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ORIGINAL ARTICLE

Calcium hypochlorite on mouse embryonic fibroblast cells (NIH3T3) in vitro cytotoxicity and genotoxicity: MTT and comet assay

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Abstract
Antimicrobial irrigation solutions are widely used under clinical settings. Their effect on dental tissue is a subject of recent research, which aims for a safer irrigant for clinical use. In this regard, here our goal was to evaluate the cytotoxicity and the genotoxicity of *calcium hypochlorite* ($Ca(OCl)_2$) solution, along with NaOCl, on Mouse embryonic fibroblast cells (NIH3T3). First, Cells were treated either with NaOCl or $Ca(OCl)_2$ in a time- and dose-dependent manner for cytotoxicity by 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, then cell viability was calculated according to cell proliferation plots. Secondly, genotoxicity was assessed by Comet assay. Data were statistically analyzed by Tukey's test ($P < .05$). NaOCl and $Ca(OCl)_2$ had similar effects on cellular viability at 3 and 6 h treatments. Cell viability of $Ca(OCl)_2$ at concentrations of 0.0125%, 0.025%, 0.05%, or 0.125% was significantly lower than that of NaOCl at 24 h treatment ($P < .05$). Comparing $Ca(OCl)_2$ and NaOCl treatments at all time points and concentrations, the damaged cell number of $Ca(OCl)_2$ was almost fourfold higher than that of NaOCl. In conclusion, both, NaOCl and $Ca(OCl)_2$ solutions were cytotoxic and genotoxic to NIH3T3, however, $Ca(OCl)_2$ had a significantly higher damaged cell percentage than NaOCl at all time points and concentrations investigated.

Keywords Sodium hypochlorite · Calcium hypochlorite · Cytotoxicity · Genotoxicity · Irrigation

Introduction

The mechanical instrumentation of the root canal and its irrigation with an antibacterial agent is referred as chemo-mechanical preparation. This preparation is fundamental, since basic mechanical instrumentation of a canal wall is not sufficient to reduce the number of bacteria significantly (1,2). Aside fromtheir antibacterial effect, dissolving effect on organic and inorganic contents is also desired for an ideal endodontic irrigation solution used for root canal instrumentation (3). However, sometimes liquids may be unintentionally forced beyond the apex during root canal treatment procedures (4). Thus, irrigation solutions with a trilateral effect may lead to severe injuries, if they are not biocompatible with the host tissue (5).

One of the main reasons for acute apical periodontitis is bacteria. Therefore, root canal irrigants are commonly chosen according to their antibacterial potential to increase their efficiency. Sodium hypochlorite (NaOCl) at different concentrations is the most preferred endodontic irrigation solution due to its broad-spectrum of antimicrobial activity. NaOCl functions as a surface disinfectant, thus its tissue dissolving and antimicrobial efficacy is attributed to its contact capacity, which can be locally administered based on this specific feature (6, 7). Nevertheless, NaOCl leads highly irritating effects against host cells such as periapical tissues when it extrudes apically (8). Moreover, it can reduce the resistance of teeth to fracture (9), and can adversely interfere with the bond strength of dentin adhesive restorations (10, 11). In this context, recent studies have focused on searching for an alternative solution for root canal irrigation due to the adverse effects of this irrigant (12, 13).

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Calcium hypochlorite ($Ca[OCl]_2$) is used in industrial sterilization and water decontamination treatment and is considered as an alternative intracanalirrigant (14). According to previous studies, this substance had antibacterial properties (15, 16) and the ability to promote organic matter dissolution (17). However, $Ca(OCl)_2$ has been reported to have similar antimicrobial potential compared to NaOCl against *Enterococcus faecalis* in the endodontic milieu (18). To our knowledge this is the first study employing both MTT and Comet assays to evaluate the cytotoxicity and genotoxicity of this substance.

In our study, we aimed to evaluate the cytotoxicity and the genotoxicity of $Ca(OCl)_2$with respect to NaOCl solution.

Materials and methods

Cell culture

Mouse embryonic fibroblast cells (NIH3T3) were a kind gift from GulsenAkalinÇiftci (Osmangazi University, Eskisehir, Turkey). NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, New York, NY) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin (Gibco). Experiments were conducted at passages 27 to 30. At 70% confluency, cells were treated with either NaOCl or $Ca(OCl)_2$ in a time- and dose-dependent manner for 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Comet assays.

