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Cytotoxicity analysis of EDTA and citric acid applied on murine resident macrophages culture

K. F. Amaral, M. M. Rogero, R. A. Fock, P. Borelli & G. Gavini
Department of Endodontics, Dental School, University of São Paulo, São Paulo, Brazil

Abstract

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Aim To assess the *ex vivo* cytotoxicity of EDTA and citric acid solutions on macrophages.

Methodology The cytotoxicity of 17% EDTA and 15% citric acid was evaluated on murine macrophage cultures using MTT-Tetrazolium method [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide]. A total of 5×10^5 cells were plated in medium culture with 17% EDTA or 15% citric acid. Fresh medium was used as a control. Toxicity values were analysed statistically by ANOVA and Tukey's test ($P < 0.05$) at short (0, 6, 12, 24 h) and medium periods (1, 3, 5, 7 days), using ELISA absorbance.

Results On the short term, both EDTA (0.253 nm) and citric acid (0.260 nm) exhibited cytotoxic effects on macrophage cultures ($P < 0.05$). On the medium term, statistical differences were observed ($P < 0.05$) between the groups. EDTA (0.158 nm) and citric acid (0.219 nm) were cytotoxic when compared with the control group; EDTA-reduced macrophage viability significantly more than citric acid ($P < 0.05$).

Conclusions Both EDTA and citric acid had effects on macrophages cells *ex vivo*, but citric acid was less toxic in periods from 1 to 7 days of use.

Keywords: cell culture, citric acid, cytotoxicity, EDTA, macrophages, root canal irrigants.

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Introduction

The use of chelating agents in combination with sodium hypochlorite is recommended during root canal treatment to disinfect the root canal system and remove organic and inorganic debris (Teixeira *et al.* 2005). Both EDTA and citric acid enhance chemo-mechanical enlargement of canals (Garberoglio & Becce 1994), remove inorganic compounds of the smear layer (Yamaguchi *et al.* 1996, Malheiros & Gavini 1998) and increase dentinal permeability (Scelza *et al.* 2000). The concentration and pH of these solutions influences directly the dissolution of the smear layer (Hülsmann *et al.* 2003).

During root canal preparation, unintentional extrusion of debris and irrigants into the periapical area is likely to occur (Al-Omari & Dummer 1995, Ferraz *et al.* 2001) and cause time reactions. Such reactions may promote vascular alterations, with activation of inflammatory cells (neutrophils, macrophages), production of chemical mediators and reduction in cellular repair (Kopp & Schwarting 1989, Piatelli *et al.* 1991, Metzger 2000). Thus, chemical agents employed should be biologically compatible, especially when in contact with the periapical region.

Biocompatibility should be evaluated by laboratory tests (cytotoxicity tests and subcutaneous implantation), then followed by *in vivo* experiments (Kawahara *et al.* 1968, Schmalz 1994). According to Freshney (1990), biocompatibility studies may be divided into

Correspondence: Giulio Gavini, Faculdade de Odontologia da Universidade de São Paulo, Disciplina de Endodontia, Departamento de Dentística, Cidade Universitária São Paulo, Av. Prof. Lineu Prestes, 2227, CEP 05508-900, SP, Brazil (Tel./fax: +55 11 5549 6114; e-mail: ggavini@usp.br).

immediate or short-term cellular responses, associated with alterations in membrane permeability or a specific damage to cell metabolism, and long-term responses, with loss of cellular self-renewal and/or survival abilities when in altered environments.

Malheiros *et al.* (2005) and Scelza *et al.* (2001) described the effects of EDTA and citric acid on cultured NIH 3T3 fibroblasts suggesting that citric acid was more biocompatible. Segura *et al.* (1996) reported that EDTA could modify macrophage functions by blocking their binding to vasoactive intestinal peptides (VIP). Different concentrations of EDTA-promoted inhibition of macrophages binding ability between 15 and 30 min, and higher concentrations showed more significant effects (Segura *et al.* 1997, Segura-Egea *et al.* 2003).

The aim of the present study was to evaluate the short and medium-term cytotoxic effects of 17% EDTA and 15% citric acid when applied on cultured murine macrophages.

Material and methods

Macrophage cells were obtained from the peritoneal exudate of male Swiss mice, aged 8–12 weeks (according to the Ethics Committee on Animal Experimentation – ECAE 19/2003). Cell content (consisting of 95% macrophages) was resuspended in McCoy's 5A medium (Sigma Chemical Co., St Louis, MO, USA), supplemented with 10% of foetal bovine serum (Cultilab, Campinas, Brazil) and 1% antibiotic-antimycotic solution (Sigma Chemical Co.), and maintained in an humidified incubator at 37 °C and 5% CO_2. Experimental groups were composed of McCoy's medium containing (i) 17% EDTA (Fórmula e Ação, São Paulo, Brazil), (ii) 15% citric acid (Fórmula e Ação) and (iii) a control group, containing only culture medium. Effects were evaluated in the short and medium-term. All experimental procedures were carried out in duplicates.

