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DOI: https://doi.org/10.1111/iej.13110

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Abstract

Aim To test whether the incorporation of a chelation powder marketed for root canal irrigation (Dual Rinse (DR) HEDP) into a sodium hypochlorite (NaOCl) solution induced additional cytotoxic and genotoxic effects not observed with the NaOCl alone.

Methodology Fresh and 24 h-old mixtures of 0.9 g of DR HEDP in 10 mL of 2.5% NaOCl were assessed for their basic chemical features including pH and the ability to chelate Ca^{2+} from hydroxylapatite. Pure NaOCl and phosphate-buffered saline (PBS) with/without DR HEDP served as control solutions. Cytotoxic and genotoxic effects of diluted solutions (1:10, 1:100, and 1:1000) were assessed on Chinese hamster lung fibroblast (V79) using the MTT, clonogenic, and micronucleus assays, respectively. One-way ANOVA and Tukey's HSD test was applied with an alpha type error of 5% ($P < 0.05$).

Results In mixtures of NaOCl and DR HEDP, the free available chlorine was lost completely after 24 h, and the pH dropped by more than 3 units. However, the ability of the HEDP to chelate Ca^{2+} was maintained. The fresh mixtures of NaOCl and DR HEDP were not more toxic than NaOCl alone ($P > 0.05$), whilst the 24 h-old mixtures were less toxic ($P < 0.05$) and statistically similar to pure HEDP. DR HEDP *per se* showed little cytotoxicity and no genotoxicity at the tested dilutions.

Conclusions The ability of DR HEDP to chelate calcium is not affected by NaOCl. Cytotoxicity and genotoxicity of mixed solutions is dictated by the presence of free available chlorine therein.

Introduction

The idea to combine an oxidation-resistant chelator directly with a sodium hypochlorite solution to expedite and simplify root canal cleansing during endodontic procedures dates back more than 10 years (Girard *et al.* 2005). Etidronic acid or 1-hydroxyethane 1,1-diphosphonic acid (HEDP) was identified to exhibit short-term compatibility with NaOCl solutions at clinical strength, thus retaining the desired antimicrobial and proteolytic effects of NaOCl whilst adding an element of decalcification to the mixture (Zehnder *et al.* 2005, Tartari *et al.* 2015). As of 2016, a commercial CE-marked HEDP product for endodontic usage has become available (Zollinger *et al.* 2018). Its chemistry is based on the sodium etidronate, i.e. the salt of HEDP (Biel *et al.* 2017). This is supposed to be added to the NaOCl solution for clinical use immediately before treatment to receive an all-in-one root canal irrigant with combined proteolytic and chelating properties. However, the concept invariably results in a slow-onset chemical interaction between NaOCl and HEDP (Biel *et al.* 2017). Since it cannot be ruled out those dentists prior to usage keep the mixture for periods longer than recommended by the manufacturer, experiments are necessary to exclude any untoward effects from doing so. It has been shown that, after 24 h on the bench top, all the available chlorine has reacted with the HEDP contained in the Dual Rinse HEDP powder (Zollinger *et al.* 2018). The stoichiometry of this reaction would dictate that in this high-pH environment, the Dual Rinse HEDP is at least partly degraded to phosphoric acid, and the available chlorine to Cl^- (Zollinger *et al.* 2018). Consequently, this reaction should not result in any products that are more cytotoxic than the active chlorine (OCl^-) contained in the pure NaOCl solution itself. However, experiments to verify this assumption are lacking and were thus performed in the experiments presented within here.

The current study was performed to investigate how the extended presence of NaOCl in solution affected the ability of the Dual Rinse HEDP to chelate Ca^{2+} from hydroxylapatite. Furthermore, it was assessed whether any cytotoxic or genotoxic impacts of the Dual Rinse HEDP occur under the influence of the uniquely concentrated NaOCl present in endodontic solutions. As has been recommended by this journal (Peters 2013), two tests of the international standard 10993-5 were used to assess cytotoxicity: MTT and clonogenic assay.

