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ORIGINAL ARTICLE

Assessment of genotoxic effect of maleic acid and EDTA: a comparative in vitro experimental study

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Abstract
Objectives This study aims to evaluate and compare the genotoxic and apoptotic effect of aqueous solutions of ethylenediaminetetraacetic acid (EDTA) with that of maleic acid (MA) using Chinese hamster lung fibroblast (V79) cells growing in vitro.
Materials and methods Exponentially growing V79 cells were treated with various concentrations of EDTA or MA alone for 30 min, and genotoxic effect was analyzed by micronucleus as well as comet assays and the type of cell death by apoptotic cell measurements using microscopic and flow cytometric methods. For all the experiments, H_2O_2 was used as a positive control.
Results Treatment of V79 cells with H_2O_2 resulted in significantly ($P<0.001$) increased micronuclei and levels of DNA damage, whereas, EDTA/MA alone treated cells did not show significant increase of MN frequencies and comet parameters even at their higher concentrations when compared with that of untreated control. V79 cells treated with EDTA/MA for 30 min showed a nonsignificant increase in the percentage of apoptotic and necrotic cells at their lower concentrations (0.025 and 0.05 % for EDTA and MA, respectively). However, at higher concentrations, i.e., $>IC_{50}$ (0.1 and 0.5 %) for EDTA and MA resulted in increased number of apoptotic and necrotic cells when compared with the untreated group.
Conclusions This study clearly demonstrates that MA and EDTA are not potentially genotoxic agents and MA induced lesser apoptotic/necrotic death than that of EDTA at their clinically relevant doses.
Clinical relevance MA may have a better clinical acceptability with comparable smear layer removal ability. Hence, the results presented here might be an additional supporting evidence for the use of MA in endodontic practice.

Keywords Maleic acid · EDTA · Micronuclei · Comet · Genotoxicity

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Introduction

Removal of pulp tissue and elimination of microorganisms and their toxins from the root canal system is one of the most important objectives of root canal therapy [1]. This is usually achieved by a combination of mechanical instrumentation and irrigation. Mechanical instrumentation of the root canal system produces an amorphous irregular layer known as smear layer on the root canal walls and to some extent into the dentinal tubules. The smear layer consists of remnants of ground dentin, odontoblastic processes, pulp tissue, and bacteria in the case of infected teeth [2]. Recent ex vivo studies suggested that the smear layer must be completely removed because it can hinder the penetration of intracanal medicaments and sealers into the dentinal tubules and has the potential to compromise the formation of an appropriate seal between the filling materials and the root canal walls [3]. A systematic review and meta-analysis of in vitro leakage studies by Shahravan and coworkers [4] concluded that smear

layer removal improves the fluid tight seal of the obturated root canal system. The use of chemicals, ultrasonics, and lasers alone or in combination has been practiced to remove the smear layer with varying results. Among the chemicals, alternating use of ethylenediaminetetraacetic acid (EDTA) and sodium hypochlorite has been recommended for the efficient removal of the smear layer [5, 6]. The biocompatibility of EDTA has been investigated by various studies at different concentrations. Silveira and coworkers [7] reported severe irritating effect of 15 % EDTA solution. Segura and coworkers [8] demonstrated time as well as a dose-dependent inhibition of the substrate adherence capacity of macrophages by EDTA. Furthermore, several of the earlier studies reported the cytotoxic effects of 17 % of EDTA [9, 10].

Maleic acid (MA) is a mild organic acid used as an acid conditioner in adhesive dentistry [11]. Ballal and coworkers [12] reported MA to possess a better smear layer removal ability than EDTA in the apical third of the instrumented root canal system. Furthermore, MA has been reported to be non-toxic on pulpal healing when used as an acid etchant [13]. Our previous study, comparing the cytotoxicity of 17 % EDTA and 7 % MA demonstrated MA to be less cytotoxic when compared with that of EDTA [14]. However, there are hardly any studies reported in endodontic literature regarding the genotoxicity of EDTA and MA. The aim of the present study was to evaluate and to compare the genotoxic and apoptotic effect of aqueous solution of EDTA with that of MA using micronuclei (MN) and comet assays as an indicator of genotoxicity and apoptotic assay as an indicator of cytotoxicity using Chinese hamster lung fibroblast (V79) cells growing in vitro.

Materials and methods

Chemicals

The chemicals used such as EDTA was purchased from Merck (Darmstadt, Germany), MA, trypsin (0.1 %), minimum essential medium (MEM), L-glutamine, penicillin, streptomycin, RNAse, low-melting agarose (LMA), normal-melting agarose (NMA), cytochalasin B, propidium iodide (PI), ethidium bromide (EtBr), Tris–HCl, and acridine orange (AO) were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Biowest (Miami, FL). Disodium hydrogen phosphate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, sodium carbonate, and all other chemicals were obtained from Qualigens Fine Chemicals (A Division of GlaxoSmithKline Pharmaceuticals), Mumbai, India.