Short-term assay

Aliquots of 5×10^5 cells were plated in triplicate in polystyrene 24-well plates, immediately after being collected and processed, and filled with culture medium, resulting in a final volume of 1 mL.

After intervals of 0, 6, 12 and 24 h, cell viability was determined through the MTT-Tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] assay.

Medium-term assay

Aliquots of 5×10^5 cells were plated in triplicate in polystyrene 24-well plates, immediately after being collected and processed, and filled with culture medium, with a final volume of 1 mL.

After intervals of 1, 3, 5 and 7 days, cell viability was determined through MTT assay.

Evaluation of light absorbance through MTT assay

After the experimental periods, 50 μL of a MTT colorimetric solution was added to the groups and kept in the incubator for 8 h. Then, 500 μL of 10% sodium dodecyl sulphate (SDS) was added to each well. The solution was allowed to solubilize overnight in 100% humidity. The absorbance of each well was determined using ELISA microplate reader at 550 nm.

Determination of standard samples and cell viability curves

Duplicates of crescent cell dilutions were distributed in 24-well plates: blank, 1×10^3, 1×10^4, 1×10^5, 5×10^5, 7.5×10^5 and 1×10^6, completed with culture medium.

Absorbance was obtained by MTT. Absorbance values were converted into cell concentrations through a logarithmic equation, and then the average line chart for the standards was determined.

The curves representing the cell viability of the experimental groups were then determined.

Statistical analysis

Data were arranged in tables and analysed using two-way ANOVA ($P < 0.05$). When a significant difference was found, Tukey's test was used to evaluate the significance amongst the mean values. Values were considered significant when $P < 0.05$.

Results

Short-term assays

Two-way ANOVA values showed significant differences ($P < 0.05$) amongst groups. Tukey's test revealed that EDTA (0.253 nm) and citric acid (0.260 nm) decreased cell density confirming their cytotoxic effect when compared with the control group (0.355 nm). No

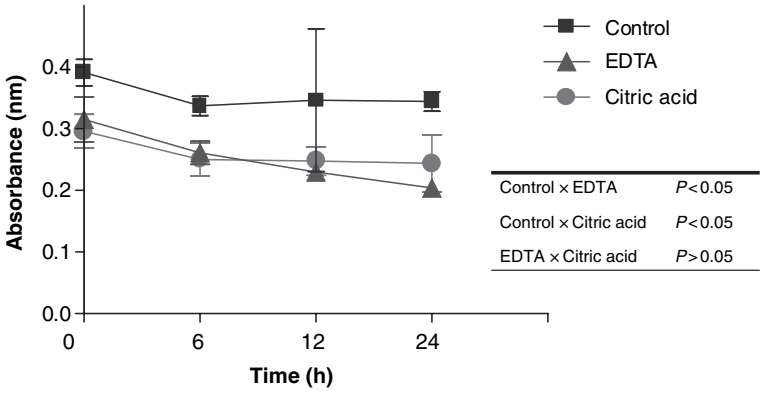

Figure 1 Short-term absorbance values (nm).

significant differences ($P > 0.05$) were observed between EDTA and citric acid (Fig. 1).

Time observations showed statistical differences ($P < 0.05$). At the 0 h observations (0.334 nm), cell viability was significantly better when compared with the other periods ($P < 0.05$). For 6 h (0.269 nm), 12 h (0.275 nm) and 24 h (0.244 nm) no statistical significance was noted ($P > 0.05$).

Medium-term assay

Cytotoxic effects were observed using the test solutions ($P < 0.05$). Tukey's test indicated that EDTA (0.158 nm) and citric acid (0.219 nm) had a negative effect on the viability of macrophage culture. Thus, EDTA resulted in intense reduction in cell viability ($P < 0.05$) compared with citric acid (Fig. 2).

The two-way ANOVA analysis of the experimental periods revealed significant differences ($P < 0.05$). Tukey's value showed significant differences ($P < 0.05$) for the average absorbance of 1, 3, 5 and 7 days (0.264, 0.237, 0.212 and 0.203 nm respectively).

The reduction in cellular viability was carried out over time. At the 1 day experimental period, the macrophage density was significantly better than that observed at 3, 5 and 7 days ($P < 0.05$). The results at day 3 showed that cellular concentration was statistically higher than at the 5 or 7 day period ($P < 0.05$).