Materials and methods

Test and control solutions

The chelating powder, Dual Rinse HEDP, used in this study was obtained from a commercial source (Medcem, Weinfelden, Switzerland). As suggested by the manufacturer, the powder contained in 1 capsule (0.9 g) was dissolved in 10 mL of phosphate-buffered saline, pH 7.4 (PBS, Thermo Fisher Scientific, Waltham, MA, USA) or 2.5% NaOCl. The NaOCl used in this study was diluted in deionized water and adjusted to its used concentration from a concentrated solution (PanReac Applichem, Darmstadt, Germany). Mixtures of Dual Rinse HEDP and NaOCl were either tested fresh or after storage in 10-mL polypropylene syringes (Omnifix, REF4617100V, B.Braun, Melsungen, Germany) for 24 h on the bench top at ambient temperature (23 °C).

Basic chemical assessments

Experiments described in this subchapter were performed in triplicate using fresh solutions/chemicals for each individual assessment. Density of the solutions was measured using a precision balance (Mettler AT261 DeltaRange, Mettler Toledo, Columbus, OH, USA). Available chlorine was assessed using a standard iodine titration method by a titration apparatus (665 Dosimat; Deutsche Metrohm, Filderstadt, Germany). The pH of these solutions was measured using a calibrated microelectrode (827 pH lab; Deutsche Metrohm); osmolarity using an osmometer (Fiske Model 110, Advanced Instruments, Norwood, MA, USA). Because the upper limit of the measuring range of the latter device is 2000 mOsm, solutions containing NaOCl and/or Dual Rinse HEDP were diluted in deionized water. The ability of the test and control solutions to dissolve calcium from hydroxylapatite was assessed by immersing 1 g of hydroxylapatite powder (Berkeley Advanced Biomaterials, Berkeley, CA, USA) in 10 mL of solution in 15-mL centrifugation tubes (Greiner Bio-One, Frickenheim, Germany). Tubes were rotated in a vertical plane for 10 min (Rotator SB 3, Carl Roth, Arlesheim, Switzerland). Subsequently, tubes were centrifuged at 10,000 ×*g* (Sorvall RC 5C Plus, Kendro Laboratory Products, Newton, CT, USA) for 10 min. Supernatants were assessed for their content of dissolved Ca^{2+} in an atomic absorption spectroscopy apparatus (Model contrAA 300, Analytik Jena, Jena, Germany) equipped with an air-acetylene flame. Measurements were obtained against a standard dilution series of $Ca(NO_3)_2$ in HNO_3 (Certipur, Merck, Darmstadt, Germany). Phosphate was masked using strontium chloride hexahydrate (Emsure, Merck).

Cytotoxicity & Genotoxicity assessment

Cell culture and maintenance

Chinese hamster lung fibroblast (V79) cells were procured from NCCS, Pune, India and maintained as per ATCC guidelines. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; AT151, Himedia Laboratories, Mumbai, India) supplemented with fetal bovine serum (final concentration 10 %; RM9955, Himedia Laboratories) and 1× antibiotic antimycotic mix (A002, Himedia Laboratories) and maintained at 37 °C in a 5 % CO_2 incubator (Galaxy 170S, Eppendorf, Hamburg, Germany).

Assessment of cell viability by MTT assay

MTT assay was performed as described earlier (Kumar *et al.* 2009). Exponentially growing V79 cells were seeded at a density of 10^4 cells per well in a 96 well plate (Nest Biotechnology Co., Jiangsu, China) and incubated at 37 °C in a 5% CO_2 incubator for overnight attachment. Next, the cells treated with 100 µL of different dilutions (1/10, 1/100 and 1/1000) of the test agents (as described above) prepared in DMEM for 15 min. Subsequently, the tested media was removed and the cells were treated with MTT reagent (M5655, Sigma Chemical, St. Louis, MO, USA) prepared in DMEM (without serum and phenol red) at a concentration of 1 mg/mL and further incubated for 4 h at 37 °C in a 5 % CO_2 incubator. The MTT-containing medium was then discarded and DMSO (100 µL) was added to each well in order to dissolve the purple colored formazan crystals formed. Absorbance was read at 570 nm using a multiplate reader (Infinite M200, Tecan, Männedorf, Switzerland). The extent of formazan formed is directly proportional to cell viability, which was calculated by the following formula:

