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Effects of Calcium Hypochlorite and Octenidine Hydrochloride on L929 And Human Periodontal Ligament Cells

Hernán Coaguila-Llerena[1], Elisandra Márcia Rodrigues[1], Mário Tanomaru-Filho[1], Juliane Maria Guerreiro-Tanomaru[1], Gisele Faria[1]

[1]Department of Restorative Dentistry, Araraquara School of Dentistry, UNESP – Universidade Estadual Paulista, Araraquara, SP, Brazil

Correspondence: Gisele Faria, Rua Humaitá, 1680, 14.801-903 Araraquara, SP, Brasil. Tel: +55-16-3301-6398. e-mail: giselefaria@foar.unesp.br

The aim of this study was to assess cytotoxicity and cell migration of calcium hypochlorite [$Ca(OCl)_2$] and octenidine hydrochloride - OCT (Octenisept®, Schülke & Mayr, Norderstedt, Germany) in L929 and human periodontal ligament (hPDL) cells. The cells were exposed to different doses of different solutions: 2.5% and 5% $Ca(OCl)_2$, 0.1% OCT, 2.5% NaOCl and 2% CHX for 10 min. Cell viability was assessed by methyl-thiazol-tetrazolium (MTT) and neutral red (NR) assays, and cell migration was determined by wound-healing assay. Statistical analysis was performed by two-way ANOVA and Bonferroni tests (α=0.05). The MTT and NR assays revealed that 0.1% OCT was less cytotoxic in hPDL cells ($p<0.05$), followed by 2% CHX and 2.5% $Ca(OCl)_2$ ($p<0.05$). There was no significant difference between 2.5% NaOCl and 5% $Ca(OCl)_2$ ($p>0.05$), but these solutions showed greater cytotoxicity than the others. The result was the same for L929 cells, except that there was no significant difference between 2% CHX and 2.5% $Ca(OCl)_2$ ($p>0.05$). Wound-healing assay in L929 and hPDL cells showed that cell migration of 0.1% OCT, 2% CHX and 2.5% $Ca(OCl)_2$ groups was higher than 5% $Ca(OCl)_2$ and 2.5% NaOCl groups at 24 h ($p<0.05$). In conclusion, 0.1% OCT had lower cytotoxicity in tested cell lines than CHX, $Ca(OCl)_2$ and NaOCl. Cell migration was higher for 0.1% OCT, 2% CHX and 2.5% $Ca(OCl)_2$. Therefore, in terms of cytotoxicity, OCT and $Ca(OCl)_2$ have the potential to be used as root canal irrigants.

Key Words: calcium hypochlorite, cytotoxicity, fibroblasts, octenidine, root canal treatment.

Introduction

Current scientific evidence indicates sodium hypochlorite (NaOCl) as the most widely used irrigant solution due to its potent antimicrobial activity (1) and organic dissolution capacity (2). However, NaOCl is cytotoxic at high concentrations (3), and has a pronounced negative effect on the survival and differentiation of stem cells of the apical papilla, factors which may hinder periapical repair and pulpal regeneration (4). CHX is a potent antiseptic used in endodontic treatment due to its antimicrobial efficacy (1) and substantivity (5). However, CHX cannot dissolve organic tissues (6). Currently, there is no root canal irrigant considered ideal, and alternative solutions continue to be studied.

Calcium hypochlorite [$Ca(OCl)_2$] has been studied as a root canal irrigant (7,8). It has tissue dissolution capacity (2) and higher chlorine content than NaOCl at the same concentration (9). The preparation of a $Ca(OCl)_2$ solution may be more accurate than that of NaOCl, because $Ca(OCl)_2$ powder can be weighed and incorporated into water prior to use. On the other hand, a NaOCl solution is prepared by diluting a more concentrated and therefore unstable solution, thus making it difficult to obtain an accurate concentration of NaOCl (9). When used as an irrigant solution during biomechanical preparation of *Enterococcus faecalis*-infected teeth, 2.5% $Ca(OCl)_2$ showed antibacterial efficacy similar to 2.5% NaOCl (7). Regarding cytotoxic effects, Blattes et al. (8) found no difference between $Ca(OCl)_2$ and NaOCl in 3T3 embryonic mouse fibroblast cells.