Cell line and culture

The Chinese hamster lung fibroblast cells (V79) obtained from the National Center for Cell Sciences (Pune, India). Cells were grown exponentially in 25 cm^2 flasks (Nunc, Roskilde, Denmark) with MEM supplemented with 10 % FBS, L-glutamine (2 mM), 100 units/ml of penicillin, and 100 μg/ml of streptomycin and were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 in air.

Preparation of MA/EDTA solutions

The stock solutions of 7 % MA (7 g of MA powder dissolved in 100 ml of distilled water) and 17 % EDTA (17 g of EDTA powder dissolved in 100 ml of distilled water) were diluted with MEM and prepared freshly whenever required, at different required concentrations immediately before use.

Experimental design

A fixed number (5×10^5) of exponentially growing V79 cells were seeded into several individual 25-cm^2 culture T-flasks and after 24 h, cells with 70 % confluence were treated as follows:

1. *Control*—cells not treated with EDTA or MA.
2. *EDTA*—cells were treated with varying concentrations of EDTA (0.0025, 0.005, and 0.01 %) alone for 30 min.
3. *MA*—cells were treated with varying concentrations of MA (0.005, 0.01, and 0.025 %) alone for 30 min.
4. *Positive control*—the cultures of these groups were treated with 50 μM H_2O_2 for 5 min.

After the treatment with the EDTA or MA, media was removed and cells were trypsinized for 1 min to dislodge the cells to get single cell suspensions. The genotoxic effect of EDTA or MA was analyzed by micronucleus and comet assays as explained below.

On the basis of the IC_{50} values of EDTA (0.07 %, i.e., 0.08 μM) and MA (0.3 %, i.e., 0.31 μM) from our earlier experiment [14], noncytotoxic concentrations were selected to assess the genotoxic effects, while to assess apoptotic and necrotic cell death, we used both lower and higher concentrations of these agents.

Micronucleus assay

The presence of MN in a binucleated cell was assayed by blocking the cells at their cytokinesis stage as originally described by Fenech and Morley [15] with some minor modifications [16]. Briefly, after the various treatments with or without EDTA/MA, cells were incubated with medium containing cytochalasin B at a final concentration of 4.0 μg/ml followed by incubation at 37 °C for further 24 h. After the incubation period, the cells were washed with phosphate-buffered saline (PBS) and detached from the culture plates by exposure to 0.1 % trypsin for 2–3 min. Trypsin activity was terminated by the addition of a few drops of MEM, and the

cells were subsequently centrifuged at 155×*g* for 10 min. The resultant pellet was resuspended in 0.5-ml 0.75 % KCl, and the centrifugation was repeated. The cells were then fixed by 1–2 ml of Cornoy's fixative (methanol/acetic acid, 3:1). The pellet was dispersed in a few drops of fixative and dropped onto the slides, air dried, and stained with 0.01 % AO in Sorensen's buffer (pH 6.8). The buffer-mounted slides were observed under a fluorescent microscope equipped with 450–490 nm bandpass filter set with excitation at 453 nm (Photomicroscope III, Carl Zeiss, Aalen, Germany) using a ×40 neofluar objective for the presence of MN in the binucleate lymphocytes (BNC). A minimum of 1,000 BNC with well-preserved cytoplasm from each culture were counted, and the frequency of micronucleated binucleate cell was determined. The MN identification was done according to the criteria of Fenech and coworkers [17], and the values were expressed as mean±standard deviation (SD) from three independent experiments.

Comet assay (single cell gel electrophoresis) for DNA damage

DNA damage was estimated by the alkaline single-cell gel electrophoresis (comet assay) method according to the procedure of Singh and coworkers [18] with minor modifications [19]. Briefly, the frosted microscope slides for comet assay were first layered with 1.5 % NMA in PBS. The adherent cells from the various treatment groups were then trypsinized, centrifuged, counted, and mixed with 1 % (*w*/*v*) LMA in PBS and layered onto NMA. The agarose was then allowed to set at 4 °C for 5–10 min, and the slide was immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM trizma base, 0.2 M NaOH, 1 % Triton-X, and 10 % DMSO) and kept at 4 °C for 60 min to remove cellular proteins and membranes. Slides were then placed in a horizontal electrophoresis tank containing alkaline electrophoresis buffer at pH 13 and left for 25 min to allow unwinding of DNA. The slides were then subjected to electrophoresis for 18 min (300 mA, 20 V) at an ambient temperature of 4 °C. The slides were then washed with neutralization buffer (0.4 M Tris, pH 7.4) for 5 min and stained with EtBr. For the assessment of DNA damage, slides were visualized using an epifluorescence microscope (Olympus BX51, Olympus Microscopes, Tokyo, Japan). Images were captured by using a CCD camera (Cool SNAP-Pro Digital Color Camera Kit Version 4.1, Media Cybergenetics, Silver Spring, MD) at ×20 magnification. Fifty comets on duplicated slides per sample were captured, and comet analysis was performed using Komet 5.5 software (Kinetic Imaging Limited, Liverpool, UK). Individual comets were analyzed using percentage of DNA in the tail and mean olive tail moment (OTM). The final values were the results of three independent experiments, and the values were expressed as mean±SD. The OTM was calculated using the following formula:

$$\mathrm{OTM} = (\text{head mean}) \times \text{tail DNA}(\%)/100$$

Apoptosis assays

Experimental design

Exponentially growing (10^6) V79 cells were inoculated into several individual culture flasks and then divided into following groups. Group I (control)—untreated. Group II (EDTA alone)—the cultures of this group were treated with different concentrations of EDTA (0.025, 0.05, and 0.1 %) for 30 min. Group III (MA alone)—the cultures of this group were treated with different concentrations of MA (0.05, 0.25, and 0.5 %) for 30 min. Group IV (positive control)—the cultures of these groups were treated with 50 μM H_2O_2 for 1 h. Twenty-four hours after incubation, cells were processed for microscopic (for apoptotic and necrotic cell populations) and flow cytometric analysis (apoptotic cells).

Detection of apoptotic and necrotic cells by fluorescence microscopy

Microscopic EtBr/AO staining was carried out according to the protocol described by Renvoize and coworkers [20] to detect morphological evidence of both apoptosis as well as necrosis. Briefly, after various treatments cells were trypsinized, centrifuged at 155×*g* for 10 min and washed twice in cold PBS. After washing with PBS, the cell pellet were further resuspended in a 1:1 ratio of EtBr (10 μg/ml) and AO (50 μg/ml) staining solution for 1 min. Two hundred stained cells were analyzed under the fluorescence microscope.

The classification of the stained cells was performed according to the following criteria: (I) live cells with intact membrane and green staining of the nucleus; (II) cells undergoing initial apoptosis with intact membrane but with DNA fragmentation, showing green staining in the nucleus and cytoplasm, with a visible marginalization of the nuclear contents; (III) cells in final apoptosis showing areas stained orange in the cytoplasm as well nuclear sites where the chromatin was condensed, distinguishing them from necrotic cells; and (IV) necrotic cells with uniform orange staining of the nucleus.

Analysis of sub-G_1 cell population by flow cytometry

Flow cytometry was performed in order to determine the apoptotic sub-G_1 hypodiploid cells according to the protocol as described by Nicoletti and coworkers [21] with minor modifications. After the various treatments, cells from the

above groups were harvested by trypsinization and fixed in cold 70 % ethanol, kept overnight at 4 °C. The next day, cells were washed twice with PBS and treated with RNAse (100 μg/ml) for 1 h at 37 °C. Then, 5 μl of PI (1 mg/ml) was added in dark on ice, and cells were further incubated with the dye for 20 min. Using the CellQuest software the cells (10^4) were analyzed after appropriate gating in a FACSCalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ) and the histograms were analyzed using WinMDI, version 2.9 software (USA).

Statistical analysis

All the values are expressed as mean±SD, and the statistical analysis was performed using one-way analysis of variance followed by Bonferroni's post-hoc test to determine the significance between the various groups. The differences between the groups were compared and $P<0.05$ was considered significant.

Results

Micronucleus assay

The representative images of binucleate cell and micronucleated binucleate cells are shown in Fig. 1. The changes in the levels of MN frequencies in V79 cells upon treatment with various concentration of EDTA/MA are shown in Fig. 2. Treatment of V79 cells with a positive control, H_2O_2 resulted in significantly

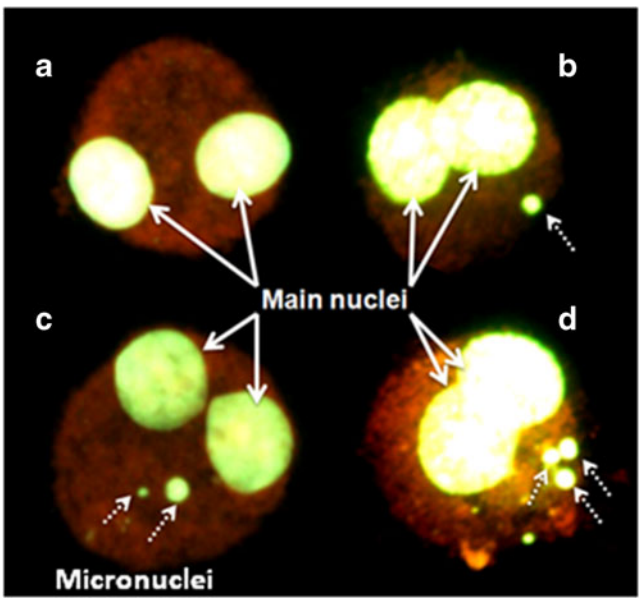

Fig. 1 Typical representative photographs of AO-stained V79 binucleate cells with one/two or multiple micronuclei. *A* BNC, *B* BNC with one micronuclei, *C* BNC with two micronuclei, *D* BNC with multiple micronuclei (*small dotted arrows* indicate micronuclei)