Preparation of test materials

$Ca(OCl)_2$ solution was made fresh from granules (Jianghan Salt and Chemical Complex, Yuicun, Qianjiang, Hubei, China) at the time of each experiment. Different concentrations were prepared with distilled water (weight/volume [w/v] ratio) to strength of 5% and mixed using a magnetic stirrer for 10 min. The commercial solutions of NaOCl tested were at 5.25% concentration (Chlorax; Cerkamed Group, Nisko, Poland).

3-(4,5-Dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

NIH3T3 were treated with NaOCl or $Ca(OCl)_2$ at concentrations of 4%, 2%, 1%, 0.5%, 0.25%, 0.125%, 0.05%, 0.025%, 0.0125%, 0.0075%, or 0.0025% for 3, 6, and 24 h, respectively. MTT assays were performed according to Kozanoglu et al. (19). Briefly, 1×10^4 cells/well were seeded into 96-well plates containing 100 µL of the growth medium and cultured for one day. The following day, the medium was changed to a treatment medium in the absence or presence of varying concentrations of NaOCl or $Ca(OCl)_2$and incubated at 37 °C of 5% CO_2 for different time periods. After the incubation period, cells were treated with 10 µL MTT (5 mg/mL) for 4 h. Then, the medium was removed from the plates, and the MTT crystals were homogenized by adding 100 µL DMSO into each well. To homogenize the pellets more efficiently, the plates were shaken for 5 min on a shaker. Afterwards, the plates were read under 570/630 nm wavelengths by an ELISA reader (Thermo Electron Corporation Multiskan Spectrum, Finland). Finally, cell viability was calculated according to the cell proliferation plots.

Data were compared by one-way variance analysis. Statistical analysis was performed using the SPSS for Windows 11.5 software. Multiple comparisons were performed by Tukey's test. $P < 0.05$ was considered the level of significance.

Comet assay (single-cell gel electrophoresis)

NIH3T3 cells were seeded into 12-well plates at a concentration of 1×10^5 cells/well and treated with NaOCl or $Ca(OCl)_2$ at concentrations of 4%, 2%, 1%, 0.5%, 0.25%, 0.125%, 0.05%, 0.025%, 0.0125%, 0.0075%, or 0.0025% for 3, 6, and 24 h. At the end of each treatment period, cells were trypsinized and suspended in the medium to be used for the comet assay. The comet assay was performed under alkaline conditions according to the method of Singh et al. (20) with slight modifications.

First normal melting agarose (NMA) was prepared in calcium and magnesium free PBS (Phosphate buffer saline). Then slides were covered with NMA and incubated at room temperature. Cells suspended with DMEM were added to preheated microfuge tubes at 40 °C, which later on mixed with NMA (250 µL of 0.5%). Afterwards, this blend (100 µL) was distributed over NMA-coated slides, which was immediately covered with a coverslip and kept in + 4 °C for 15 min.

Then the coverslips were removed and placed into lysis solution for 90 min at + 4 °C. Slides were rinsed with precooled distilled water and located in a horizontal gel electrophoresis chamber filled with freshly prepared buffer (300 mMNaOH + 1 mM EDTA) for DNA to unwind ahead of electrophoresis for 20 min. Electrophoresis was performed at 25 V and 185 mA for 20 min at room temperature. In order to avoid DNA damage these steps must be performed in dark. At the end of electrophoresis, slides were rinsed with precooled distilled water and incubated with neutralizing buffer (0.048 g/ml) for 5 min.

Slides were then washed with cold distilled water and put in chilled ethanol for 10 min and stained with ethidium bromide (0.1 mg/ml at 1 to 4 ratio). Slides were finally examined with a fluorescent microscope (BX51, Olympus, Japan). Each group consisted of six analogous samples.

Comet imaging

Comet images were scored as per Collins et al. (21). One hundred comet images were visually evaluated for each treatment in a double blind fashion, the observers had no knowledge on the nature of the slide. A damage score starting from class 0 (undamaged) to class 4 (ultra-high damage) was employed for evaluation (22).

