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Cytotoxic effect of endodontic irrigants *in vitro*

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Background: Cytotoxicity of root canal irrigants is important due to their close contact with host tissues. The aim of this study was to assess the cytotoxic effect of NaOCl 3%, Chx 2%, and MTAD on rat periodontal ligament fibroblasts, at 0.1 and 100 µl/mL, using WST-1 colorimetric method.

Material/Method: Rat ligamental fibroblasts were exposed to the irrigants and their viability was assessed after 1, 24, 48, and 72 h. The measurements were determined using WST-1 assay, using a micro ELISA reader.

Results: At 100 ml/L all 3 irrigants were strongly cytotoxic, although CHX was less so than NaOCl and MTAD. At the 0.1 ml/L concentration, NaOCl and MTAD were only moderately cytotoxic, whereas Chx was highly deleterious to cell viability at all time points. There was a significant influence of the dilution rate of the substance, because the odds ratio for cell viability being over 50% was increased 51 times between the 100 ml/L and 0.1 ml/L dilutions.

Conclusions: It seems that irrigating solutions should be used at lower concentrations to enhance cell viability.

MeSH Keywords: Sodium Hypochlorite – toxicity • Chlorhexidine – toxicity

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Background

The use of root canal irrigant solutions is essential in endodontic treatment because they help to disinfect and lubricate the root canal, flush out debris from the canal system, and dissolve organic and inorganic tissues [1]. Sodium hypochlorite (NaOCl) and chlorhexidine gluconate (Chx) are commonly used for these purposes in endodontic treatment. NaOCl is widely recommended as a root canal irrigant for its antibacterial effects and its capacity to dissolve organic tissues. Concentrations ranging from 0.5–5.25% have been reported in the literature, with higher concentrations having better antibacterial efficacy [2] but also higher toxicity [3,4]. Chx is used extensively in periodontal therapy because of its substantive and broad-spectrum antimicrobial effects. For these same reasons, it is also used as a root canal irrigant and intracanal medication in endodontics.

Recently, a new root canal irrigant, known as MTAD, has been introduced for use as a final irrigation solution, comprising a tetracycline isomer, an acid, and a detergent. This is reported to eliminate microorganisms that are resistant to conventional root canal irrigants [5].

An ideal root canal irrigant should be biocompatible, because of its close contact with the periodontal tissues during endodontic treatment. The biocompatibility of dental materials has frequently been analyzed using the WST-1 test, in which the conversion of tetrazolium dye into formazan crystals in the mitochondria of living cells is proportional to their viability [6]. The purpose of this study was to evaluate the cytotoxicity of NaOCl, Chx and MTAD on rat periodontal ligament fibroblasts using the WST-1 colorimetric method.

Material and Methods

Materials

The materials tested in this study were: 3% NaOCl (ChlorCid; Ultradent, USA); 2% chlorhexidine gluconate solution (Consepsis; Ultradent, USA); and BioPure MTAD (DENTSPLY Tulsa Dental, USA).

Irrigants were diluted to 0.1 and 100 µl/mL with Dulbecco's Modified Eagle's medium (DMEM), following a previously reported protocol [7].

Cell culture

Primary cell cultures of rat periodontal fibroblasts were established from rat periodontal ligament (obtained from the Histology and Embryology Department of the Faculty of Medicine, Hacettepe University, Ankara, Turkey). Rat periodontal ligament tissues were minced to 1 mm^3 pieces and placed in 25 cm^2 tissue culture flasks with DMEM containing 10% fetal bovine serum (FBS), 1% penicillin, 1% L-glutamine, and cultured in the same media at 37°C, 95% air, and 5% CO_2. Fibroblasts were isolated from the explants within 2 weeks. Cells were trypsinized and passaged once they covered at least 75% of the flask surface. Gingival fibroblast cultures reached confluence in ~7 days and were subsequently sub-cultured (diluted 1:3 with tissue culture medium) until the start of the experiment. These cultures were used down to the third passage.

Measurement of cytotoxicity

Cytotoxicity was determined using the WST-1 assay (Roche Diagnostics GmbH, Mannheim, Germany; Cat. No.: 11644807001). Rat periodontal ligament fibroblasts were plated at 1×10^4 cells/well in 96-well plates (Falcon Biosciences, Milano, Italy) in 100 µl of culture medium, and cultured for 24 h prior to use.