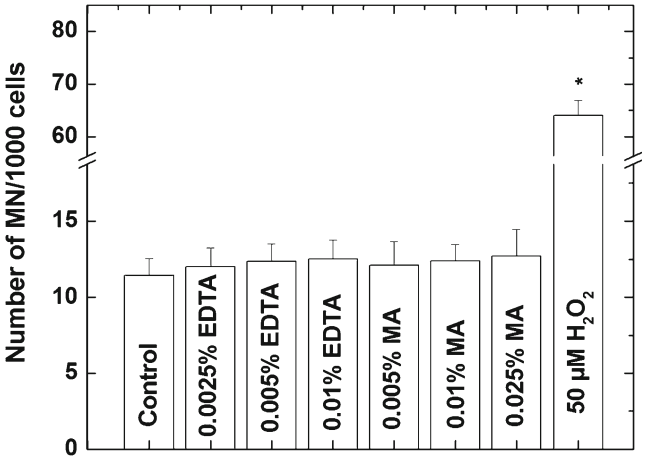

Fig. 2 Induction of MN in V79 cells after exposure with various concentrations of EDTA or MA. H_2O_2 (50 μM) was used as positive control. Values represent means and SD of three independent experiments. *$P<0.001$, level of significance when compared with control, EDTA, and MA groups

($P<0.001$) increased in number of MN, which was 5.3-fold more than that detected in the untreated control. Whereas, EDTA/MA groups did not show any significant increase in the number of MN frequencies even at highest concentrations of these agents (0.01 and 0.025 % for EDTA and MA, respectively) when compared with the untreated control (Fig. 2).

Comet assay

The effects of EDTA/MA on DNA in the form of single- and double-strand breaks along with the other types of DNA damage in V79 cells was observed by using single cell gel electrophoresis assay under alkaline conditions. The comet parameters OTM and percentage of DNA in the tail indicates the increased DNA damage when treated with any genotoxic agent (Fig. 3).

Figure 4 shows that treatment with H_2O_2, as positive control resulted in a significant ($P<0.001$) increase in the levels of DNA damage. Whereas, V79 cells treated with different concentrations of EDTA/MA did not result in a significant increase in the comet parameters even at the highest concentrations (0.01 and 0.025 % for EDTA and MA, respectively), when compared with the baseline damage in untreated cells.

Apoptosis assays

Morphological analysis of apoptosis

The morphological changes in V79 cells after various treatments were observed by dual staining with AO/EtBr. The typical representative images (×40) of live/apoptotic and necrotic cells are shown in Fig. 5 along with the images after various treatments (×20). The microscopic analysis

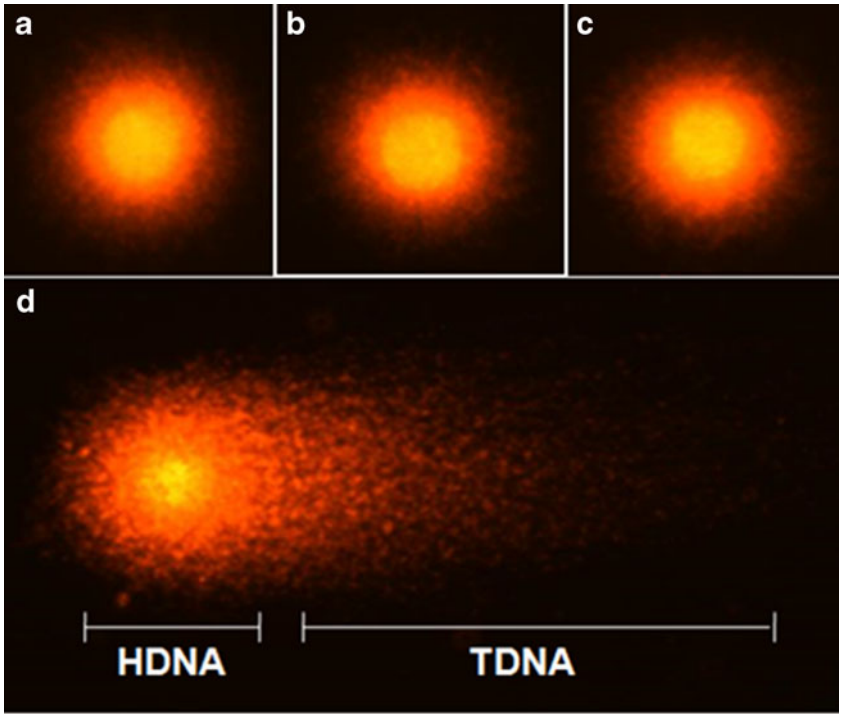

Fig. 3 Representative comet images: *A* control (untreated), *B* EDTA alone, *C* MA alone, and *D* positive control (H_2O_2 50 μM). DNA was stained with ethidium bromide, and images were of ×20 magnifications. *HDNA* head DNA, *TDNA* tail DNA