Octenisept® - OCT (Schülke & Mayr, Norderstedt, Germany) contains 0.1% octenidine hydrochloride (an antimicrobial agent) and 2% phenoxyethanol, a derivative of ethanol, which serves as a preservative (10). OCT is used primarily for antisepsis of burns and wounds, and as a mouthwash (10). As a root canal irrigant, OCT has showed similar efficacy to 2.5% and 5.25% NaOCl, and to 2% CHX against *Candida albicans* (11) and *E. faecalis* (12). Regarding cytotoxic effects, another 0.1% octenidine hydrochloride-based antiseptic, Octenidol®, has presented cytotoxicity lower than 0.2% CHX in human gingival fibroblasts and nasal epithelial cells (13).

Therefore, OCT and $Ca(OCl)_2$ have the potential to be used as alternative root canal irrigants to NaOCl and CHX. However, further studies comparing the cytotoxicity of OCT and $Ca(OCl)_2$ with that of other root canal irrigants in different cell lines are needed to indicate these solutions for endodontic treatment. The aim of this study was to assess effects of OCT and $Ca(OCl)_2$, in comparison with NaOCl and CHX, on viability and proliferation/migration of human periodontal ligament (hPDL) cells and L929 fibroblasts. The

null hypothesis was that there would be no difference in effects of solutions on viability and proliferation/migration of the cells tested.

Material and Methods

Preparation of Irrigant Solutions

The solutions evaluated were 2.5% and 5% $Ca(OCl)_2$ (Sigma-Aldrich, St. Louis, MO, USA), 0.1% OCT (Octenisept®, Schulke & Mayr), 2.5% NaOCl (AraQuímica, Araraquara, SP, Brazil) and 2% CHX (Reactive Manipulation Pharmacy, Araraquara, SP, Brazil). $Ca(OCl)_2$ solution was prepared immediately prior to use by diluting $Ca(OCl)_2$ powder in distilled water, and 2.5% NaOCl was prepared by diluting 9% NaOCl solution in distilled water. The available chlorine content in NaOCl and in $Ca(OCl)_2$ solutions was determined by the physicochemical spectrophotometric method (14). The $Ca(OCl)_2$ concentrations of 2.5% and 5%, as well as 0.1% OCT, 2.5% NaOCl and 2% CHX, were considered grade 1 dilutions, and were serially diluted in saline solution (0.9% sodium chloride) using a 1.5 dilution factor (15). The cells were incubated with solutions in the following dilutions: 1/111, 1/166, 1/250, 1/375, 1/562, 1/844, 1/1266 and 1/1898, which corresponded to doses/concentrations of 0.9%, 0.6%, 0.4%, 0.26%, 0.18%, 0.12%, 0.08% and 0.05%, respectively.

Cell Culture and Treatment Protocol with the Irrigant Solutions

Permanent cell lines of L929 mouse fibroblasts (American Type Culture Collection) and human hPDL cells were used. All procedures conformed to the applicable ethical guidelines and regulations of the dental school's Research Ethics Committee which approved the project, with written informed consent obtained from all subjects. Human third molars with no evidence of carious lesions or periodontal disease were obtained from healthy patients aged 16-25 year, who were being treated at the dental school's surgery clinic. After extraction, the teeth were immediately stored in Dulbecco's Modified Eagle's Medium - DMEM (Sigma-Aldrich). The periodontal ligament was removed from the middle third of the root surface with a #15 scalpel blade, and then fragmented and cultured in 100-mm culture dishes using the explant technique (16). hPDL cells from the 3rd through 6th passages were used, and assays were performed in triplicate, using cells from 3 donors.

Both hPDL and L929 cells were cultured with DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco/Invitrogen, Waltham, MS, USA), 1% penicillin and streptomycin (Sigma-Aldrich) (100.00 U/mL penicillin, 100.00 mg/mL streptomycin), and were incubated at 37°C in an atmosphere containing 5% CO_2 and 95% humidity.

The assays were performed by culturing cells in 24- or 96-well culture plates (Corning Inc., Corning, NY, USA), containing DMEM with 10% FBS, and incubating them for 24 h in order for them to adhere to the wells. Then, the culture medium was removed, and the cells were incubated with different doses of the solutions, for 10 min (15). The solutions were then removed, and the cells were incubated with culture medium containing 10% FBS for 4 h (15). Saline and DMEM were used as controls.

Cell Viability Evaluation by Methyl-Thiazol-Tetrazolium (MTT) and Neutral Red (NR) Assays

The hPDL ($8x10^4$ cells/mL) and L929 ($6.5x10^4$ cells/mL) cells were cultured in 96-well plates (Corning). The cell treatment protocol was performed with the tested solutions at doses of 0.05-0.9% and with the controls, after which the culture medium was removed and the MTT and NR assays were performed.

