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Cytotoxic effects of alkaline tetrasodium EDTA irrigating solutions

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Abstract: The aim of this study is to determine the cytotoxic effects of tetrasodium ethylenediaminetetraacetic acid ($EDTANa_4$) when used alone or when combined with sodium hypochlorite (NaOCl), with and without the addition of cetrimide (CTR). Human pulmonary fibroblast cell line was exposed to the following irrigating solutions: group 1, 2.5% NaOCl; group 2, 10% $EDTANa_4$; group 3, 20% $EDTANa_4$; group 4, 2.5% NaOCl/5% $EDTANa_4$; group 5, 2.5% NaOCl/10% $EDTANa_4$; group 6, 2.5% NaOCl/5% $EDTANa_4$/0.2% CTR; group 7, 2.5% NaOCl/10% $EDTANa_4$/0.2% CTR; group 8, control, cells in Dulbecco's modified Eagle's medium. Methyl thiazol tetrazolium assay was used to determine the viability of cells after 1 and 24 h. Viability percentages were analyzed for global comparison using the Welch test followed by the Games-Howell test to determine groups with similar viability, and the Student's *t* test was used to compare the two times. The lowest viability was obtained with a 2.5% NaOCl solution at both time periods. The association of NaOCl with $EDTANa_4$ increased the cellular viability in direct relation with the concentration of the chelating agent. Globally, after 24 h of exposure, cell viability reduced. The solutions of $EDTANa_4$ showed moderate cytotoxic effects when compared with NaOCl alone.

Keywords; alkaline $EDTANa_4$, cetrimide, cytotoxicity, irrigating solutions, NaOCl

Introduction

The conventional and alternating irrigation protocols applied in endodontics to dissolve organic matter, kill bacteria, and remove the smear layer involve the use of sodium hypochlorite (NaOCl) and calcium-chelating agents [1,2]. To simultaneously promote the elimination of organic and inorganic remains during root canal preparation and minimize the interaction between irrigating solutions [3], mixtures of alkaline chelating agents with NaOCl have recently been proposed. This new protocol permits a continuous chelation [4] that also prevents the accumulation of inorganic residue in areas that are inaccessible to instruments [5,6].

Combined solutions of etidronate (HEDP) and alkaline tetrasodium EDTA ($EDTANa_4$) with NaOCl maintain the proteolytic and antibacterial effects of NaOCl [7,8] as well as the ability of NaOCl to remove the smear layer [5,9]. Incorporating surfactant agents with irrigating solutions improves the disinfecting efficacy [10,11] and wetting properties of the solutions [12,13].

The biocompatibility of endodontic materials can be characterized using many parameters including cytotoxicity. This is related to the degree of specific destructive action an agent has on cells [14]. NaOCl is more cytotoxic than EDTA in murine fibroblasts [15], human lung fibroblasts [16], and human peripheral blood mononuclear cells [17].

A recent publication [18] evaluated the cytotoxicity of mixtures containing etidronate powder (Dual Rinse HEDP) in NaOCl solutions on hamster lung fibroblasts. The mixtures of NaOCl and etidronate were not more toxic than NaOCl alone. However, the toxicity of alkaline $EDTANa_4$ and NaOCl mixed solutions remains unknown.

Therefore, the aim of this study is to determine the cytotoxic effects of $EDTANa_4$ solutions, alone and combined with NaOCl, with and without the addition of cetrimide (CTR), on the human pulmonary fibroblast (HPF) cell line.

Materials and Methods

This study protocol was approved by the Ethics Committee of the University of Granada, Spain (783/CEIH/2019).

Cell culture

HPFs were obtained from ScienCell Research Laboratories (CA, USA). Cells were grown in a 75-cm^2 culture flask in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Paisley, UK) supplemented with 10% inactivated fetal bovine serum (FBS; Gibco), 2 mM glutamine, and antibiotics (100 U/mL penicillin and 100 Pg/mL streptomycin; Gibco). To avoid changes in the pH of the medium HEPES buffer (pH 7.2) was added at a final concentration of 2 mM. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. The confluent cells were detached using EDTA solution (0.5 mM EDTA pH 8.0 in PBS), the supernatant was centrifuged (1,000 rpm for 10 min), and the pellet was resuspended in DMEM containing 10% FBS. Thereafter, the cells were counted in a Neubauer chamber (Brand GmbH + CO KG, Wertheim, Germany). Adherent cells in a logarithmic growth phase were seeded (100 µL $well^{-1}$) in 96-well flat-bottom microtiter plates (Jet Biofil, Guangzhou, P. R. China) at a 10^4 cells/well concentration and incubated for 24 h at 37°C with 5% CO_2.

