



CYTOTOXICITY AND GENOTOXICITY OF ENDODONTIC IRRIGANTS ON HUMAN CELLS

Citotoxicidade e genotoxicidade de irrigantes endodônticos em células humanas

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Abstract

OBJECTIVE: Endodontic irrigants solutions with antibacterial activity have been used in treatment of teeth with infected root canals; however, these solutions can irritate periapical tissues. The aim of this study was to evaluate the cytotoxicity and genotoxicity of different endodontic irrigants solutions – sodium hypochlorite (1% and 2%), calcium hydroxide (0.2%), and HCT₂₀ in human KB cells. **MATERIAL AND METHOD:** Cells were incubated with solutions for 2 and 24 hours. The cell viability was assessed after the *trypan blue* exclusion and the frequency of cell death mechanism (apoptotic or necrotic) was determined by acridine orange/ethidium bromide fluorescent dyeing test. The genotoxicity effects were assessed by the micronucleus assays. **RESULTS AND DISCUSSION:** The results showed that Ca(OH)₂ alone or in combination with tergentol (HCT₂₀), and NaOCl induced cytotoxicity in KB causing death cells by apoptosis. The micronuclei test showed that KB treated with NaOCl (1%) present an increase in the frequency of micronucleus compared to the control group.

Keywords: Endodontic irrigants solutions. Micronuclei. Cytotoxicity.

Resumo

OBJETIVO: Soluções irrigadoras com atividade antibacteriana têm sido usadas no tratamento de dentes com canais radiculares infectados; entretanto, essas soluções podem irritar os tecidos periapicais. O objetivo deste estudo foi avaliar a citotoxicidade e genotoxicidade de diferentes soluções irrigadoras – hipoclorito de sódio (1% e 2%), hidróxido de cálcio (0,2 %) e HCT em células humanas KB. **MATERIAL E MÉTODO:** As células foram incubadas em soluções por 2 e 24 horas. A viabilidade celular foi determinada após exclusão do tripan blue e a frequência de mecanismo de morte celular (apoptótica ou necrótica) foi determinada pelo teste acridine Orange/ethidium bromide fluorescen dyeing. Os efeitos de genotoxicidade foram determinados pelo ensaio de micronúcleos. **RESULTADOS E DISCUSSÃO:** Os resultados demonstraram que o $\text{Ca}(\text{OH})_2$, isoladamente ou em combinação com Tergentol™ (HCT_{20}) e NaOCl , induziram citotoxicidade em KB, causando morte celular por apoptose. O teste de micronúcleos demonstrou que KB tratado com NaOCl (1%) apresentou aumento na frequência de micronúcleos quando comparado com o grupo controle.

Palavras-chave: Soluções irrigantes endodônticas. Micronúcleos. Citotoxicidade.

INTRODUCTION

Biomechanical canal preparation during the root-canal treatment involves cleaning and shaping procedures with endodontic instruments and irrigants solutions. In general, these irrigants solutions remove dentine debris, dissolve organic materials, disinfect the canal system, and provide lubrication during biomechanical preparation (1). One of the most popular endodontic irrigant solutions is sodium hypochlorite; it is an effective antimicrobial agent (2) and an excellent organic solvent for vital and necrotic tissues. Chemical and toxicological concerns related to sodium hypochlorite solution use has been discussed, including different approaches to enhance local efficacy without increasing the caustic potential (3). Another antimicrobial agent used as endodontic irrigant is calcium hydroxide solution; it has been widely used as an intracanal disinfectant (4), and as a physical barrier to prevent bacterial recontamination (5). The calcium hydroxide is also used in combination with tergentol (lauril-diethylene-glycol-ether-sodium sulphate) – HCT_{20} as disinfecting root canals (6).

Since irrigating solutions can get contact with surrounding soft and hard tissues during endodontic treatment, they should have an acceptable biocompatibility. Recent findings about root canal medicaments and irrigating solutions showed that some irrigant solutions may be potentially toxic and may cause damage to the surrounding tissue (7). Thus, biocompatibility data concerning genotoxicity and cytotoxicity are needed for complete risk assessment of these compounds.

The aim of current study was to evaluate the genotoxic and cytotoxic effects of three different irrigant solutions – sodium hypochlorite, calcium hydroxide, and calcium hydroxide associated with Tergentol™ (HCT_{20}) – over KB cells.

