

Cytotoxicity assessment of 1% peracetic acid, 2.5% sodium hypochlorite and 17% EDTA on FG11 and FG15 human fibroblasts

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ABSTRACT

The aim of this study was to evaluate the cytotoxic effects of 2.5% sodium hypochlorite (NaOCl), 17% ethylenediamine tetraacetic acid (EDTA), and 1% peracetic acid (PAA) on human fibroblasts. FG11 and FG15 cell lines were cultured in 24-well cell culture plates for cell proliferation assessment and 96-well cell culture plates for the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay; Dulbecco's modified Eagle's medium (DMEM) was used as control data. The experimental solutions were used at 0.01%, 0.05%, and 0.1% dilutions and assessed at 1-, 2-, and 4-hour intervals. Data were subjected to statistical analysis by two-way analysis of variance (ANOVA), followed by the Bonferroni test at a significance level of p

< 0.05 . The assessment of cell proliferation in this study showed cytotoxicity to the fibroblasts with 2.5% NaOCl for all three dilutions at all time intervals, 17% EDTA for the 0.05% and 0.1% dilutions at the 2- and 4-hour intervals, and 1% PAA for all three dilutions at the 4-hour interval. The cell viability assay (MTT assay) for fibroblasts showed 2.5% NaOCl to be cytotoxic at the 0.05% and 0.1% dilutions at all time intervals, 17% EDTA to be cytotoxic at the 0.1% dilution at the 2- and 4-hour intervals, and 1% PAA to be cytotoxic at the 0.1% dilution at the 2- and 4-hour intervals. In conclusion, 1% PAA was less cytotoxic than 2.5% NaOCl and 17% EDTA.

Key words: EDTA, peracetic acid, sodium hypochlorite, toxicity.

Avaliação da citotoxicidade de ácido peracético a 1%, hipoclorito de sódio a 2,5% e EDTA a 17% em fibroblastos humanos FG11 e FG15

RESUMEN

O objetivo do presente estudo foi o de avaliar os efeitos citotóxicos de hipoclorito de sódio (NaOCl) a 2,5%, ácido etilenodiaminotetracético (EDTA) a 17% e ácido peracético (PAA) a 1% em fibroblastos humanos. As linhagens celulares FG11 e FG15 foram colonizadas em 24-well cell plates para avaliação da proliferação celular e em 96-well cell plates para o ensaio de MTT; O médio modificado Dulbecco's Eagle's (DMEM) foi usado como controle. As soluções experimentais foram usadas com diluições de 0,01%, 0,05%, e 0,1% e avaliadas com 1, 2 e 4 horas de intervalo. Os dados foram submetidos à análise estatística pelo two-way ANOVA, seguido do teste de Bonferroni com nível de significância de $p < 0.05$. A avaliação da

proliferação celular neste estudo mostrou o NaOCl a 2,5% como citotóxico nas 3 diluições e 3 intervalos de tempo, o EDTA a 17% nas diluições de 0,05% e 0,1% nos intervalos de 2 e 4 horas, e o PAA a 1% em todas as diluições no intervalo de 4 horas. O teste de viabilidade celular (MTT) mostrou o NaOCl a 2,5% citotóxico a 0,05% e 0,1% em todos os intervalos, o EDTA a 17% citotóxico nas diluições de 0,1% nos intervalos de 2 e 4 horas e o PAA a 1% citotóxico na diluição de 0,1% nos intervalos de 2 e 4 horas. Como conclusão o PAA a 1% mostrou-se menos citotóxico que o NaOCl a 2,5% e o EDTA a 17%.

Palavras Chave: EDTA, ácido peracético, hipoclorito de sódio, toxicidade.

INTRODUCTION

Removal of bacteria is the key to successfully treating necrotic teeth. Root canal instrumentation alone cannot render a root canal completely disinfected, but needs to be complemented by the use of irrigants to achieve disinfection and prevent or heal apical periodontitis¹.

The ideal irrigant should meet the following requirements: effectiveness against a broad spectrum of bacteria, ability to dissolve vital and necrotic pulp tissue and remove the smear layer, and biological compatibility with periodontal tissues. Cytotoxicity is important because during irrigation procedures a solution may extrude and contact periodontal

tissues^{2,3}. For many years, sodium hypochlorite (NaOCl) has been the most commonly used irrigant solution. Despite its antibacterial and tissue dissolution characteristics, NaOCl is not able to remove the smear layer, so chelant solutions have been used for this purpose. Ethylenediamine tetraacetic acid (EDTA) and Citric Acid (CA) have been recommended for smear layer removal^{4,5}.

