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Nitric oxide detection in cell culture exposed to LPS after Er:YAG laser irradiation

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Abstract

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Aim To evaluate *in vitro* the effect of calcium hydroxide [$Ca(OH)_2$] and Er:YAG laser on bacterial endotoxin [also known as lipopolysaccharide (LPS)] as determined by nitric oxide (NO) detection in J774 murine macrophage cell line culture.

Methodology Samples of LPS solution (50 μg mL^{-1}), $Ca(OH)_2$ suspension (25 mg mL^{-1}) and LPS suspension with $Ca(OH)_2$ were prepared. The studied groups were: I – LPS (control); II – LPS + $Ca(OH)_2$; III – LPS + Er:YAG laser (15 Hz 140 mJ); IV – LPS + Er:YAG laser (15 Hz 200 mJ); V – LPS + Er:YAG laser (15 Hz 250 mJ), VI – Pyrogen-free water; VII – $Ca(OH)_2$. Murine macrophage J774 cells were plated and 10 μL of the samples were added to each well. The supernatants were collected for NO detection by the Griess reaction. Data were analysed statistically by one-way ANOVA and Tukey's test at 5% significance level.

Results The mean and SE (in μmol L^{-1}) values of NO release were: I – 10.48 ± 0.58, II – 6.41 ± 0.90, III – 10.2 ± 0.60, IV – 8.35 ± 0.40, V – 10.40 ± 0.53, VI – 3.75 ± 0.70, VII – 6.44 ± 0.60; and the values for the same experiment repeated after 1 week were: I – 21.20 ± 1.50, II – 9.10 ± 0.60, III – 19.50 ± 1.00, IV – 18.50 ± 0.60, V – 21.30 ± 0.90, VI – 2.00 ± 0.20, VII – 6.80 ± 1.70. There was no significant difference ($P > 0.05$) between the control and the laser-treated groups (III, IV and V), or comparing groups II, VI and VII to each other ($P > 0.05$). Group I had significantly higher NO release than group II ($P < 0.05$). Groups II and VI had similar NO release ($P > 0.05$).

Conclusions Calcium hydroxide inactivated the bacterial endotoxin (LPS) whereas none of the Er:YAG laser parameter settings had the same effectiveness.

Keywords: bacterial endotoxin, calcium hydroxide, Er:YAG laser, LPS, nitric oxide.

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Introduction

A large number of studies have demonstrated that infection in teeth with apical periodontitis is of polymicrobial origin and that anaerobic microorganisms, particularly gram-negative bacteria (Assed *et al.* 1996), are highly prevalent in these teeth (Abou-Rass & Bogen 1998, Leonardo *et al.* 2005).

In addition to their different virulence factors and generation of products and byproducts that are toxic to the periapical tissues, gram-negative microorganisms have endotoxin as an integral part of the outer layer of their cell wall (Rietschel & Brade 1992). This is especially important because endotoxin, also known

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as LPS due to its lipopolysaccharide constitution, is released upon bacterial multiplication or death causing a series of relevant biological effects (Mc Gee *et al.* 1992, Barthel *et al.* 1997). These events induce inflammatory reaction (Rietschel & Brade 1992, Silva *et al.* 2002) and bone and cementum resorption (Nelson-Filho *et al.* 2002, Jiang *et al.* 2003), which contribute to the formation and/or persistence of chronic periapical lesions.

Furthermore, LPS acts as a potent stimulator of nitric oxide (NO) production (Blix & Helgeland 1998, Bellows *et al.* 2006). During inflammation, NO becomes readily activated (Bellows *et al.* 2006) and acts together with numerous additional mediators, including cytokines and other proinflammatory mediators, which trigger immune and non-immune cells (Hibbs *et al.* 1988). The overproduction of NO is detrimental to the host (Bellows *et al.* 2006) and may cause changes that lead to tissue damage or direct injury in both acute and chronic inflammation (Knowles & Moncada 1994).