Cellular viability

Cellular viability of the experimental groups and periods of time is presented in Table 1 and Fig. 3.

Discussion

Macrophages play a role in the immune response of human inflammatory and infectious reactions. They are involved in the innate immune responses and have the capability to recognize and then phagocyte any antigenic agent. Besides, they produce several biological active substances, such as enzymes, prostaglandins and cytokines, such as IL-1α, IL-1β, TNF-α, related to bone resorption (Kopp & Schwarting 1989, Piatelli *et al.* 1991, Metzger 2000).

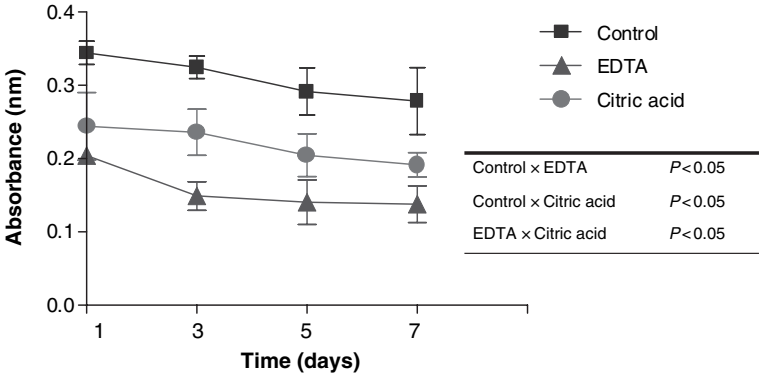

Figure 2 Medium-term absorbance values (nm).

Table 1 Mean absorbance (nm) and macrophage concentration, according to groups and experimental time

	Control		EDTA		Citric acid	
Time	Mean absorbance (nm)	Cell concentration (cells mL^{-1})	Mean absorbance (nm)	Cell concentration (cells mL^{-1})	Mean absorbance (nm)	Cell concentration (cells mL^{-1})
Initial (−1 h)	0.391	679 050	0.391	679 050	0.391	679 050
0 h	0.391	679 050	0.315	489 050	0.296	441 550
6 h	0.337	544 050	0.261	354 050	0.250	326 550
12 h	0.347	569 050	0.230	276 550	0.248	321 550
24 h	0.344	556 250	0.204	206 250	0.244	306 250
1 day	0.344	556 250	0.204	206 250	0.244	306 250
3 days	0.324	506 250	0.149	68 750	0.236	286 250
5 days	0.292	421 250	0.141	43 750	0.205	203 750
7 days	0.278	386 250	0.138	36 250	0.191	168 750

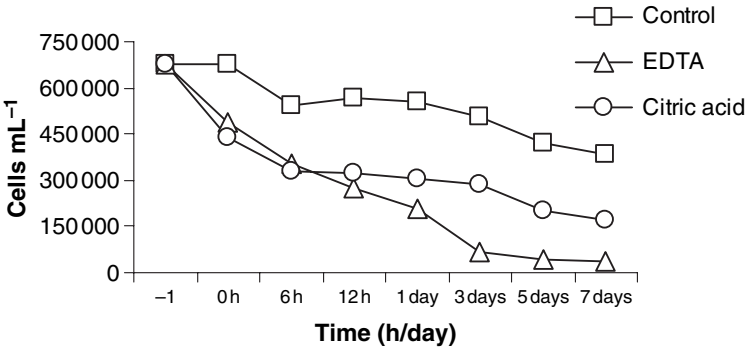

Figure 3 Viability cell curve (macrophage concentration), according to tested solutions and time.

The present study showed that EDTA and citric acid solutions had cytotoxic effects on macrophage cultures, confirmed by a 50–70% reduction of viability during 0–24 h.

The irrigant solutions probably exerted a direct effect on macrophages, promoting alterations on their cell membranes caused by chelator ions, such as Ca^{2+} and Mg^{2+}, and accelerating the apoptotic process as these divalent cations are considered co-factors to several enzymatic reactions. These findings are in agreement with Segura *et al.* (1996), who reported an inhibitory effect on VIP caused by EDTA. They concluded that EDTA reduced the VIP binding to macrophage membranes that are responsible for the modulation of periapical immune response.

Moreover, it seems that EDTA and citric acid could cause indirect effects on cell metabolism. The findings of the present study indicate that the presence of the irrigant solutions decreased the pH of the culture medium and reduced the available cell nutrients causing a significant reduction in macrophage viability. If macrophage functions were altered, the phagocyte activity of inflammatory cells will not occur, consequently periapical healing will be impaired.