Irrigating solution

The solutions tested were NaOCl (PanreacQuımica SA, Castellar del Vallés, Spain), $EDTANa_4$ (Sigma-Aldrich Chemie, Steinheim, Germany), and CTR (Sigma-Aldrich Chemie).

The final irrigating solutions evaluated were as follows: group 1, 2.5% NaOCl; group 2, 10% $EDTANa_4$; group 3, 20% $EDTANa_4$; group 4, 2.5% NaOCl/5% $EDTANa_4$; group 5, 2.5% NaOCl/10% $EDTANa_4$; group 6, 2.5% NaOCl/5% $EDTANa_4$/0.2% CTR; group 7, 2.5% NaOCl/10% $EDTANa_4$/0.2% CTR; and group 8, control, cells in DMEM.

All solutions were freshly prepared before the experiments. For the 2.5% NaOCl/5% $EDTANa_4$ and 2.5% NaOCl/10% $EDTANa_4$ association, both irrigation solutions were prepared at double concentration and mixed in a 1:1 ratio. When CTR was added, the solutions were prepared at triple concentration and mixed in a 1:1:1 ratio.

Evaluation of cytotoxicity

Methyl thiazol tetrazolium (MTT) assay (Sigma-Aldrich Chemie) was used to determine the viability of cells in contact with the solutions. After 1 and 24 h of exposure to irrigating solutions and control (100 µL/each), the solutions were removed and the cells were incubated with 10 µL of the MTT reagent (Sigma-Aldrich Chemie) added to each well, and the plates were incubated for 4 h. Then, 100 µL of dissolving agent (HCl: isopropyl alcohol, 0.04 N) was added to dissolve the formazan precipitate. The optical density (OD) was measured at 570 nm using a spectrophotometer (FLUOstar Optima, Ortenberg, Germany). The values of OD were expressed as the percentage of cell viability using the following formula: Viability (%) = MeanOD (test)/MeanOD (control) × 100.

The assay was performed in triplicate and repeated at three different

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Table 1 Viability percentage of human pulmonary fibroblasts determined by optical density after contact with irrigating solutions

Groups	Viability %		
	1 h	24 h	**Comparison *P* value
1 2.5% NaOCl	27.75 (0.79)[a]	20.97 (0.63)[a]	<0.001
2 10% $EDTANa_4$	60.62 (11.40)[b,c]	44.11 (8.97)[b]	0.004
3 20% $EDTANa_4$	73.52 (5.50)[c]	54.76 (10.33)[b]	<0.001
4 2.5% NaOCl/5% $EDTANa_4$	40.39 (11.10)[a,d]	41.47 (3.10)[b]	0.724
5 2.5% NaOCl/10% $EDTANa_4$	55.42 (10.35)[b,d]	43.05 (6.72)[b]	0.008
6 2.5% NaOCl/5% $EDTANa_4$/0.2% CTR	55.95 (11.03)[b,d]	44.75 (5.49)[b]	0.016
7 2.5% NaOCl/10% $EDTANa_4$/0.2% CTR	70.44 (19.38)[b,c]	46.53 (4.72)[b]	0.011
*Global comparison, *P* value	<0.001	<0.001	

Mean (standard deviation), $n = 9$ per group. *Global comparison between groups by Welch test, previously subjecting data to the Logit transformation. Read vertically, the same superscript letters show no statistically significant differences determined with the Games-Howell test. **Comparison between times by Student *t* test. NaOCl, sodium hypochlorite; $EDTANa_4$ tetrasodium EDTA; CTR, cetrimide

