ABSTRACT

Objectives: The aim of this study was to assess the physicochemical properties, cytotoxicity and penetration into dentinal tubules of ChlorCid™ Surf (3% sodium hypochlorite [NaOCl] with surfactant) in comparison to ChlorCid™ (3% NaOCl without surfactant).

Materials and Methods: The physicochemical properties evaluated were pH, surface tension, free available chlorine (FAC) and contact angle. Cytotoxicity was evaluated in L929 fibroblasts exposed to the solutions by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and neutral red assays. Assessment of penetration into dentinal tubules was performed by staining single-rooted permanent human teeth with crystal violet (n = 9), which were irrigated with the solutions and analyzed in cervical, middle and apical segments. Data were analyzed by one-way analysis of variance (ANOVA) and Tukey’s post-test, 2-way ANOVA and Bonferroni’s post-test or t-test (α = 0.05).

Results: ChlorCid™ Surf and ChlorCid™ FAC values were close to those indicated by the manufacturer. ChlorCid™ Surf showed lower surface tension and contact angle on dentin, and higher pH than ChlorCid™ (p < 0.05). The penetration of ChlorCid™ Surf was higher in cervical and middle segments, compared with ChlorCid™ (p < 0.05). There was no difference in irrigant cytotoxicity (p > 0.05).

Conclusions: ChlorCid™ Surf showed lower surface tension, lower contact angle on root canal dentin, higher penetration into dentinal tubules and more alkaline pH, compared with ChlorCid™. However, both solutions showed similar cytotoxicity and FAC content.

Keywords: Dentin permeability; Materials testing; Physicochemical analysis; Sodium hypochlorite; Surface-active agents

INTRODUCTION

The complex anatomy of the root canal system hinders the elimination of microorganisms in areas untouched by endodontic files, including the dentinal tubules [1,2]. Therefore, the penetration depth of irrigants into dentinal tubules may affect the effectiveness and
consequent prognosis of root canal treatment [3]. The penetration of sodium hypochlorite (NaOCl) into dentinal tubules is reportedly affected by its concentration, contact time, temperature, ultrasonic activation, gel form and surface tension [3-5].

Surface tension is defined as the force between surface molecules, which causes a drop of liquid to spread or concentrate when placed on a surface [6]. This property is related to the contact angle, whose measurement may provide a better understanding of the interaction between solids and liquids, and, consequently, irrigant wettability [7]. It has been reported that the addition of surfactants can decrease NaOCl surface tension, contact angle and improve its penetration into the root canal system [3,8-14]. However, there is still no definitive consensus regarding the improvement of the NaOCl penetration into dentinal tubules, since some studies did not report this effect [5,15].

NaOCl contains hypochlorite ions and hypochlorous acid in different proportions. Together, they constitute free available chlorine (FAC), which promotes organic dissolution and antimicrobial activity [16,17]. The pH of NaOCl influences its organic dissolution and antimicrobial activity; organic dissolution is greater at an alkaline pH [18]. More research is warranted on the effect of a surfactant on the physicochemical properties of NaOCl, such as surface tension, contact angle, FAC and pH, as well as on NaOCl penetration into dentinal tubules. The cytotoxicity of new endodontic irrigants should also be evaluated, since NaOCl may come into contact with periapical tissues, and the tissue response to the irrigant may influence the root canal treatment prognosis [19]. It was previously reported that the addition of benzalkonium chloride, a cationic surfactant, did not alter the cytotoxicity of 2.4% NaOCl [10].

Recently, 3% NaOCl with a surfactant, called ChlorCid™ Surf (Ultradent Products, South Jordan, UT, USA), was introduced. The manufacturer does not declare what surfactant is contained in its composition but claims that it modifies the surface tension, thus allowing better penetration into anatomical irregularities [20]. To the best of our knowledge, no studies have evaluated the physicochemical properties, cytotoxicity and penetration into dentinal tubules of ChlorCid™ Surf. Therefore, this study aimed to assess the physicochemical properties, cytotoxicity and penetration into dentinal tubules of ChlorCid™ Surf (Ultradent Products) compared with surfactant-free 3%NaOCl (ChlorCid™, Ultradent Products). The null hypothesis was that there would be no difference between the solutions regarding the parameters evaluated.