% Viability = [(test OD- blank OD) / (control OD- blank OD)]*100

Clonogenic assay

This experiment was performed as described earlier (Franken *et al.* 2006) with slight modifications. In brief, a known number of exponentially growing V79 cells (200 cells) were seeded on 60-mm culture dishes (Nest Biotechnology Co., Wuxi, China) and incubated overnight for attachment. Subsequently, cells were treated with the different dilutions (1/10, 1/100, 1/1000) of the test and control agents for 15 min as mentioned above for the MTT assay. After incubation, the test agent containing media was aspirated aseptically and the cultures were rinsed with warm growth medium twice in order to ensure complete removal of the chemicals under investigation. The cultures were replenished with 5 mL of growth

medium and further incubated at 37 °C in a 5% CO_2 incubator for 12 days in order to allow colony formation. Subsequently, the media was discarded and the cultures were rinsed with sterile PBS. Colonies were stained using a fixing-staining solution containing 0.1% (w/v) crystal violet in absolute methanol. The excess stained was removed by rinsing with tap water and the petri-dishes were then allowed to air-dry. Blue stained colonies were then counted manually for the determination of the surviving fraction as mentioned below. A group of 50 or more cells was considered to be a viable colony.

Surviving fraction (SF) = $\left[\text{no.of colonies counted}\right)/\left(\text{no.of cells seeded} \times \left(\frac{\text{PE}}{100}\right)\right)]$;

where PE = plating efficiency; calculated form the number of colonies obtained from untreated/control cells as indicated below:

PE = $[(\text{no.of colonies counted})/(\text{no.of cells seeded})] \times 100$

This assay was performed as three independent experiments on different experimental days with each of the dilutions of the test agents examined in triplicate ($N = 3$).

Genotoxicity assay

A micronucleus (MN) test was performed as described earlier (Kumar *et al.* 2009). In brief, exponentially growing V79 cells were seeded at a density of 6×10^5 cells in 60-mm culture dishes (Nest Biotechnology Co., Jiangsu, China). Following overnight attachment, cells were exposed to 5 mL of each of the diluted test solutions (1:1000 diluted in DMEM). After 15 min of incubation the drug-containing media were discarded, and the cultures were treated with 3 µg/mL cytochalasin B (C6762, Sigma Chemical) in DMEM and further incubated for 24 h to obtain binucleated cells. Subsequently, cells were dislodged by trypsinization and collected into 10 mL glass tubes and centrifuged at 155× *g* for 10 min. Cells were washed twice with sterile PBS to remove media content. A hypotonic shock was then provoked for 1 min using 0.075 % KCl, and cells were fixed with ice-cold Carnoy's fixative (methanol/acetic acid; 3/1 (v/v)). Fixed cells were smeared into cleaned glass slides, allowed to air dry, and stained using 20 µg/mL of Acridine orange dye (A6014, Sigma Chemical), prepared in Sorensen's buffer (pH 6.8). After staining, the slides were dipped in fresh Sorensen's Buffer to remove excess dye, and observed and manually scored under a fluorescence microscope (BX 51, Olympus Microscopes, Tokyo, Japan) at 400× magnification. A minimum of 1000 binucleated cells with well-preserved cytoplasm were scored from each group for the presence of one or more micronuclei.

Statistical analysis

Chemical data are presented using descriptive statistics where applicable. Some triplicate values e.g. related to osmolarity showed no variance when rounded according to the measurement error of the individual experiment. Data is presented as mean values ± standard deviations (SD). The graphs were plotted and analysed for statistical significance using GraphPad Prism-Instat software Version 5 (GraphPad Software, La Jolla, CA, USA). Mean values were compared between groups using one-way analysis of variance (ANOVA) followed Tukey's HSD test for individual comparisons. The alpha-type error was set at 5%.

