Original Research
Dental Materials

Assessment of toxicity and oxidative DNA damage of sodium hypochlorite, chitosan and propolis on fibroblast cells

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Abstract: The objective of this study was to evaluate and compare the cytotoxicity and genotoxicity on human fibroblast cell lines of sodium hypochlorite (NaOCl), chitosan and propolis as root canal irrigating solutions. Human fibroblast cells were exposed to chitosan, propolis and NaOCl for 4 and 24 h. Cell viability was assessed by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, and oxidative DNA damage was assessed by determination of 8-hydroxydeoxyguanosine (8-OHdG) level with an ELISA kit. The data of cell cytotoxicity were analysed statistically using a test of one-way analysis of variance at a significance level of $p < 0.05$. In the NaOCI group, the 8-OHdG level was higher than in the chitosan group, but there was no statistical difference when compared with the other groups ($p < 0.05$). It was determined that the irrigation solutions were cytotoxic, depending on the dose and time. NaOCl was the most toxic solution after both 4and 24 h of exposure ($p < 0.05$). Chitosan and propolis may be alternatives to NaOCl for irrigation solutions, because they are both less toxic and produce less oxidative DNA damage.

Keywords: Chitosan; Propolis; Sodium Hypochlorite.

Declaration of Interest: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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Introduction

The removal of pulp tissue, microorganisms and microorganism toxins from the root canal system is one of the most important goals of endodontic treatment. To achieve this goal, root canal irrigation is an important step in endodontic treatment.[1]

In endodontic treatment, many irrigation solutions with different contents are used for root canal irrigation.[1] The solutions have a risk of contact with the surrounding soft and hard tissues such as dentin and periapical tissues. For this reason, a lack of biocompatibility of irrigation solutions can lead to degeneration of cell proliferation, adhesion and enzyme systems in the area where there is contact.[2] In this context, assessment of the biocompatibility of materials used in endodontic treatment is of great importance, and biocompatibility is accepted as one of the basic requirements for the use of any dental restorative material in clinical practice.[2,3]

Sodium hypochlorite (NaOCl) (0.5%–6.15%) is the most commonly used irrigation solution, because it has a broad antibacterial spectrum and the ability to dissolve organic tissues; however, it has high toxicity. For this

reason, the search continues for an ideal irrigating solution that may be an alternative to NaOCl.[4,5]

Natural compounds as alternative irrigation solutions are commonly preferred.Therefore, the use of natural chitosan or chitosan-containing compounds has become increasingly widespread.[6,7] Chitosan is a polymer obtained by deacetylation from a chitin that is a linear amino polysaccharide composed of β-(1 → 4) -dependent D-glucosamine units on the outer skeletons of various types of seafood, on the wings of butterflies and on the cell walls of fungi.[8,9] Chitosan is used for many purposes including haemostatic agents, chelation agents, filling materials and periodontal tissue regeneration material indentistry.[10,11,12] It has been reported that chitosan scaffolds, fibroblasts and basic fibroblast growth factorcan be useful materials for tissue regeneration. Some studies have also reported that chitosan-containing composites used for tissue regeneration in periodontics significantly increase cell adhesion and proliferation, thereby increasing the success of periodontal treatment.[13]

Because of the positive effects on the immune system, as well as containing natural polymers such as chitosan, treatment with bee products is also accepted in medicine. Such treatments are called 'apitherapy',and propolis is among the most popular of the bee products. Propolis is a product obtained from bees by mixing resin from the buds and sprouts of plants, beeswax and saliva.[14] In addition to traditional uses of propolis, it is also used in different forms for different purposes in dentistry. Propolis has been reported to be used as a root canal irrigation solution, as a root canal sealer, as pulp capping material, to prevent dentin hypersensitivity, to protect the vitality of periodontal cells in avulsed teeth, to improve periodontally induced tissue regeneration, to heal oral mucosal ulcerations, and in many other functions.[15,16,17,18,19]

The periodontal ligament is anatomically closely related to the root canal system, so it is the first contact site where irrigation solutions are not restricted within the root canal. Fibroblasts are common cells in this region, which may be due to direct and indirect contact with irrigation solutions.[20] For this reason, a human fibroblast cell line was used in this study.

In this study, the cytotoxicity and oxidative DNA damage of propolis, chitosan and NaOClwere evaluated *in vitro* on the fibroblast cell line. The null hypothesis was no difference between cytotoxicity and the oxidative DNA damage of irrigation solutions.