For the MTT assay, 100 µL of the 0.5 mg/mL MTT solution (Sigma-Aldrich) was added, and the cells were incubated for 3 h at 37 °C, in an atmosphere containing 5% CO_2 and 95% relative humidity. Then, 100 µL of acidified isopropyl alcohol (HCl: isopropyl alcohol, 0.04N) was added to the extract to solubilize the formazan crystals. The optical densities of the solutions were measured by spectrophotometer with a 570 nm wavelength filter (ThermoPlate, Nanshan District, Shenzhen, China). The absorbance readings were normalized with cells exposed to the saline, and represented succinate dehydrogenase activity (cell metabolism).

For the NR assay, 100 µL of 0.05 mg/mL NR solution (Sigma-Aldrich) was added, and the cells were incubated at 37 °C, in an atmosphere containing 5% CO_2 and 95% relative humidity for 3 h. Then, the solution was discarded and 100 µL of 1% acetic acid solution in 50% ethanol was added to each well. The optical densities of the solutions were measured by spectrophotometer with 570 nm wavelength filters (ThermoPlate). The absorbance readings were normalized with cells exposed to saline solution, and represented the ability to incorporate the dye into lysosomes of viable cells. The assays were performed in triplicate and repeated at three different times.

Wound-Healing Assay

The wound-healing assay is an established *in vitro* model to investigate proliferation and migration characteristics of cells maintained under different culture conditions (17). The hPDL ($5x10^5$ cells/mL) and L929 ($2.5x10^5$ cells/mL) cells were cultivated in 24-well culture plates (Corning). Then, the cells were removed with a P200 tip (TPP, Techno Plastic Products, Trasadigen, Switzerland), by creating an artificial gap, or so-called "scratch" or "wound," through the center of each well. Immediately afterwards, the cells

were washed twice with phosphate-buffered saline (PBS) and exposed to the treatment protocol with the tested solutions at a dose of 0.05% and the controls. To determine the cell growth area, the wells were photographed at 0, 8, 16 and 24 h by EVOS F1 microscope (AMC, Bothell, WA, USA), and the images were analyzed by two blinded and calibrated examiners, using 145 ImageJ software (National Institutes of Health, NIH, Bethesda, Maryland, USA). The experiments were performed in quadruplicate and repeated at two different times. Eight different fields per well were photographed and analyzed.

Statistical Analysis

The data were analyzed with Graph Pad Prism 5 statistical software (GraphPad Software, La Jolla, CA, USA), by using two-way ANOVA and the Bonferroni post-test (α=0.05).

Results

Cell Viability Evaluation by MTT and NR Assays

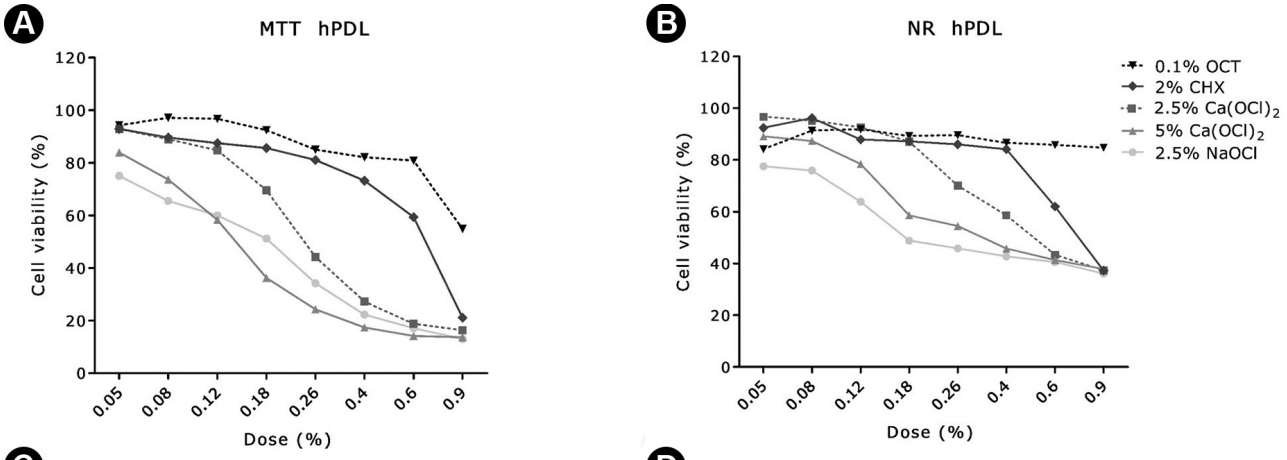
Cell viability is shown in Figures 1 and 2. All the solutions had a dose-dependent effect on cell viability; the higher the dose, the greater the cytotoxicity. The MTT and NR assays on hPDL cells revealed that 0.1% OCT was the least cytotoxic ($p<0.05$), followed by 2% CHX and 2.5% $Ca(OCl)_2$ ($p<0.05$). There was no significant difference between 2.5% NaOCl and 5% $Ca(OCl)_2$ ($p>0.05$), but these solutions had greater cytotoxicity than the others. The result was the same for the L929 cells, except that there was no significant difference between 2% CHX and 2.5% $Ca(OCl)_2$ ($p>0.05$). There was also no significant difference ($p>0.05$) in the response of the cell lines to saline and DMEM (controls).

