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Toxicity of irrigating solutions and pharmacological associations used in pulpectomy of primary teeth

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Abstract

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Aim To evaluate the *in vitro* toxicity of irrigating solutions and pharmacological associations used in the pulpectomy of primary teeth.

Methodology The cell viability (MTT), lipid peroxidation (TBARS), alkaline comet assay and GEMO tests were performed to evaluate the cytotoxicity and genotoxicity of solutions: sodium hypochlorite (1% and 2.5%), 2% chlorhexidine, 6% citric acid and 17% EDTA, which were tested, individually and in association, exposing human peripheral blood mononuclear cells (MTT, TBARS and alkaline comet assay), at 24 and 72 h, and dsDNA (GEMO). After performing the Kolmogorov–Smirnov test, data were analysed by ANOVA followed by Dunnett's *post hoc* test, and Kruskal–Wallis followed by Dunn *post hoc* test. A significance level was established at $P < 0.05$.

Results All irrigating solutions and pharmacological associations reduced cell viability at 24 h ($P < 0.05$). These reductions were maintained after 72 h, except for EDTA and associations of sodium hypochlorite (1% and 2.5%) with EDTA and of chlorhexidine with EDTA. Lipid peroxidation at 24 h was caused by EDTA and by 2.5% sodium hypochlorite with EDTA; it was also caused at 72 h by sodium hypochlorite (1% and 2.5%) and the three associations with citric acid ($P < 0.05$). All groups caused DNA damage when assessed by the alkaline comet assay, at 24 h and 72 h ($P < 0.05$). In the GEMO assay, all groups caused dsDNA damage ($P < 0.05$), except for chlorhexidine with EDTA.

Conclusion All groups showed some level of toxicity. Amongst the main solutions, chlorhexidine presented less cytotoxic potential. EDTA was the least cytotoxic of the auxiliary irrigant solutions, and the association of these two solutions showed the lowest toxicity potential amongst all groups.

Keywords: biocompatibility, deciduous tooth, pulpectomy, root canal irrigants, toxicity tests.

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Introduction

When primary teeth require a pulpectomy, due to decay or trauma, the complex morphology of the canal system of these teeth makes instrumentation challenging (Ahmed 2013, Wang *et al.* 2013). Therefore, irrigating solutions play a significant role in eliminating microorganisms, dissolve and remove tissue debris, including the smear layer (Rodd *et al.* 2006, Barcelos *et al.* 2012). These solutions came into intimate contact with periapical tissues when used in pulpectomy (Poornima & Subba Reddy 2008),

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especially in primary teeth with root resorption, when the identification of the anatomical apex is difficult (Camp 2008). In addition, deciduous molars often have accessory canals in the floor of the pulp chamber, with the chance of irrigants extruding into the furcation region, where the permanent tooth germ lies, which is an additional concern (Kumar 2009). Thus, it is essential to evaluate the biocompatibility of these solutions (Nishimura *et al.* 2008).

Sodium hypochlorite (NaOCl) and chlorhexidine digluconate (CHX) are widely used as main irrigants with demonstrated efficacy in antimicrobial capacity and specific properties such as the dissolution of tissue (NaOCl) and substantivity (CHX) (Ercan *et al.* 2004, Bulacio *et al.* 2006, Sassone *et al.* 2008, Carrilho *et al.* 2010). However, they are not able to remove the smear layer, which is essential for dissemination of disinfectants into dentinal tubules (Shahravan *et al.* 2007). Thus, to fulfil this need associations amongst main solutions with auxiliary solutions, which are chelants, are essential, for example 17% ethylenediaminetetraacetic acid (EDTA) and 6% citric acid (Pitoni *et al.* 2011, Barcelos *et al.* 2012).

To establish biocompatibility and safety in the use of such solutions and pharmacological combinations, it is desirable to conduct a variety of tests that analyse several parameters (Tang *et al.* 1999), such as *in vitro* cytotoxicity and genotoxicity (International Standard Organization, ISO 10993-5 2009). Cytotoxicity is related to the degree to which an agent has specific destructive action on certain cells (Peters 2013), whilst genotoxicity is the potential damage of certain substances to DNA (Ribeiro 2008). There is no consensus in the literature on the potential toxicity of these solutions. The contradictory findings may be related to differences in the concentrations of the solutions that have been studied, the different cell types and methodologies used in the tests (Lee *et al.* 2010) and, moreover, there are few studies testing the genotoxicity effect of these irrigants, as well as the toxicity of associated solutions having not yet been investigated.

