$Ca(OH)_2$ action on TNF-alpha and NO release in macrophage culture stimulated by *Pseudomonas aeruginosa* LPS

Ação do $Ca(OH)_2$ sobre a produção de TNF-alfa e NO de cultura de macrófagos estimulada por LPS de *Pseudomonas aeruginosa*

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Abstract

Purpose: Calcium hydroxide [$Ca(OH)_2$] interaction with the immune system to destroy or neutralize bacteria and their by-products is not completely understood. This study evaluated the calcium hydroxide ability to neutralize *Pseudomonas aeruginosa* lipopolysaccharides (LPS) using two different methods: nitric oxide (NO) stimulation and Tumoral Necrosis Factor-alpha (TNF-alpha) release in mice macrophage culture.

Methods: For the NO assay, peritoneal exudate cells (PECs) were placed in contact with 25μg/mL and 50μg/mL LPS and LPS/$Ca(OH)_2$ suspensions (50μg/25mg and 25μg/25mg). After incubation for 8h, Griess reagent was used, and the NO release was quantified. In the TNF-alpha assay the LPS solution was tested in 25μg/mL, and the LPS/$Ca(OH)_2$ suspension was tested in 25μg/25mg concentration. After incubation for 24 hours, cells were fixed and stained with crystal violet. Absorbance values were obtained. Results were expressed in micromols. All tests were performed in triplicate.

Results: The presence of $Ca(OH)_2$ in both tested concentrations significantly reduced NO and TNF-alpha release.

Conclusion: It can be concluded that bacteria LPS is a strong stimulus for NO and TNF-alpha release, but calcium hydroxide can neutralize it.

Key words: Peritoneal macrophages; nitric oxide; lipopolysaccharides; tumor necrosis factor-alpha

Resumo

Objetivo: A ação do hidróxido de cálcio [$Ca(OH)_2$] com o sistema imune e o mecanismo de neutralização das bactérias e seus subprodutos ainda não foi completamente esclarecida. Neste estudo foi avaliada a capacidade do $Ca(OH)_2$ em neutralizar o lipopolissacarídeo (LPS) de *Pseudomonas aeruginosa*, utilizando-se duas metodologias: liberação de Óxido Nítrico (NO) e Fator de Necrose Tumoral Alfa (TNF-alfa) em cultura de macrófagos peritoneais de camundongos.

Metodologia: No ensaio do NO, as células peritoneais foram expostas a uma solução de LPS (25mg/mL e 50mg/mL); e à suspensão de LPS/$Ca(OH)_2$ em duas concentrações (50mg/25mg e 25mg/25mg). Após 8 horas de incubação, foi utilizado reagente de Griess, e a liberação de NO foi quantificada. No ensaio do TNF-alfa, a solução de LPS foi usada na concentração de 25mg/mL e o LPS/$Ca(OH)_2$ a 25mg/25mg. Após 24 horas, as células foram fixadas e coradas com cristal violeta, e os valores de absorbância foram obtidos. Os resultados foram expressos em micromols. Todos os testes foram realizados em triplicata.

Resultados: A presença de $Ca(OH)_2$ nas duas concentrações avaliadas reduziu significativamente a liberação de NO e TNF-alfa.

Conclusão: Pode-se concluir que o LPS bacteriano representa um forte estímulo para liberação destas citocinas, mas o hidróxido de cálcio foi capaz de neutralizar este efeito.

Palavras-chave: Macrófagos peritoneais; óxido nítrico; lipopolissacarídeos; fator de necrose tumoral alfa

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Introduction

Total elimination of bacteria is the main purpose of root canal therapy in infected teeth. Complete chemomechanical preparation remains the most important step in root canal disinfection. However, even after cleaning and shaping, total elimination of bacteria is unlikely to achieve (1-3). Pataky et al. (4) infected the root canals of extracted teeth and examined the number of remaining bacteria after mechanical preparation; reduction of bacteria only occurred after chemomechanical preparation. Current regimens of chemomechanical debridment are not predictably effective for root canal disinfection (3). Therefore, a root canal dressing containing calcium hydroxide is essential to optimize disinfection of the root canals system. Calcium hydroxide ($Ca(OH)_2$) pastes are the most commonly used canal dressings (5).

The inflammatory reaction caused by bacteria stimulates the defense cells at the periapical region. Among such cells, the macrophages, in response to those stimuli, may release cytokines (*i.e.*, interleukin-1, tumoral necrosis factor alpha), peroxidase, hydrogen peroxide (H_2O_2), and nitric oxide (NO) (6,7).