A comparison of the response of both cell lines in the MTT assay showed that 0.1% OCT, 2.5% and 5% $Ca(OCl)_2$ were more cytotoxic to hPDL than L929 cells ($p<0.05$), and that 2% CHX was more cytotoxic to L929 than hPDL cells ($p<0.05$). There was no significant difference between the response of cell lines exposed to 2.5% NaOCl ($p>0.05$).

Wound-Healing Assay

In regard to the L929 cells (Fig. 3), at 8 h, the controls (culture medium and saline), as well as 0.1% OCT and 2% CHX showed higher wound closure than 2.5% $Ca(OCl)_2$ ($p<0.05$). Lower wound closure was observed for 5% $Ca(OCl)_2$ and 2.5% NaOCl ($p<0.05$). At 16 and 24 h, there was no significant difference among the controls, 0.1% OCT, 2% CHX and 2.5% $Ca(OCl)_2$ ($p>0.05$). These groups had higher wound closure than the 5% $Ca(OCl)_2$ and 2.5% NaOCl groups ($p<0.05$).

A similar pattern was observed in hPDL cells (Fig. 4), but at 16 h, the controls showed higher wound closure than the other solutions ($p<0.05$). At 24 h, no differences were found among the controls, 2% CHX, 0.1% OCT and 2.5% $Ca(OCl)_2$ ($p>0.05$), showing that these solutions had higher wound closure than 5% $Ca(OCl)_2$ and 2.5% NaOCl ($p<0.05$).



C

	0.05	0.08	0.12	0.18	0.26	0.4	0.6	0.9
0.1% OCT	a	c	b	c	c	b	b	b
2% CHX	a	bc	b	bc	c	b	c	a
2.5% $Ca(OCl)_2$	a	bc	b	b	b	a	a	a
5% $Ca(OCl)_2$	a	ab	a	a	a	a	a	a
2.5% NaOCl	a	a	a	a	ab	a	a	a

