Archives of Oral Biology 73 (2017) 34–39

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/aob

Cell migration, viability and tissue reaction of calcium hypochlorite based-solutions irrigants: An *in vitro* and *in vivo* study

Gabriela Bess Ferraz Blattes, DDS, MSc[a],
Leticia Boldrin Mestieri, DDS, MSc, Post-Graduation Student[a],
Daiana Elisabeth Böttcher, DDS, MSc, PhD, Post-Doctural Research Fellow[b],
Anna Cristina Medeiros Fossati, DDS, MSc, PhD[c], Francisco Montagner, DDS, MSc, PhD[a],
Fabiana Soares Grecca, DDS, MSc, PhD[a,*]

[a] *Department of Conservative Dentistry, School of Dentistry, Federal University of Rio Grande do Sul, Porto Alegre. Av. Ramiro Barcelos, 2492, Zip Code: 90035-003, Porto Alegre/RS, Brazil*
[b] *Department of Endodontics, School of Dentistry, Pontifical Catholic University of Rio Grande do Sul, Av. Ipiranga, 6681, Zip Code 90619-900, Porto Alegre/RS, Brazil*
[c] *Department of Morphological Sciences, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul. Rua Sarmento Leite, 500, Zip Code 90050-170, Porto Alegre/RS, Brazil*

ARTICLE INFO

Article history:
Received 14 December 2015
Received in revised form 22 August 2016
Accepted 31 August 2016

Keywords:
Biocompatibility
Calcium hypochlorite
Cytotoxicity
Endodontics
Scratch assay

ABSTRACT

Objective: This study aimed to analyze *in vitro* cytotoxicity to cultured 3T3 fibroblasts and *in vivo* inflammatory reaction in rats by calcium hypochlorite ($Ca(OCl)_2$) solutions compared with sodium hypochlorite (NaOCl) solutions.
Design: Cultured 3T3 fibroblasts were exposed to different concentrations of ($Ca(OCl)_2$) and NaOCl solutions, and a scratch assay was performed. The viability rate was analyzed with trypan blue assay. Both solutions of 1% and 2.5% concentrations were injected into the subcutaneous tissue of 18 male Wistar rats aged 18 weeks. The inflammatory tissue reaction was evaluated at 2 h, 24 h, and 14 days after the injections. The samples were qualitatively analyzed using a light microscope. Statistical analysis was performed with ANOVA and Tukey post hoc tests for *in vitro* assays and Kruskal–Wallis and Dunn post hoc tests for *in vivo* assays ($\alpha = 0.05$).
Results: In the scratch assay, $Ca(OCl)_2$ showed no significant difference compared with the control group (culture medium) at 24 h ($p < 0.05$). Solutions of 0.0075% and 0.005% NaOCl and $Ca(OCl)_2$ concentrations presented similar results compared with those in the positive control group (hydrogen peroxide) ($p > 0.05$) in the trypan blue assay. In the *in vivo* assay, 1% $Ca(OCl)_2$ group showed a significant decrease in neutrophils at 2 h and 24 h ($p = 0.041$) and 2 h and 14 days ($p = 0.017$). There was no statistically significant difference for lymphocyte/plasmocyte and macrophage counts among the different concentration groups.
Conclusions: $Ca(OCl)_2$ showed favorable results of viability and induced a low-level inflammatory response. $Ca(OCl)_2$ presented acceptable cytotoxicity and biocompatibility as an irrigant solution.

1. Introduction

In endodontics, chemomechanical preparation aims to promote root canal cleansing and shaping by using endodontic instruments and auxiliary chemical solutions (Schilder, 1974; Stojicic, Zivkovic, Qian, Zhang, & Haapasalo, 2010). Owing to the anatomical complexity of the root canal system, irrigating solutions are fundamental to promote an effective cleansing during treatment (Gernhardt, Eppendorf, Kozlowski, & Brandt, 2004; Guneser, Arslan, & Usumez, 2015; Önçağ et al., 2003; Villas-Bôas et al., 2011). The most frequently employed substance is sodium hypochlorite (NaOCl) (Gernhardt et al., 2004; Gomes-Filho, Aurélio, Costa, & Bernabé, 2008; Mohammadi, 2008).

