Original Article

Effects of chlorhexidine, ethylenediaminetetraacetic acid, and sodium hypochlorite on cell viability of human gingival fibroblasts *in vitro*

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Abstract

Introduction: The aim of this *in vitro* study was to evaluate the cytotoxic effects of chlorhexidine (CHX), ethylenediaminetetraacetic acid (EDTA), and sodium hypochlorite (NaOCl) irrigants on human gingival fibroblasts.

Materials and Methods: Gingival fibroblasts were cultured in Dulbecco Modified Eagle Medium for 24 h. Then, the cells were exposed for 1 min to different concentrations of CHX, EDTA, and NaOCl and the cell viability was assessed using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide tetrazolium reduction assay. The percentage of gingival fibroblast viability was compared to control (100% viability).

Results: All concentrations of CHX, EDTA, and NaOCl were cytotoxic to gingival fibroblasts compared to control group and this effect was dose-dependent ($P < 0.0001$). There was an inverse relationship between the concentrations of these irrigants and cell viability. The highest clinically used concentrations of CHX (2%), EDTA (17%) and NaOCl (2.5%) reduced the cell viability to 2.2%, 3.2%, and 1.9%, respectively, compared to the 100% viability of control. However, the lowest concentration of CHX (0.00002%), EDTA (0.00017%), and NaOCl (0.000025%) increased the cell viability to 39.8%, 30.2%, and 44.2%, respectively, in comparison to control. There were no significant differences between the irrigants at clinically used concentrations ($P > 0.05$).

Conclusion: The cytotoxic effect of CHX, EDTA, and NaOCl on gingival fibroblasts was dose-dependent. Further studies are needed to assess and optimize the safety and efficacy of these irrigants *in vivo*.

Keywords: Chlorhexidine, cytotoxicity, ethylenediaminetetraacetic acid, fibroblasts, sodium hypochlorite

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INTRODUCTION

The endodontic root canal treatment is common dental procedure aims to eliminate infection and remove all the inflamed and necrotic tissues in the pulp through the use of intracanal medicaments during the mechanical cleaning and shaping of the involved root canal.[1,2]

Spreading of the endodontic infection into periapical tissues cause inflammation of the periapical tissues where the periodontal ligament and bone are damaged and replaced by inflamed tissue which is characteristically fibrous and infiltrated by inflammatory cells.[3]

The ideal irrigation solution should eliminate or reduce the intracanal microbial population and serve as a lubricant during instrumentation.[4] It should also have low toxic effect to the periodontium to avoid the destructive sequela of irrigant extrusion through apical foramen to the surrounding tissues which prevent regeneration and recovery.[5]

A widely diverse irrigating solutions are available for endodontic treatment, including sodium hypochlorite (NaOCl), chlorhexidine gluconate (CHX), ethylenediaminetetraacetic acid (EDTA), QMix, and normal saline.[6]

NaOCl is the most frequently used endodontic irrigant due to its efficiency in dissolving the remnants of necrotic tissue and its antimicrobial activity but is not able to remove the smear layer.[7] It is usually used at a concentration range of 0.5%–5.25%.[8] EDTA is a powerful efficient chelating agent used in endodontic treatment,[9] because of its ability to remove the mineralized portion of smear layer but not soft tissue.[10] CHX is commonly used as an endodontic irrigant because it has the range of antibacterial and antifungal activity.[11] Several *in vivo* studies demonstrated the lingering effect of CHX and its anti-microbial activity with residual effects in the root canal system.[12,13] QMix solution (combination of 17% EDTA and 2% CHX), removes the smear layers and has antimicrobial activity.[14]

Irrigant extrusion into the periapical area may influence the survival of the cells of pulpal and periapical tissues.[15] NaOCl can cause injury if it comes in contact with vital tissues.[16] The fibroblasts, osteoblasts, and endothelial cells are the most affected cells. The periodontal ligament fibroblasts present in the apical constriction are the main cells that first react to irrigant extrusion.[17] These cells as well as gingival fibroblasts are commonly used in evaluating the toxicity of endodontic materials. Histological studies have shown that the morphology, and growth rates of gingival fibroblasts and periodontal ligament fibroblasts are similar.[18,19]

Previous studies evaluating cytotoxicity of intracanal irrigants have showed contradicting results. Vouzara *et al*.[20] have found 17% EDTA to be less cytotoxic to fibroblasts than 2.5% NaOCl, while Karkehabadi *et al*.[21] showed that EDTA had the highest toxicity to human periodontal ligament fibroblasts compared to NaOCl and CHX. In addition, Farhad Mollashahi *et al*.[22] have shown that CHX had the lowest cytotoxicity to stem cells from the human apical papilla compared to EDTA, and NaOCl. However, Vouzara *et al*.[20] showed CHX to be significantly more cytotoxic to human lung fibroblasts than NaOCl and EDTA. Hence, the aim of this study was to compare the cytotoxicity of CHX, EDTA, and NaOCl irrigants on human gingival fibroblast cells using ten-fold serial dilutions with a starting concentration of 2%, 17%, and 2.5%, respectively.