Statistical analysis of the comet assay

The arbitrary unit (AU) was used to express the extent of DNA damage, which was calculated using the formula below:

Genetic damage index (GDI) : [(0 × Type 0) + (1 × Type 1) + (2 × Type 2) + (3 × Type 3) + (4 × Type 4)].

Damaged cell percentage (DCP): Type 2 + Type 3 + Type 4.

The AU values indicating the comet assay scores showed levels of UD (undamaged, 0), Type 1 (low damaged, 1), Type 2 (moderate damaged, 2), Type 3 (high damaged, 3), and Type 4 (ultra-high damaged, 4) (Fig. 1).

Data were compared by one-way analysis of variance. Statistical analysis was performed using the SPSS for Windows 11.5 software. Multiple comparisons were performed by Tukey's test. $P < 0.05$ was considered the level of significance.

Results

Cytotoxicity of $Ca(OCl)_2$ and NaOCl

The effect of $Ca(OCl)_2$ and NaOCl at concentrations of 0.0025–2% on the cell viability of NIH3T3 cells is

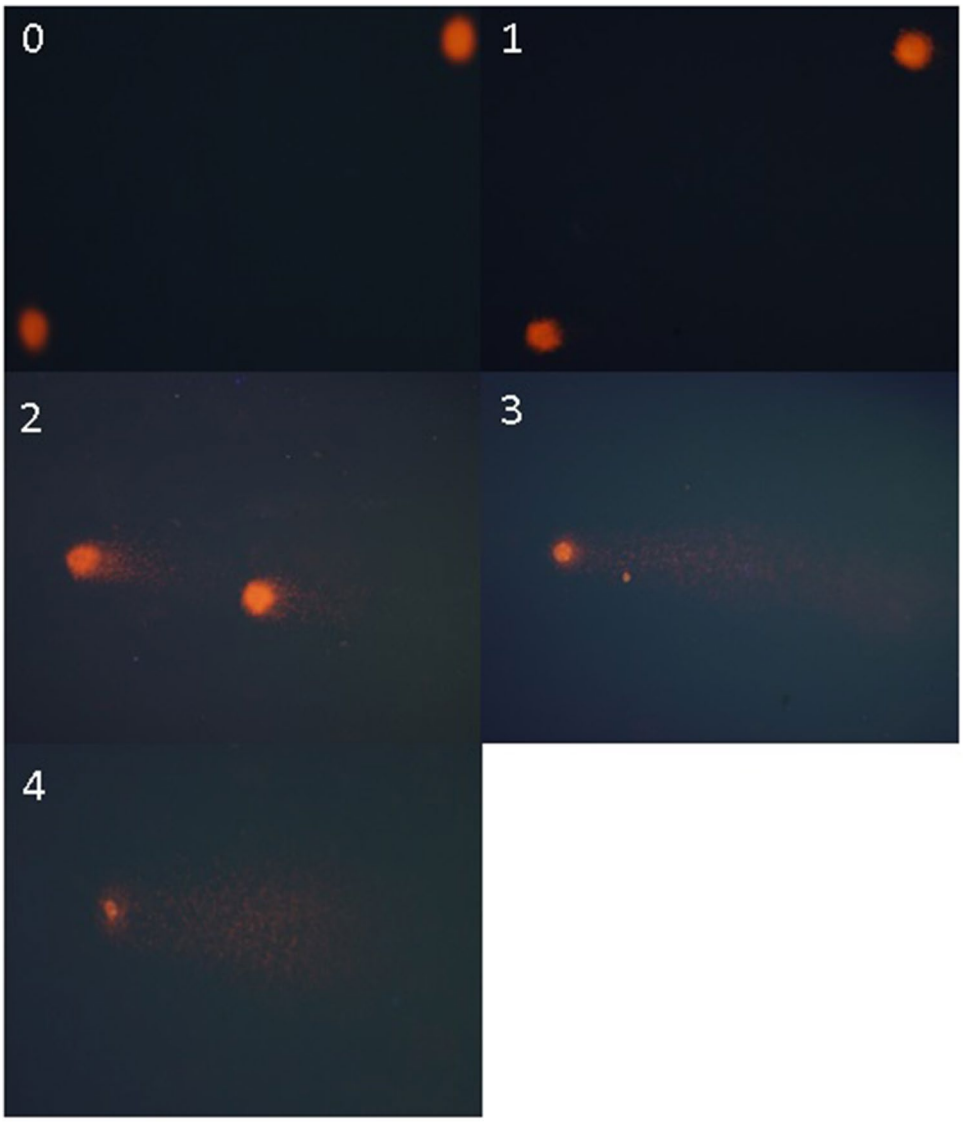

Fig. 1 Classification of DNA damage, which is scored as UD (undamaged, **0**), Type 1 (low damaged, **1**), Type 2 (moderate damaged, **2**), Type 3 (high damaged, **3**), and Type 4 (ultra high damaged, **4**)

presented in Fig. 2. The viability of untreated cells remained unchanged throughout the experimental period.

The cell viability of $Ca(OCl)_2$ in concentrations of 0.0125%, 0.025%, 0.05%, and 0.125% was significantly lower than that of NaOCl specifically in the 24 h treatment ($P < 0.05$). No statistical difference was found between the tested solutions at all concentrations in the 3 and 6 h treatments. Moreover, a progressive decrease in cell viability was observed at 3, 6, and 24 h in all tested concentrations of NaOCl and $Ca(OCl)_2$.