It is well-known that the use of a $Ca(OH)_2$ intracanal dressing between sessions is capable to inactivate LPS (Safavi & Nichols 1994, Barthel *et al.* 1997, Nelson-Filho *et al.* 2002, Jiang *et al.* 2003, Oliveira *et al.* 2005). However, over the last years, the antimicrobial effect of Er:YAG laser irradiation has been investigated in an attempt to develop a treatment protocol that can increase the success rate of endodontic therapy in these cases (Jelinková *et al.* 1999, Mehl *et al.* 1999, Moritz *et al.* 1999, Dostálová *et al.* 2002, Schoop *et al.* 2002, Perin *et al.* 2004, Leonardo *et al.* 2005). Although intracanal laser irradiation has been shown to have an antimicrobial effect, its action on bacterial endotoxin (LPS) has not yet been addressed.

The purpose of this study was to assess *ex vivo* the effect of $Ca(OH)_2$ and Er:YAG laser irradiation on LPS as determined by NO detection in J774 murine macrophage cell line culture.

Materials and methods

Preparation of LPS solution

In a laminar flow cabinet, lyophilised *Escherichia coli* lipopolysaccharide (LPS) (Lipopolysaccharide B *E. coli* 055 : B5; Sigma Aldrich Corp., St. Louis, MO, USA) was suspended in phosphate-buffered saline, diluted in pyrogen-free water in a concentration of 50 μg mL^{-1} and stored in non-pyrogenic plastic tubes. The absence of endotoxin in the pyrogen-free water (Milli-Q; Millipore Corporate, Billerica, MA, USA) was determined by the limulus amebocyte lysate kinetic assay (Kinetic-QCL™ kit; Bio-Whittaker, Cambrex Co., East Rutherford, NJ, USA) followed by filtration in a Millex GV Durapore® polyvinylidene flouride (PVDF) membrane filter (pore size: 0.22 μm; Millipore Corporate).

The LPS concentration used in this study was defined based on the results of a dose–response pilot experiment in which different concentrations were tested (1 ng mL^{-1}, 10 ng mL^{-1}, 100 ng mL^{-1}, 1 μg mL^{-1}, 5 μg mL^{-1} and 50 μg mL^{-1}). The 50 μg mL^{-1} LPS concentration showed the best results in the activation of J774 murine macrophage cell line for NO production.

Preparation of calcium hydroxide suspension

Two hundred and fifty milligrams of $Ca(OH)_2$ p.a. (Merck, Darmstadt, Germany) was diluted in 10 mL of pyrogen-free water to obtain a $Ca(OH)_2$ suspension with a final concentration of 25 mg mL^{-1}. The tube was sealed with parafilm 'M®' (American National Can Company, Chicago, IL, USA) and homogenized in an automatic stirrer (Mixtron®; Toptronix, São Paulo, SP, Brazil) for 10 min.

Preparation of LPS/calcium hydroxide suspension

Fifty microliters of LPS solution (concentration of 50 μg mL^{-1}) were added to 950 μL of $Ca(OH)_2$ suspension in non-pyrogenic plastic tubes, which were stored in an incubator at 37 °C under rotational agitation (126 rpm) for 24 h and thereafter placed in a microcentrifugal unit (2 000 rpm) at 20 °C for 7 min. The supernatants of the samples were then collected.

Macrophage cell line culture

Murine J774 macrophage cell lines obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) were kindly donated by Dr Célio Lopes e Silva from the Department of Biochemistry and Immunology of the Medical School of Ribeirão Preto, University of São Paulo, São Paulo, Brazil. For the *ex vivo* stimulation assay, the cells were defrosted at room temperature, dispersed in 750-cm^2 cell culture flasks in RPMI medium supplemented with 10% foetal bovine serum (RPMI-C) and placed in a humidified incubator containing 95% air and 5% CO_2 mixture at 37 °C.

After the formation of a monolayer, the cells adhered to the flask were suspended in a medium and the total number of macrophages in a suspension of 1 mL diluted 100 times in Trypan blue solution was counted

in a Neubauer chamber under light microscopy to assess cell viability.

The cells were seeded in 24-well plates (Corning Glass Works, New York, NY, USA) in a concentration of 2×10^5 cells/well and incubated for 2 h in 5% CO_2-containing complete RPMI medium at 37 °C, for cell adherence to the plates. Thereafter, the supernatant was discarded and 990 μL of RPMI-C was added to each well.