Results

Density of the solutions increased according to their salt content (Table 1). The addition of Dual Rinse HEDP to NaOCl lowered the pH of the combined solution by 0.2 to 0.3 units. Because of the increased specific weight of the NaOCl/Dual Rinse HEDP solution, its weight content of available chlorine dropped already in the fresh mixture. After 24 h of storage on the bench top, the available chlorine was used up, and the pH had dropped to 8.7/ 8.8. However, the ability of the combined solution to dissolve Ca^{2+} from hydroxylapatite was maintained (Table 1).

The effect of three different dilutions (1/10, 1/100 and 1/1000) of NaOCl or Dual Rinse HEDP alone or in combination (fresh and 24 h-old) on cell viability was assessed by MTT assay (Fig. 1). Differences in the cytotoxicity between solutions were most pronounced at the 1/10 dilution: the pure NaOCl solution and its fresh mixture with Dual Rinse HEDP were the most cytotoxic ($P < 0.05$ compared to all other groups), whilst the Dual Rinse HEDP in PBS and in NaOCl that was left on the bench top for 24 h were less cytotoxic and statistically similar ($P > 0.05$). With the 1/100 dilutions, these differences became less pronounced. The Dual Rinse HEDP in PBS did not differ anymore from the pure PBS control ($P > 0.05$), whilst the pure NaOCl and its fresh mixture with Dual Rinse HEDP were still statistically the most cytotoxic. No differences were detected between solutions in the 1/1000 dilutions.

The plating efficiency of the clonogenic assay was calculated to be 82.2 ± 1.9 and the surviving fraction (SF) is summarized in Table 2. Representative crystal violet stained colonies are shown in the supplement. Pure NaOCl dilutions of 1/10 and 1/100 were highly toxic and no colony formation was observed. Freshly prepared Dual Rinse HEDP in NaOCl revealed the same results for the 1/10 dilution but the 1/100 dilution showed a SF of 0.10 ± 0.16. Dual Rinse HEDP in PBS was not significantly different to the mixtures of Dual Rinse

HEDP/NaOCl at 24 h ($P > 0.05$) for any dilution. All tested mixtures were non-toxic and showed no significant difference at a dilution of 1/1000.

The genotoxic potential of NaOCl or Dual Rinse HEDP alone or in combination was assessed by micronucleus assay. A 1/1000 dilution (dose selected from MTT assay) of each of the test agents was investigated for their genotoxic potential. Data indicated that NaOCl-treated cells exhibited a significant increase (4-fold; $P < 0.001$) in the frequency of micronucleus (MN) formation when compared to control (untreated) cells indicating its genotoxic potential even though it did not cause a significant change in viability at this tested dilution (Fig. 2). Cells treated with freshly prepared Dual Rinse HEDP/NaOCl also showed a significant increase (2.3 fold, $P < 0.05$) in MN frequency, but the frequency was low when compared to NaOCl alone. Cells exposed to Dual Rinse HEDP prepared in PBS or Dual Rinse HEDP/NaOCl-24h did not exhibit a significant increase in MN frequency indicating their non-genotoxic nature at this tested dilution.

Discussion

The current study confirmed some known facts and offered some new insights into the interaction between Dual Rinse HEDP and NaOCl in aqueous solution. In mixtures of NaOCl and Dual Rinse HEDP, the free available chlorine got lost over time. However, the ability of the chelator or its reaction products with NaOCl to chelate calcium was maintained. The fresh mixtures of NaOCl and Dual Rinse HEDP were not more toxic than NaOCl alone, and the 24 h-aged Dual Rinse HEDP/NaOCl solution indicated similar effects like Dual Rinse HEDP/PBS. These potentially problematic effects of NaOCl solutions can thus solely be attributed to their content of free available chlorine.