Several solutions that are effective against bacteria while removing the smear layer have recently been proposed. Chlorhexidine (CHX), tetraacetythylenediamine with sodium perborate (TAE+P), maleic acid (MA), and iodine potassium Iodide (IPI) have been suggested as alternative irrigant solutions, although none of them possess all of the desired properties to be an ideal single irrigant solution^{1,6}. CHX has been widely recommended; however, it cannot dissolve pulp tissue, and recent studies have shown it to be cytotoxic⁷. In addition, several new endodontic irrigants have failed to significantly remove or kill biofilm⁶. Citric acid has been used for root canal irrigation due to its ability to remove the smear layer and effectiveness against several bacterial species⁸⁻¹⁰. However, it is not an efficient antimicrobial solution, requiring more contact time to kill bacteria *in vitro*; therefore, its use as a single irrigant should be evaluated carefully¹¹.

Peracetic acid (PAA) has been used to disinfect medical devices¹² and has low levels of cytotoxicity¹³. Furthermore, 4% PAA has demonstrated efficacy in the reduction of live bacteria in biofilm⁶, and 2% PAA has been shown to be effective against *Enterococcus faecalis*¹⁴. A low concentration of PAA has been shown to be useful in dissolving the smear layer¹⁵, disinfecting gutta-percha cones¹⁶, removing calcium hydroxide from the apical third¹⁷, and providing antimicrobial activity against *Enterococcus faecalis* biofilm¹⁸. Additionally, recent studies have shown that 2.25% PAA increases dislodgment resistance of Biodentine filling material¹⁹ and does not affect the push-out strength of MTA Fillapex²⁰,

Some studies have shown the appropriate properties of a low concentration of PAA. In fact, a previous study of our research group demonstrated the efficacy of 1% PAA against *Enterococcus faecalis* biofilm; however, little is known about the cytotoxicity of 1% PAA on human fibroblasts. The aim of this study is therefore to assess and compare the cytotoxicity of 1% PAA, 2.5% NaOCl and 17% EDTA on human fibroblasts.

MATERIALS AND METHODS

FG11 and FG15 fibroblast cells (110/mm²) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Srl, Milan, Italy) with 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT) and 1% penicillin-streptomycin solution (Invitrogen). The cells were kept in a humidified atmosphere containing 5% CO₂ at 37°C until they reached confluence. Then they were enzymatically removed from the wells, and 10 uL of trypan blue (Sigma-ALdrich, St. Louis, MO) was added to 10 uL of the cells; 1 uL of this solution was counted by microscope in a Neubauer chamber. Cell proliferation, with the experimental solutions at 0.01%, 0.05%, and 0.1% dilutions, was evaluated by the trypan blue exclusion method in a hemocytometer (Neubauer Improved Bright-line, HBG, Western Germany) at 1-, 2-, and 4-hour intervals. Each dilution was assessed in sextuplicate per test.

Cytotoxicity was examined for the 0.01%, 0.05%, and 0.1% dilutions for each experimental solution after the 1-, 2-, and 4-hour intervals using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay (Sigma Chemical Company, St Louis, MO). For the cytotoxicity assay, 110 cells/mm² in 96-well plates were cultured with the experimental solutions after 1, 2, and 4 hours. Then the cultured cells were added to 10 uL of the MTT assay (5 mg/mL) diluted in 90 uL of DMEM for 3 hours at 37°C. After this period, 100 uL of dimethyl sulfoxide (DMSO) was added; and after 15 minutes, the reading was performed. Cellular viability was determined using a microplate spectrophotometer reader (Epoch; BioTek Instruments Inc., Winooski, VT) at 590 nm, and the percentage of cell viability at each concentration was compared to the control. All the experimental procedures were conducted in sterile conditions under a laminar flow hood (Nuair, Fernbrook Lane, Plymouth, MN).

Data were evaluated by two-way analysis of variance (ANOVA) followed by the Bonferroni test using Prism 5 software (GraphPad Software, San Diego, CA) at a significance level of $p < 0.05$.

RESULTS

The trypan blue and MTT assays showed that the untreated cells remained vital at all time intervals. The trypan blue assay showed values for cell proliferation of the control group of ($0.53 \pm 0.03 \times 10^4$) at 1 hour, ($0.60 \pm 0.05 \times 10^4$) at 2 hours, and

($1.29 \pm 0.16 \times 10^4$) at 4 hours (Fig. 1). The MTT assay showed absorbance values for the control group of (0.34 ± 0.01) at the 1-hour interval, (0.34 ± 0.02) at the 2-hour interval, and (0.34 ± 0.04) at the 4-hour interval (Table 1).