demonstrated that the control cells possessed intact green nuclei, while the H_2O_2 (50 μM) treated cells with green fragmented nuclei, characteristic feature of early apoptosis (cells with green cytoplasm and green fragmented nuclear bodies) or as late apoptotic stages (cells with green/orange cytoplasm with orange stained fragmented nuclear bodies) along with an increased number of necrotic cell population (orange/red staining for both cytoplasm as well as nuclei). The cells treated with various concentrations of EDTA/MA for 30 min showed a nonsignificant increase in the percentage of apoptotic and necrotic cells at the lower concentrations (0.025 and 0.05 % for EDTA and MA, respectively) while at higher concentrations, i.e., >IC_{50} (0.1 and 0.5 % for EDTA and MA, respectively) of both these test agents resulted in increased number of apoptotic and necrotic cells when compared with the untreated group. The total percentage of cell death after various treatments is shown in Fig. 6a, b.

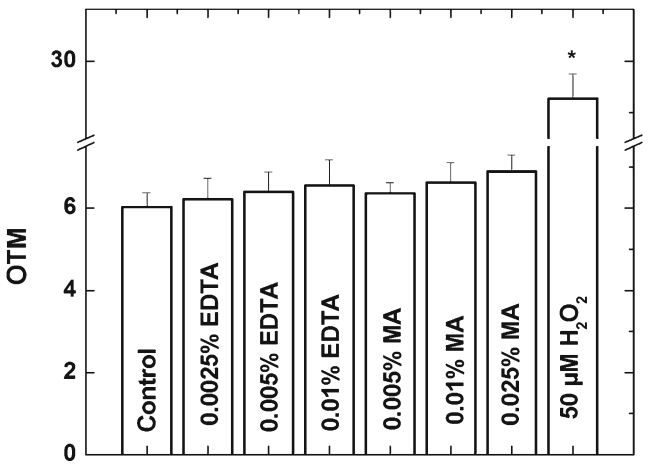

Fig. 4 DNA damage induction in V79 cells measured by comet assay after incubation with various concentrations of EDTA or MA. H_2O_2 (50 μM) as positive control; DNA damage is expressed as OTM; values represent means±SD of three independent experiments. *$P<$ 0.001, level of significance when compared with control, EDTA, and MA groups

Flow cytometric analysis

Furthermore, to confirm the apoptotic cell death observed by DNA fragmentation, we analyzed the changes in DNA content by flow cytometry. Increase in the population of cells with sub-G_1 DNA content indicates increase in the apoptotic cells. The univariate cell cycle analysis after staining the cells with PI indicated that the proportion of cells in the sub-G_1 region increased from 1.05 % in control (untreated cell) to 18.75 % with H_2O_2 (50 μM) treatment and there was no significant increase in the number of apoptotic cells at the lower concentrations of EDTA or MA (<IC_{50} doses). As observed in the microscopic analysis, cells treated with the higher concentration (>IC_{50} doses) of EDTA or MA for 30 min resulted in higher percentage of apoptotic cells. When EDTA or MA was compared, a maximum apoptosis was seen at 0.1 % EDTA concentration (Fig. 7).

Discussion

The clinical use of chelating agents should be applied with caution to prevent their extrusion to the periapical tissues. As they are administered directly into the oral cavity, these