The current study was an *in vitro* investigation. Results can thus not necessarily be extrapolated to the clinical situation. Moreover, it is also not legitimate to extrapolate cytotoxicity data related to HEDP when mixed with NaOCl from food hygiene or water treatment studies. The concentration of NaOCl solutions used in endodontics is uniquely high, and thus chemical reactions with HEDP are stronger than in the highly diluted solutions applied in other fields (Zollinger *et al.* 2018). Interestingly, NaOCl showed cytotoxicity in the presented experiments that would probably prevent it from clinical applicability under current legislature. From the *in vitro* experiments conducted using V79 cells, we observed that NaOCl, at ten-fold dilution showed maximum toxicity. NaOCl is a strong oxidant that readily generates highly reactive species like OCl^-, HOCl and OH^- that not only cause an increase in the pH of the medium but also act as causative factors for cell death. This observation is in line with previously published reports that too advocate the cytotoxic nature of NaOCl in

various model system (Gul *et al.* 2009, Aubut *et al.* 2010, Marins *et al.* 2012). On the other hand, Dual Rinse HEDP too at ten-fold dilution showed decreased cell viability. The chelator *per se* may not be toxic to cells but its chelating property (chelation of divalent metal ions like Ca^{2+}/Mg^{2+} which are vital for cell adhesion *in vitro*) may be the causative factor that allows cells to get detached resulting in lesser formazan formation.

The reproductive capacity of a cell to grow into a colony (at least 50 cells) after being treated with a test agent, such as a cytotoxic drug or radiation, is described by the clonogenic assay (Franken *et al.* 2006). It is a gold standard assay and also required for the cytotoxic evaluation according to the international standard for medical devices. Nowadays, the MTT assay should be confirmed by a second cytotoxic assay (Peters 2013), which investigates the proliferation at a later stage. NaOCl and Dual Rinse HEDP/NaOCl (freshly prepared) exhibited a long-term impact on cell viability and inhibited colony formation at 1/10 and 1/100 dilutions. Although, MTT assay showed a viability of > 80% at 1/100 dilution, the absence or few colonies formed indicated the longer lasting impact of these solutions on cell viability. On the contrary, cells treated with Dual Rinse HEDP/PBS and Dual Rinse HEDP/NaOCl (24 h-aged) demonstrated greater SF at the 1:10 dilution compared to the other NaOCl solutions.

Data obtained from the genotoxic experiment showed that freshly prepared NaOCl even at non-cytotoxic concentration could induce a significant increase in MN frequency when compared to the other solutions tested. This is in concordance with previously published literature that also reported the genotoxic property of NaOCl (Gul *et al.* 2009, Aubut *et al.* 2010). A MN is a marker for a genotoxic event and can be produced due to chromosomal fragmentation, improper chromosomal segregation or can also be parts of mis/unrepaired DNA (Fenech *et al.* 2011). The genotoxic potential of freshly made NaOCl can be attributed to the formation of HOCl which can directly interact with DNA bases leading to oxidation mediated damage (Hawkins & Davies 2002).

However, NaOCl solutions have a long tradition of successful use in endodontics, and are the most frequently applied irrigants in clinics. A web-based survey among members of the American Association of Endodontists showed that over 90% of respondents use NaOCl as their main root canal irrigant, with over 50% using it at a highly caustic concentration of over 5% (Dutner *et al.* 2012). Although severe complications from inadvertently irrigating concentrated NaOCl solutions over the root apex have been reported (Hulsmann & Hahn 2000), this has not prevented clinicians from using these irrigants. Their reason for doing so is the unique effect of NaOCl on necrotic tissue dissolution and biofilm disintegration