Among cytokines, tumoral necrosis factor alpha (TNF-alpha) presents activity for macrophages and neutrophils and cytotoxic activity through the induction of oxygen radicals and NO production, both presenting strong antimicrobial activity. This cytokine also participates in the proliferation and differentiation of human B cells and acts in synergy with interleukin-1 (IL-1) to activate osteoclasts and regulate root resorption (8,9). The release of chemical mediators when cells suffer morphological changes, degeneration or death due to aggression, stimulus or stress may allow the cytotoxicity evaluation of lipopolysaccharides (LPS), as well as the effectiveness of drugs to neutralize these toxic products. The higher the toxicity, the greater the release of TNF-alpha and NO (6,7).

The aim of this study was to evaluate the calcium hydroxide ability to neutralize *Pseudomonas aeruginosa* LPS by assessing NO and TNF-alpha release in a culture of mice peritoneal macrophages.

Methods

Animals and macrophages culture

Eighteen Swiss mice from the Faculty of Pharmaceutical Sciences of Araraquara, UNESP, in Araraquara, SP, Brazil, weighting 18-25g, were used.

Thioglycollate-elicited peritoneal exudate cells (PECs) were harvested from Swiss mice using 5.0mL of sterile phosphate buffered saline (PBS) (Sigma-Aldrich Brasil, São Paulo, SP, Brazil), pH 7.4. The cells were washed twice in PBS by centrifugation at 200xg for 5min at 4°C and resuspended in appropriate medium for each test.

Complete tissue culture medium (CTCM) consisted of RPMI-1640 containing 5%(v/v) heat-inactivated fetal bovine serum (Cutilab, Campinas, SP, Brazil), 100U of penicillin (Sigma-Aldrich Brasil, São Paulo, SP, Brazil), 100µg of streptomycin (Sigma-Aldrich Brasil, São Paulo, SP, Brazil) and $5x10^{-2}$M mercaptoethanol (Sigma-Aldrich Brasil, São Paulo, SP, Brazil) per mL.

LPS solution

Fifty mL of 50µg/mL *Pseudomonas aeruginosa* LPS (L7018) (Sigma-Aldrich Brasil, São Paulo, SP, Brazil) solution in saline (Glicolabor, Ribeirão Preto, SP, Brazil) was prepared.

$Ca(OH)_2$ and LPS/$Ca(OH)_2$ suspensions

$Ca(OH)_2$ powder (Glicolabor, Ribeirão Preto, SP, Brazil) and saline were mixed to prepare 100mL of 25mg/mlL$Ca(OH)_2$ suspension. LPS/$Ca(OH)_2$ suspension was prepared in two different concentrations: 50mg/25mg and 25mg/25mg.

NO measurement

NO synthesis was determined by measuring the accumulation of nitrite (NO_2-), a stable NO metabolite, in culture supernatants using the Griess reaction, previously described by Green et al. (10).

The PECs were resuspended in CTCM at a concentration of $5x10^6$ cells/mL, and 100mL of this suspension was added to each well of a 96-well tissue culture dish (Corning, São Paulo, SP, Brazil) with 100mL of LPS/$Ca(OH)_2$ suspension (25mg/25mg and 50mg/25mg) or LPS solution (50mg/mL). These concentrations were established according to compatibility parameters following MTT tests.

The cells were incubated for 8 hours at 37°C, and 50mL aliquots of culture supernatant were mixed with 50mL of Griess reagent (1%w/v sulphanylamide (Sigma-Aldrich Brasil, São Paulo, SP, Brazil), 0.1%w/v naphthylethylenediamine (Sigma-Aldrich Brasil, São Paulo, SP, Brazil), and 3% H_3PO_4 (Sigma-Aldrich Brasil, São Paulo, SP, Brazil)), which were added to the plate. After 10 minutes, the reading was performed in an Organon ELISA 2001 reader (Organon Teknika, Durham, NC, USA) with a 540nm filter. Results were expressed in micromols from a standard curve formed by the known micromol NO concentrations for each assay.

TNF-alpha assay

In the TNF-alpha assay, LPS solution was tested at 25mg/mL concentration, and LPS/$Ca(OH)_2$ suspension was tested at 25mg/25mg concentration.

TNF-alpha activity in supernatants from macrophages stimulated with *Pseudomonas aeruginosa* LPS and LPS/$Ca(OH)_2$ suspension was assayed on a TNF-sensitive L929 cell line by means of a previously described photometric crystal violet staining method (11). Briefly, L929 cells in RPMI-1640 medium containing 5% (v/v) foetal calf serum (Sigma, St. Louis, MO, USA) were seeded at 4×104 cells per well in 96-well microtiter plates (Corning-Costar Corp., Cambridge, MA, USA) and incubated overnight at 37°C in atmosphere of air/CO2 (95:5, v/v).

