Biological properties of a neutralized 2.5% sodium hypochlorite solution

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Objectives. The objective of this study was to evaluate the influence of neutralizing a 2.5% NaOCl solution on its cytotoxicity, genotoxicity, and tissue-dissolving potential.
Study design. The cytotoxicity and the genotoxicity of Dakin, a 2.5% NaOCl solution, and a neutralized 2.5% NaOCl solution were assessed according to ISO 10993 standards. The weight of palatal mucosa samples placed in neutralized 2.5% NaOCl, 2.5% NaOCl was recorded over time as well as the pH of the solutions.
Results. The neutralized 2.5% NaOCl solution was 10-fold more cytotoxic than the 2.5% NaOCl solution. None of the solutions was genotoxic. The 2.5% NaOCl solution had a better tissue-dissolving capacity than the neutralized 2.5% NaOCl solution. The pH of the 2.5% NaOCl solution and neutralized 2.5% NaOCl solution decreased from 12 to 9 and from 7.5 to 5.6, respectively.
Conclusion. Neutralizing a 2.5% NaOCl solution increased its cytotoxicity, did not induce any genotoxic effect, and reduced its tissue-dissolving ability. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;109:e120-e125)**

It has been shown in vivo and in vitro that a chemomechanical preparation of the root canal is necessary because the simple mechanical instrumentation of canal walls is not sufficient to reduce the number of bacteria significantly.[1,2] The root canal irrigants are often chosen according to their antibacterial potential to increase their efficiency.[3] However, the liquid may be forced inadvertently beyond the apex and lead to severe injuries if not biocompatible.[4] Therefore, a balance must be found between the antibacterial properties and the biocompatibility of the root canal irrigants. The type of irrigant and the ideal concentration of a sodium hypochlorite solution are still a matter of debate but a 2.5% NaOCl solution may be considered as a good compromise between Dakin (0.5%) and the 5.25% NaOCl solution commonly used in the United States.[5] Beyond this concentration, the NaOCl solution is not considered as biocompatible and is not used for endodontic purposes.

The manufacturers add sodium hydroxide to the NaOCl solution to increase its shelf life. The high pH of the commercial NaOCl solutions induces a shift of the hypochlorous acid (HClO) toward its alkaline form: the hypochlorite ion (ClO^-). Since HClO has a better antibacterial efficiency than ClO^-, using a neutralized NaOCl solution should theoretically increase its antibacterial efficiency, but also its biocompatibility because of its neutral pH.[6] In addition, the lack of sodium hydroxide in the neutralized solution eliminates the risks of possible chemical burns beyond the apex.[7] This was not the case when a 0.5% NaOCl solution was buffered with sodium carbonate at pH 9.[8] However, it has recently been shown that a neutralized 2.5% NaOCl solution at pH 7.5 might be a good alternative to its unbuffered counterpart.[9] It has been demonstrated that the 2.5% NaOCl neutralized solution, used within 2 hours upon mixing because of the lack of NaOH, presented the same pulp tissue–dissolving capacities after 5 minutes as the unbuffered 2.5% NaOCl solution and displayed a higher antibacterial efficiency.[9] Nevertheless, other properties of the 2.5% NaOCl neutralized solution, such as its biocompatibility, should be evaluated because the root canal irrigant may be inadvertently forced beyond the apex and cause severe inju-

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ries.[10] The biocompatibility of medical devices should be assessed according to ISO standards, which specify that cytotoxicity and genotoxicity are the 2 mandatory primary tests for dental materials.[11] Besides the biocompatibility issue, it should also be kept in mind that lowering the pH of sodium hypochlorite may also modify its tissue-dissolving potential and therefore its aggressiveness toward host tissues in cases of iatrogenic perforation.[12]

Therefore, the purpose of this study was to evaluate the cytotoxicity, the genotoxicity, and the tissue-dissolving potential on porcine palatal mucosa of a neutralized 2.5% NaOCl solution.

MATERIALS AND METHODS

Cytotoxicity test

The test was performed according to ISO standards.[13] L 929 fibroblasts were cultivated in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (BioScience, Walkersville, MD). These cells were plated at 30,000 cells cm^{-2} in 96-well plates (Falcon 3072, Becton Dickinson, Oxford, UK). The 96-well dishes were then placed into a humid incubator with an atmosphere of 5% CO_2, 95% air, before use. After 24 hours, the medium from the 96-well plates was removed and replaced by 100 μL of the test medium. The test medium was obtained by serial dilutions (vol/vol) of 3 root canal irrigants in culture medium:

- Neutralized 2.5% NaOCl solution obtained mixing 3 volumes of 0.2 M HCl (Sigma Chemical Co., St. Louis, MO) with 1 volume of 10% NaOCl (Sigma).[9]
- 2.5% NaOCl solution (Sigma)
- Dakin (Cooper, Melun, France)

The 3 liquids were tested undiluted and diluted to 1/10, to 1/100, or to 1/1000. The medium was left 10 minutes in contact with the target cells before performing the test. Each dilution was tested in a separate plate because chloride is volatile and may evaporate, modifying the outcome of the study. A succinyl dehydrogenase assay (MTT) was performed on the 96-well plates after the 10 minutes of incubation (i.e., 24 hours + 10 minutes after the beginning of the experiment). The medium was removed and immediately replaced with 100 μL/well of 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,(-diphenyl tetrazolium bromide) dissolved in the medium (Sigma). After incubation for 2 hours at 37°C, the supernatants were discarded, and the formazan crystals were solubilized with dimethylsulfoxide (100 μL/well) (Sigma). The absorbance of each 96-well dish was determined using an automatic microplate spectrophotometer (E 960, Bioblock, Strasbourg, France) at 550 nm. The absorbance of the wells containing the same medium was calculated against that of the control medium to determine the percentage of cell viability. The positive control was phenol (0.64 mg/mL) and the negative control was the medium itself. Experiments were done in triplicate.

A 2-way analysis of variance (ANOVA) (factor 1: root canal irrigant, factor 2: dilution) was performed to compare the cytotoxicity of each tested dilution. A post hoc Duncan test was performed to compare the 3 liquids within each dilution. The significance level was set at 5% for both tests.

Genotoxicity test

The study was performed according to ISO standards[14] on human lymphocytes because of its sensitivity.[15] Fresh whole blood collected by venipuncture (0.7 mL) was added to vials containing 9.3 mL of culture medium (X-VIVO 10), supplemented with 1% phytohemagglutinin (Life Technologies, Paisley, UK), which is a specific mitogen to induce T-lymphocyte proliferation and 1% heparin (Life Technologies) to prevent blood coagulation. These vials were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 24 hours, serial dilutions of the 3 root canal irrigants were added to the vials. The 3 liquids were tested diluted to 1/250, 1/500, and to 1/1000. These concentrations were determined from a cytotoxicity study performed on human lymphocytes (data not shown). The positive control was mytomycin C (5 μg/mL) and the negative control was the medium itself. The vials were then incubated for 20 hours. Then, 50 μL of cytochalasin B (1 mg/mL) (Sigma) was added to each vial. The vials were gently shaken and incubated again for 24 hours.

Sixty-eight hours after starting the experiment, the cells were harvested by centrifugation at 1200 rpm at 20°C for 10 minutes. The supernatant was discarded and the T-lymphocytes underwent a mild hypotonic treatment: 5 mL of 0.075 M potassium chloride was added and vortexed before a second centrifugation at 1200 rpm and 20°C for 1 minute. Once the supernatant was discarded, 5 mL of the fixative solution (3 parts methanol + 1 part acetic acid) were added gradually to fix the cells. The fixation step was repeated twice after a 20-minute storage at 4°C. The vials were then centrifuged a third time for 10 minutes at 1400 rpm and the cells were smeared on precleaned microscope glass slides and air dried. The human T-lymphocytes were colored with GIEMSA and 1000 cells with or without micronluclei were counted per slide under a light microscope. The number of micronuclei per 1000 cells was recorded in a double-blind manner by an experi-

enced operator and used for statistical analysis. Each experiment was performed in triplicate.

A chi-square test was performed for each dilution to compare the number of micronuclei after exposure to the 3 root canal irrigants and to the negative control.

Tissue-dissolving capacity

Palatal mucosa was used to simulate the histological effects of a NaOCl solution forced in an iatrogenic perforation and coming in contact with gingiva. Full-thickness palatal mucosa samples were dissected from 2 pigs within 2 hours after slaughtering. Twenty-four rectangular pieces (10 × 5 mm) were obtained and weighted with a precision balance for standardization purpose (0.25 ± 0.02 g). The samples were randomly soaked for 120 minutes in microfuge tubes containing 1 mL of the following solutions (n = 6 per liquid): neutralized 2.5% NaOCl, 2.5% NaOCl, NaOH (pH adjusted to that of the 2.5% NaOCl), and saline as negative control. Dakin was replaced by NaOH in this part of the study because the influence of HClO concentration on tissue-dissolving potential has already been reported. On the opposite, the relative importance of each of the 3 components present in NaOCl solutions (HClO, ClO^-, and NaOH) has never been tested.