MATERIAL AND METHODS

Cells and reagents

The calcium hydroxide – $\text{Ca}(\text{OH})_2$ and Tergentol™ (lauryl-diethylene-glycol-ethersodium sulphate) were purchased from Sigma™. The HCT_{20} is an aqueous solution of $\text{Ca}(\text{OH})_2$ 0.2% and 0,025g/ml Tergentol. The sodium hypochlorite solutions – NaOCl were obtained from a pharmacology manipulation laboratory, Brasília, DF. KB cells were obtained from cell culture bank of Universidade Federal do Rio de Janeiro (UFRJ), Brazil.

Cell and culture conditions

In order to test the effects genotoxicity and cytotoxicity of sodium hypochlorite, calcium hydroxide, and HCT_{20} solution, KB were grown at 37°C, at an atmosphere of 5% of CO_2 in Dulbecco's modified Eagle's medium, DMEM, (GIBCO-BRL), pH 7.4, supplemented with 10% fetal calf serum, and 100 U/ml penicillin and 100 U/ml streptomycin.

For cytotoxicity (cell viability after the trypan blue exclusion test and acridine orange/

ethidium bromide double staining) test, KB (3×10^6) were placed in 6-wells culture plates and stabilized for 24 hours. The irrigating solution concentration used was based on clinical concentration. For each treatment NaOCl (1% and 2%), $\text{Ca}(\text{OH})_2$ (0.2%) and, HCT₂₀ cultures in triplicate were treated for 2 and 24 hours, washed, harvested with 0.25% trypsin-EDTA, re-suspended in phosphate-buffer saline (PBS) and used for the test.

Untreated cultures correspond to negative control. Cyclophosphamide – Enduxan™ at 6.0 µg/mL, was used as positive control. For micronuclei assay, KB cells were treated with the three irrigant solutions and incubated for 24h. For analysis, cells were washed, harvested with 0.25% trypsin-EDTA, resuspended in phosphate-buffer saline (PBS) and used for the test.

Analysis of cell viability by light microscopy and fluorescence microscopy

Viability of KB cells after incubation was determined by *trypan blue* dye exclusion staining. In order to assess the frequency death mechanism of cell (apoptotic or necrotic), cells were collected, centrifuged and re-suspended in phosphate buffer saline (PBS). Briefly, 20 µL of the cell suspension (10^6 cells) was stained with a fresh solution of acridine orange (100 µg/mL)/ethidium bromide (100 µg/mL) for 5 min. The cells were then examined using a fluorescence microscope (Olympus BX-40) equipped with barrier filter (590-nm). Apoptosis or necrosis and cell survival were evaluated by visual analysis according to the criteria established by others (8, 9).

Micronuclei assay

At harvesting time, cell suspension were spread over microscopy slides, fixed in methanol for 10 minutes and stained with 10% Giemsa in PBS (pH 6.8) for 7 min. The micronuclei (MN) frequency was analyzed in 1000 cells per treatment group. The criteria for the identification of micronuclei were as follows:

- the nuclei and micronuclei should be round;
- the micronuclei should be smaller than 1/3 of the main nuclei;

- the micronuclei must not touch the main nuclei;
- the micronuclei must be the same color and intensity as the main nuclei (10).

Statistical analysis

Statistical analysis of acridine data was based on cells/group after arcsine \sqrt{x} transformation (x = number of death cells). Transformed data (arcsine) were subjected to analysis of variance (ANOVA) and a Fisher's test was used when there was statistically significant difference. The mean of MN frequency was calculated from the two independent experiments for each treatment and differences between defined groups were tested for significance using analysis of variance (ANOVA) and a Fisher's test was used when there was statistically significant difference. Differences were considered significant at $p < 0.05$.

RESULTS

Trypan blue dye exclusion showed that NaOCl (2%), $\text{Ca}(\text{OH})_2$ alone or in combination with Tergentol – HCT₂₀ induced cytotoxicity in KB cells compared to the control group. However, the cytotoxicity these solutions were time or dose-dependent; while $\text{Ca}(\text{OH})_2$ solution showed cytotoxicity only at 2 hours, NaOCl (2%) was cytotoxic at 2 and 24 hours. When HCT₂₀ treated cells was evaluated, no viable cells were observed after two hours. This cells fragmentation, after HCT₂₀ treatment, didn't allow the others cytotoxicity and genotoxicity assays in this experimental group. It is important to notice that NaOCl (1%) was similar to negative control group, and didn't presented cytotoxicity to KB cells in *trypan blue* assay (Figure 1).