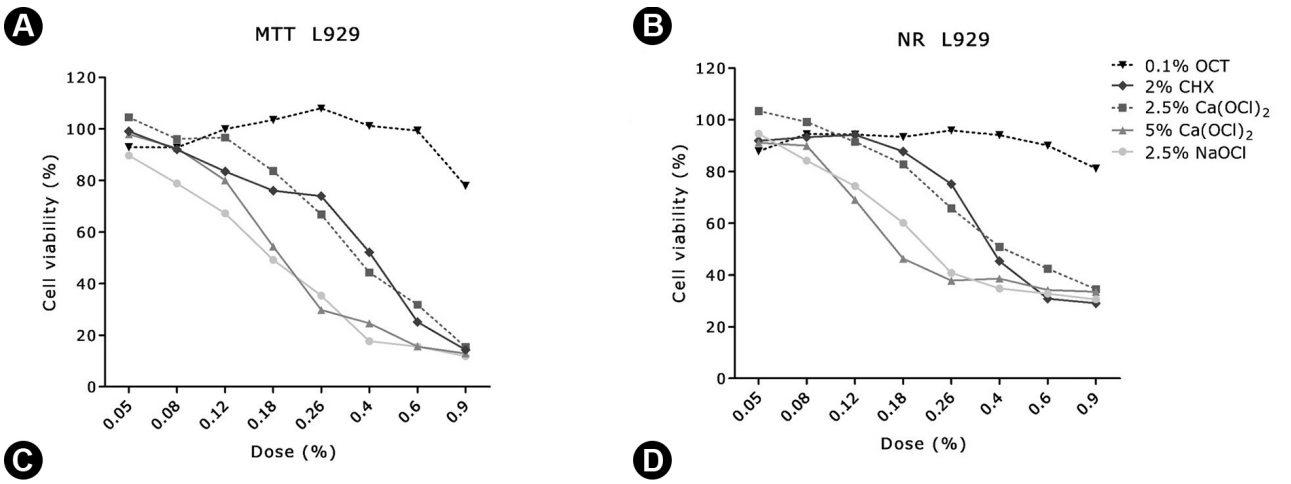
D

	0.05	0.08	0.12	0.18	0.26	0.4	0.6	0.9
0.1% OCT	ab	b	b	b	c	c	b	b
2% CHX	bc	b	b	b	c	c	c	a
2.5% $Ca(OCl)_2$	bc	b	b	b	b	b	a	a
5% $Ca(OCl)_2$	ab	ab	ab	a	a	a	a	a
2.5% NaOCl	a	a	a	a	a	a	a	a

Figure 1. Viability of hPDL cells after exposure to solutions tested at different doses by (A) methyl-thiazol-tetrazolium (MTT) and (B) neutral red (NR) assays. Statistical comparison of MTT (C) and NR (D) results: different letters in columns indicate statistically significant differences among the solutions.

Discussion

The aim of this study was to assess the effects of OCT and Ca $(OCl)_2$, in comparison with NaOCl and CHX, on viability and proliferation/migration of L929 and hPDL cells. The null hypothesis was rejected because there were differences between solutions. This kind of study is important because the root canal irrigant may reach the periapical tissues (18) and influence the prognosis of endodontic therapy, mainly in teeth with destroyed apical constriction due to root canal instrumentation or root resorption. This becomes more



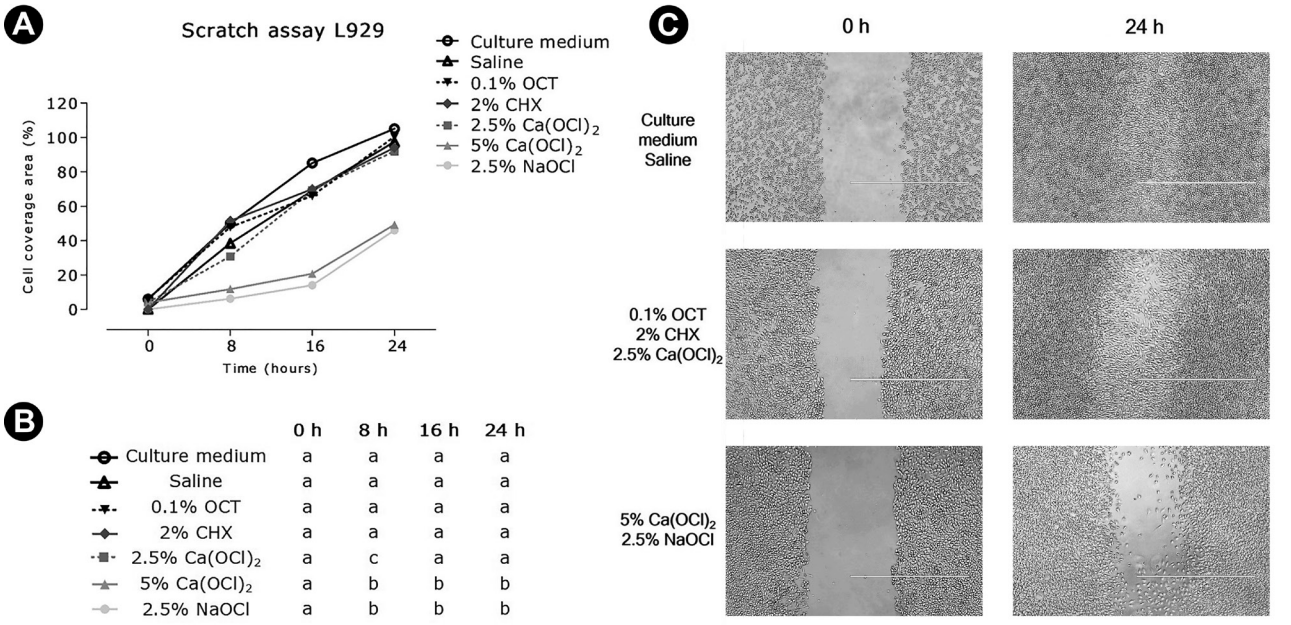
C

	0.05	0.08	0.12	0.18	0.26	0.4	0.6	0.9
0.1% OCT	a	ab	c	c	c	c	c	b
2% CHX	a	ab	bc	b	b	b	ab	a
2.5% $Ca(OCl)_2$	a	b	c	b	b	b	b	a
5% $Ca(OCl)_2$	a	ab	ab	a	a	a	a	a
2.5% NaOCl	a	a	a	a	a	a	a	a