Ex vivo macrophage cell line stimulation assay

Different stimuli (10 μL) were added to the adhered J774 macrophage cell lines, obtaining the following groups (n = 7/group): Group I: LPS solution; Group II: LPS/$Ca(OH)_2$ suspension; Group III: LPS solution irradiated with Er:YAG laser (Kavo Key Laser 1242; Kavo Dental GmbH, Biberach, Germany) with 15 Hz pulse repetition rate and 140 mJ output energy for 20 s, delivered by an E2055 handpiece connected to a 50/28 optical fibre (0.285 mm diameter) submitted to dry heat sterilisation and depyrogenation (Lab-Line/Barnstead Imperial V Laboratory Oven Model 3481M, Boston, MA, USA) at 200 °C for 2 h. The optical fibre was placed inside the plastic tubes and the laser beam was delivered in two 10-s applications with a 5-s interval between irradiations, according to a helicoidal kinematics that started from the bottom of the plastic tube towards its upper opening; Group IV: LPS solution irradiated with Er:YAG laser (15 Hz 200 mJ); Group V: LPS solution irradiated with Er:YAG laser (15 Hz 250 mJ); Group VI: pyrogen-free water; Group VII: $Ca(OH)_2$ suspension. To assess the reproducibility of the results, the experiment was repeated after 1 week (n = 4/group), with total of 11 samples for each group.

After addition of the stimuli, the plates were incubated in an environment with 5% CO_2 at 37 °C for 48 h. The supernatants were then collected and stored at −20 °C for NO detection.

NO detection in macrophage cell line culture supernatants

Nitric oxide production was measured indirectly by a spectrophotometric method based on the Griess reaction (Ding *et al.* 1988) for nitrite detection. The supernatant of the macrophage cell line culture (100 μL) was dispensed into the wells of a 96-well plate followed by the addition of 100 μL of Griess reagent containing one part of 1% NEED solution [*N*-(1-naphthyl)ethylenediamine dihydrochloride] and one part of 1% sulfanilamide in 5% aqueous phosphoric acid. After 5-min incubation at room temperature, the optical density was measured with a 550-nm filter absorbance microplate reader (μQuant; Bio Tek Instruments Inc., Winooski, VT, USA). A quantitative analysis was undertaken with nitrite standard curves.

Statistical analysis

The tabulated data and numerical results from nitrite dosage (in μmol L^{-1}) were subjected to statistical analysis of the differences amongst the groups by one-way ANOVA, as the data had a normal distribution, and Tukey's multiple-comparison test using the GRAPHPAD PRISM 4 software (GraphPad Inc., San Diego, CA, USA). Significance level was set at 5%.

Results

The results of NO concentration in the supernatants of the macrophage cell line cultures in all groups are given in Fig. 1.

The mean and SE values (in μmol L^{-1}) of NO release were: Group I (LPS): 10.48 ± 0.58; Group II (LPS + $Ca(OH)_2$): 6.41 ± 0.90; Group III (Er:YAG laser/140 mJ): 10.2 ± 0.60; Group IV (Er:YAG laser/200 mJ):8.35 ± 0.40; Group V (Er:YAG laser/250 mJ laser): 10.40 ± 0.53; Group VI (pyrogen-free water): 3.75 ± 0.70; Group VII ($Ca(OH)_2$): 6.44 ± 0.60.

There was no statistically significant difference ($P > 0.05$) between Group I and the laser-irradiated groups (III, IV and V), regardless of the parameter settings. No statistically significant difference ($P > 0.05$) was found amongst Groups II, VI and VII. Significant difference ($P < 0.05$) was found between Groups I and II, with greater NO release in Group I.

The reproducibility of the repeated experiment after 1 week was confirmed. The mean and SE values (in μmol L^{-1}) of NO release were: Group I: 21.20 ± 1.50; Group II: 9.10 ± 0.60; Group III: 19.50 ± 1.00; Group IV: 18.50 ± 0.60; Group V: 21.30 ± 0.90; Group VI: 2.00 ± 0.20; Group VII: 6.80 ± 1.70. The statistical analysis of the results obtained in the control and test groups in the second experiment was similar to those of the first experiment.