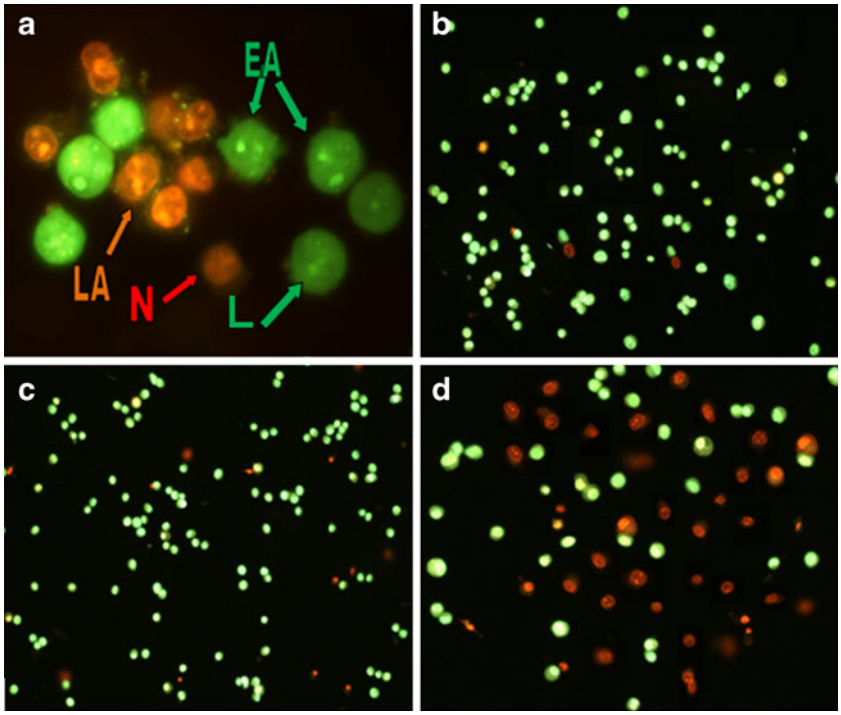

Fig. 5 Morphological analysis of apoptosis and necrosis using AO/EtBr staining. *A* Magnified (×40) images of typical representative cells (*L* live cell, *EA* early apoptotic, *LA* late apoptotic, *N* necrotic cell) and ×20 magnified images of *B* control, *C* EDTA/MA (<IC_{50}) indicating more live cells along with few apoptotic cells, and *D* EDTA/MA (>IC_{50}) indicating more percentage of necrotic cell population following apoptotic and very few live cells

agents should have low or clinically acceptable toxicity. One approach to safe treatment is to assess their cytotoxic and genotoxic effects prior to their clinical recommendation. Various organisms and genetic end points, including the induction of gene mutations as well as chromosomal damage in mammalian cells, comprise a battery of tests for analyzing the mutagenic activity of a chemical [22]. Among these assays, the micronucleus test and comet assay are considered as gold standard end point tests. Furthermore, the cytotoxic effects of an agent could be either by the apoptotic mode or by necrotic cell death. Therefore, in the present investigation, micronucleus and comet assay was performed to elucidate the genotoxic effect of these test agents while apoptotic/necrotic cell death was determined to understand the mode of cell death. The results from our earlier observation [14] clearly indicated that, the lowest concentration at 0.05 % for both MA/EDTA MTT assay showed 90 and 60 % cell viability, respectively. Based on these findings, the various noncytotoxic MA/EDTA concentrations were selected for assessing genotoxicity as the concentrations used for these assays should be noncytotoxic. However, in the present study, to assess the apoptosis/necrosis, the MA/EDTA concentrations used were in the cytotoxic range.

In order to prevent the undesirable ill effects of the chemicals used in the removal of the smear layer from the human root canal system, it is advised, materials exerting least hazardous effects are to be exploited, as a part of positive clinical outcome. In view of this in our study, we employed V79 fibroblast cells to appraise the biocompatibility of EDTA and MA. Our preliminary study clearly demonstrated that among both the chemicals MA was more efficient than EDTA with respect to its action on smear layer removal [14]. Also, we suggested the potential of MA being

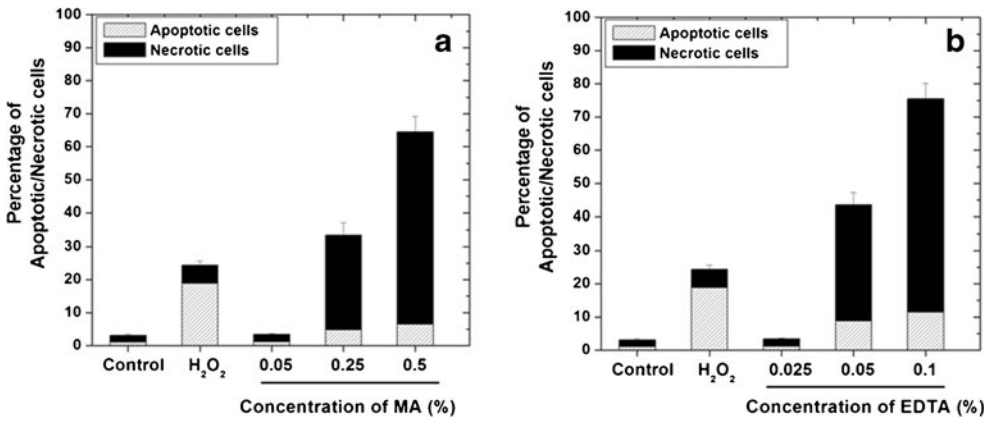

Fig. 6 Total percentage of apoptotic and necrotic V79 cells treated with different concentrations of **a** MA and **b** EDTA. H_2O_2 (50 μM) as positive control. All the results are shown as mean±SD from the data of a minimum of three separate experiments