NaOCl is bactericidal owing to its high pH. It has low surface tension, detergent action, and tissue-dissolution capacity (Gernhardt et al., 2004; Guneser et al., 2015; Mohammadi, 2008;

* Corresponding author at: 2492, Ramiro Barcelos Av. Bom Fim – Porto Alegre, Rio Grande do Sul, Zip Code 90035-003, Brazil.
E-mail address: fabiana.grecca@ufrgs.br (F.S. Grecca).

http://dx.doi.org/10.1016/j.archoralbio.2016.08.037
0003-9969/

Schilder, 1974; Stojicic et al., 2010). Although higher concentrations of NaOCl significantly improve its antimicrobial and tissue-dissolving effects, it can become more cytotoxic and induce inflammatory response when in contact with periapical tissues (Gomes-Filho et al., 2008; Mohammadi, 2008; Önçağ et al., 2003; Spangberg & Engström, 1973; Yesilsoy, Whitaker, Cleveland, Phillips, & Trope, 1995). Another important limitation to the use of NaOCl is its chemical instability. External agents such as temperature, light, and storage conditions influence the availability of chlorine ions, which in turn affect the maintenance and preservation of its properties and influence the outcome of endodontic treatment (Aparecida & Fernandes, 1996). Therefore, other strategies need to be considered.

Calcium hypochlorite ($Ca(OCl)_2$) is a halogenated compound, used for industry sterilization, bleaching, and water purification (Dutta & Saunders, 2012; Whittaker & Mohler, 1912). In contrast with NaOCl, $Ca(OCl)_2$ is relatively stable with an available chlorine ion percentage higher than NaOCl (Dutta & Saunders, 2012; Whittaker & Mohler, 1912). $Ca(OCl)_2$ has the ability to promote soft-tissue dissolution (Dutta & Saunders, 2012) and presents similar antibacterial properties when compared with NaOCl on *Enterococcus faecalis* colony-forming units in infected bovine teeth (de Almeida et al., 2014). Nonetheless, there are no available data concerning cytotoxicity and tissue reaction to $Ca(OCl)_2$.

The aim of the present study was to compare the cytotoxicity and biocompatibility of $Ca(OCl)_2$ and NaOCl *in vitro*, using scratch and trypan blue assays on cultured 3T3 fibroblasts and, *in vivo*, by inflammatory reactions evaluation in rat connective tissue. Our objective was to verify if $Ca(OCl)_2$ can be employed as auxiliary chemical substance in root canal system preparation.

2. Material and methods

2.1. Study design

The present study was approved by the Research Committee of the School of Dentistry of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil (no. 26578).

The biocompatibility study was approved by the Research Board and by the Ethics Committee for Animal Use in Research (protocol number 26102), following the guidelines from the National Council for Ethics in Animal Experiments (CONCEA).

2.2. Cell culture

3T3 fibroblasts (ATCC CRL-1658) were cultured at 37 °C in a 100% humidified atmosphere containing 5% CO_2. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich) and 1% penicillin and streptomycin (PenStrep, Gibco, Invitrogen, Life Technologies, Canyon City, Oregon, USA). At 80% confluence, cells were treated with 0.25% trypsin–EDTA solution (Sigma-Aldrich) in the well-plates.