(Naenni *et al.* 2004, Tawakoli *et al.* 2017). The whole concept of chemomechanical root canal cleansing evolved around these features of NaOCl solutions (Grossman 1943). Dual Rinse HEDP may add an element of decalcification to this concept, which may have some desirable clinical effects such as the reduction of smear layer and hard tissue accumulation during root canal instrumentation. As shown here and in earlier investigations (Zehnder *et al.* 2005), the overall effect on decalcification by Dual Rinse HEDP is not affected by the interaction with the NaOCl. The reason for this may be two-fold: first, at the ratio that Dual Rinse HEDP is mixed with NaOCl as recommended by the manufacturer, the etidronate main structure is mostly maintained (fresh mixtures) and the amount of the chelator (in moles) is similar to the NaOCl. However, as the chemical reaction proceeds two NaOCl react with one etidronate (Zollinger *et al.* 2018), thus resulting in an excess of chelator after 24 h. Hence the stoichiometry would dictate the amount of residual Dual Rinse HEDP. Second, the etidronate that is lost during that interaction with the NaOCl is compensated that Dual Rinse HEDP binds Ca^{2+} more effectively at neutral compared to alkaline pH (communication submitted elsewhere). As the OCl^- is lost during the interaction of NaOCl solutions with the Dual Rinse HEDP contained therein, the pH drops to a more neutral pH level (Table 1), and the remaining chelator becomes more effective than its counterpart in a high-pH environment.
Combinations of etidronate and oxidizing agents are used for food disinfection (Mattia *et al.* 2006), and it should thus not surprise that Dual Rinse HEDP by itself has a low toxicity. However, whether any untoward effects would evolve from combining Dual Rinse HEDP with the highly concentrated NaOCl solutions uniquely used in endodontics was not known and, thus, studied here. The current results suggest that at least from the standpoint of the experiments reported here, no additional dangers to the patient that are not already inherent in NaOCl irrigation should evolve by using a combination of NaOCl and Dual Rinse HEDP in clinics.

Conclusions

The ability of Dual Rinse HEDP to chelate calcium from hydroxylapatite is not affected by NaOCl. In mixtures of NaOCl and Dual Rinse HEDP, cytotoxicity and genotoxicity of the respective solutions is dictated by the presence of free available chlorine therein. Therefore, apart from losing one desired ingredient (the chlorine), no untoward effects should be expected from combining Dual Rinse HEDP and NaOCl at the concentrations described here.

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Figure Captions

Figure 1 Changes in cell viability of V79 cells upon treatment with different dilutions of NaOCl or Dual Rinse HEDP alone or in combination. (Control indicates that these cells received DMEM only)

Figure 2 Effect of 1/1000 dilutions of NaOCl or Dual Rinse HEDP alone or in combination on induction of micronucleus in V79 cells. (*$P < 0.05$, ***$P < 0.001$ when compared to control group)

Supplement Representative photographs of the clonogenic assay used in this study.