Cell death quantification, distinguishing the necrotic and the apoptotic cells, was achieved by acridine orange/ethidium bromide double staining assay. NaOCl promoted a cytotoxicity to KB cells depended of the solution concentration (1 or 2%). While NaOCl (1%) was similar to negative control; NaOCl (2%) presented cytotoxicity activity to KB cells causing cell death mainly by apoptosis (Figure 2).

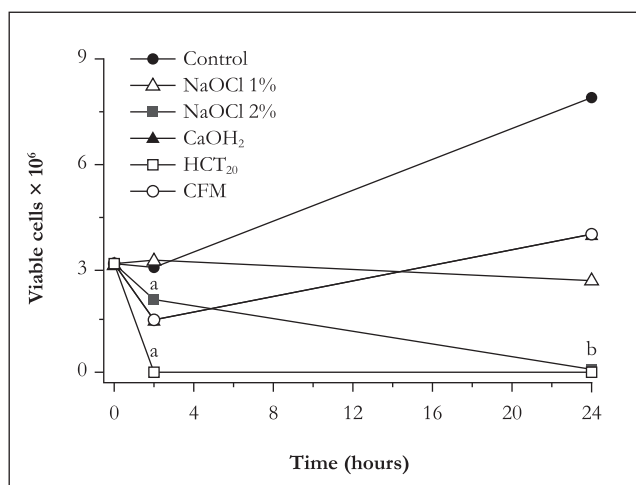


Figure 1 - Curve viability of KB cells submitted to different treatments after 2 and 24 hours

Note: a) Indicates significant difference to 2 hours control ($p < 0.05$);
 b) Indicates significant difference to 24 hours control ($p < 0.05$).
 CFM means cyclophosphamide group.

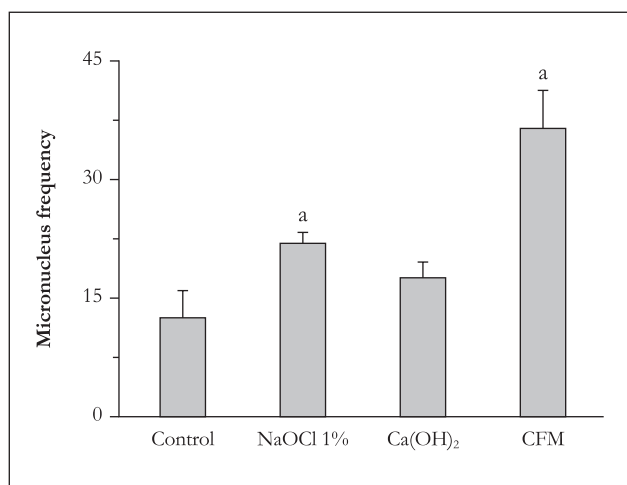


Figure 3 - Frequency of cells with micronucleus after 24 hours of different treatments. (a) Significant difference to control ($p < 0.05$). CFM means cyclophosphamide group

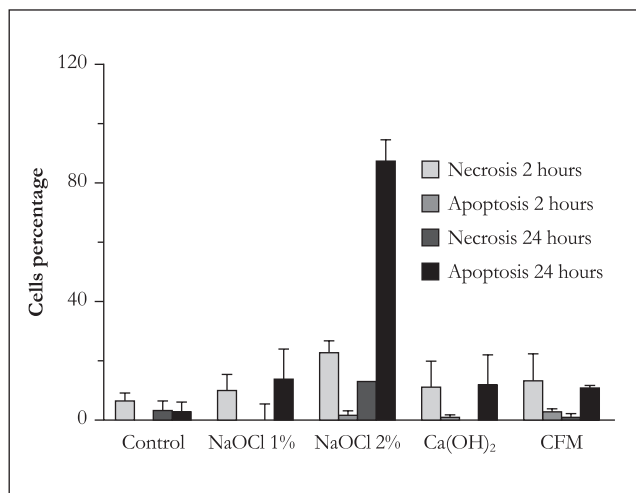


Figure 2 - Differential cell death percentage (necrosis/apoptosis) of KB cells submitted to different treatments

Apoptotic cells were characterized by morphologic alterations such as chromatin fragmentation or condensation and apoptotic bodies.

The genotoxicity, represented by the micronuclei frequency, showed that KB cells treated with NaOCl at lower concentration (1%) present an increase in the frequency of micronucleus compared to the control group (Figure 3).

NaOCl (2%) genotoxicity activity wasn't evaluated due to the in cells fragmentation in this group after 24 hours.