Methodology

Preparation of propolis samples

Propolis samples were manually collected from the Central Anatolia region (Sivas) and stored in the dark until use. Crude propolis weighed 30 g/100 mL and was dissolved in 70% ethanol (Merck, Darmstadt, Germany) for 72 h on a shaker. The resulting mixture was filtered through Whatman No. 1 paper and centrifuged (MSE Mistral 1000, Leicestershire, England) for 5 min at 1,000 rpm. Then, the ethanol remaining at 50°C was evaporated in a vacuum evaporator at 50–60°C for 5–10 min. The 15% propolis extract was stored at −20°C until use in experiments.[21]

Test solutions

NaOCl of 5.25% (Caglayan Kimya San., Konya, Turkey) was used in accordance with the manufacturer's instructions in several steps of the assay after preparation under aseptic conditions. Chitosan solution at 0.2% (pH 3.2 crystalline homogenous solution) was obtained by using 0.2 g of chitosan and 100 mL of 1% acetic acid (Sigma-Aldrich Chemie, Steinheim, Germany) for 2 h using a magnetic stirrer (Heidolph Elektro, Kelheim, Germany).

Cell culture

The cells used in the study were obtained from the human gingival fibroblast (HGF) (ATCC® CRL-20114™) cell line, and the cytotoxicity of the irrigation solutions and the oxidative DNA damage that they produced were determined. As a culture medium, Dulbecco's modified Eagle's medium (DMEM) (Biochrom GmBh, Berlin, Germany) was supplemented by 100 units/mL streptomycin–penicillin, 10% (v/v) fetal bovine serum (Gibco Invitrogen, Karlsruhe, Germany) and 2 mM glutamine. The HGF cells were cultured at 37°C in a 100% humidified environment (NuAire) containing 5% carbon dioxide (CO_2). Confluent cells were separated with 0.25% trypsin or trypsin/EDTA

mixture. The culture medium was changed every day until it reached enough confluence. Cells were examined under a 100× magnification microscope (Leica Microsystems, Germany). Cells were seeded at a density of 10^4 for each dish of a 96-well sterile plate and incubated for 24 h at 37°C with 5% CO_2. Cell cultures between the fourth and sixth passages were used in all experimental procedures.

After incubation, the culture media in each well were removed, and 150 μL of sterile test solutions (NaOCl, chitosan or propolis) and 150 μL DMEM were added to the wells. As a control group, only 300 μL of DMEM was added to the polyethylene wells. The polyethylene platelets were incubated for 4 and 24 h at 37°C with two groups of 5% CO_2 containing a reagent. Each specimen was used for each rinse solution, and eight specimens were prepared for the control group.

Assessment of oxidative DNA damage

To assess DNA damage, HGF cells were incubated for 15 min on ice with hydrogen peroxide (H_2O_2) at a final concentration of 75 μM (positive control). Then, the cells were treated with the NaOCl group, chitosan group and propolis group (1.0 mg/mL) to a final concentration of 0.1% phosphate-buffered saline (PBS) in the culture medium for 24 h. The control cells (negative control) were incubated with the same final amount of 0.1% PBS in the culture medium. Oxidative DNA damage ELISA kit (Oxiselect™,Cell Biolabs STA-320, San Diego, USA) was used to determine the oxidative DNA damage.The process was broken into three main steps: DNA extraction, DNA digestion and 8-hydroxydeoxyguanosine (8-OHdG) detection. DNA was extracted from HGF cell samples by using DNA extraction kit (DNeasyBlood& Tissue kit, QIAGEN), and the purity and quantities were determined spectrophotometrically (Nanodrop,Thermo Fisher Scientific,Wilmington, USA). The isolated DNA samples were converted to single-stranded DNA by rapid cooling on ice immediately after being incubated at 95°C for 5 min. Then, 10 μL of nuclease P1 was added to 100 μL of DNA. The samples were incubated at 37°C for 2 h to hydrolyse DNA. Alkaline phosphatase (10 μL) was added to each sample of hydrolysed DNA and incubated for an additional 1 h at 37°C. To remove all impurities, the hydrolysates were filtered and centrifuged at 12,000 rpm for 5 min. The resulting supernatant was used for the 8-OHdG ELISA test. The test was performed following the supplier's instructions. In summary, the ELISA plate was coated with 8-OHdG conjugate (100 μL of 1 μg/mL to each well) and incubated overnight at 4°C. The coated wells were washed with 200 μL of assay diluents and blocked with the assay diluent at room temperature for 1 h. Unknown samples (50 μL) and supplied 8-OHdG standards (ranging from 0 to 20 ng/mL) were added to the resembling coated wells and incubated for 10 min at room temperature on an orbital shaker. Next, 50 μL of anti-8-OHdG antibody was added to each well and incubated again at room temperature on an orbital shaker for 1 h. Microwell strips were washed three times with 250 μLWash Buffer (1×) per well to allow aspiration to occur correctly between each wash. The wells were thoroughly cleaned with a paper towel to remove the Wash Buffer after all wash cycles.The secondary antibody-enzyme conjugate (100 μL) was added and incubated for 1 h. After washing again threetimes with Wash Buffer, 100 μL substrate was added to each buffer and incubated for 2 to 30 min until colour formation wasobserved. After colour fixation developed, 100 μL of stop solution was added, and the absorbance was measured at 450 nm in a 96-well ELISA plate reader (Multiskan FC Microplate Photometer,Thermo Fisher Scientific, Boston, MA, USA). The experiment was performed with three different samples, each with duplicate.