Genotoxicity of $Ca(OCl)_2$ and NaOCl

In the comet assay, two different parameters, namely, GDI and DCP, were measured. The effect of $Ca(OCl)_2$ and NaOCl at concentrations of 0.00025–0.000025% on DCP is presented in Fig. 3.

Cells were treated with different concentrations of NaOCl or $Ca(OCl)_2$ at varying time points (3, 6, and 24 h). The DCP of either treatment was higher than that of the control (mock treatment) at all-time points and concentrations. The highest DCP was observed at a 0.025% $Ca(OCl)_2$ concentration. The DCP of $Ca(OCl)_2$ was almost fourfold higher than that of NaOCl at all-time points and concentrations.

Assessment of the GDI showed that maximum damage was observed in the 24 h treatment at 0.025% $Ca(OCl)_2$ concentration. Comparing the $Ca(OCl)_2$ and NaOCl treatments at, the damaged cell number of $Ca(OCl)_2$ was higher than that of NaOCl for all the parameters examined.

Discussion

Irrigation solutions can cause complications, such as tissue damage, allergic reactions, and variable degrees of discomfort to patients depending on the type and volume of the irrigant used on periradicular tissues (23). Therefore, biocompatibility issue is as important as the antibacterial or tissue-dissolving property of an intracanal irrigation solution due to the extrusion risk beyond apex. In this perspective, this study aimed to assess the cytotoxicity and genotoxicity of $Ca(OCl)_2$ compared to NaOCl in NIH3T3 mouse fibroblasts employing MTT and Comet assays.