2.3. Scratch assay

This test was performed using a modified method proposed by Liang, Park, and Guan, 2007 (15). 3T3 fibroblasts were seeded in a 35-mm Petri dish at 2×10^6 concentration. After 24 h incubation at 37 °C in an atmosphere containing 5% CO_2 and 95% humidity, a scratch was made in the cell monolayer with a P200 pipette tip (TPP, Techno Plastic Products, Trasadigen, Switzerland). The culture medium was replaced according to the groups: control group (DMEM), 0.00075% $Ca(OCl)_2$, and 0.00075% NaOCl. After this, the plate was placed into an Axio Observer Z1 microscope (Zeiss, Göttingen, Germany) with a charge-coupled device camera (Axiocammrn, Zeiss) using a 10 × objective (Ecplan-Neofluar 10 × /0.3 aperture, Zeiss) and AxioVision Software (Zeiss). Images were taken every 10 min until the scratch was closed and were analyzed using ImageJ Software (National Institute of Health, NIH, USA).

2.4. Trypan blue assay

Cells were plated in 96-well plates (Kasvi, Curitiba, PR, Brazil) at a concentration of 1×10^5 cells/mL. After 24 h, the medium was removed and replaced by 100 μL of the tested solutions diluted with DMEM supplemented with 1% PenStrep. The following $Ca(OCl)_2$ and NaOCl concentrations were used: 0.0075%, 0.005%, 0.00075%, and 0.0005%. The number of plated cells and the determination of the concentrations used were previously determined using a cell growth curve and the half maximal inhibitory concentration (IC_{50})_ determination. The culture medium was used as the negative control (CT−); hydrogen peroxide 10 mM was used as the positive control (CT+).

After 24 h, the culture medium was removed from the plates and 100 μL of 0.25% trypsin–EDTA was added to each well for 3 min to detach the cells. The cell suspension in the well was mixed with 100 μL of 0.4% trypan blue (Sigma-Aldrich). Then, 10 μL of this suspension was counted using a hemocytometer chamber under an inverted light microscope. Cell viability rate was calculated by the following formula: % viable cells = (no live cells/total number of cells) × 100.

The assay was performed in duplicate (n = 10/group).

2.5. Biocompatibility assay

The following irrigating solutions were tested: 1% $Ca(OCl)_2$, 2.5% $Ca(OCl)_2$, 1% NaOCl, 2.5% NaOCl, and 0.9% sterile saline (NaCl). Except for the sterile saline solution, all solutions were prepared one day before the experiment.

Eighteen male *Wistar* rats aged 18 weeks were used for the experiments. The animals were divided into three groups of six rats each, according to the three experimental periods: 2 h, 24 h and 14 days.

Under anesthesia with 0.008 mL/100 g ketamine (Vetnil Indústria e Comércio de Produtos Veterinários S.A., Louveira, Brazil) and 0.004 mL/100 g 2% xylazine hydrochloride (Syntec do Brasil Ltda, Cotia, Brazil), the dorsal skin of the animals was shaved and cleaned with a 10% iodine solution. Using a paper template and indelible pen (A. W. Faber-Castell S.A., São Carlos, Brazil), six circles were demarcated on the dermis of each rat leaving at least 2 cm between each circle. With disposable insulin syringes (Injex Indústria Cirúrgica Ltda, Ourinhos, Brazil) and a standard depth needle, 0.1 mL of each solution was injected subcutaneously into one of the 5 circles. In the 6th circle the needle of an empty syringe was introduced (no irrigant: negative control group). The location of the solution was randomly assigned in each animal.

By the end of each experimental period (2 h, 24 h, and 14 d), all animals of each group were euthanized by anesthetic overdose with 120 mL/kg pentobarbital (Syntec do Brasil Ltda, Cotia, Brazil). Immediately, tissue specimens were removed and fixed in 10% formalin solution (LabSynth Produtos para Laboratório LTDA, São Paulo, Brazil) for 48 h. After fixation, specimens were embedded in paraffin, and 3-μm-thick sections were obtained and stained with hematoxylin and eosin.

Three blinded and calibrated examiners ($k = 0.79$, $P < 0.01$) analyzed the slices under a light microscope (Olympus BX41–Olympus America Inc., Melville, NY, EUA), using 40, 100, 400, and 1000 × magnification. Cellular events were analyzed according to the description of the following inflammatory cells: neutrophils, eosinophils, lymphocytes/plasmocytes, macrophages, and giant