Discussion and conclusion

The present study showed that NaOCl (2%), Ca(OH)₂ and HCT₂₀, solutions induced cytotoxicity in KB cells; in addition, NaOCl (1%) induced increased in frequency of micronuclei. The literature has a series of case related of toxicity acute manifestation after the extruding of irrigant solution to surrounding tissues. These cases are generally related with high concentrations irrigants solutions use during endodontic therapy (1, 3). However, low concentrated solutions might cause severe consequences when inadvertently extruded into periradicular tissues.

The sanitization process from infected root canal is the main objective of endodontic therapy. Thus, biomechanical cleaning and shaping added to chemical irrigant adjuvant can reduce, but not eliminate, endodontic microorganisms. Although, various irrigant solutions have been proposed to endodontic therapy, almost all of them are harmful when outside the root canal and causes damage when in contact with the periradicular tissues. In

this way, endodontic irrigant solution could damage permanent tooth follicles, peripheral tissues and oral mucosa (11).

It is important to notice that when the cells were treated with HCT₂₀ no viable cells were observed two hours after treatment. The effectiveness of calcium hydroxide on microorganisms control is attributed to the high pH; as result of dissociation and diffusion of hydroxyl ions (12). The optimum effects of these hydroxyl ions are probably due to one of the following mechanisms: damage to the cytoplasmic membrane, protein denaturation, or DNA damage induction (13). According to Seabra et al. (14), the addition of a tension-active anionic detergent (tergentol) to calcium hydroxide reduces the surface tension of the solution, which might increase the action of calcium hydroxide. The exposure of mammalian cells to sodium lauryl sulfate solutions *in vitro* increased cell membrane permeability (15). The additional effects of calcium hydroxide to sodium lauryl sulfate mechanisms could explain the high cytotoxicity of HCT₂₀ observed in this study.

NaOCl was cytotoxic only at high concentration (2%) and NaOCl at lower concentration (1%) was genotoxic observed by the increase in micronuclei observed in KB cells. It is proposed that the mechanism of this substance is based in the degradation of lipid structures and protein inactivation, promoted by the saponification and chloroamination reactions, respectively. The chloroamination reaction produces free radicals such as hydroxyl ions that create an alkaline environment that promote biological stress to different cellular regions, including nucleus compartment (16). Thus, the difference in cell responses dependent to the NaOCl concentration can be associated with great generation of free radicals by cells treated with the higher NaOCl concentration (2%). Free radicals may be responsible for the induction of damage to the cellular DNA that leads to the formation of chromosome aberrations (17). We speculate that chromosome aberrations induced by NaOCl (1%), observed by micronuclei formation, are associated with the increase generation of free radicals and the generations of free radicals are a significant component of its cytotoxicity action. Thus, cells treated with the higher NaOCl concentration (2%) were severely damaged in the first cycle. Because of that, no cells viable were observed at 24 hours. On

other hand, the lower NaOCl concentration (1%) did not cause cell death in the initially (2 hours) treatment and the continuous exposure to NaOCl (1%) to the remaining cells may have contributed to the continues free radicals generation and DNA damage formation represented by the increase in micronucleus frequency.

In our study we also showed that Ca(OH)₂ alone and NaOCl induced death cell mainly by apoptosis in KB cells. Apoptosis describes the orchestrated collapse of a cell, staging membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation and DNA degradation followed by rapid engulfment of corpses by neighboring cells. It is an important process in the maintenance of normal physiology; as part of a surveillance system that eliminates damaged or altered cells, thus reducing the potential for genetic instability and cellular dysfunction.

As reviewed by Ishikawa et al. (18), in response to genotoxic stress, cell cycle checkpoints which slow down or arrest cell cycle progression can be activated, allowing the cell to repair or prevent the transmission of DNA damaged. Checkpoint machineries can also initiate pathways leading to apoptosis and the removal of a damaged cell from a tissue. The balance between cell cycle arrest and damage repair on one hand and the initiation of cell death, on the other hand, could determine if DNA damage is compatible with cell survival or requires cell elimination by apoptosis.

Clearly, the use of the best possible irrigant solution during chemo-mechanical preparation is of great clinical importance. Any chosen solution has to be cytotoxicity to microorganism and maintain the normal architecture of the surrounding tissue; however our results showed cytotoxicity on human cells, or at least certain degree of genotoxicity at NaOCl in lower concentration (1%), of the solutions used in this study. Based on this study, in spite of a great efficacy of the higher concentrated solutions, it is imperative the use of lower concentration in order to avoid extensive DNA damage in the normal cells.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest in the present manuscript.

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