D

	0.05	0.08	0.12	0.18	0.26	0.4	0.6	0.9
0.1% OCT	b	ab	b	b	c	c	c	b
2% CHX	b	ab	b	b	b	bd	a	a
2.5% $Ca(OCl)_2$	a	b	b	b	b	b	b	a
5% $Ca(OCl)_2$	b	ab	a	c	a	ad	ab	a
2.5% NaOCl	ab	a	a	a	a	a	ab	a

Figure 2. Viability of L929 fibroblasts after exposure to solutions tested at different doses by (A) methyl-thiazol-tetrazolium (MTT) and (B) neutral red (NR) assays. Statistical comparison of MTT (C) and NR (D) results: different letters in columns indicate statistically significant differences among the solutions.



B

	0 h	8 h	16 h	24 h
Culture medium	a	a	a	a
Saline	a	a	a	a
0.1% OCT	a	a	a	a
2% CHX	a	a	a	a
2.5% $Ca(OCl)_2$	a	c	a	a
5% $Ca(OCl)_2$	a	b	b	b
2.5% NaOCl	a	b	b	b

Figure 3. Wound closure (in percentage of cell coverage area) in L929 fibroblasts after exposure to tested solutions at 0.05% for 10 min (A). Statistical comparison of results: different letters in columns indicate significant differences among the solutions (B). Representative images of cell coverage at 0 and 24 h. Bar=1000 µm (C).

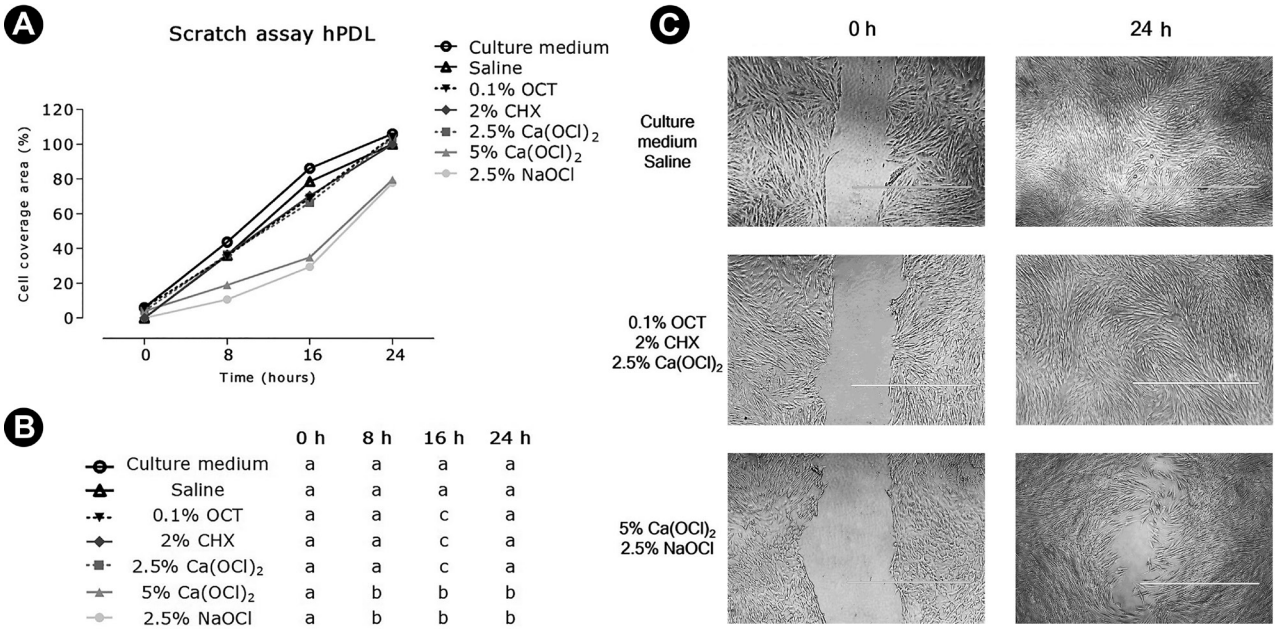
critical when regenerative endodontic protocols are used in immature teeth, since root canal irrigant contacts the periapical tissues, which are essential for regeneration (19).