MATERIALS AND METHODS

Cell culture

Human gingival tissues were collected from healthy adult gingiva at Dental Teaching Hospital after obtaining signed informed consent and approval of ethical committee of Faculty of Dentistry, Umm Al-Qura University, Makkah, Saudi Arabia. The gingival tissue was cut into small pieces and then digested in a solution of 3 mg/mL collagenase Type I and 4 mg/mL dispase (Sigma, USA) for 1 h at 37°C. Single-cell suspensions were obtained by passing cells through a 70 μM cell strainer and cultured in 25 mL flask in Dulbecco Modified Eagle Medium (DMEM, UFC Biotech, KSA) containing 10% fetal bovine serum (FBS; HyClone Thermo Scientific, USA), 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma, USA), and incubated at 37°C in a humidified atmosphere of 5% CO_2.

Gingival fibroblast treatment

Gingival fibroblasts at passage 3 were seeded at 10,000 cells/well in 96 well-plate and incubated in DMEM with fetal bovine serum and penicillin/streptomycin and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. When the cells reached 70% confluency, they were exposed for 1 min to 10-fold serial dilutions of chlorohexidine, EDTA, and NaOCl starting with concentration of 2%, 17%, and 2.5%, respectively. The control cells were left untreated. After 1 min, the cells were washed 3X in PBS and reapplication of complete DMEM and the cell viability were assessed using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay.

3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide cell viability assay

The MTT assay was used to evaluate the cytotoxic effect of CHX, EDTA and NaOCl on gingival fibroblasts. The cell viability was assessed with a MTT (Sigma). The MTT assay method developed by Carmichael *et al*.[23] was followed. Briefly, the medium was removed and replaced with 100 µl/well complete DMEM containing 0.5 mg/ml of MTT and incubated for 3 h at 37°C. At the end of the incubation period, the medium was removed and DMSO: Isopropanol (1:1) solvent solution was added to dissolve formazan crystals. The optical density was read at 570 nm by a spectrophotometric Microplate Reader (SpectroStar Nano, BMG Lab). The optical density values obtained for each irrigant were used to calculate cell viability relative to untreated control, which is set at 100%.

Statistical analysis

The data were collected, tabulated, and analyzed using one-way ANOVA using Graphpad prism 7. The cell viability experiments were performed in triplicate and the results are expressed as mean ± standard error of the mean and differences were significant if a $P < 0.05$.

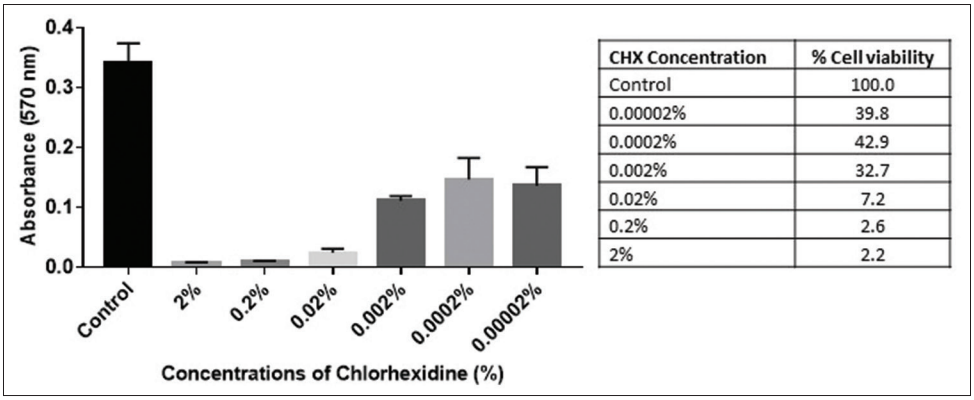
RESULTS

All tested concentrations of CHX [Figure 1], EDTA [Figure 2], and NaOCl [Figure 3] were cytotoxic to the fibroblasts and this effect was dose-dependent ($P < 0.0001$). There was an inverse relationship between the concentrations of CHX, EDTA, and NaOCl with the cell viability.