This study aimed to verify the cytotoxicity and genotoxicity by analysing different parameters of the main and auxiliary irrigating solutions and associations amongst them, used in pulpectomy in primary teeth. The solutions were evaluated at the concentrations used in clinical practice on human peripheral blood mononuclear cells (PBMCs), which has been applied for decades as biomarkers of cytotoxic and genotoxic effects (Ciapetti *et al.* 1983). The null hypothesis was that there is no difference in the parameters of toxicity in irrigating solutions and pharmacological associations evaluated.

Materials and methods

Ethical approval

This study was approved by the Ethics Committee of the Federal University of Santa Maria, RS, Brazil (No. CAAE 20457313.7.0000.5346).

Experimental groups

All solutions were manipulated by 'NOVA DERME manipulation pharmacy' (Santa Maria-RS, Brazil) with doubled concentration. Solutions and pharmacological associations were placed in contact with the cell culture in the ratio 1 : 1 (100 mL solution + 100 mL of cell culture), so the concentration of the solutions was reduced to half by dilution in the medium. Thus, the desired concentrations in the study, similar to that used in dental practice, could be obtained and the irrigating solutions were effectively tested at concentrations of NaOCl (1% and 2.5%), 2% CHX, 6% citric acid and 17% EDTA.

Experimental groups were divided into twelve groups, including the control group, as described in Table 1.

Table 1 Experimental groups

Group	Irrigating soutions and pharmacological associations
Control	Cells in culture environment and phosphate-buffered saline (PBS)
1% NaOCl	1% sodium hypochlorite
2.5% NaOCl	2.5% sodium hypochlorite
2% CHX	2% chlorhexidine digluconate
6% CA	6% citric acid
17% EDTA	17% ethylenediaminetetraacetic acid
1% NaOCl + 6% CA	1% sodium hypochlorite and 6% citric acid
1% NaOCl + 17% EDTA	1% sodium hypochlorite and 17% ethylenediaminetetraacetic acid
2.5% NaOCl + 6% CA	2.5% sodium hypochlorite and 6% citric acid
2.5% NaOCl + 17% EDTA	2.5% sodium hypochlorite and 17% ethylenediaminetetraacetic acid
2% CHX + 6% CA	2% chlorhexidine digluconate and 6% citric acid
2% CHX + 17% EDTA	2% chlorhexidine digluconate and 17% ethylenediaminetetraacetic acid

Cell culture

The PBMCs derived from blood discard samples of healthy adults. Blood specimens were subjected to the procedure of separation of its constituents by density gradient using Ficoll Histopaque-1077® reagent (Sigma-Aldrich, St. Louis, MO, USA) followed by centrifugation at 1611 ***g*** for 30 min. The PBMCs were plated in 6-well plates containing RPMI 1640 culture with 10% foetal bovine serum, 1% penicillin and streptomycin. The cells were cultured at an initial density of 2×10^5 cells mL^{-1} of material.

Treatment of PBMCs

Cell suspensions were exposed to the irrigating solutions and pharmacological associations, and incubated at 37 °C for periods of 24 h and 72 h. To avoid interference of the coloration of the culture environment in the tests, samples were collected from the culture wells and centrifuged in Falcon tubes for 10 min at 2222 ***g*** obtaining a pellet containing the treated PBMCs, which were resuspended in 2 mL phosphate-buffered saline (PBS) for later conducting the cell viability (MTT Assay), lipid peroxidation (TBARS Assay) and genotoxicity (Alkaline Comet Assay) tests.

dsDNA

In the GEMO Assay, DNA from calf thymus (dsDNA) purchased from Invitrogen (Eugene, OR, USA) was used. The dsDNA were distributed in black 96-well plate and exposed to the solutions just before the test.

Cytotoxicity evaluation

Cell Viability Assessment by MTT Assay (Mosmann 1983, Denizot & Lang 1986)
200 μL of sample was distributed in 96-well plate and 20 μL of 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide reagent – MTT was added, (5 mg mL^{-1} diluted in phosphate buffer pH 7.4). The plate was incubated for 1 h at 37 °C and was further centrifuged for 10 min at 2222 ***g***, the supernatant was removed and added to 200 μL of dimethyl sulfoxide (DMSO, Sigma®), then centrifuged again for 10 min at 2222 ***g***, so that the formazan crystals were released into the extracellular environment. The supernatant was removed, and then the reading of the data was performed. The test was performed in triplicate, and the absorbance was measured by colorimetrically spectrophotometry at a wavelength of 570 nm. The mean of the triplicate was calculated and submitted to statistical analysis.