L929 fibroblasts were used because they are recommended by ISO 10993 (20), have good reproducibility (21), and are frequently used in cytotoxicity assays of dental materials (15). However, permanent cell lines may have alterations in biological processes, components and cellular functions (22). hPDL cells were used because they are affected by irrigant solutions that could extrude to the periapical region, and the response of these cells to irrigant solutions may influence the prognosis of the endodontic therapy. Primary cells have specific metabolic potential, almost like target cells of dental materials in tissues (21) - although laborious and time-consuming to isolate – exhibit low cell profitability and have a limited number of passages (22).

The tested solutions were diluted in saline solution instead of culture medium or PBS, because the latter options contain buffering substances that can alter the pH of the solutions, and thus alter the clinical situation of use (15). The doses from 0.09% to 0.5%, determined in pilot experiments, were used to obtain a dose-response curve. This curve is important because the higher dose not always means greater cytotoxicity. Some substances in very low doses produce more cell alterations than in higher concentration (23). Moreover, the curve allows the comparison of the cytotoxicity in intermediate doses, because in the higher and lower doses the cell viability tends to equalize.

The MTT assay is based on the ability of viable cells to metabolically reduce soluble yellow MTT salt to insoluble blue-violet formazan crystals, insoluble in aqueous solutions, whose absorbance after dilution in alcohol is proportional to the number of viable cells (20). NR is a viability assay based on the ability of viable cells to incorporate this dye into their lysosomes, where it accumulates when the cell membrane is intact (24). MTT and NR assays revealed that all the solutions, in both cell lines, had an effect on cell viability in a dose-dependent manner; the higher the dose, the greater the cytotoxicity.

OCT was less cytotoxic in comparison with the other solutions. Schmidt et al. (13) demonstrated that, in relation to human gingival fibroblasts and nasal epithelial cells, another 0.1% octenidine hydrochloride-based antiseptic, Octenidol®, presented lower cytotoxicity than 0.2% CHX. $Ca(OCl)_2$ at a concentration of 2.5% was less cytotoxic than 2.5% NaOCl. Blattes et al. (8) observed no difference in cytotoxicity between $Ca(OCl)_2$ and NaOCl at the same concentration. This discrepancy could be attributed to methodology, since Blattes et al. (8) evaluated the viability in fibroblasts exposed to solutions for 24 h. In the present study, the cells were exposed to solutions for 10 min, because longer periods of exposure, such as 24 h, allow cells to recover from reversible toxic effects, considering that hypochlorite solutions present rapid breakdown kinetics.



B

	0 h	8 h	16 h	24 h
Culture medium	a	a	a	a
Saline	a	a	a	a
0.1% OCT	a	a	c	a
2% CHX	a	a	c	a
2.5% $Ca(OCl)_2$	a	a	c	a
5% $Ca(OCl)_2$	a	b	b	b
2.5% NaOCl	a	b	b	b

Figure 4. Wound closure (in percentage of cell coverage area) in hPDL cells after exposure to solutions tested at 0.05% for 10 min (A). Statistical comparison of results: different letters in columns indicate significant differences among the solutions (B). Representative images of cell coverage at 0 and 24 h. Bar=1000 µm (C).

The maximum chlorine is released within the first 4 h, and thereafter the real hypochlorous acid (HOCl) concentration decreases progressively and significantly according to the exposure time (25).

The comparison of two cell line responses to the solutions showed that OCT and $Ca(OCl)_2$ were more toxic to hPDL than L929 cells ($p<0.05$), and CHX was more toxic to L929 than hPDL cells ($p<0.05$). This can occur because different cells can be affected in varying degrees of severity, depending on the target mechanism for the cytotoxic action of the substance (26).

The results of the 2.5% NaOCl group corroborate those of the study by Viola et al. (15), which showed the same pattern of cellular metabolism lost in the MTT assay, when L929 cells were exposed to 2.5% NaOCl, using the same protocol as the present study. According to these authors, the mechanism of cytotoxicity of NaOCl includes a decrease in cellular metabolism, cytoskeletal deconstruction, accumulation of proteins in the rough endoplasmic reticulum and induction of cell death predominantly by necrosis. In hPDL and L929 cells, 2% CHX showed lower cytotoxicity than 2.5% NaOCl, which corroborates the findings of a recent study showing that 2% CHX had less cytotoxic potential than 2.5% NaOCl in mononuclear blood cells (27).