We used 3 test groups: Group I: NaOCl 3%; Group II: MTAD; and Group III: Chx 2%. All irrigants were tested at dilutions of 0.1 and 100 µl/mL. In addition to the test groups, we included a control group that contained cells and culture medium alone with no irrigant. The diluted irrigants were added to the relevant wells in 100 µl volumes and incubated with the cells at 37°C for 1, 24, 48, and 72 h. At the end of these times, 10 µl of WST-1 was added to each well and after incubation for 4 h at 37°C, the absorption of each well at 420–600 nm was measured using a micro-ELISA reader (VersaMax, Molecular Devices)

WST-1 test

The WST-1 assay was performed according to the manufacturer's recommendations. This method is based on the reduction of WST-1 to a yellow-orange soluble formazan product by mitochondrial dehydrogenases, which are a part of the mitochondrial respiratory chain. Therefore, cell viability can be determined by measuring the optical density of the formazan product with regard to cellular respiration and metabolic rate [8]. Cellular damage precludes cells from generating the energy required for metabolic function and growth, thus this assay provides a good model for cytotoxicity assessment [8–10].

Baseline absorption readings at 420–600 nm were measured using a micro-ELISA reader (VersaMax, Molecular Devices). The percent cell viability shown in our Tables represents the mean of 8 experimental replicates. 'Percent Cell viability' was calculated using the following formula:

$$\% \text{ Viability of cells} = \frac{\text{OD test compound}}{\text{OD Control}} \times 100$$

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Table 1. Cell viability in the presence of different endodontic irrigants at 100 µl/mL for different time periods, as assessed by WST-1 assay. Data are mean ±SD of eight replicates.

Irrigant	1 h	24 h	48 h	72 h
NaOCl 3%	3.18±0.68	11.49±0.46	9.26±0.49	16.13±0.52
MTAD	8.34±0.28	6.50±0.23	8.37±0.60	11.87±0.60
CHX 2%	14.48±0.11	18.53±0.92	8.34±0.97	15.98±2.82
P-value	P<0.0001	P<0.0001	P=0.002	P=0.022

Table 2. Cell viability in the presence of different endodontic irrigants at 0.1 µl/mL for different time periods, as assessed by WST-1 assay. Data are mean ±SD of eight replicates.

Irrigant	1 h	24 h	48 h	72 h
NaOCl 3%	63.06±1.85	58.74±12.60	83.89±2.55	86.80±11.87
MTAD	78.06±3.37	48.67±1.72	66.72±5.14	71.44±10.35
CHX 2%	16.86±1.16	5.25±0.61	5.61±0.16	9.88±0.72
P-value	P<0.0001	P<0.0004	P<0.0001	P=0.0005

where 'OD (optical density) test compound' is the mean absorbance of treated cells minus the baseline reading and 'OD control' is the mean absorbance of control cells minus the baseline reading.

Cytotoxicity was rated based on cell viability compared with control, as defined by Dahl et al. The different categories of cell viability were: non-cytotoxic (>90% cell viability); slightly cytotoxic (60–90%); moderately cytotoxic (30–59%); and strongly cytotoxic (<30%). The percentage cell viability for each irrigant was recorded and the results were tabulated and subjected to statistical analysis.

Statistical analysis

Cell viability values for each tested material at each time point are expressed as the mean ± standard deviation (mean ±SD). The group means were compared by analysis of variance (ANOVA) followed by a multiple comparison of means using the Student-Newman-Keuls test; if necessary, comparison of individual pairs of mean values by Student's *t*-test was used. Statistical significance was set at $p<0.05$.

Results

The cell growth and absorbance values of the negative and positive controls for each experiment (data not shown) were within the normal data range obtained in this laboratory. To normalize the data among the test materials, we calculated the percentage of proliferating cells in each group at each dose and time point compared with the positive control (100% viable cells) and negative control (0% viable cells) (see conversion formula in Materials and Methods, above). The results are summarized in Tables 1 and 2.

For the 100 µl/mL dilution of each irrigant at 1 h, Chx was least cytotoxic, while NaOCl was most cytotoxic. This difference was statistically significant (F=455.4, P<0.0001). The multiple comparison of means by the Student-Newman-Keuls test revealed differences between each of the 3 tested groups.

At 24 h, Chx remained the least cytotoxic but MTAD was significantly more toxic; this difference was statistically significant (F=116.5, P<0.0001). There was no significant difference between the NaOCl and MTAD groups.

At 48 h, NaOCl was less cytotoxic than both Chx and MTAD (F=13.47, P=0.0022). There was no difference between the Chx and MTAD groups at this time point.

At 72 h, MTAD had the lowest cell viability, so it was more cytotoxic than NaOCl and Chx (F=5.99, P=0.022). There was no significant difference between the Chx and MTAD groups.

Compared with other time points, MTAD and NaOCl at 100 µl/L concentration cell viability was higher at 72 h than at the earlier time points, while CHX was more cytotoxic at 72 h than at 24 h, a statistically significant difference (P<0.0001) (Table 1).