Lipid peroxidation by determining the thiobarbituric acid-reactive substances/TBARS Assay (Ohkawa et al. 1979)
The treated cells were centrifuged for 10 min at 2222 ***g*** to remove the culture medium. The supernatant was discarded and followed by two centrifugations with saline solution (0.9% NaCl) for 10 min at 2222 ***g***. Then, the supernatant was discarded and added to 100 mL butylhydroxytoluene (BHT 10 mmol L^{-1}), 500 μL of trichloroacetic acid (20% TCA) and left for a final centrifugation step for 5 min at 2222 ***g***. Immediately after centrifugation, 900 μL of supernatant was mixed in a reaction environment containing 0.8% TBA and incubated at 95 °C in a water bath for 1 h. After cooling the samples, the absorbance readings were made at a wavelength of 532 nm using a spectrophotometer. One malondialdehyde curve was generated to determine the equivalents of each treatment in question. The test was performed in triplicate, and the average was submitted to statistical analysis.

Evaluation of genotoxicity

Alkaline Comet Assay (Singh et al. 1988, adapted by García et al. 2004)
The treated cells suspended in low-melting agarose point (low melting) were deposited on a glass slide pre-coated with a layer of 1.5% agarose. The material was immersed in lysis solution (lysis solution of 89–10 mL of dimethyl sulfoxide and 1 mL of Triton X-100) for the removal of membranes and cytoplasm. Subsequently, the slides were incubated in alkaline electrophoresis buffer at pH 13 (300 mmol L^{-1} NaOH and 1 mmol L^{-1} EDTA in distilled water) and subjected to electrophoresis for 30 min at 25 V and 300 mA. Subsequently, the processes of neutralization (neutralizing buffer pH 7.5), fixation (a fixing solution composed of 15% trichloroacetic acid) and coloration (silver nitrate and 5% sodium carbonate) were performed, so the genetic material could be analysed. Two experienced, blinded and trained evaluators analysed one hundred cells (50 cells from each slide) in an optical microscope (400×) and classified the cells according to tail length. The cells received scores from 0 (no damage) to 4 (maximum damage). The test was performed in duplicate, and the data

were transformed into a damage index (Montagner *et al.* 2010) to be submitted to statistical analysis.

Genomodifier Capacity Assay/GEMO Assay (Cadoná et al. 2014)
In this test, performed in triplicate, 10 μL of dsDNA (1 mg mL^{-1}) was distributed in a black 96-well plate and was were exposed to 100 μL of irrigating solutions or pharmacological associations (90 μL of TE was added to obtain a final volume of 200 μL), remaining in contact for 30 min. After this treatment, the dye PicoGreen® (1 : 200 TE) was added to the wells and the fluorescence was read after five minutes, at room temperature, at a wavelength of 480 nm excitation and 520 nm emission. The average of the triplicate was calculated to be submitted to statistical analysis.

Statistical analysis

The data were tabulated and analysed according to the average and standard deviation using three statistical programs: Minitab (version 17, Inc. State College, PA, USA), GMC Basic Software (version 7.7, School of Dentistry of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil) and Statistical Package for Social Sciences (SPSS) (version 12.0, SPSS Inc., Chicago, IL, USA). First, data normality was assessed by the Kolmogorov–Smirnov test. Data generated by TBARS 72 h and alkaline comet assay 72 h were assessed using ANOVA followed by *post hoc* Dunnett tests, whilst data for all other tests were evaluated by the Kruskal–Wallis test followed by *post hoc* Dunn. The significance level was established at $P < 0.05$.

Results

The results of the cytotoxicity and genotoxicity tests are presented in Figs 1–4.

Cytotoxicity

Cell Viability – MTT Assay
In PBMCs exposure to irrigating solutions and pharmacological associations, at 24 h, a decrease in cell viability was observed in all tested groups compared to the control group ($P < 0.05$). After 72 h, the groups remained decreased, except for 17% EDTA and (1% and 2.5%) NaOCl + 17% EDTA ($P < 0.05$). The 2% CHX + 17% EDTA had cell viability similar to the control group at 72 h ($P > 0.05$) (Fig. 1).