Table 1 Basic chemical properties of the solutions under investigation

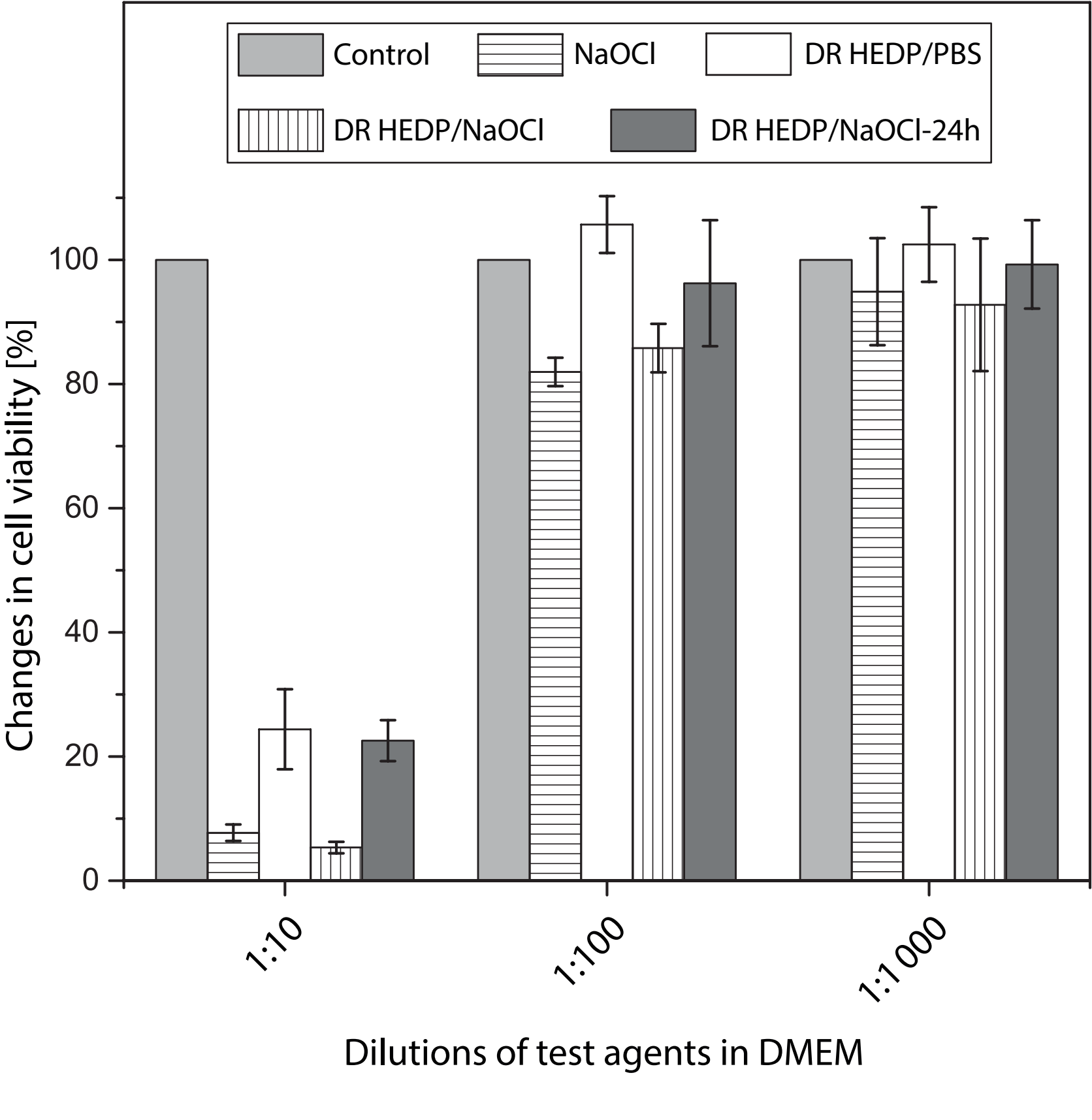
Solution	Density (g/ml)	pH	Osmolarity (mOsm)	Cl_2 (wt%)	Bound Ca* (ppm)
PBS	1.00	7.3	290	0	0
DR HEDP/PBS§	1.06	11.2	820	0	189 ± 4
2.5% NaOCl	1.10	12.3	4280	2.4	0
DR HEDP/NaOCl§ fresh	1.15	12.0-12.1	4550	2.2	156 ± 5
DR HEDP/NaOCl§ 24 h	1.15	8.7-8.8	5090	0.1	167 ± 10