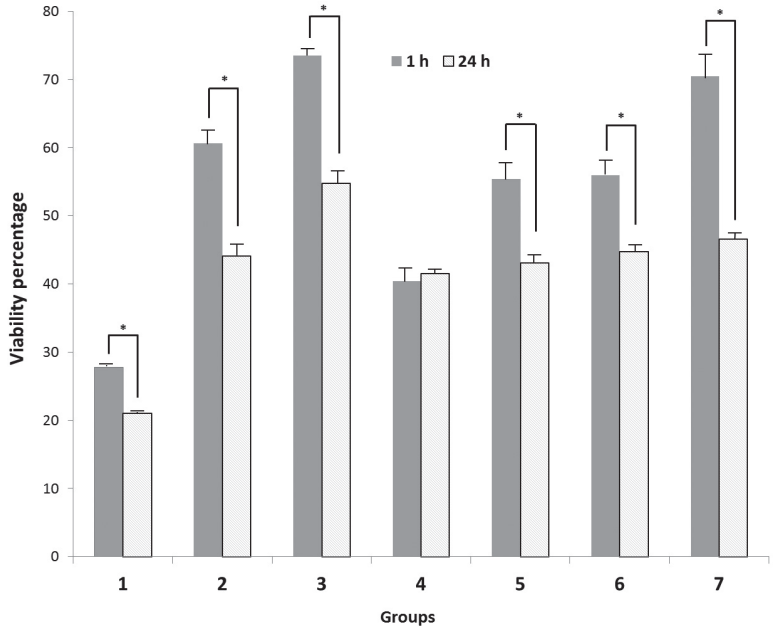

Fig. 1 Mean of cell viability percentage after 1 and 24 h exposure to irrigating solutions. *Statistically significant differences between times

times. The data were exported and submitted for statistical analysis.

Statistical analysis

For analysis of the results, the percentages were converted into proportions by dividing them by 100, and then the transformation "logit" was performed on proportions P to normalize the variables: $P = \text{Ln}\ (P/1-P)$.

The global comparison between groups for each time point was conducted using the Welch test due to the nonequality of the variances determined by the Levene test. To determine the statistical groupings at each time, the Games-Howell test was applied. For each of the groups, a comparison between times was performed using the Student's *t*-test.

All analyses were performed using SPSS software 20.0 (SPSS Inc, Chicago, IL, USA).

Results

The mean and standard deviation of OD of the controls at 1 and 24 h were 0.6145 (0.0457) and 0.7194 (0.0816), respectively. Afterwards, the viability percentages of the groups were calculated; these results and the comparisons are shown in Table 1. For both time periods the lowest viability was obtained by the 2.5% NaOCl solution with statistically significant differences. After 1 h of exposure, the highest percentage of viability was obtained by the 20% $EDTANa_4$ solution, without significant differences from the 2.5% NaOCl/10% $EDTANa_4$/0.2% CTR and the 10% $EDTANa_4$ solutions. The solution of 2.5% NaOCl/5% $EDTANa_4$ showed the second lowest percentage of cell viability, and it was the only one that did not show differences from 2.5% NaOCl. The solutions of 10% $EDTANa_4$, 2.5% NaOCl/10% $EDTANa_4$, and 2.5% NaOCl/5% $EDTANa_4$/0.2% CTR showed similar viability percentages (60.62%, 55.42%, and 55.95%, respectively).

After 24 h, cell viability was reduced in all study groups with the exception of the 2.5% NaOCl/5% $EDTANa_4$ group, which was the most cytotoxic mixture at both time points after NaOCl (Fig. 1). The 20% $EDTANa_4$ group obtained the lowest values, but without significant differences from the other groups, except NaOCl. The addition of CTR to the solutions tended to improve cell viability at both study time points.

Discussion

The biocompatibility of irrigating solutions is important because the solutions can come in contact with periradicular tissues and hinder the healing process of the apical region. *In vitro* tests offer the possibility of studying the effects of the materials in cellular systems [19]. Cell-culture studies have been performed for decades to investigate the cytotoxic reactions induced by endodontic materials [20]. Cell lines such as mouse embryonic and primary human cells, mainly fibroblasts, may be involved in these experiments [21].