Lipid peroxidation – TBARS Assay
The 17% EDTA and 2.5% NaOCl + 17% EDTA increased the levels of lipid peroxidation at 24 h when compared with the control group ($P < 0.05$). After 72 h, high levels of lipid peroxidation were observed in (1% and 2.5%) NaOCl and in the three associations with 6% citric acid ($P < 0.05$) (Fig. 2).

Genotoxicity

DNA damage – Alkaline Comet Assay
The results showed that in all groups, the irrigating solutions and pharmacological associations, promoted DNA damage of PBMCs when compared to the control group at 24 h and 72 h ($P < 0.05$) (Fig. 3).

dsDNA damage – GEMO Assay
All groups, except for 2% CHX + 17% EDTA, caused dsDNA damage compared to the control group ($P < 0.05$) (Fig. 4).

Discussion

In this study, several parameters were used to evaluate the toxicity of irrigating solutions and pharmacological associations in the cells in order to obtaining more complete information (Tang *et al.* 1999). The toxicity of pharmacological associations was evaluated for their recognized superiority in the decontamination of the canal system with the use of an antimicrobial solution followed by a chelating solution for removing the smear layer (Barcelos *et al.* 2012). Although these solutions are not mixed during use, the infiltration of dentinal tubules and the occasional diffusion to periapical tissues will cause an interaction between them (Rasimick *et al.* 2008). So far, no studies were found to have evaluated the toxicity of main and auxiliary irrigating solutions in association, as proposed in this work. In addition, although the products are generally analysed at dilutions, in this study the materials at the concentrations used in clinical practice were evaluated, as the amount and concentration in which those products reach the apical region is uncertain (Lee *et al.* 2010).

PBMCs were chosen because they are cell types present in the periapical region, where intimate contact with the solutions evaluated in this study is established (Bartelstone 1951). In addition, peripheral blood lymphocytes have been applied for decades in cytotoxicity (Ciapetti *et al.* 1983) and genotoxicity

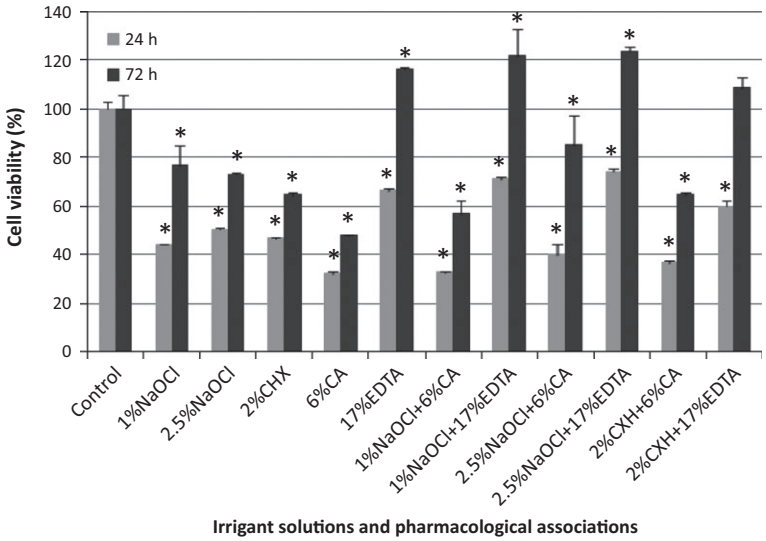

Figure 1 Cytotoxicity by MTT Assay in PBMCs after exposure to irrigating solutions and pharmacological associations. The cell cultures were exposed for 24 and 72 h. Bars represent the means (±SD). Significant differences between untreated (Control) and treated cell cultures are indicated by asterisks.