Evaluation of cytotoxicity

Cell viability was determined using cell proliferation ELISA kit 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) (Applichem A-1080) according to the manufacturer's instructions. First, the cells were replicated for 24 h with 5% CO_2 at 37 °C by adding 100 μL of growth medium in a flat-bottomed 96-well plate. The cells were treated with 5.25% NaOCl, 0.2% chitosan solution and 15% propolis extract. Control cells were incubated in culture medium only. Reagent and activation solutions of XTT were cooled in a 37 °C water bath before being used. To prepare enough reaction

solution for use in the 96-well plates, 0.1 mL of the activation solution was added to 5 mL of the XTT reagent (20 cells/well, 5 mg/mLin PBS). Reaction solution (50 µL) was added to each of the plates, and the cell density was incubated for 24 h. Plates were shaken slowly on the shaker to distribute the homogeneity evenly in the wells. Measurements were made spectrophotometrically against the control groups with at480 nm using ELISA reader (Multiskan FC Microplate Photometer,Thermo Fisher Scientific, Boston, USA). The experiment was performed with three different samples, each with duplicate.

Statistical analysis

The data were analysed using statistical software (SPSS Pc + version 22.0). Data were normally distributed (Shapiro–Wilk test) and homoscedastic (Levene test). Therefore, the effect of time and the used solution on cell viability was evaluated by two-way ANOVA tests, followed by a Tukey's post hoc test. One-way ANOVA tests followed by a Tukey's post hoc test were performed to determine the significance of the level of 8-OHdG released from the cells according to the used solution. The results are presented as mean ± standard error. Differences were considered statistically significant at $p < 0.05$.

Results

Oxidative DNA damage

The oxidative DNA damage assay was conducted on four groups (control group, NaOCl group, chitosan group and propolis group) with cultured HGF1in 96-well plates. Figure 1 shows the 8-OHdG levels released after exposure to the groups of solutions by the cells. There was no statistically significant difference between the 8-OHdG levels in the NaOCl group, the chitosan group and the propolis group when compared with the control group after the oxidative damage assay test ($p > 0.05$). The 8-OHdG levels in the chitosan group were significantly lower than in the NaOCl group ($p < 0.05$).

Cell viability

The XTT assay was conducted on four groups (control group, NaOCl group, chitosan group and propolis group) with cultured HGF1 in 96-well plates. Each group was incubated for 4 and 24 h. Figure 2 shows cell viability due to the time after application of the group solutions. The decrease in cell viability was not statistically significant with the exposure time increase in the control group and the NaOCl group ($p > 0.05$). The decrease in cell viability was found to be statistically significant with the exposure time increase in the propolis group ($p < 0.001$) and the chitosan group ($p < 0.001$).

The cell viability after exposure to groups of solutions for 4 his shown in Figure 3.The decrease in cell viability in the NaOCl group ($p < 0.001$) and the chitosan group ($p < 0.05$) was statistically significant, whereas the decrease in cell viability in

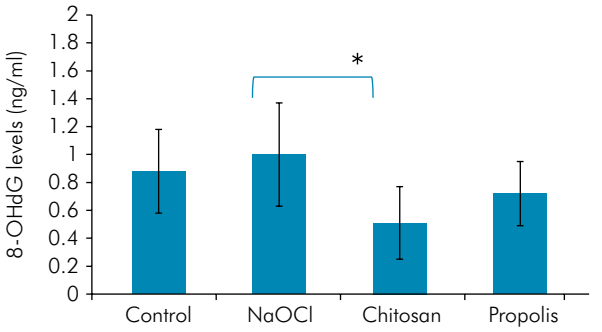

Figure 1. The level of 8-OHdG released after the application of the groups of solutions. Values represent mean and SD of three independent experiments. $p < 0.05$; *Statistically significant differences between groups.