The percentage of fibroblast cell viability of CHX, EDTA, and NaOCl were compared to control that represent 100%. The cell viability was 2.2% for CHX at the highest concentration (2%) and 39.8% at the lowest concentration (0.00002%), whereas EDTA at highest concentration (17%) reported 3.2% viability and at the lowest concentration (0.00017%) it was 30.2%. Similarly, the viability of the cells exposed to high concentration of NaOCl (2.5%) was reduced to 1.9% but the lowest concentration (0.000025%) increased the cell viability to 44.2%. When we compared the cytotoxic effect of the three irrigants at clinically used concentrations we found that all irrigants were cytotoxic to the fibroblasts ($P < 0.0001$) and there were no significant differences among them ($P > 0.05$).

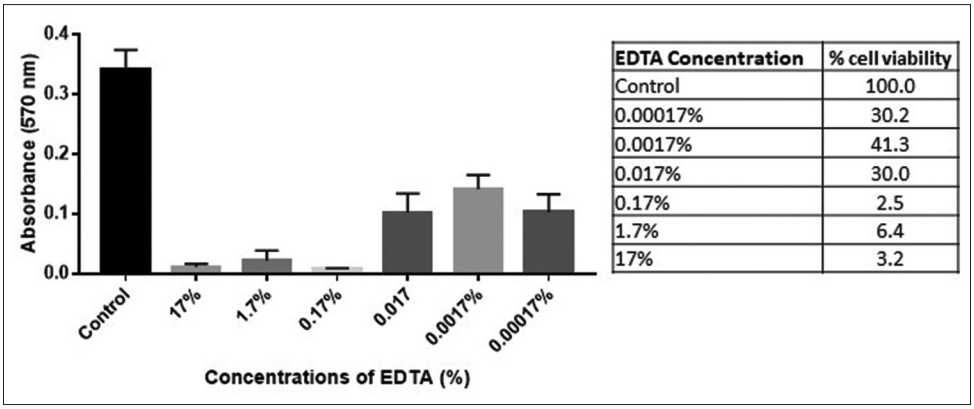
DISCUSSION

Cell viability assays are used to determine the cytotoxic effect of test material. The frequently used assays include the tetrazolium or resazurin reduction and protease activity assays that measure general metabolism or an enzymatic activity of the viable cells. Another commonly used assay is the luminogenic ATP assay which is the fastest and most sensitive, whereas tetrazolium or resazurin reduction assays are cheap with satisfactory performance. The most commonly used compounds in Tetrazolium reduction assays include viable MTT, MTS, XTT, and WST-1.[24] In this study, MTT assay was used to assess the cytotoxic effects of three endodontic irrigation solutions, CHX, EDTA, and NaOCl on the human gingival fibroblasts. It was found that all concentrations were cytotoxic to gingival fibroblasts compared to control group and this effect was dose-dependent. The results of the current study confirmed previous findings that NaOCl, EDTA, and CHX are cytotoxic to periodontal ligament fibroblasts[21] and fibroblast cell line in dose-dependent manner.[20,25] It has been found that higher concentrations of NaOCl (1%, 3%, and 5%) were cytotoxic to periodontal ligament fibroblasts[26] and NaOCl concentrations >0.05% caused complete death of human dermal fibroblasts.[27]



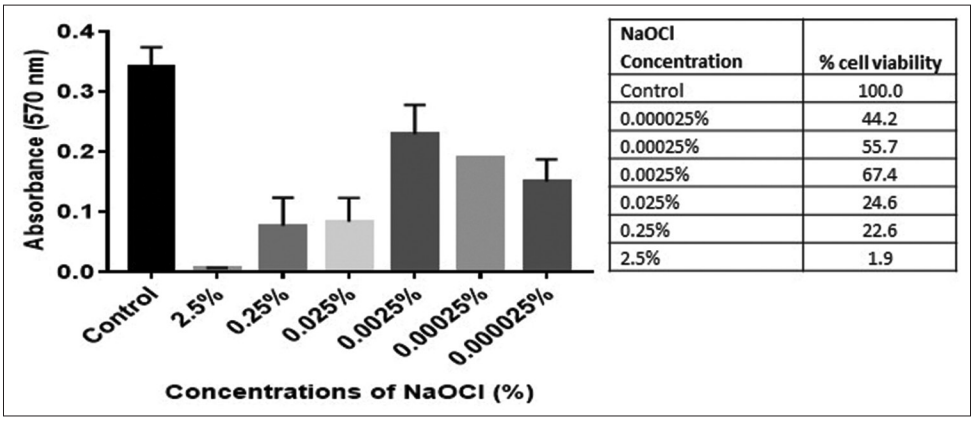
CHX Concentration	% Cell viability
Control	100.0
0.00002%	39.8
0.0002%	42.9
0.002%	32.7
0.02%	7.2
0.2%	2.6
2%	2.2