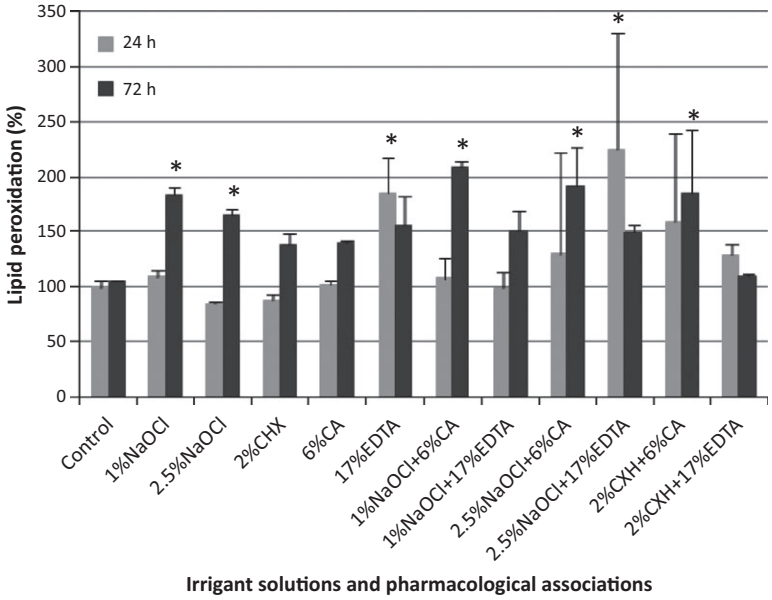

Figure 2 Cytotoxicity by Oxidative Stress/TBARS Assay (Lipid Peroxidation) in PBMCs after exposure to irrigating solutions and pharmacological associations. The cell cultures were exposed for 24 and 72 h. Bars represent the means (±SD). Significant differences between untreated (Control) and treated cell cultures are indicated by asterisks.

assays, as biomarkers of the primary genotoxic effects (Maluf & Riegel 2011).

The MTT assay evaluates cell viability of a sample, and the level of mitochondrial metabolic activity of viable cells (Ciapetti *et al.* 1983, Chan *et al.* 1999). In the present study, there was a reduction in cell viability at both time periods with the main irrigating solutions (1% and 2.5%) NaOCl and 2% CHX. These results are in agreement with Navarro-Escobar *et al.* (2010), who evaluated cell viability by MTT at 24 h of 2.5% NaOCl in two dilutions, 0.1% and 0.5% on fibroblasts (NIH/3T3) and also found a significant

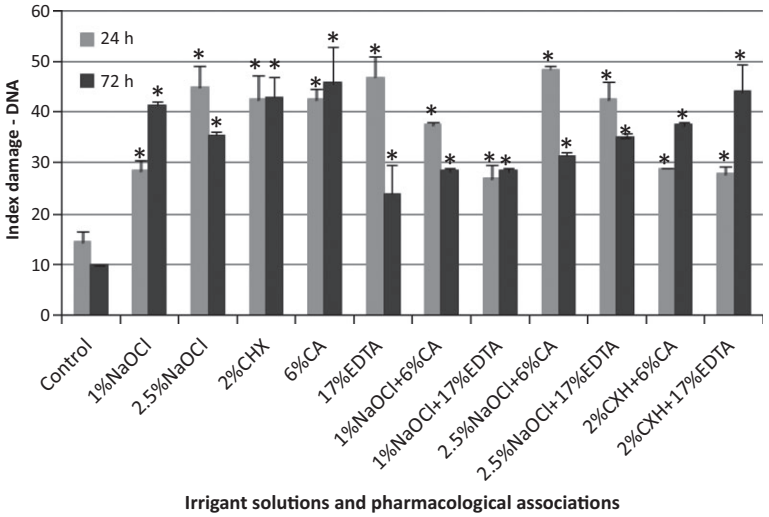

Figure 3 Genotoxicity by Alkaline Comet Assay in PBMCs after exposure to irrigating solutions and pharmacological associations. The cell cultures were exposed for 24 and 72 h. Bars represent the means (±SD). Significant differences between untreated (Control) and treated cell cultures are indicated by asterisks.