MATERIALS AND METHODS

Physicochemical properties

1. pH

Two 3% NaOCl solutions were stirred, one with surfactant (ChlorCid™ Surf, Ultradent Products) and the other surfactant-free (ChlorCid™, Ultradent Products). The 10 mL of each solution was placed in plastic flasks and the pH of each solution (n = 5 per group) was measured using a digital pH-meter (DM-22, Digimed, São Paulo, SP, Brazil), which was previously calibrated according to manufacturer’s instructions. The readouts were obtained at 22°C room temperature according to the requirements of the European Pharmacopoeia [21]. Each sample was measured 3 times and the mean value was considered as the value of each sample.
2. FAC content
The FAC content of the solutions was determined following the Standard Methods for the Examination of Water and Wastewater (SMEWW) using the N,N-diethyl p-phenylene diamine (DPD) colorimetric method [22]. The 10 mL of each solution was mixed with 500 µL of phosphate buffer and 500 µL of DPD was added. The FAC reacts immediately with DPD and forms a pink color. The results were obtained using a calibrated spectrophotometer (DR6000, HACH, Loveland, CO, USA) at 515 nm, and expressed in percentage (%) of mass/mass (m/m). Five samples of each group were used for the assay (n = 5).

3. Surface tension
An optical technique, the pendant drop method, was performed at 22°C–24°C room temperature. Each solution was placed in a syringe coupled to OCA-20 system (DataPhysics Instruments GmbH, Filderstadt, Germany), wherein it forms a drop, which is digitally captured by a camera, and the surface tension is calculated automatically using SCA-20 software (DataPhysics Instruments GmbH), as described by Andrade et al. [23]. This test was performed in triplicate and repeated at 3 times.

4. Contact angle
The sample size was calculated using the G*Power 3.1 software for Windows (Franz Faul, Christian-Albrechts-Universität zu Kiel, Kiel, Germany). The calculation, based on an effect size = 1.01 (based on pilot studies), test power (β) = 0.95 and α = 0.05, using “F-test family”, showed that 21 specimens (7 per group) were needed. However, since losses could occur during the experimental phase, 10 teeth per group were used. After approval by the research ethics committee (CAAE: 09798919.3.0000.5416), 15 single-rooted permanent human teeth stored in 0.1% thymol solution at 4°C were used to assess the contact angle. The teeth were decoronated and sectioned longitudinally to obtain 30 hemisections. Each hemisection was mounted on a 20-mm diameter and 15-mm wide polyvinyl chloride ring, observing that the dentin surface of the root canal was left facing the bottom of the device, so that it could later be filled with acrylic resin. After the filling set, the root canal dentin surface and the resin were polished with sequential abrasive paper granulations (120 to 1,200-grit) (3M ESPE, St. Paul, MN, USA) to obtain a smooth surface. The hemisections were distributed into the following 3 groups (n = 10): ChlorCid™, ChlorCid™ Surf and water. The specimens were kept in 4°C distilled water until the test. Before performing each measurement, the dentin surface was dried with absorbent paper to remove excess water [10]. The sessile drop method was performed using the OCA-20 system (DataPhysics Instruments GmbH) and SCA-20 software (DataPhysics Instruments GmbH) to determine the contact angle (θ). Three 5 µL aliquots of each solution were pipetted at 3 different points on the dentin surface, and the images were captured after 30 seconds until each drop was stable [24]. The average of the 3 values was considered the final value for each specimen (Figure 1).

Cytotoxicity
ChlorCid™ and ChlorCid™ Surf solutions were considered grade 1 dilutions, serially diluted in saline using a dilution factor of 1.5 [25]. The cells were incubated with the solutions at dilutions from 1/250 to 1/1,898, which corresponded from 0.4% to 0.05% doses/concentrations. L929 mouse fibroblasts were cultured (5 × 10⁴ cells/mL) in 96-well culture plates (Corning, Corning, NY, USA) containing Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin (100 U/mL penicillin, 100 mg/mL streptomycin) (Sigma-Aldrich, St. Louis, MO, USA). The cells were kept in an oven for 24 hours and incubated with the solutions at different doses for 10
minutes to simulate clinical contact time during chemo-mechanical procedures [25]. Saline and DMEM were used as controls. After this first incubation, the solutions were removed from the oven, and the cells were incubated with DMEM for 4 hours [25].