Cell proliferation in the 2.5% NaOCl group was different from the control group with the 0.01% dilution at the 1-hour interval ($0.30 \pm 0.07 \times 10^4$), the 2-hour interval ($0.22 \pm 0.05 \times 10^4$), and 4-hour interval ($0.92 \pm 0.06 \times 10^4$); the values for the 0.05% and 0.1% dilutions were the same (0×10^4) at all time intervals and differed significantly from the control group ($p < 0.05$). The MTT assay showed

differences in cell viability for the 0.05% and 0.1% dilutions at all time intervals; no difference was found for the 0.01% dilution ($p < 0.05$).

Cell proliferation in the 17% EDTA group was different from the control group with the 0.05% dilution at the 2-hour interval ($0.40 \pm 0.06 \times 10^4$) and the 4-hour interval ($0.73 \pm 0.06 \times 10^4$) and with the 0.1% dilution at the 2-hour interval ($0.37 \pm 0.04 \times 10^4$) and the 4-hour interval ($0.22 \pm 0.11 \times 10^4$). No difference was found for the 0.01% dilution at any time interval. The MTT assay showed differences in cell viability for the 0.1% dilution at the 2-hour interval (0.24 ± 0.03) and the 4-hour interval ($0.21 \pm$

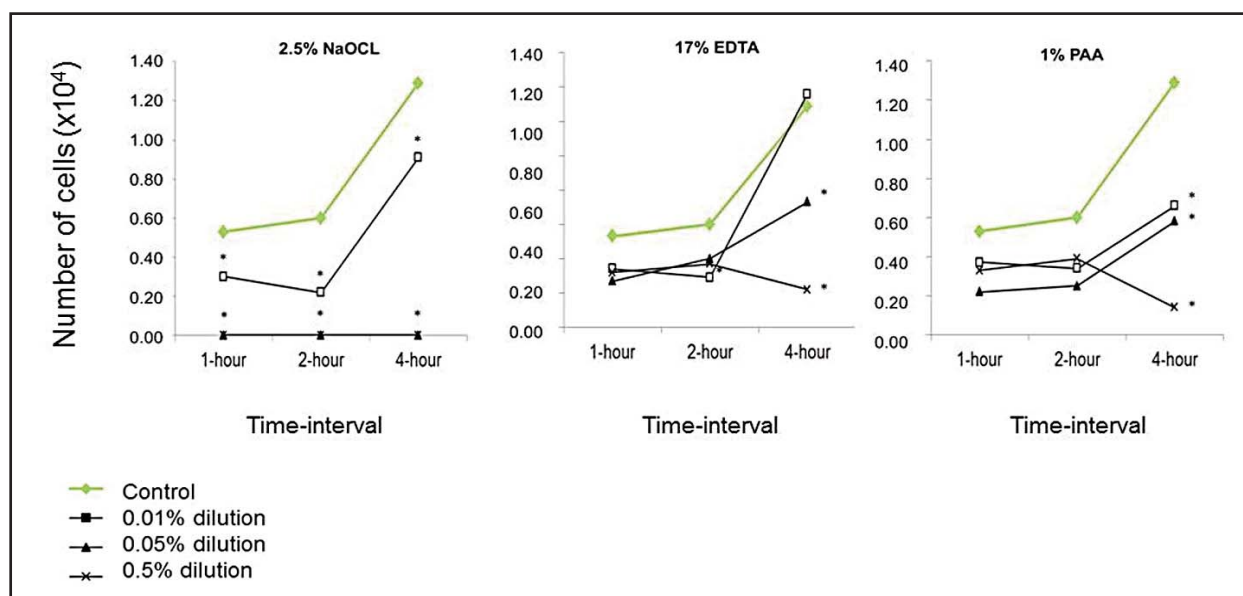


Fig. 1: Number of cells ($\times 10^4$) for each experimental solution at different time intervals and dilutions. *Values statistically different from control group at $p < 0.05$.

Table 1: Cell Viability for the Experimental Solutions at Different Time Intervals and Dilutions According to Optical Absorbance (590 nm).