Every 10 minutes, the samples were blotted dry for 5 seconds and weighted using a precision balance, the pH of the solution was recorded at room temperature using a pH meter.

After 120 minutes, the samples were placed in neutral buffered 10% formalin. After dehydration, the specimens were embedded in paraffin. Routine histological techniques were used to obtain 5 μm serial sections, which were stained with hematoxylin-eosin. Histological findings were recorded by 2 independent operators in a blind manner.

Values obtained from the dissolution test and from the pH analysis were compared using a nonparametric Kruskall and Wallis test. Post hoc analysis was performed using a Tukey test adapted to nonparametric data. The significance level was set at 5% ($P < .05$).

RESULTS

Cytotoxicity

The 2-way ANOVA showed that the difference was statistically significant for both factors: root canal irrigant ($P < .01$) and dilution ($P < .001$) (Table I). The Duncan test showed that Dakin was the least cytotoxic when tested undiluted ($P = .04$) or diluted to 1/10 and that that the neutral 2.5% NaOCl solution was more cytotoxic than the 2 other liquids when tested diluted to 1/100 ($P = .01$). However, no difference was found among the 3 liquids when tested after their dilution to 1/1000 (nonsignificant).

Table I. Cell viability of L 929 exposed to serial dilutions of 3 root canal irrigants

Cell viability (% negative control)	*Dakin*	*2.5% NaOCl*	*Neutralized 2.5% NaOCl*
Undiluted	19 ± 3[a]	9 ± 3[b]	11 ± 5[b]
1/10	92 ± 9[a]	11 ± 4[b]	16 ± 5[b]
1/100	100 ± 8[a]	99 ± 7[a]	20 ± 6[b]
1/1000	100 ± 9[a]	113 ± 8[a]	115 ± 9[a]

The analysis of variance showed statistically significant differences among dilutions ($P < .001$) and liquids ($P < .01$). Within the same dilution, the groups with the same superscript letter[a,b] were not statistically different.

Table II. Frequencies of binucleated micronucleated human lymphocytes (‰) after exposure for 48 hours to 3 root canal irrigants

Root canal irrigant	*Positive control*	*Negative control*	*Dakin*	*2.5% NaOCl*	*Neutralized 2.5% NaOCl*
1/250	27 ± 8	7 ± 2	8 ± 2	6 ± 2	8 ± 2
1/500	26 ± 9	7 ± 3	9 ± 2	7 ± 3	7 ± 2
1/1000	27 ± 6	7 ± 2	7 ± 2	6 ± 2	8 ± 2

There was no statistically significant difference between the negative control and the 3 root canal irrigants.

Genotoxicity

None of the root canal irrigant was genotoxic. The binucleated micronucleated rate of lymphocytes of the negative control showing 7% of multinucleated cells was in agreement with results reported in a meta-analysis.[16] The positive controls were performed by addition of mitomycin C, which is a well-known clastogen, showed chromosome damage and showed 27% of multinucleated cells. The chi-square tests did not show any statistically significant difference among the root canal irrigants and the negative control (ns): none were genotoxic (Table II).

Tissue-dissolving capacity

No weight or pH variation was observed when the samples were stored in saline showing that the weighing technique was adequate.

A weight loss was observed in the samples stored in 2.5% NaOCl and in the neutralized 2.5% NaOCl solution, but an increase both in weight and volume was reported for the samples stored in NaOH. The Kruskall and Wallis test showed a statistically significant difference among the sample weights ($P < .01$) (Fig. 1). The Tukey test ranked the samples by order of increasing weight from the 2.5% NaOCl solution (loss of 60%), followed by the neutralized 2.5% NaOCl solution (loss of 8%), to saline (no weight change) and finally to NaOH (gain of 28%).

The pH of the NaOH solution as well as that of the saline solution did not vary over time and remained stable respectively at 12 and 7 (Fig. 2). The pH of the

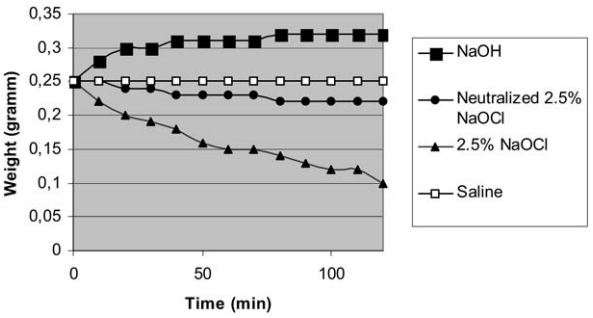

Fig. 1. Weight variation over 120 minutes of palatal mucosa samples placed in 2.5% NaOCl, neutralized 2.5% NaOCl, NaOH, and saline. The Kruskall and Wallis test showed a statistically significant difference among the 4 liquids. The samples stored in NaOH gained weight, those placed in saline did not vary, and those soaked in 2.5% NaOCL lost more weight than in neutralized 2.5% NaOCl.