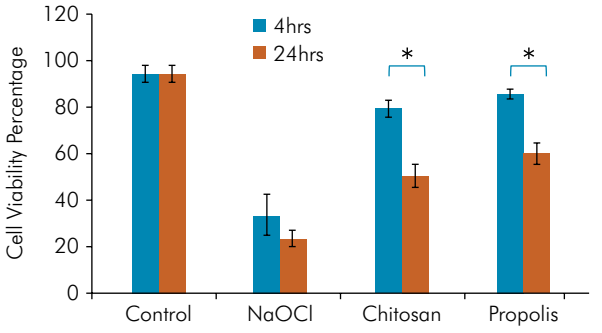

Figure 2. Changes in cell viability after 4 and 24 h exposure of groups of solutions. Values represent mean and SD of three independent experiments.$p < 0.05$; *Statistically significant differences between groups.

the propolis group ($p > 0.05$) was not statistically significant when compared with the control group. There was no statistically significant difference in cell viability in the chitosan group and the propolis group ($p > 0.05$). In the NaOCl group, cell viability was lower than both the chitosan group and the propolis group ($p < 0.001$).

The cell viability after exposure to groups of solutions for 24 his shown in Figure 4. Compared with the control group, there was a statistically significant decrease in cell viability in the NaOCl group, the chitosan group and the propolis group ($p < 0.001$). There was no difference in cell viability in the chitosan group and propolis group ($p > 0.05$). In the NaOCl group, however, less cell viability was observed than in both the chitosan group and the propolis group ($p < 0.001$).

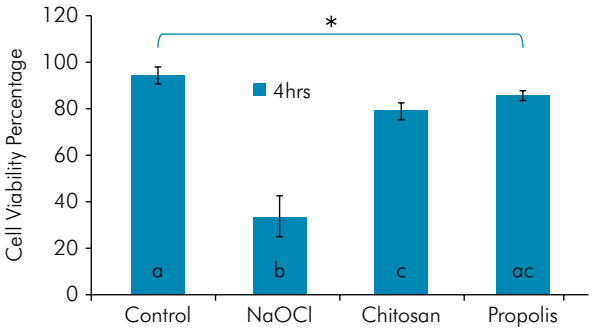

Figure 3. Changes in cell viability after 4 h exposure of groupsof solutions. Values represent mean and SD of three independent experiments. $p < 0.05$;* and different letters indicate statistically significant difference at 5%.

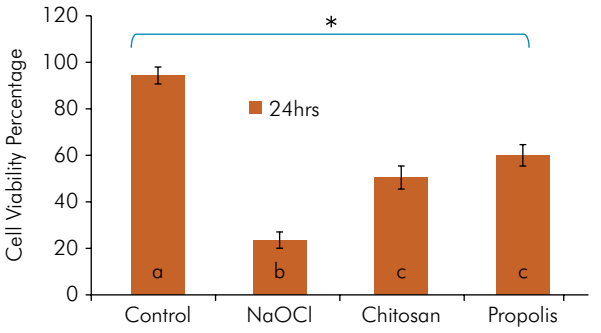

Figure 4. Changes in cell viability after 24 h exposure of groupsof solutions. Values represent mean and SD of three independent experiments. $p < 0.05$;* and different letters indicate statistically significant difference at 5%.

Discussion

Root canal irrigation solutions will be extruded into periapical tissues, no matter how many attempts are made to prevent this during the performance of root canals in endodontic treatment.[22]

It is known that irrigation solutions are not limited to the root canal space but make contact with the periapical tissues composed of cement, periodontal ligament and alveolar bone through the apical foramen.[23] Therefore, biochemical analyses assessing the genetic and toxic effects of irrigation solutions on the tissues that interact are important to minimize future risks for both the patient and the clinician.[24]

One of the most important disadvantages of NaOCl is the toxic effect; however, this irrigation solutionis frequently used by clinicians and is the main topic of various studies. In many studies, it has been found that NaOCl is more cytotoxic and produces more oxidative DNA damage compared with the control group and other test solutions. This toxicity is increased in direct proportion to increased exposure time.[25,26] In the current study, NaOCl was found to be more toxic than other tested solutions. For this reason, the results obtained from this study confirm the toxic effect of NaOCl. Toxicity of NaOCl has been reported to be detrimental to cellular cytoplasmic membrane integrity and to high pH (hydroxyl ion action), leading to irreversible enzymatic inhibition. It has also been shown that the ability to dissolve and eradicate organic tissues contributes to this situation.[27] In some studies, NaOCl has been shown to have no cytotoxic effect, although it has a genotoxic effect.[28] This result is thought to be due to some external influences that may lead to cell stress, even if the production conditions are the same. Additionally, studies tend to use different concentrations of the solution and different cell lines. However, based on the existence of a limited number of studies on propolis toxicity, data on the cytotoxic and genotoxic effects of propolis have been obtained in the current study. Al-Shaheret al.[29] assessed calcium hydroxide and propolis toxicity on periodontal ligament cells by spectrophotometric analysis. In that study, cell viability was >75% after propolis exposures at 4 mg/mL or less, which is less than in the current study.