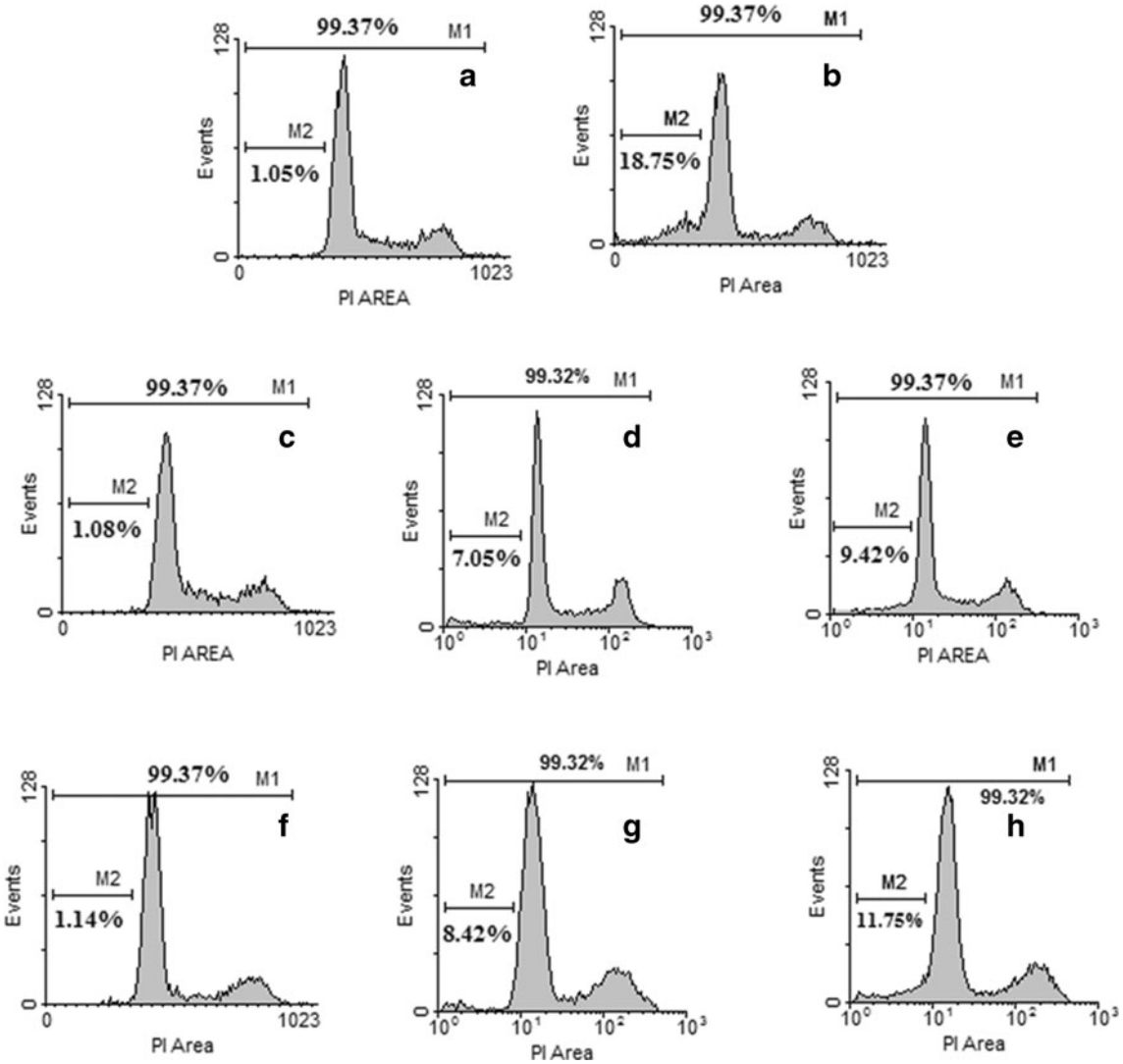

Fig. 7 Flow cytometric analysis of cellular DNA content indicating percentage of apoptotic cells (gate M2, representing sub-G_1 cells in terms of DNA content indicating apoptotic population) after treatment with various concentrations of EDTA/MA. **A** Control (untreated, M2/apoptotic=1.059 %); **b** H_2O_2 (50 μM) alone (M2/apoptotic=18.75 %); **c** 0.05 % MA (M2/apoptotic=1.08 %); **d** 0.25 % MA (M2/apoptotic=7.05 %); **e** 0.50 % MA (M2/apoptotic=9.42 %); **f** 0.025 % EDTA (M2/apoptotic=1.14 %): **g** 0.05 % EDTA (M2/apoptotic=8.42 %); and **h** 0.1 % EDTA (M2/apoptotic=11.75 %)

less toxic over to that of EDTA with equivalent smear layer removal potential. However, so far the genotoxicity of these chemicals has not been studied convincingly. In the present study, we endeavored the genotoxic effects of these two chemicals on V79 cells. The main purpose of incorporating the in vitro cell culture is to study the toxicity and not the tissue response. This in vitro system help in ranking the chemicals regarding their toxicity under given test conditions, and it also enables us to choose the compound exhibiting minimum toxicity if there is a choice of other chemicals. As there is no single test that is capable of detecting the mechanisms by which compounds induce genetic damage, we used a battery of such tests including those that asses DNA damage (alkaline comet assay), MN, and apoptosis study by microscopic and flow cytometric analysis.

