in dental literature, which might be due to its short-term use in root canal therapy and the fact that it is generally associated with other solutions such as sodium hypochlorite. Nevertheless, there are reports on the irritating potential of EDTA to the connective tissue of rats (36) and induced inflammatory responses to the periapical tissues of dogs (37). In addition, deaths were reported after intravenous injections of aqueous solutions of EDTA (0.6%) used for medical purposes in the so-called chelating therapy (38, 39).

Few studies used NO expression as a marker of drug-induced inflammation (40–42) but none of them to test irrigants. Considering that biocompatibility tests should be performed before *in vivo* experiments, the present experiment would help to add information for initial evaluation of root canal irrigants.

In conclusion, clinically used endodontic chelators displayed elevated proinflammatory effects on murine cultured macrophages. Citric acid–based solutions induced lower NO release than EDTA-based solutions. LPS- and EDTA-based solutions presented comparable

macrophage-induced NO expression, and, therefore, care should be taken to avoid apical extrusion of those solutions during root canal treatment.

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