Figure 1: Cell viability of gingival fibroblasts after treatment with CHX. The cells were incubated with different concentrations of CHX for 1 min and the cell viability was measured by MTT assay. All concentrations of CHX were cytotoxic to the fibroblasts and this effect was dose-dependent ($P < 0.0001$). CHX: Chlorhexidine, MTT: 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide



EDTA Concentration	% cell viability
Control	100.0
0.00017%	30.2
0.0017%	41.3
0.017%	30.0
0.17%	2.5
1.7%	6.4
17%	3.2

Figure 2: Cell viability of gingival fibroblasts after treatment with EDTA. The cells were incubated with different concentrations of EDTA for 1 min and the cell viability was measured by MTT assay. All concentrations of EDTA were cytotoxic to the fibroblasts and this effect was dose-dependent ($P < 0.0001$). EDTA: ethylenediaminetetraacetic acid, MTT: 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide



NaOCl Concentration	% cell viability
Control	100.0
0.000025%	44.2
0.00025%	55.7
0.0025%	67.4
0.025%	24.6
0.25%	22.6
2.5%	1.9

Figure 3: Cell viability of gingival fibroblast after treatment with NaOCl. The cells were incubated with different concentrations of NaOCl for 1 min and the cell viability was measured by MTT assay. All concentrations of NaOCl were cytotoxic to the fibroblast and this effect was dose-dependent ($P < 0.0001$). NaOCl: Sodium hypochlorite, MTT: 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide

The results of the present study showed that the used concentrations of CHX (2%), EDTA (17%), and NaOCl (2.5%) reduced the cell viability dramatically and there were no significant differences between these irrigants. These findings are in accordance with other *in vitro* studies that have shown that the used concentration of CHX (2%), EDTA (17%), and NaOCl (2.5%) CHX (2%) are cytotoxic to fibroblasts.[20,25,28] On the contrary, Karkehabadi *et al.*[21] showed that EDTA had the highest toxicity to human periodontal ligament fibroblasts compared to NaOCl and CHX, while Vouzara *et al.*[20] found CHX to be significantly more cytotoxic to human lung fibroblasts than NaOCl and EDTA.

The cytotoxicity of CHX is associated with the inhibition of protein synthesis[29] or the induction of H_2O_2 and superoxide radicals,[30] whereas the cytotoxic effect EDTA might be related to its ability to penetrate cell membrane and intracellular chelation of ions.[31] On the other hand, NaOCl cytotoxicity can be attributed to its high pH which interferes with the integrity of cytoplasmic membrane[32] or inhibition of DNA synthesis leading to growth arrest.[27]

Cytotoxicity of irrigation solutions may differ due to variation in the concentration of the solution, exposure length, composition of the exposure medium, and cell type and the evaluation methods used.[33] Teixeira *et al.*[25] have shown that NaOCl was able to maintain human fibroblasts cell viability only with the 0.01% dilution while 0.05% and 0.1% dilutions was cytotoxic. A recent study has demonstrated that CHX at low concentration (0.002%) did not affect the proliferation of human gingival fibroblasts but at higher concentration (≥0.04%) suppressed cell proliferation.[34] In addition, Babich *et al.*[35] demonstrated that cytotoxicity values were 0.106, 0.011, and 0.0045 mmol/L after 1-, 24-, and 72-h exposures of CHX to gingival epithelial cell, respectively. Furthermore, the cytotoxicity was reduced when fetal bovine serum in the exposure medium was increased from 2% to 8% and the medium amended with albumin, lecithin, and heat-killed *Escherichia coli*.[35]

In general, *in vitro* cytotoxicity tests are only indicative of what may happen in clinical situations when dealing with root canal treatment and they cannot be directly implemented in clinical practice.[20]

This study has some potential limitations. One of these limitations is that only MTT assay was used to examine cell viability and not using apoptosis and necrotic cell death pathways that remain to be studied. Furthermore, the experiment was conducted on cultured cells, and the outcome represents the response of these cells without considering the host defense mechanism.

CONCLUSION

The cytotoxic effect of CHX, EDTA and NaOCl on gingival fibroblasts was dose-dependent. Further studies are needed to assess and optimize the safety and efficacy of these irrigants *in vivo*.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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