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed by adding 100 µL of 0.5 mg/mL MTT solution (Sigma-Aldrich) to each well, and incubating the cells for 3 hours. Afterward, 100 µL of acidified isopropyl alcohol was added. The neutral red (NR) assay was performed by adding 100 µL of the 0.05 mg/mL NR solution (Sigma-Aldrich) to each well, and incubating the cells for 3 hours. Then, 100 µL of 1% acetic acid solution in 50% ethanol was added to each well. The optical densities were measured using a spectrophotometer (ThermoPlate, Shenzhen, China) with a 570 nm wavelength filter. The percentage of cell viability was calculated from the absorbance of the control (saline), considered as 100%. The experiments were performed in triplicate and repeated at 3 different times.

**Penetration into dentinal tubules**

The sample size was calculated using the G*Power 3.1 software for Windows. The calculation, based on an effect size = 1.5 (based on pilot studies), test power (β) = 0.80 and α = 0.05, using “t-test family” (differences between 2 independent groups), showed that 14 specimens (7 per group) were needed. However, since losses could occur during the experimental phase, 9 teeth per group were used. The methodology was performed according to Faria et al. [5], with modifications. The working length of 20 single-rooted permanent human teeth was established 1 mm short of the apical foramen using a size 10 type K-file. Then, the root apex was sealed with composite resin, and the root canals were instrumented using the ProDesign Logic system (Easy Equipamentos Odontológicos, Belo Horizonte, MG, Brazil) up to a 40/.05 file at 350–600 rpm speed and 1–4 Ncm torque, using an electric motor (VDW Silver, VDW GmbH, Munich, Germany). The root canals were irrigated with 2 mL of 2.5% NaOCl for 1 minute at each instrument change, followed by irrigation with 5 mL of 17% EDTA for 3 minutes and 5 mL of distilled water. The root canals were then filled with 1% crystal violet solution (Labsynth, Diadema, SP, Brazil) and kept at 37°C and 95% humidity for 3 days.
changing the crystal violet every 12 hours. Afterward, the root canals were irrigated with 20 mL of distilled water. The roots were randomly distributed into 2 groups (n = 9): ChlorCid™ Surf and ChlorCid™. Two teeth were irrigated with distilled water, and served as the control of the reaction. The specimens were irrigated with 5 mL of the irrigants for 2 minutes using a 5 mL syringe (Ultradent Products) attached to a 27 G side-vented needle (Endo-Eze®, Ultradent Products) positioned 2 mm short of the working length. Next, the root canals of all the groups were irrigated with 5 mL of distilled water, and were sectioned transversely along their longitudinal axis at 3, 7 and 12 mm from the apex. The cervical surface of each segment was polished using 1,000-grit abrasive paper (3M ESPE). A stereomicroscope (LeicaM80, Leica Microsystems, Wetzlar, Germany) and Leica Application Suite EZ 3.0 software (Leica Microsystems) were used to obtain the images. Penetration depth analysis was performed by measuring crystal violet bleaching effects in the cervical surface of each segment, using Image J software (National Institutes of Health, Bethesda, MD, USA). Linear measurements, from the root canal border to the maximum extent of crystal violet bleaching, were made in 10 equidistant regions. The mean value, in micrometers (μm), of 10 linear measurements was considered the final value of the penetration depth for each specimen. A previously calibrated and blinded examiner conducted the analysis twice at a 2-week interval (intraclass correlation coefficient > 0.9).

Statistical analysis
The data were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) and SPSS 20.0 (IBM, Armonk, NY, USA) statistical software. The statistical tests consisted of one-way analysis of variance (ANOVA) and Tukey’s post-test (surface tension and contact angle), 2-way ANOVA and Bonferroni’s post-test (cytotoxicity and penetration into dentinal tubules) or t-test (pH and FAC content), at a significance level of 5%.

RESULTS
ChlorCid™ and ChlorCid™ Surf solutions had an alkaline pH of 12.27 ± 0.05 and 13.08 ± 0.02, respectively, and were significantly different (p < 0.05). ChlorCid™ had 2.78% ± 0.047% FAC, whereas the ChlorCid™ Surf FAC was 2.85% ± 0.044% (Table 1). Deionized water had the highest surface tension (71.95 ± 0.46 mN/m), followed by ChlorCid™ (70.20 ± 0.38 mN/m) and ChlorCid™ Surf (46.10 ± 0.22 mN/m), with significant difference among the 3 solutions (p < 0.05). Regarding the contact angle, deionized water had the highest values (50.78° ± 8.85°), followed by ChlorCid™ (36.99° ± 3.34°) and ChlorCid™ Surf (29.18° ± 3.75°) (p < 0.05) (Figure 2).