Interval	Dilution	Control	2.5% NaOCl	17% EDTA	1% PAA
1-hour	0.01%	0.34 ± 0.03	0.40 ± 0.09	0.33 ± 0.03	0.33 ± 0.03
	0.05%		$0.20 \pm 0.02^*$	0.31 ± 0.03	0.35 ± 0.03
	0.1%		$0.10 \pm 0.03^*$	0.35 ± 0.06	0.32 ± 0.02
2-hour	0.01%	0.34 ± 0.02	0.32 ± 0.04	0.34 ± 0.02	0.34 ± 0.04
	0.05%		$0.09 \pm 0.004^*$	0.35 ± 0.07	0.35 ± 0.03
	0.1%		$0.08 \pm 0.003^*$	$0.24 \pm 0.03^*$	$0.25 \pm 0.03^*$
4-hour	0.01%	0.34 ± 0.04	0.30 ± 0.06	0.43 ± 0.06	0.38 ± 0.08
	0.05%		$0.07 \pm 0.004^*$	0.40 ± 0.09	0.34 ± 0.03
	0.1%		$0.08 \pm 0.01^*$	$0.21 \pm 0.05^*$	$0.13 \pm 0.007^*$

*Values statistically different from the control group at $p < 0.05$.

0.05). No difference was found for the 0.01% and 0.5% dilutions at any time interval ($p < 0.05$).

Cell proliferation in the 1% PAA group at the 4-hour interval was different from the control group with the 0.01% ($0.66 \pm 0.11 \times 10^4$), 0.05% ($0.58 \pm 0.25 \times 10^4$), and 0.1% ($0.14 \pm 0.12 \times 10^4$) dilutions. No difference was found at the 1-hour and 2-hour intervals ($p < 0.05$). The MTT assay showed differences from the control group at the 2-hour (0.25 ± 0.03) and 4-hour intervals (0.13 ± 0.007) for the 0.1% dilution ($p < 0.05$).

DISCUSSION

The aim of this study was to assess the cytotoxicity of different irrigant solutions used in endodontics. The trypan blue assay stains dead cells and is largely applied as a confirmatory test for measuring changes in viable cell number caused by a drug. The MTT assay measures the mitochondrial function and is commonly used to detect the loss of cell viability²¹. It is important to emphasize that the dilutions used in the present study (0.01%, 0.05%, and 0.1%) are based on the fact that *in vitro*, these cells do not present other shields such as phagocytic cells, lymphatic system or blood to decrease the cytotoxic effect of the experimental solutions²⁰. Different cells have been used to evaluate cytotoxicity^{12,21}; our study used FG11 and FG15 human periodontal fibroblasts for better simulation of cells that might be clinically affected by irrigant solutions.

The results of the present study confirm previous findings that NaOCl is cytotoxic to fibroblasts cells⁷. NaOCl was able to maintain cell viability only with the 0.01% dilution. These findings are in accordance with Simbula et al.²³, who have shown that higher concentrations are related to lower percentages of cell viability. It has also been suggested that low concentrations of NaOCl

possess the antibacterial characteristics required to be an appropriate solution²⁴. The present study is in agreement with a previous study that showed 17% EDTA to be less cytotoxic than 2.5% NaOCl⁷.

PAA has been shown to be efficient against Ebola virus in culture plates and in dried blood²⁵; and in its different dilutions has been suggested for use as a disinfectant solution for dental devices due to its rapid action against all microorganisms and absence of harmful products¹². A previous study recommended PAA as a single irrigation solution in the treatment of teeth presenting necrotic pulp tissue²⁶. Another study showed that 0.3% PAA was not cytotoxic when polyvinyl chloride (PVC), polyurethane, silicone tubes and polytetrafluoroethylene (PTFE) tubes were soaked in the solution and immersed in a culture of Henrietta Lacks cells (HeLa)¹³. To the best of our knowledge, no study has yet evaluated the cytotoxic effects of 1% PAA against human periodontal fibroblasts. Our results showed that 1% PAA presented lower cytotoxicity than 2.5% NaOCl and 17% EDTA. While our results suggest that 1% PAA appears to be an appropriate solution, another recent study showed 1% PAA to be more cytotoxic than 2.5% NaOCl, in disagreement with our findings²⁷. PAA has been used in the past as a single irrigant solution for root canal therapy. Recently, PAA has attracted the attention of the endodontic community. Further clinical studies using current instrumentation, irrigation and filling protocols are necessary to evaluate whether 1% PAA can be used as a single irrigant solution. The present study evaluated only short-term cytotoxicity, but long-term evaluations are also recommended.

Within the limitations of this study, 1% PAA is less cytotoxic than 2.5% NaOCl and 17% EDTA.

ACKNOWLEDGMENTS

The authors deny any conflict of interest related to this study.

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