For the 0.1 µl/mL dilution of each irrigant at 1 h, MTAD was the least cytotoxic while Chx was the most cytotoxic (F=193.8,

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P<0.0001). There were statistically significant differences between all 3 groups.

For the 3 later time points, there was a pattern of cell viability being highest with NaOCl, with MTAD comparable but slightly lower, and lowest with Chx. This difference was significant in the 24 h, 48 h, and 72 h groups.

At these irrigant dilutions, cell viability was significantly (P<0.0001) increased between 1 h and 72 h with NaOCl and MTAD, but not with Chx (P>0.05) (Table 2).

Finally, we performed a multiple logistic regression analysis to evaluate the dose-dependence of the toxicity effect. Results of this analysis showed a significant influence of the dilution rate of the substance, because the odds ratio for cell viability being over 50% was increased 51 times between the 100 µl/L and 0.1 µl/L dilutions (OR–51.18, 95% CI 8.16–21.09).

Discussion

In vitro cytotoxicity tests are usually carried out for new materials before applying them clinically. *In vitro* model assays enable experimental factors and variables to be controlled, which often is an important complication of performing experiments *in vivo*. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is well established for cytotoxicity analysis of dental materials, being used initially for cell viability analysis in the 1980s. Although the MTT assay is sensitive, it requires more time to be completed, but recent developments of other metabolic activity-based tests such as the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay have accelerated this process. In principle, WST-1 works similarly to MTT, by acting as a substrate for the mitochondrial succinate-tetrazolium reductase enzyme, which converts it to the formazan dye [6]. The WST-1 reagent produces a water-soluble formazan, in contrast to the product of the MTT assay, which is water-insoluble. Thus, the WST-1 analysis is easier and faster than the MTT test and is considered to be a more sensitive index for evaluating the cytotoxicity of dental materials [11].

This study evaluated *in vitro* the cytotoxic damage induced by NaOCl, Chx and MTAD at dilutions of 0.1 and 100 µl/mL using the WST-1 colorimetric test. The solutions were diluted because cultured cells are far more susceptible than periapical tissues to toxic effects of drugs [12]. *In vivo*, the substances are rapidly neutralized and removed by phagocytes and the lymphatic and vascular systems, and are thus less harmful in clinical situations than at equivalent concentrations in *in vitro* studies [13].

According to our results, NaOCl, CHX, and MTAD solutions all induced cytotoxicity in rat periodontal ligament fibroblasts cells, and these effects were time- and dose-dependent. At 100 µl/L and 1 h and 24 h, all 3 irrigants were strongly cytotoxic, although CHX was less so than NaOCl, and MTAD was more cytotoxic (Table 1). Nevertheless, cell viability was significantly higher at 72 h than at earlier time points for all irrigants. At the 0.1 µl/L concentration, NaOCl and MTAD were only moderately cytotoxic, whereas Chx was highly deleterious to cell viability at all time points (Table 2). Indeed, Chx was more cytotoxic at the higher dilution than at the lower dilution. The finding that NaOCl and MTAD were less toxic upon dilution to 0.1 µl/L is in agreement with the findings from Zhang et al. (2003), which showed a correlation between NaOCl concentration and its cytotoxicity [14]. The study also demonstrated that MTAD was less cytotoxic than 5.25% NaOCl but more cytotoxic than 2.63% NaOCl. In our study, MTAD showed similar cytotoxicity to 3% NaOCl at all time points and at both dilutions. Furthermore, our results indicated that 2% Chx was more cytotoxic than the other 2 irrigants.

Yasuda et al. (2010) reported that MTAD has minimal cytotoxicity against MC3T3- E1 and periodontal ligament cells compared with conventional irrigants [15].

The cytotoxicity of Chx has been shown in several studies, with one report demonstrating that these effects were dependent on the exposure dose, length of exposure, and the composition of the exposure medium [14]. While chlorhexidine does not appear to cause any long-term damage to host tissues, it may still cause an inflammatory response in these tissues if expressed beyond the root canal [16].

In vitro measurements of toxicity are purely cellular, so our results cannot be directly compared with those obtained in *in vivo* studies. New studies on root canal irrigants should be done in animals and then in humans to evaluate their cytotoxicity and *in vivo* biocompatibility.

Conclusions

All of the tested irrigants showed some level of cytotoxicity towards rat periodontal ligament fibroblasts, and these effects were time- and dose-dependent. We conclude that these irrigating solutions should be used at lower concentrations to enhance cell viability and protect the tissues from toxicity damage, irrespective of their increased efficacy at higher concentrations.

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