Serial 1:2 dilutions of the culture supernatants previously obtained with 25mg/mL LPS solution and 25mg/25mg LPS/$Ca(OH)_2$ were made in the above medium containing 1.0µg actinomycin D (Sigma, St. Louis, MO, USA) per mL, and the cell culture medium was replaced with 100-µL volumes of the dilutions in triplicate. The next day, cell survival was assessed by fixing and staining the cells with crystal violet (0.2% in 20% methanol), dissolving the stained cells with 0.1mL 1% (w/v) SDS per well and reading the absorbance of each well at 490nm with an ELISA reader Multiskan Ascent (Thermo Labsystems, Franklin, MA, USA). TNF-alpha units were calculated using a standard curve obtained with recombinant TNF-alpha (Sigma, St. Louis, MO, USA) constructed for each test run. To prove the presence of TNF-alpha in the culture supernatants, those preparations were previously incubated with rabbit anti-rTNF-alpha (Sigma, St. Louis, MO, USA) immune serum, and the test samples were added to L929.

Results

Table 1 shows the values of NO and TNF-alpha release, expressed in micromols, in a culture of mice peritoneal macrophages stimulated by *P. aeruginosa* LPS solution and LPS solution treated with $Ca(OH)_2$.

The confidence intervals (Table 1) show that there was no significant difference between the mean NO release in relation to LPS concentration. This result was confirmed by Student t test, considering the pairs of NO release values in the same animal. The presence of $Ca(OH)_2$ reduced the NO release significantly, and the results were equivalent at both tested concentrations. There was a twentyfold decrease of TNF-α release when *P. aeruginosa* LPS was treated with $Ca(OH)_2$.

Discussion

Although $Ca(OH)_2$ is widely used and studied, its mechanisms of action are not totally understood, mainly its interaction with the immune system to destroy or neutralize bacteria and their by-products (12). Even after bacteria death, endotoxin is released. Thus, the use of substances that promote only bacteria death is not sufficient for the complete treatment of teeth with pulp necrosis. It may be necessary to use intracanal dressings to accomplish bacterial endotoxin inactivation. Calcium hydroxide hydrolyzes lipid A, which is a highly toxic agent, and releases nontoxic free-hydroxy fatty acids (13,14). Thus calcium hydroxide is able to neutralize bacterial endotoxin (8) and its toxic effects (15).

To analyze the reaction to *P. aeruginosa* LPS treated with $Ca(OH)_2$, mice peritoneal macrophages were chosen because they are essential to inflammatory and healing processes, and strongly present in the peritoneum. NO and TNF-alpha release in macrophage culture has been used as a parameter to evaluate cytotoxicity and maintenance of cell integrity (6-8,16,17). After stimulation, cytokines, which act in autocrina or paracrina ways, are some of the several substances secreted by macrophages.

TNF-alpha presents functions that are closely linked with endodontic therapy because of its role in bone and root resorption (18,19), as well as fibroblastic proliferation (20), macrophage activation (7,19), and collagen synthesis (21). Bone and root resorption after stimulation with bacteria LPS is closely related to IL-1b and TNF-alpha release (18). Also, TNF-alpha is responsible for tumor cell destruction by macrophages and is strongly synergic with IFNg to destroy tumors. Thus, a typical biological assay to determine TNF is based on the lysis of L929 tumor cells treated with Actinomicin D (22).

P. aeruginosa LPS stimulates NO release as detected by Queiroz et al. (6), who used root canal sealers to stimulate macrophages. The treatment of *P. aeruginosa* LPS with $Ca(OH)_2$ caused its neutralization. This fact was observed in the present study through the NO production at physiologic levels (Table 1). Queiroz et al. (6) also found that a $Ca(OH)_2$ endodontic sealer was less cytotoxic than other sealers.

In summary, the present study showed that *P. aeruginosa* LPS proved to be a strong stimulator of TNF-alpha production by macrophages. The addition of $Ca(OH)_2$ to LPS prevented TNF-alpha release or promoted it at physiological levels.

Table 1. Values of NO and TNF-alpha release (in micromols), stimulated by *P. aeruginosa* LPS solution and LPS treated with $Ca(OH)_2$.

	NO				TNF-α	
	LPS		LPS/$Ca(OH)_2$		LPS	LPS/$Ca(OH)_2$
	50µg	25 µg	50µg/25mg	25µg/25mg	25µg	25µg/25mg
Mean values	23.07	22.76	7.45	7.30	236.61	3.03
Standard deviation	3.20	3.31	0.29	0.19	40.54	4.77
95%CI lowest limit	20.62	20.22	7.22	7.15	205.46	0.00
95%CI highest limit	25.53	25.31	7.67	7.44	267.77	6.70

Conclusions

Based on the methodology used in the present study, it can be concluded that bacteria LPS is a strong stimulator of the production of NO and TNF-alpha. The treatment of *Pseudomonas aeruginosa* LPS with calcium hydroxide neutralized the effects of LPS on TNF-alpha and NO release.

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