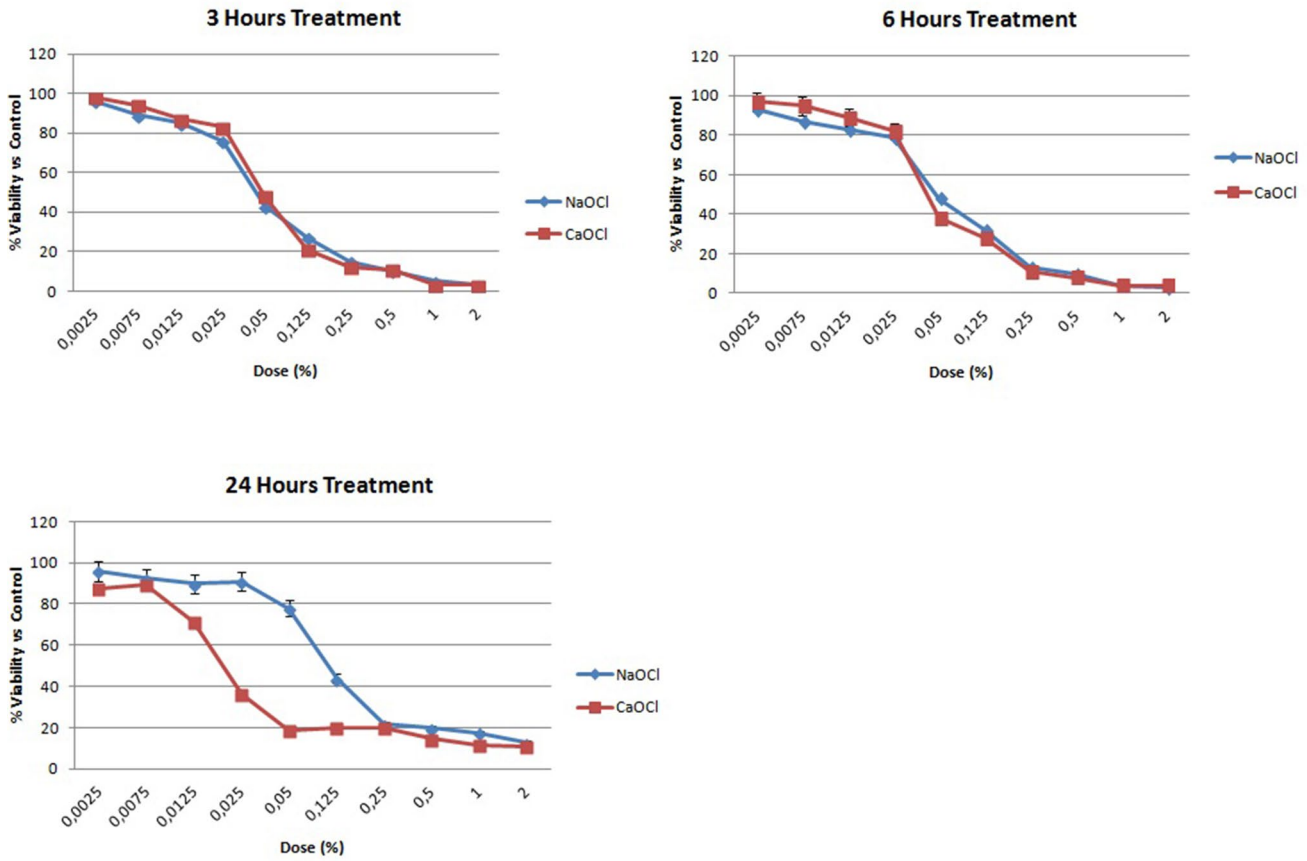

Fig. 2 The effect of NaOCl and $Ca(OCl)_2$ on cell viability determined by MTT assay. Results are expressed as the means ± standard deviations. *Represent statistically significant differences between the tested materials ($P < .05$)