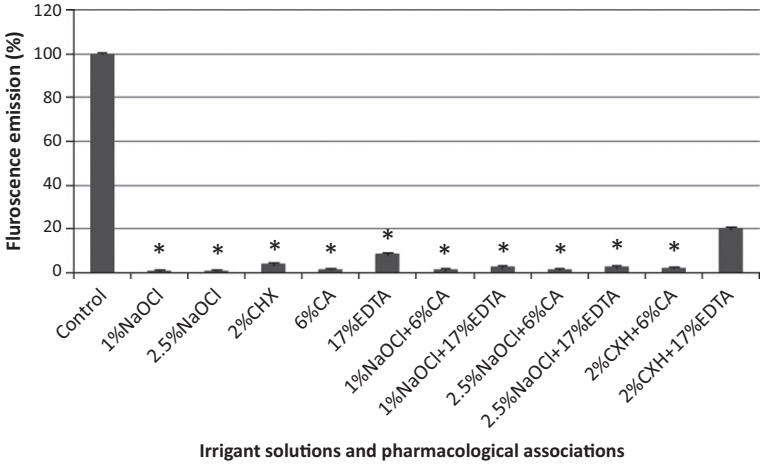

Figure 4 Genotoxicity by GEMO Assay in dsDNA after exposure to irrigating solutions and pharmacological associations. Bars represent the means (±SD). Significant differences between untreated (Control) and treated cell cultures are indicated by asterisks.

reduction in 0.5%, only 0.1% dilution had higher levels of cell viability. Regarding CHX, Lessa *et al.* (2010) also found that 2% CHX, decreased cell viability, using the MTT test, in odontoblasts (MDPC-23), in accordance with the findings of this research.

Although reactive oxygen species (ROS) are observed in various physiological conditions, as an integral part of human metabolism there is strong evidence of an overproduction ratio of ROS in processes such as DNA and cellular function change (Yang *et al.* 2008). Lipid peroxidation is the damage to the cell membrane as the result of oxidative stress, a consequence of the increased production of ROS. In the oxidative stress induction test (TBARS), CHX did not cause lipid peroxidation at any of the time periods. However, NaOCl (1% and 2.5%) caused lipid peroxidation at 72 h, indicating that long-term contact with the cells can cause oxidative stress. Saghiri *et al.* (2011) suggested that the high pH of sodium hypochlorite interferes with the integrity of the cytoplasmic membrane and is related to the destruction of its phospholipids, agreeing with the findings of the

present study. Thus, it can be suggested that NaOCl in both concentrations has greater potential for toxicity to PBMCs than CHX.

The alkaline comet assay is a standard method for evaluating DNA damage, of simple execution and high sensitivity (García *et al.* 2004), whilst the GEMO assay also allows the analysis of the potential mutagenic ability of a product, but without the interference of cellular metabolic and physiological variables. It is an ultrasensitive, simple, fast and accurate test (Cadoná *et al.* 2014). The two main solutions (1% and 2.5% NaOCl and 2% CHX) had genotoxic potential in both tests. Genotoxicity studies on the CHX as an endodontic antimicrobial agent are scarce and most studies investigated the adverse effects of this solution for mouthwash use. Ribeiro *et al.* (2005) tested the genotoxicity of CHX at various concentrations (0.1% to 1%) by the alkaline comet assay in Chinese hamster ovary cells. They found no DNA damage, in contrast to the results of the present study. This difference between the results may be related to the concentrations of tested solutions, which were lower than in this study. Marins *et al.* (2012) also found no DNA damage with NaOCl (1.25%, 2.5% and 5%) in the alkaline comet assay, but the exposure time was lower (3 h).

In evaluating the auxiliary irrigating solutions, 17% EDTA caused a reduction in cell viability and lipid peroxidation at 24 h, which was not maintained after 72 h. This recovery of cell viability in culture was also verified by Saghiri *et al.* (2011), which evaluated the cell viability of fibroblasts (human gingiva) exposed to 17% EDTA by MTT at 1, 6 and 12 h. Despite the different time periods, these authors observed a reduction in fluorescence in the cultures during the first hours (1–6 h) followed by an increase in fluorescence after 12 h, reflecting a reduction of cytotoxicity in long-term results. These findings were associated with factors related to nutritional reserves, dispersion capacity of substances in the environment and the adaptation of cells to the environment (Saghiri *et al.* 2011).

The 6% citric acid caused a decrease in cell viability at both time periods, which may be related to the change of the pH in the culture exposed to acidic pH solution. This justification was suggested by Chan *et al.* (1999) who also observed strong cytotoxicity of this solution in the MTT assay and reported high rates of cell death (human dental pulp cells) starting at 0.5%. Navarro-Escobar *et al.* (2010) also reported a reduction in cell viability evaluating 15% citric acid with the MTT assay at 24 h, with fibroblasts (3T3L1) exposed to the diluted solution of 0.1% and 0.5%. Although the two auxiliary solutions have toxic effects on PBMCs, it is possible to suggest a less cytotoxic potential of EDTA, especially since the culture showed high levels of cell viability in the long-term (72 h).