Aliyazicioglu et al.[30] showed that propolis reduced the DNA damage caused by H_2O_2 on the fibroblast cell line. Montoro et al.[31] evaluated the radioprotective effect of propolis against chromosomal damage induced by irradiation *in vitro*. In the results of that study, propolis decreased chromosomal damage due to radiation. Santos et al.[32] reported that propolis reduced DNA damage (5–100 μg/mL) on mice over cell lines. Also, it has been shown that the propolis concentration of 50 μg/mL reduced the percentage of necrotic cells and provided a significant proliferative effect.

In the current study, propolis does not increase oxidative DNA damage when compared with the control group.This is in concordance with the results of the other studies, showing that cell viability is not decreased in the control group after 4 h of exposure.

Many studies have confirmed that the pharmacological properties of propolis are mainly due to the presence of flavonoids. Flavonoids have been proven to be topoisomerase inhibitors. Topoisomerase is an enzyme that has the ability to regulate the superhelical area of chromosomal DNA, playing a crucial role in chromosome replication, transcription, recombination, segregation, consolidation and repair.[33] Therefore, the fact that propolis does not cause DNA damage can be explained by this information. Montoro et al.[33] found that propolis was cytotoxic and genotoxic in high concentrations on the human lymphocyte cell line. Tsai et al.[34] reported that the induction of oxidative DNA damage was related to H_2O_2 produced by propolis. H_2O_2 decomposes in the presence of a large number of substances, such as iron, copper, manganese, nickel and chromium salts, when heated or decomposed to separate the water and oxygen. In addition, flavonoids in propolis, such as galangin, crysin and pinocembrin, have the capacity to induce oxidative DNA damage, which is related to H_2O_2 production. The current study found that propolis was more toxic than the control group solution after 24 h of exposure. Conversely, propolis did not cause oxidative DNA damage and did not have cytotoxic effects after 4 h of exposure. Differences in the effects of the different species of propolis used in various studies may explain this difference. In the current study, where propolis was obtained from the Central Anatolia region, a decrease in cell viability and DNA damage occurred. Experimental differences, such as the cell line and exposure time, can be effective on the changes in the results.

Many other studies have evaluated the toxicity of chitosans on various cells. Fernandes et al.[35] found that chitosan showed a toxic effect on the human red blood cell line depending on the concentration (> 0.1 mg/mL). Wiegandet al.[36] found that chitosan increased apoptosis on human keratinocyte cell lines due to exposure time and concentration. In the current study, chitosan showed a toxic effect on the human gingival cell line due to the exposure time. This can be explained by the release of anti-inflammatory cytokines by chitosan and the induction of caspases associated with apoptosis by chitosan.[36] Chellat et al.[37] found that chitosan did not show toxicity on the fibroblast cell line. This is most likely a result of methodological differences such as concentration, cell line and test method.

Fernandeset al.[38] found that the chitosan used on the lymphocyte cell line at 0.07 mg/mL and lower doses did not have a genotoxic effect. Hu et al.[39] found that chitosan combined with the graft model did not produce genotoxicity. Similarly, Yoon et al.[40] found that chitosan did not produce genotoxicity in mice. The lack of genotoxic effects of chitosan maybe explained by the inhibition of DNA damage by the chitosan, but this mechanism is not yet clear.[35]

The use of different cell lines in research studies causes variation in the results. The only factor that is effective in the selection of an irrigation solution used for treatment is lack of biocompatibility. It is expected that the irrigation solution will have broad antibacterial activity and the ability to remove the smear layer. At the same time, the solution must also be able to adequately dissolve the necrotic and vital pulp tissue.

Conclusions

According to the result of this study, chitosan and propolis are more reliable in terms of toxicity than NaOCl. However, these results alone are not enough to predict the success of the irrigation

solutions used in endodontic treatment. More *in vitro* and *in vivo* studies are needed to provide a more comprehensive interpretation of the biocompatibility of these solutions.

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