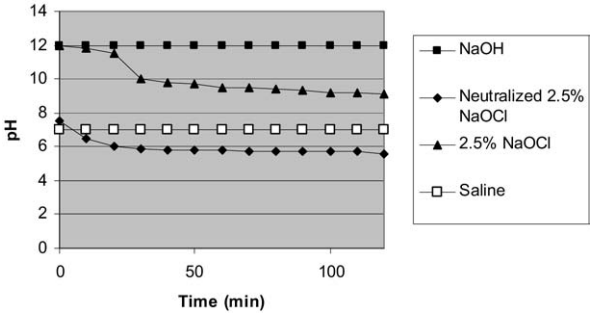

Fig. 2. pH variations over 120 minutes of solutions containing pig palatal mucosa samples. The pH of saline and NaOH did not vary but that of the 2.5% NaOCL and neutralized 2.5% NaOCl solutions decreased respectively from 12 to 9 and from 7.5 to 5.6.

2 NaOCl solutions decreased over time from 12 to 9 for the 2.5% NaOCl solution and from 7.5 to 5.6 for the neutralized 2.5% NaOCl solution.

The histological study of the samples stored in the saline solution showed classical images of a keratinized stratified epithelium with projections into the mucosa underneath. The samples placed in NaOH showed that the epithelium layer was detached from the underlying connective tissue. It looked like in vesiculo-bullous lesions or superficial burning, but the underlying structures were left intact. This explains that the samples placed in the NaOH solution gained weight. The samples in 2.5% NaOCl and neutralized 2.5% NaOCl showed that the epithelium had been totally dissolved. This is directly correlated to the weight loss reported here. For both liquids, the remaining connective tissue was covered by a necrotic layer. The thickness of this necrotic layer was twice larger in the neutralized 2.5% NaOCl solution than that in the 2.5% NaOCl solution.

DISCUSSION

Cytotoxicity test

This work showed that the neutralized 2.5% NaOCl solution was the most cytotoxic root canal irrigant because it started to be noncytotoxic after dilution to 1/1000 while Dakin and the 2.5% NaOCl solution started to be noncytotoxic when tested diluted respectively to 1/10 and 1/100. In other words, this indicates that there is 1 level of magnitude between the cytotoxicity of Dakin and the 2.5% NaOCl solution and also another level of magnitude between the cytotoxicity of the 2.5% NaOCl solution and the neutralized 2.5% NaOCl solution.

Several studies evaluating the cytotoxicity of various root canal irrigants have already been performed using L 929.[17-19] The contact time between the liquids and the target cells was reduced to 10 minutes because preliminary studies had shown a great cytotoxic potential of these liquids. Our results are in agreement with previous results according to which a 5% NaOCl solution kills 100% of the target cells within 15 minutes.[20] Another work had demonstrated that a 0.01% NaOCl solution still had effect on the mitochondrial function of human fibroblasts.[21]

The cytotoxicity of the NaOCl solution increased with concentration because the 2.5% NaOCl solution was more cytotoxic than Dakin (0.5% NaOCl solution). This difference was no longer observable with low dilutions (1/1000) because cytotoxicity is a dose-dependent phenomenon that does not exist below a threshold specific to each chemical. The reported difference in cytotoxicity, owing to higher concentrations, was expected because it is a general feature of cytotoxicity studies and in accordance with previously reported data.[21] In case of iatrogenic perforation, the available chlorine concentration of a neutralized 2.5% NaOCl solution forced beyond the apex is likely reduced thanks to bleeding caused by tissue dilacerations and to tissue clearance. This likely results in a lower cytotoxicity of the neutralized 2.5% NaOCl solution toward the surrounding tissues. More interestingly, the neutralized 1/1, 1/10, and 1/100 2.5% NaOCl solutions were more cytotoxic than the unbuffered counterparts. Despite the same available chlorine concentration, the chemical composition of the 2 liquids is different in 2 points. First, the neutralized solution does not contain sodium hydroxide and second, the available chlorine is under the form of hypochlorous acid (HClO) in the neutral solution and under the form of hypochlorite ion (ClO^-) in the unbuffered solution. Sodium hydroxide may act by saponification of fatty acids,[22] thus participating in the cytotoxic action of the 2.5% NaOCl solution. However, the results of the present work showed that this

effect is limited compared to the action of hypochlorous acid, which easily permeates cell membranes and induces chloramination of proteins resulting in the presence of intracellular organic chloramines that are themselves cytotoxic. A recent and interesting study investigated the intracellular effects of HClO and clearly demonstrated that it leads to cellular protein aggregation and unfolding.[23]