The wound-healing assay is based on the creation of an artificial gap in the cell monolayer and in the capture of images at certain periods, and serves to analyze cell migration and proliferation to close the wound (17). The main objective of endodontic treatment is the periapical repair, and the root canal irrigants can accelerate or retard this process (8). In L929 and hPDL cells, the wound closure in the 0.1% OCT, 2% CHX and 2.5% $Ca(OCl)_2$ groups was higher than that of the 5% $Ca(OCl)_2$ and the 2.5% NaOCl groups at 24 h. These results corroborate those of Blattes et al. (8), who showed higher wound closure in the $Ca(OCl)_2$ group than in the NaOCl group, and also those of Jenull et al. (28), who observed that the wound in fibroblasts exposed to OCT for 2 min had closed completely within 24 h, as was shown in the present study.

It is important to note that cell culture models have limitations because the non-physiological conditions to which cells are maintained: only one cell type without cell-cell interaction, no elimination of toxic substances, and the lack of biotransformation capacity and defense mechanisms (29). For these reasons, a direct extrapolation of results from cytotoxicity tests to the periapical tissue is not possible (23). Further *in vivo* researches evaluating the biocompatibility of these solutions are necessary to confirm their use in endodontic therapy.

In conclusion, 0.1% OCT had lower cytotoxicity in tested cell lines than CHX, $Ca(OCl)_2$ and NaOCl. $Ca(OCl)_2$ at concentrations of 2.5% and 5% showed cytotoxicity lower than or similar to 2.5% NaOCl, respectively. The 0.1% OCT, 2% CHX and 2.5% $Ca(OCl)_2$ groups had higher cell proliferation/migration than 5% $Ca(OCl)_2$ and 2.5% NaOCl groups. Therefore, in terms of cytotoxicity and cell proliferation/migration, OCT and $Ca(OCl)_2$ have the potential to be used as root canal irrigants.

Resumo

Para a seleção do irrigante endodôntico deve-se considerar os possíveis efeitos citotóxicos. O objetivo foi avaliar os efeitos do hipoclorito de cálcio [$Ca(OCl)_2$] e do cloridrato de octenidina (OCT) em células L929 e do ligamento periodontal humano (hPDL). As células foram expostas a diferentes doses das soluções: $Ca(OCl)_2$ 2,5% e 5%, OCT 0,1%, hipoclorito de sódio (NaOCl) 2,5% e clorexidina (CHX) 2%. A viabilidade celular foi avaliada pelos ensaios de metil-tiazol-tetrazólio (MTT) e vermelho neutro (NR), e a proliferação/migração pelo teste de cicatrização. Os resultados foram analisados por ANOVA de duas vias e Bonferroni (α=0,05). Os ensaios MTT e NR mostraram que OCT 0,1% foi menos citotóxico nas células do hPDL ($p<0,05$), seguido da CHX 2% e $Ca(OCl)_2$ 2,5% ($p<0,05$). Não houve diferença entre NaOCl 2,5% e $Ca(OCl)_2$ 5% ($p>0,05$). No entanto, estas soluções foram mais citotóxicas que as demais. O resultado foi o mesmo nas células L929, exceto que não houve diferença significativa entre CHX 2% e $Ca(OCl)_2$ 2,5% ($p>0,05$). A proliferação/migração das células L929 e do hPDL às 24 h nos grupos OCT 0,1%, CHX 2%, e $Ca(OCl)_2$ 2,5% foi maior que nos grupos $Ca(OCl)_2$ 5% e NaOCl 2,5% ($p<0,05$). Concluiu-se que OCT foi menos citotóxico que CHX, $Ca(OCl)_2$ e NaOCl. $Ca(OCl)_2$ 2,5 e 5% apresentaram citotoxicidade menor ou similar ao NaOCl 2,5%, respectivamente. Os grupos OCT, CHX e $Ca(OCl)_2$ 2,5% apresentaram maior proliferação/migração celular do que os grupos do $Ca(OCl)_2$ 5% e NaOCl 2,5%. Portanto, OCT e $Ca(OCl)_2$ têm potencial para serem utilizados como irrigantes endodônticos.

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