There was no difference in the cytotoxicity between ChlorCid™ and ChlorCid™ Surf according to both MTT and NR assays (p > 0.05). There was no difference between saline and DMEM groups (Figure 3). It was found that the higher the dose, the higher the cytotoxicity.

Table 1. Mean and standard deviation of pH and free available chlorine in percentage (% of weight/mass (w/w)) of ChlorCid™ and ChlorCid™ Surf

| Solution       | pH       | Free available chlorine (% [m/m]) |
|----------------|----------|-----------------------------------|
| ChlorCid™      | 12.27 ± 0.05* | 2.788 ± 0.047                     |
| ChlorCid™ Surf | 13.08 ± 0.02  | 2.854 ± 0.044                     |

*p < 0.0001.
In cervical and middle segments, ChlorCid™ Surf had higher penetration depth (156.22 ± 24.40 µm cervical, 127.52 ± 31.89 µm middle) than ChlorCid™ (92.86 ± 34.44 µm cervical, 88.61 ± 35.34 µm middle) \((p < 0.05)\). In the apical segment, penetration depth was 50.47 ± 17.60 µm for ChlorCid™ and 51.44 ± 14.42 µm for ChlorCid™ Surf, with no significant difference \((p > 0.05)\). Comparison between the segments showed that both ChlorCid™ and ChlorCid™ Surf had higher penetration depth in the cervical and middle segments than the apical segment \((p < 0.05)\), and that there was no difference between cervical and middle segments \((p > 0.05)\) (Figure 4).

In cervical and middle segments, ChlorCid™ Surf had higher penetration depth (156.22 ± 24.40 µm cervical, 127.52 ± 31.89 µm middle) than ChlorCid™ (92.86 ± 34.44 µm cervical, 88.61 ± 35.34 µm middle) \((p < 0.05)\). In the apical segment, penetration depth was 50.47 ± 17.60 µm for ChlorCid™ and 51.44 ± 14.42 µm for ChlorCid™ Surf, with no significant difference \((p > 0.05)\). Comparison between the segments showed that both ChlorCid™ and ChlorCid™ Surf had higher penetration depth in the cervical and middle segments than the apical segment \((p < 0.05)\), and that there was no difference between cervical and middle segments \((p > 0.05)\) (Figure 4).

**Figure 2.** Mean and standard deviation of (A) surface tension in millinewton/meter (mN/m) and (B) contact angle (θ) of ChlorCid™, ChlorCid™ Surf and deionized water (control). Different letters in columns indicate a significant difference between solutions. (C) Representative images of the contact angle on the dentin surface.

**Figure 3.** Viability of L929 fibroblasts after exposure to ChlorCid™ and ChlorCid™ Surf solutions at different doses by (A) MTT and (B) NR assays. No significant differences were founded between the solutions \((p > 0.05)\).

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NR, neutral red.
DISCUSSION

This study aimed to assess the physicochemical properties, cytotoxicity and penetrability into dentinal tubules of a 3% NaOCl solution with surfactant (ChlorCid™ Surf), compared with surfactant-free 3% NaOCl (ChlorCid™). The null hypothesis was rejected because ChlorCid™ Surf had lower surface tension, lower contact angle on dentin, and higher pH and penetration into dentinal tubules than ChlorCid™.

The determination of surface tension of NaOCl solutions has been performed using the ring, glass slide, or pendant drop methods [9-12,26]. Studies that performed the pendant drop method reported that 2.4% NaOCl and 2.5% NaOCl showed a surface tension similar to water, which is approximately 72 mM/m, and equivalent to dyn/cm or mJ/m [10,26]. ChlorCid™ Surf showed lower surface tension than ChlorCid™ (46.10 ± 0.22 and 70.20 ± 0.38 mN/m, respectively). Previous studies showed that the addition of surfactants reduced surface tension of NaOCl solutions, whether the NaOCl concentration was higher or lower than 3% [10-12,14,27,28].