In the present investigation, hydrogen peroxide was used as the positive control which easily goes through the cell membrane and is known to generate hydroxyl radicals by a nonenzymatic process in the presence of metal ions occurring in the cytoplasm by a process known as the Fenton reaction. These resultant hydroxyl radicals induce single-strand breaks, double-strand breaks, alkali-labile sites, and various species of oxidized purines and pyrimidines in DNA [23] and therefore used as a positive control in many of the earlier studies [24, 25].

DNA damage plays an important role in the development of diseases like hereditary deformities, degenerative

diseases and cancer. The study of DNA damage at the chromosome level is an essential part of genetic toxicology because chromosomal mutation is an important event in carcinogenesis. The cytokinesis-block MN assay is a well-known cytogenetic technique used to quantify DNA damage induced by any chemical compounds [26], which ultimately results in chromosomal aberrations [27] and could be detected as small MN in cytokinesis-blocked binucleate cells that are able to successfully divide at least once. Increased frequency of micronucleated cells is a biomarker of genotoxic effects that can reflect exposure to agents with clastogenic and a eugenic mode of action [28]. To our knowledge, so far there is no such study evaluating the genotoxicity of neither MA nor EDTA using these standard genotoxicity tests. Our results showed that as expected H_2O_2 treatment, the positive control induced MN indicating its genotoxic effect while EDTA and MA were found to be nongenotoxic at the clinically relevant doses suggested for efficient smear layer removal.

Furthermore, the alkaline comet assay used additionally in our study also considered as a very sensitive method to assess a wide variety of DNA lesions like single and double-strand breaks, oxidative DNA base damage, alkali-labile sites [23]. OTM is a virtual measure calculated by the computerized image analysis system considering both the length of DNA migration in the comet tail and the head. This parameter is one of the best indices of induced DNA damage among the various parameters calculated by this method. On the basis of OTM data, the results of this study indicated out that the alkaline single-cell gel (comet) assay in the experimental conditions used, did not detect the presence of DNA damage after a treatment by either MA or EDTA in all concentrations tested. However, H_2O_2 at a dose of 50 μM induced a significant DNA damage as indicated by increased MN counts and OTM without any observable immediate cytotoxic effects, as substantiated by the nonsignificant values of apoptotic and necrotic cells compared with the untreated control V79 cells.

Cell death occurs mainly by two major mechanisms, necrosis and apoptosis. Necrotic cell death is an unregulated process resulting from severe damage to the cell and is characterized by ATP depletion, cell swelling, lysis, and the release of intracellular contents resulting in tissue inflammation [29, 30]. Whereas, apoptosis is a nonrandom process characterized by cell shrinkage, nuclear condensation and fragmentation, cleavage of chromosomal DNA into internucleosomal fragments, and cytoplasmic budding containing intact organelles [31, 32]. In the present study, V79 cells exposed to H_2O_2, EDTA or MA exhibited the distinct features of apoptosis, necrosis, at higher tested concentrations.

Our data from the present study suggest that the type of cell death following EDTA or MA exposure is also dependent upon concentration in V79 cells. Furthermore, the flow cytometric observations revealed apoptotic mode cell death (sub-G_1 group) treated with EDTA or MA at higher concentrations used in this study. We also accomplished morphological examination which showed cell shrinkage, membrane blebbing and chromatin condensation elicited by increasing EDTA or MA concentration. Our study indicated that increasing the concentration of EDTA or MA eventually lead to a shift from apoptotic to necrotic mode of cell death.

The concentrations of EDTA and MA used in this study was far below than those used in clinical practice because in the body, the phagocytic cells, lymph, and blood, all of these help to dilute and carry away the drug when it comes out of the apical foramen. Therefore, EDTA and MA used in the present study were diluted to simulate the clinical scenario. The results of the present study are in agreement with previous studies in which the concentration of 17 % EDTA salt was found to be toxic in vitro [33], whereas lower concentrations have displayed reduced cytotoxic effect [34]. Similar observation was demonstrated with the MA-treated group, wherein all the tested concentrations used in this study showed much lower percentage of apoptotic and necrotic cell death than that of EDTA indicating the clinical advantage over that of EDTA with equivalent smear layer removal efficacy. Our present findings further confirmed and substantiated the data already published showing a good biocompatibility of MA over EDTA in dental practice [14].

Conclusions

Based on the results of the present study, it may be concluded that (1) both MA and EDTA are not potentially genotoxic agents at their clinically relevant doses and (2) MA induced lesser apoptotic/necrotic death than EDTA; therefore, MA may have a better clinical acceptability with comparable smear layer removal ability. Hence, the results presented here might be an additional supporting evidence for the use of MA in endodontic practice.

Conflict of interest The authors declare that they have no conflict of interest.

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