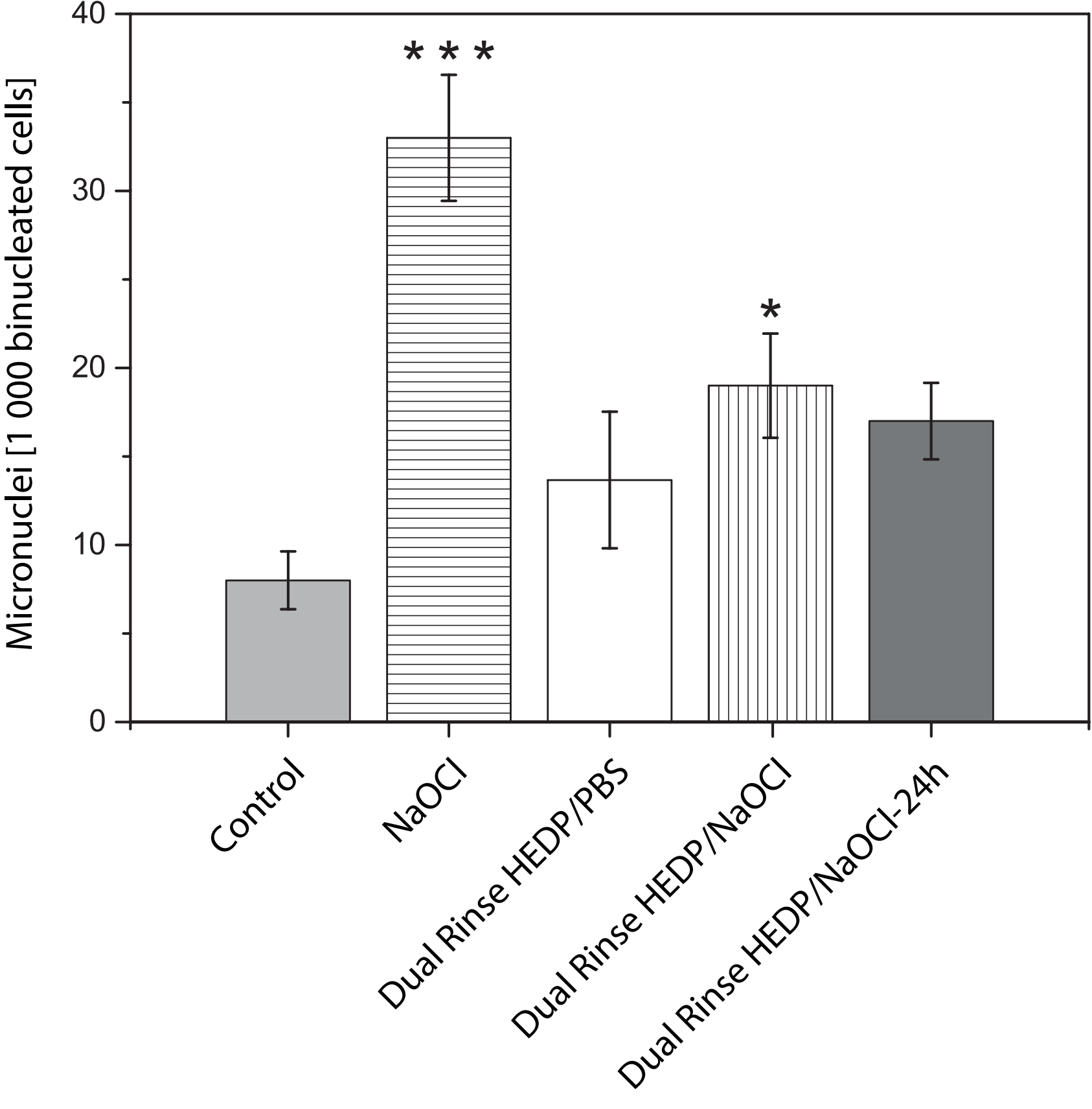
§0.9 g of Dual Rinse HEDP (etidronate) powder was added to 10 mL of solution;
* 1 g of hydroxylapatite powder was suspended in 10 mL of solution for 10 min and Ca^{2+} content of supernatant was determined by atomic absorption spectroscopy.

Table 2 Surviving fraction of V79 cells after treatment with the different tested solutions

Tested solution*	1:10	1:100	1:1000
Control	1.00 ± 0.00^{A}	1.00 ± 0.00^{A}	1.00 ± 0.00^{A}
NaOCl	0 ± 0.00^{C}	0 ± 0.00^{B}	0.98 ± 0.12^{A}
Dual Rinse HEDP/PBS	0.76 ± 0.18^{B}	0.98 ± 0.12^{A}	1.08 ± 0.06^{A}
Dual Rinse HEDP/NaOCl (fresh)	0 ± 0.00^{C}	0.10 ± 0.14^{B}	0.97 ± 0.10^{A}
Dual Rinse HEDP/NaOCl (24 h)	$0.91 \pm 0.23^{A,B}$	1.05 ± 0.10^{A}	1.06 ± 0.10^{A}

* 1:10, 1:100, 1:1000 indicate dilutions of the different tested solutions. Data sets ($N = 9$) that share a superscript letter did not differ at the 5% level for a specific dilution (ANOVA, Tukey's HSD).


Control
NaOCl
DR HEDP/PBS
DR HEDP/NaOCl
DR HEDP/NaOCl-24h
Changes in cell viability [%]
0
20
40
60
80
100
1:10
1:100
1:1000
Dilutions of test agents in DMEM


Micronuclei [1 000 binucleated cells]
40
30
20
10
0
* * *
*
Control
NaOCl
Dual Rinse HEDP/PBS
Dual Rinse HEDP/NaOCl
Dual Rinse HEDP/NaOCl-24h