Discussion

In the present study, a NO dose–response curve was constructed after stimulation of the macrophage cell line culture with LPS solutions either subjected or not

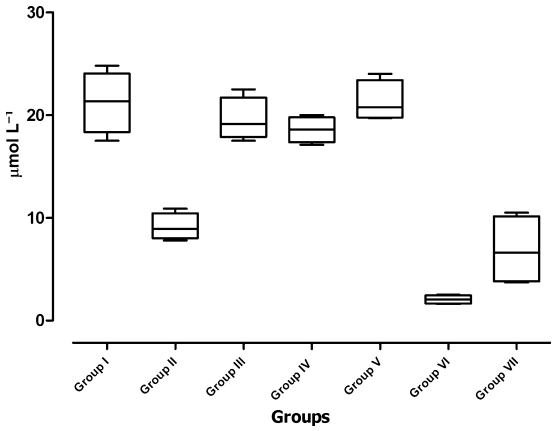

Figure 1 Box plot diagrams illustrating NO release after stimulation of macrophages in the LPS (I–V) and controls (VI and VII).

to previous treatment with $Ca(OH)_2$ or Er:YAG laser. NO is a multifunctional mediator and participates in inflammatory tissue destruction (Van't Hof & Ralston 2001). Studies have suggested that NO is also involved in bone resorption in periapical lesions (Takeichi *et al.* 1999), and plays a key role in regulating inflammatory reaction in apical periodontitis with the association of cytokines. However, the precise mechanism of how NO is related to periapical lesion remains unclear. It has been hypothesized that specific inhibitors of NO production could be used as pharmacological agents in the management of periapical lesions (Hama *et al.* 2006).

Cell culture studies have demonstrated that LPS stimulates macrophages to produce NO (Blix & Helgeland 1998, Bellows *et al.* 2006). In the present study, greater NO release was observed in Group I (LPS). There was a significant difference between Groups I (LPS) and II [LPS plus $Ca(OH)_2$], with greater NO release in Group I, whilst Group II presented similar NO release to Group VI (pyrogen-free water). These results are in agreement with those of other authors (Barthel *et al.* 1997, Nelson-Filho *et al.* 2002, Jiang *et al.* 2003, Oliveira *et al.* 2005), who reported that $Ca(OH)_2$ is capable of hydrolysing the bacterial LPS molecule, inactivating its toxic effects *in vitro* and *in vivo*. This is an important finding from a clinical standpoint because teeth with apical periodontitis have a great amount of LPS causing chronic inflammation. Inactivation of bacterial endotoxin is required in these cases because LPS produces a chronic inflammatory status that stimulates excessive NO production, which induces pathological and physiological changes that will cause tissue damage (Knowles & Moncada 1994, Bellows *et al.* 2006).

The antimicrobial effect of Er:YAG laser has been extensively demonstrated, especially in endodontics (Jelinková *et al.* 1999, Mehl *et al.* 1999, Moritz *et al.* 1999, Dostálová *et al.* 2002, Schoop *et al.* 2002, Perin *et al.* 2004, Leonardo *et al.* 2005). Nevertheless, there is no standardisation regarding the laser parameter settings (pulse repetition rate, output energy and irradiation time). A pulse repetition rate of 15 Hz was chosen for the present study because it has been widely used in studies that assessed the antimicrobial effect of Er:YAG laser (Mehl *et al.* 1999, Schoop *et al.* 2002). Er:YAG laser energy and irradiation time settings in studies that evaluated its antimicrobial effect range from 4 to 250 mJ and from 15 to 60 s, respectively (Jelinková *et al.* 1999, Mehl *et al.* 1999, Moritz *et al.* 1999, Dostálová *et al.* 2002, Schoop *et al.* 2002, Perin *et al.* 2004, Leonardo *et al.* 2005). A total of three energy intensities (140, 200 and 250 mJ) were chosen for a 20-s irradiation time to evaluate whether energy variation would interfere with Er:YAG laser effect on LPS. However, it was observed that, regardless of the parameter settings, the laser-treated groups (III, IV and V) had similar NO production to that of Group I (LPS), which indicates that, in spite of its antimicrobial activity, Er:YAG laser was not able to inactivate the toxic effects of LPS.

Regarding the high cost of some laser devices and their ineffectiveness on bacterial endotoxin, the use of the laser in endodontics, in relation to this propriety, has no advantage compared with $Ca(OH)_2$.

Further studies are required to assess the effects of Er:YAG laser on LPS using other parameter settings and additional *in vitro* and *in vivo* methodologies, to substantiate its clinical use in endodontic therapy of teeth with apical periodontitis, which exhibit high levels of LPS in the root canal system.

Conclusion

Calcium hydroxide inactivated the bacterial endotoxin (LPS) whereas none of the Er:YAG laser parameter settings had the same effect.

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