Genotoxicity test

Several studies compared the cytotoxicity of various root canal irrigants but few studies reported on the other factors of biocompatibility. One study assessed the inflammatory effects of Dakin when injected in the peritoneal cavity,[24] whereas another work evaluated the genotoxicity of sodium hypochlorite and concluded to a lack of genotoxicity of the root canal irrigant.[25] Nevertheless, according to ISO standards, the genotoxicity of dental materials should be evaluated before in vivo testing.[11] In addition, some studies reported that sodium hypochlorite[26] and hypochlorous acid[27] may present a genotoxic potential.

The cytokenesis-blocked micronuleus assay was used here because it is more relevant to work on mammalian cells. Furthermore, the Ames test is not really suitable to test antibacterial substances that are, by essence, cytotoxic toward procaryotic cells. In addition, the cytokenesis-blocked micronuleus assay allows the detection of both clastogenic or aneugenic events. Other authors already used the micronucleus test to assess the genotoxicity of dental materials such as resinous monomers,[28] siloranes,[29] root canal filling materials,[30] and dental amalgams.[31] This test, performed on human lymphocytes, is known to be a robust test for genotoxic assays and provides a reliable index of chromosome breakage and loss.[32] A guideline for the testing of chemicals using the in vitro mammalian cell micronucleus test, like in the present work, is in preparation by the Organization for Economic Co-operation and Development. This part of the study suggests that the host cells in contact with a neutralized 2.5% NaOCl solution are not subject to chromosome alterations.

Tissue-dissolving assay and histological study

The protocol used for the tissue-dissolving study is derived from previous works using pig palates to assess the tissue-dissolving action of various root canal irrigants.[8,33] This is clinically relevant because the root canal irrigant may come in contact with gingival or palatal mucosa in cases of iatrogenic perforation of maxillary teeth. Dakin was not used for this part of the work because its histological effects on pig palatal mucosa have already been explored.[8] However, NaOH was introduced to evaluate the relative effects of each of the 3 major components of the NaOCl solutions: HClO, ClO^-, and NaOH. The pH of the NaOH solution was adjusted to that of the 2.5% NaOCl solution in order to have exactly the same amount of OH^- ions.

This part of the work showed that the dissolving action of the 2.5% NaOCl solution on pig palatal mucosa is about 5 times higher than that of the neutralized 2.5% NaOCl. This corroborates the results of nonendodontic studies that have already shown that the optimal pH region of the antibacterial activity of NaOCl is around 7.5 but that of the dissolving activity is higher.[34] The 2.5% NaOCl solution is composed mainly of ClO^- ions with a low antibacterial potential but a high dissolving action. On the opposite, the neutralized 2.5% NaOCl solution contains HClO with a high antibacterial potential but a low cleaning activity.[35] Therefore, the loss of 60% of weight obtained with the 2.5% NaOCl solution compared with the loss of 8% recorded with the neutralized 2.5% NaOCl solution are in agreement with the literature. This study also confirms the results given by Christensen et al.,[12] who demonstrated that a 5.25% and a 2.6% NaOCl solutions presented the same low tissue-dissolving potential when their pH was adjusted to 6. At pH 6, ClO^- no longer exists and the NaOCl solution only contains HClO, which has a low dissolving potential even at high concentration.[35]

The histological study showed that the thickness of the necrotic layer was inversely related to weight loss: it was twice larger on the samples placed in the neutralized 2.5% NaOCl solution than in the 2.5% NaOCl solution. This is likely due to the higher cytotoxicity of the neutralized solution reported in the first part of the study. The histological findings of the study demonstrated that NaOH has no dissolving potential but causes chemical burnings that cause swelling and protect the underlying tissues. This has already been shown in ophthalmology[36] and gastroenterology.[37]

No pH variation was observed with NaOH because no interaction occurred between the biological tissue and the liquid apart from the initial burning of the superficial epithelium layer. The decrease in pH was higher for the 2.5% NaOCl solution than for the neutralized 2.5% NaOCl solution. A high weight loss is associated with a high consumption of ClO^-, which is not the case of the H^+ ions, which remain unchanged. This combination led to the accelerated decrease in pH reported here.

This part of the present work suggests that a neutralized 2.5% NaOCl solution in contact with palatal mucosa presents a limited aggressiveness toward the surrounding tissues.

The conclusions of the present work are limited to the in vitro conditions of the study and should be confirmed by further in vivo investigations.

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