In this study, the undiluted irrigating solutions were used as well as at concentrations that are used in clinical practice. This allowed the determination of possible cell damage caused by the solutions when in direct contact with periapical tissues because when reach the apical region, the amount and concentration are uncertain [22]. Time periods of 1 and 24 h made it possible to evaluate the cytotoxicity during short and medium terms.

The irrigating solutions were applied to the HPF cell line and cytotoxicity was measured using MTT assay [23] because this method evaluates the

ability of viable cells to convert water-soluble tetrazolium salts to insoluble formazan crystals through the activity of mitochondrial dehydrogenase enzyme. In addition to its speed, accuracy, and reproducibility, an additional advantage is that it does not require a washing step, which could cause variations in the sample [24].

In the short term, the results of the present study were not surprising; the results confirmed the greater cytotoxicity of a 2.5% NaOCl solution (27% cellular viability) when compared with 10% and 20% $EDTANa_4$ solutions (60% and 73%, respectively). Studies with these solutions have shown coincident results in terms of the greater cytotoxicity of NaOCl with respect to EDTA solutions, regardless of the method and cell population used for its determination [15-17,25]. Recent studies on cytotoxicity with a 5.25% NaOCl solution report a viability percentage of approximately 30% in 4 h on human gingival fibroblasts [26] and 22% in 10 min on human periodontal ligament cells [27]. Such variability in percentages could be due, in the same way, to the use of different cell lines, times, and/or concentrations.

The combined solutions of $EDTANa_4$ with NaOCl were less cytotoxic than 2.5% NaOCl. The caustic potential of NaOCl is affected by available chlorine rather than pH or osmolarity [28]. The mixture of $EDTANa_4$ solutions with NaOCl causes a reduction in the available amount of free chlorine [9]. This loss, which is also concentration-dependent, is responsible for the lower toxicity seen when $EDTANa_4$ is combined with NaOCl. Such a finding suggests an extra advantage in using this combination because the antibiofilm activity is not reduced with respect to 2.5% NaOCl alone [8].

The greater viability obtained by the 20% $EDTANa_4$ solution (either alone or in combination with NaOCl) with respect to the 10% $EDTANa_4$ solution could be related to the amount of sodium ions present in the chelating agent. The exposure of organic samples, such as bovine muscle [9] or the biofilm of *E. faecalis* [8], to these solutions favors hydration by deposition on the surface of sodium ions in a concentration-dependent manner, which could be linked to the lower toxicity found for the 20% $EDTANa_4$ solution.

The cytotoxicity results after 24 h of exposure to the solutions demonstrated a global reduction in the percentage of cell viability compared with the results after the exposure time of 1 h. This effect can be explained by a lack of nutrients, given that the solutions, unlike in other studies [17], were not prepared in culture medium. Therefore, all the study groups show similar viability, without statistically significant differences among them. NaOCl also reduced viability over time (from 27.75% to 20.97%), although in this case, the effect can be attributed to its powerful and direct cytotoxic action.

The incorporation of CTR to $EDTANa_4$/NaOCl solutions showed a tendency, without statistically significant differences, to improve cell viability at both study time periods. The addition of surfactants to the preparations of NaOCl accelerated the degradation of free available chlorine [29], most likely because of the reaction between NaOCl and the surfactants that are organic compounds [30]. These combined solutions did not modify the antibiofilm activity [8], which could be explained by the action of CTR disrupting the biofilms in addition to its antimicrobial activity.

The use of new irrigating solutions for root canal preparation calls for testing any possible undesirable effects as a prerequisite for the recommendation of these solutions [25]. Although the results obtained in this investigation cannot be extrapolated to the clinical setting, one might expect a reduction in the cytotoxicity of the solutions because of the dilution of these solutions by the periapical tissues [31]. Future studies are needed to evaluate the outcomes on inflammatory host response.

ln conclusion, within the limitations of the present study, the $EDTANa_4$ irrigating solutions used alone and combined with NaOCl, with and without CTR, showed moderate cytotoxic effects when compared with NaOCl alone.

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Conflict of interest

The authors declare that they have no conflict of interest.

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