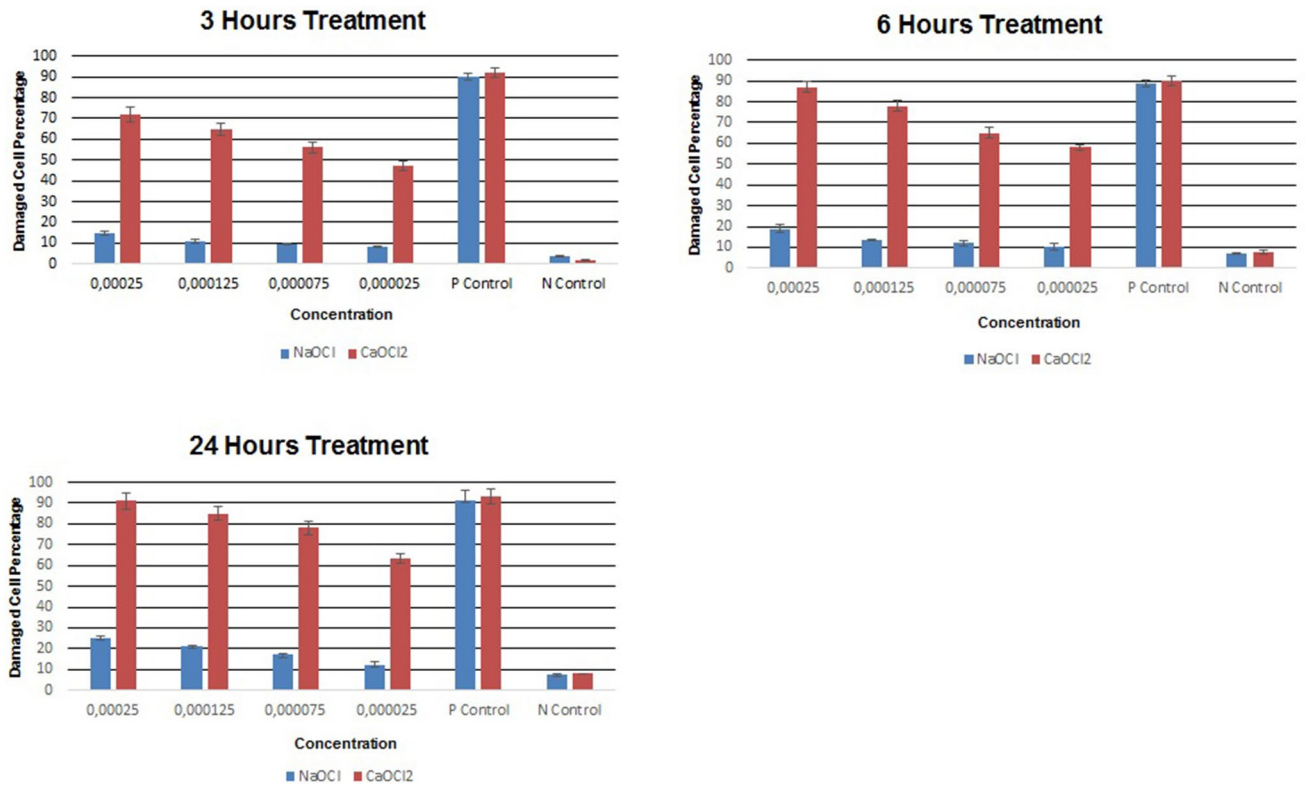

Fig. 3 DCP of NIH3T3 cells treated with different concentrations of NaOCl or $Ca(OCl)_2$ for 3, 6 and 24 h

In case of a possible extrusion from the apex, the intracanalirrigants may come in contact with the periapical tissues, which include periodontal ligament cells, osteoblasts and connective tissue cells. Human bone marrow mesenchymal stem cells (24) and fibroblasts (25) can be used to assess the cytotoxicity of intracanalirrigants. Various studies also employ preosteoblast cells (26), human dental pulp cells (27), human gingival fibroblasts (28) and periodontal ligament fibroblast cells (29) for cytotoxic assessment of root canal sealers or pastes. However, the most common used cell to evaluate the cytotoxic effect of a chemical is the mouse fibroblasts (25, 30).

The MTT assay is a cell viability assessment technique used to test the biocompatibility of intracanal irrigants (25, 30), endodontic sealers (31,32), or bioactive cements (33). The present studysuggested that NaOCl and $Ca(OCl)_2$ were both cytotoxic in a dose- and time-dependent manner as revealed by MTT assay. The tested irrigants had similar cell viability at 3 and 6 h treatments regardless of the dose. However, the MTT assay indicated that $Ca(OCl)_2$ significantly reduced cell viability compared to NaOCl at concentrations of 0.0125%, 0.025%, 0.05%, or 0.125% at 24 h treatment.

In terms of relative cytotoxicity between the two tested irrigants, our results are in accordance with the previous studies (24, 25, 30), which indicated that NaOCl is cytotoxic. However, the differences in cell line, assay methodology, and concentrations used make the cytotoxicity data difficult to compare.

The assay methodology and employed concentrations of the present study are comparable with those of Simbula (30) and Aubut et al. (25) to a certain extent, in which they reported that NaOCl concentrations were detrimental to cell viability in a time- and dose-dependent manner. However, NaOCl was found to be less cytotoxic in the 24 h but not in the 3 and 6 h treatments in our study. One possible explanation for this difference might stem from the fact that we employed a different mouse cell line for our MTT assays.