The contact angle evaluation was performed by storing the specimens in distilled water and immediately removing the excess water before the assay since dentin is always wet during root canal treatment [10,14]. ChlorCid™ Surf showed a lower contact angle on root canal dentin than ChlorCid™. This observation agrees with studies showing that the addition of surfactants to NaOCl solutions can reduce this value [10,13]. The contact angle of ChlorCid™ on dentin was lower than that of water. Studies have shown that contact angles of 1% and 2.5% NaOCl on dentin show no difference from the contact angle of water on dentin [13,14]. Differences can be attributed to the methodology, considering that Iglesias et al. [14] polished the specimens up to 180-grit abrasive paper, whereas the present study specimens were polished up to 1,200-grit abrasive paper, as previously described [29]. Different granulations may affect dentin surface irregularities, and interfere in contact angle evaluation [30]. Differences from the study by Stojicic et al. [13] may be related to...
the low water contact angle on dentin observed in their study (39.33°), adopted to make comparisons with NaOCl solutions.

The crystal violet staining technique was used because NaOCl bleaching ability to act on this dye allows penetration depth evaluation. Dentin exposure time to NaOCl was 2 minutes as previously reported. ChlorCid™ Surf penetration was higher in cervical and middle segments, compared with ChlorCid™, indicating that the surfactant in the ChlorCid™ Surf composition led to higher penetration into dentinal tubules. A higher penetration depth of NaOCl with surfactants was also observed in root canal dentin blocks stained with crystal violet. Conversely, Faria et al. showed that the addition of surfactant did not influence the penetration of 2.5% NaOCl into dentin. The difference between their study and ours is that they left the irrigants in contact with dentin for a longer time (10 and 15 minutes). Our hypothesis is that the greater exposure time in their study produced an effect in which the results for the solutions with and without surfactant were the same.

ChlorCid™ penetration depth is reported to be similar at both 3 and 7 mm from the apex, using manual irrigation for 2 minutes. In sum, the addition of surfactants to 3% NaOCl (ChlorCid™ Surf) resulted in decreased surface tension and contact angle on dentin. Consequently, in increased penetration into dentinal tubules.

The assessment of pH is important since it may have a critical effect on the reactivity of chlorine in NaOCl solutions. Additionally, commercially available NaOCl products, at different concentrations, have an alkaline pH, which is directly related to a higher organic dissolution capacity. ChlorCid™ Surf and ChlorCid™ pH is alkaline, above 12; however, ChlorCid™ Surf showed significantly higher pH than ChlorCid™, indicating that the surfactant influenced this parameter. Previous studies reported that the surfactant had no significant influence on NaOCl pH at different concentrations. These divergent results may be attributed to the surfactant type and/or concentration in the ChlorCid™ Surf composition.

The determination of FAC content was performed according to the most recent edition of the SMEWW, which includes all the recognized methods, among them the iodine/sodium thiosulfate titration method (SMEWW 4500-Cl B) and DPD colorimetric method (SMEWW 4500-Cl G). The DPD colorimetric method is considered simple and fast, which is an advantage. Although the iodine/sodium thiosulfate titration method has been widely performed, the DPD colorimetric method has also been performed in endodontic research. ChlorCid™ Surf and ChlorCid™ had a lower FAC content than that reported by the manufacturer (2.85% and 2.78%, respectively). Comparatively, that declared by the manufacturer of ChlorXtra was also lower (6% NaOCl with Triton X-100 surfactant), but that established by the manufacturer of Hypocelle was higher (4% NaOCl with an undeclared surfactant). This demonstrates that FAC may be irregular in commercial presentations of NaOCl.

The cytotoxicity of irrigating solutions is important since toxic effects may harm cells in the periapical region and prejudice the repair process. In this regard, the MTT assay has been widely used; however, it is necessary for more than one assay to evaluate the cytotoxicity. The evaluation of different cellular parameters allows a more reliable interpretation of the cytotoxic effect of the material. For this reason, 2 assays were
performed, the MTT, which allows evaluation of cellular metabolism, and the NR assay, which allows evaluation of cell viability based on the ability of live cells to incorporate NR in the membrane of their lysosomes [37,38]. ChlorCid™ Surf and ChlorCid™ induced similar cytotoxicity of L929 cells in both MTT and NR assays, corroborating a study showing that 2.4% NaOCl in association with benzalkonium chloride showed cytotoxicity similar to 2.4% NaOCl [10]. Although ChlorCid™ Surf showed higher penetration into dentinal tubules than ChlorCid™, further research is needed to assess whether the 2 products are different regarding their antimicrobial effect along with the extension of dentinal tubules.

CONCLUSIONS

It can be concluded that ChlorCid™ Surf showed lower surface tension, lower contact angle on root canal dentin, higher penetration depth into dentinal tubules and more alkaline pH, compared with ChlorCid™. However, both showed similar cytotoxicity and FAC content.

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