The two solutions (17% EDTA and 6% citric acid) had genotoxic potential, causing DNA and dsDNA damage. This contrasts with the results of Marins *et al.* (2012), who evaluated genotoxicity by alkaline comet assay with murine fibroblasts exposed to 17% EDTA for 3 h and to citric acid (10.5% and 21%) and did not observe DNA damage in any of these solutions. It is believed that the difference in results is related to the difference in the time of exposure of the cells to the tested solutions.

In associations of the main solution, NaOCl (1% and 2.5%) with auxiliary solutions (17% EDTA and 6% citric acid), DNA and dsDNA damage were observed in all combinations. In the cytotoxicity tests, the toxicity potential of NaOCl associated with citric acid was higher than NaOCl with EDTA. The NaOCl associations with EDTA revealed reduced cell viability by MTT assay at 24 h, which was not maintained after 72 h, and lipid peroxidation occurred only at the highest concentration of NaOCl at 24 h and it was also not maintained after 72 h. Meanwhile the NaOCl associations with citric acid decreased cell viability in both time periods and still induced lipid peroxidation at 72 h, demonstrating the long-term oxidative stress induction.

These results may be related to a by-product of the reactions of these associations, chlorine gas, which has potentially harmful effects to humans and that is produced in the reduction in pH values of NaOCl when associated with chelating solutions (Baumgartner & Ibay 1987). These authors evaluated the formation of chlorine gas in the association of 5.25% NaOCl with 15% EDTA or 50% citric acid and found that chlorine gas formation was more detectable, and present at a longer distance, in the NaOCl interaction (pH = 12.12) with citric acid (pH = 1.28) than in the combination of NaOCl with EDTA (pH = 7.51), reporting that the lowest pH of the citric acid increased the initial reaction energy. Prado *et al.* (2013) also evaluated the combination of 2.5% NaOCl with 17% EDTA and 10% citric acid and observed the formation of bubbles, that were essentially chlorine gas and more intense with citric acid.

In the association of the main solution, 2% CHX with auxiliary solutions (17% EDTA and 6% citric acid), the

interaction with citric acid also showed greater toxicity than with EDTA. Whilst a interaction of CHX with citric acid generated a reduction of cell viability at 24 and 72 h, lipid peroxidation at 72 h and DNA damage, the combination of CHX with EDTA had the lowest toxic potential of all pharmacological associations. Although this combination caused DNA damage in the alkaline comet assay and reduced cell viability at 24 h, recovery of cell viability occurred after 72 h to rates similar to the control and oxidative stress or dsDNA damage in GEMO assay were not observed.

There are few studies evaluating the interaction between these solutions. In the association of 2% CHX with 10% citric acid, no by-product was observed and in the association of 2% CHX with 17% EDTA a milky white precipitate was noticed, probably related to an acid base reaction (Prado *et al.* 2013). This probable electrostatic neutralization reaction of CHX (cationic) and EDTA (anionic), as reported by Rasimick *et al.* (2008), could be related to the lower toxicity of this association in the present study. Meanwhile, the absence of precipitate in the interaction of CHX with citric acid (Prado *et al.* 2013) could be an absence of reaction between the solutions, which would preserve their original properties and toxicity. Therefore, there is need for more tests to test these hypotheses.

In vitro tests are useful to understand the basic biological effects of dental materials, but are limited in their ability to simulate clinical conditions. In the human body, cytotoxic and genotoxic effects can be different because of the cell repairing process. However, the *in vitro* toxicity evaluation generated valuable information about the toxic potential of the tested materials (Koulaouzidou *et al.* 1999, Malheiros *et al.* 2005). Thus, a sequence of standardized tests to continue the evaluation of the biocompatibility of these solutions and associations are suggested by the International Standard Organization ISO 10993-5 2009.

Conclusion

All the tested irrigating solutions and pharmacological associations demonstrated some level of cytotoxicity or genotoxicity. However, by comparing the results obtained, it was observed that amongst the main solutions CHX had less cytotoxic potential, and in the auxiliary solutions EDTA was less cytotoxic to PBMCs. Analysing the pharmacological associations, the interaction of CHX with EDTA had the least potential for toxicity amongst all groups, for it showed cell viability recovery levels at 72 h, and did not cause damage to dsDNA in the GEMO test. From the results, the biocompatibility of the CHX and EDTA combination was demonstrated.

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