Currently, there have been two studies conducted on the cytotoxicty of $Ca(OCl)_2$, of these Sedigh-Shams et al. (31), found that $Ca(OCl)_2$ was less toxic compared to NaOCl, which was different from our findings. Our group and they used similar cell lines, which was mouse fibroblast, however, the tissue origins of these lines that were established from was different. They used L929 cells of subcutaneousconnective tissue origin, we on the other hand used mouse embryonic origin. In this regard, these two cell lines may have differential gene expression patterns and different responses to environmental cues. Another point to be considered is that as the passage number changes the cellular characteristics of a cell might also change, which includes response to certain chemical. The other possible reason for the difference in their and our data is the dose used, in our experimental

settings we could not achieve 5% solubility due the high rate of precipitation, which was also suggested as 4% maximum solubility by the manufacturer. In another study by Blattes et al. (36) with lower concentrations, found favorable viability results for $Ca(OCl)_2$ compared to NaOCl. Since they used trypan blue assay, which is less sensitive compared to MTT, our results may not be correlated.

Various tests are now commonly used to evaluate apoptotic effects of a substance at a cellular level. These tests are mostly rely on Fluorometric or Colorimetric methods. Fluorometric methods employ flow cytometry, calorimetric methods on the other hand utilize spectrophotometry. Flow cytometry applications such as Annexin V and Tunel assay yields information on the apoptotic stage of a cell, spectrophotometry assays, such as MTT, however, gives us information mostly on the metabolic activity of a cell, inferring that cells with lower cellular activity tend to enter apoptotic phase.

The comet assay is used to determine the genotoxicity of a biomaterial. The comet assay (single-cell gel electrophoresis) is a versatile, sensitive yet simple, and economical technique used to measure DNA damage and repair in an individual cell. It helps to measure single-/double-strand DNA breaks at alkali labile sites (apurinic/apyrimidinic sites), DNA cross-links, base/base–pair damages, and apoptotic nuclei in the cells (34).

As for genotoxicity assays, Bacterial Reverse Mutation Assay can also be used evaluate genotoxic effects. Compared to comet assay, it can be used to detect mutagenic agents causing point mutations at a nucleotide level in prokaryotes, comet assay, however, detects DNA damage occurring at a larger scale causing DNA fragmentation by employing eukaryotic cells.

The results of the present study indicated that the DCP of both NaOCl and $Ca(OCl)_2$ treatments was higher than that of the control at all time points and concentrations evaluated. The DCP of $Ca(OCl)_2$ was almost fourfold higher than that of NaOCl at all time points and concentrations. The assessment of the GDI showed that maximum damage was observed in the 24 h treatment of 0.025% $Ca(OCl)_2$ concentration. When $Ca(OCl)_2$ and NaOCl treatments were compared, the damaged cell number of $Ca(OCl)_2$ was higher than that of NaOCl for all the parameters investigated.

The present findings are in contrast to those of Aubut (25) and Marins et al. (37), who found no genotoxic effect of NaOCl. The employed concentrations in the aforementioned studies were not tested in the present study because of their high toxicity. Moreover, both groups utilized different evaluation methods with a single time point.

Under clinical conditions, extrusion of the irrigant beyond the apex is a common issue, which is not always avoidable. It has been reported that accidental extrusion of NaOCl may cause some drawbacks ranging from severe pain and swelling (38) to nerve damage (39). In this regard, it is possible that $Ca(OCl)_2$ may cause similar or worse drawbacks, since it is moregenotoxic than NaOCl. However, host defense mechanisms of the periapical tissues and neutralizing effects against the irrigants by dentine have been reported in previous studies (40, 41). These are the limitations that are not adapted to the methodology of this study. Additionally, the concentration of the tested solutions in this study was much lower and the time of the treatment was much longer compared to clinical settings. The clinical concentration of the irrigants were not tested, because in the cell culture the cells are oriented as individuals, which are readily exposed to outer environment without a protective sheet, therefore the cytotoxic and genotoxic effects of irrigants on individual cells are much worse compared to tissue itself.

In conclusion, the NaOCl and $Ca(OCl)_2$ solutions are both cytotoxic and genotoxic to NIH3T3. NaOCl and $Ca(OCl)_2$ have similar effects on cellular viability, but $Ca(OCl)_2$ has a significantly higher DCP. Therefore, based on these observations, NaOCl is a relatively safer root canal irrigant than $Ca(OCl)_2$ specifically in terms of genotoxicity. In this study, the effects of NaOCl and Ca(OCl)2 on cell toxicity and genotoxicity in general were investigated. Our further goal will be to explain how these substances affect the molecular mechanisms and cell cycle in our future projects, using the data from this study.

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