As substrate adherence of monocytes is the first step in the phagocytic process, the EDTA had negative effects on macrophage activity (Segura *et al.* 1997). Segura-Egea *et al.* (2003) tested inhibitory effects of EDTA and EGTA (Ethylene glycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid) substances on substrate adherence capacity of macrophages and repeated that EGTA-reduced substrate adherence in a time–concentration-dependent manner whilst EDTA had more potent effects on the reduction of adherence capacity. These results could be explained because EGTA binds Ca^{2+} ions more specifically than EDTA. In accordance with the results of the present study, it is supposed that both EDTA and citric acid would be able to retard phagocytosis in macrophages, but the exact mechanism is unknown.

To observe the residual effects, as well as the interference of these irrigant solutions on macrophage growth, EDTA and citric acid were also analysed at medium-time (Freshney 1990). EDTA and citric acid revealed significant cytotoxic patterns, but citric acid had a less irritant effect on cell cultures. Citric acid reduced the initial macrophage population (5×10^5cells mL^{-1}) to 25% of the total cells whilst EDTA caused 95% of cellular death.

Based on these results, it is suggested that the clinical use of EDTA and citric acid in endodontic therapy must be performed carefully to avoid apical extrusion (Al-Omari & Dummer 1995, Ferraz *et al.* 2001). These substances not only cause a decalcifying action on periapical bone, but they also could affect inflammatory and neuroimmune regulation.

According to other cytotoxic studies, 17% of EDTA-T produced more aggressive effects in NIH 3T3 fibroblast cultures than 10% citric acid (Scelza *et al.* 2001). These results are in agreement to those reported by Malheiros *et al.* (2005), who showed 10% and 15% citric acid to be more biocompatible than 17% EDTA.

The elevated cytotoxicity of EDTA might be related to the chelating reaction itself, and also to the accentuated decrease in pH. When in contact with metallic ions, two chemical reactions take place: formation of a complex (eqn 1) and protonation (eqn 2):

$$EDTAH^{3-} + Ca^{2+} = EDTACa^{2-} + H \quad (1)$$

$$EDTAH^{3-} + H = EDTAH_2^{2-}. \quad (2)$$

As the reaction occurs, acids accumulate, the protonation of EDTA prevails (eqn 2) and the rate of demineralization decreases. On the other hand, the EDTA molecule is formed by four carboxylic groups and ionic dissociation occurs in four steps with its own dissociation constant (pK). This means that EDTA dissolution takes place over a broad range of different pH values. As the pH becomes permanently acid, the reaction of EDTA with hydroxyapatite matrix affects directly dentinal solubility (Hülsmann *et al.* 2003).

The final irrigant solutions may improve dentinal permeability because of the chelating action of metallic ions. However, according to the literature, it seems that apical leakage of EDTA during root canal preparation has a higher negative influence on apical cells than citric acid. This could be an effect of the EDTA calcium chelating property; it reacts with magnesium, iron, copper, zinc and other divalent ions and affects negatively the cellular membrane structure, as well as macrophage function and viability.

In the present study, the initial periods of observation demonstrated more intense levels of decrease in cell viability (5.4×10^5 to 3.2×10^5 cells mL^{-1}) than the medium-term assay (3.5×10^5 to 1.9×10^5 cells mL^{-1}). The absorption of the medium's nutrients could also explain the decrease in cell viability. McCoy's 5A medium (Sigma Chemical Co.) supplemented with 10% foetal bovine serum (Cultilab) reproduces the ideal conditions for maintaining *ex vivo* cultures, but the consumption of the nutrients and accumulation of metabolic wastes cannot be excluded.

The choice of an irrigating solution is based on its chemical, physical and biological properties. Until now, there is no single solution that is capable of promoting complete removal of the organic and inorganic components into root canals. Consequently, the association of sodium hypochlorite and a chelating or decalcifying agent is necessary. Both EDTA and citric acid have great efficiency in terms of root canal cleaning and increasing dentinal permeability (Garberoglio & Becce 1994, Yamaguchi *et al.* 1996, Malheiros & Gavini 1998, Scelza *et al.* 2000, Teixeira *et al.* 2005).

Root canal preparation should retain endodontic agents in the root canal space. Considering its acid chelating effect on calcium ions, the level of injury caused to the periapical tissues by agents such as EDTA is likely to be high, and interfere with the healing process.

Conclusions

Both EDTA at 17% and citric acid at 15% had cytotoxic effects on murine macrophage cultures. However, in medium-term evaluation, the citric acid solution was less cytotoxic, according to the cell viability tests. Thus, citric acid may be considered as an alternative irrigant solution in root canal treatment.

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