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Antimicrobial effect of a hyperosmotic solution on endodontic microorganisms in planktonic state.

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Key words: hyperosmotic solution; endodontics; *E. faecalis; C. albicans;* antimicrobial.

Abstract. To achieve the elimination of microorganisms inside root canals with endodontic treatment, the use of a suitable mechanical and chemical biopreparation is necessary. The chemical irrigation is performed with endodontic irrigation solutions, the most commonly used is sodium hypochlorite; however, this has harmful effects as cytotoxicity and thus, the research of new solutions is essential in this area. The aim of this study was to evaluate the anti-microbial effect *ex-vivo* of a hyperosmotic solution based on salts of potassium and sodium. A mixture of endodontic microorganisms (*Enterococcus faecalis* and *Candida albicans*) was employed at different concentrations and with 5, 10 and 15 minutes of contact time with the hyperosmotic solution and subsequently, counts of colony forming units were carried out. In addition, the percent of microbial inhibition of the hyperosmotic solution was evaluated in comparison to 5.25% sodium hypochlorite. A significant difference was found in the amount of bacterial load after the use of the hyperosmotic solution, and the percent of microbial reduction of this solution was similar to 5.25% sodium hypochlorite. This study showed that the hyperosmotic solution has a potent anti-microbial effect against endodontic microorganisms in planktonic state, thus it could be used as endodontic irrigation agent, following further *ex-vivo* investigations.

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Efecto antimicrobiano de una solución hiperosmótica sobre microorganismos endodónticos en estado planctónico.

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Palabras clave: solución hiperosmótica; endodoncia; *E. faecalis; C. albicans;* antimicrobiano.

Resumen. Uno de los objetivos del tratamiento de endodoncia es la eliminación de los microorganismos del interior de los conductos radiculares, para lograr esto, una adecuada preparación tanto química como mecánica es necesaria. La preparación química es realizada con irrigantes endodónticos, el más común es el hipoclorito de sodio, sin embargo, este puede causar efectos dañinos debido a su citotoxicidad, por lo tanto, la búsqueda de nuevos irrigantes es esencial en esta área. En este estudio evaluamos el efecto antimicrobiano de una solución hiperosmótica basada en sales de potasio y sodio. Empleamos una mezcla de microorganismos endodónticos (*Enterococcus faecalis* y Cándida albicans) a diferentes concentraciones y con 5, 10 y 15 minutos de tiempo de contacto con la solución hiperosmótica y posteriormente se llevó a cabo el conteo de unidades formadoras de colonias. Además, se evaluó el porcentaje de reducción microbiana de la solución hiperosmótica en comparación con el hipoclorito de sodio al 5,25%. Se encontraron diferencias significativas en la reducción de la carga microbiana con el uso de la solución hiperosmótica, y el porcentaje de reducción microbiana de esta misma resultó similar al del hipoclorito de sodio al 5,25%. Este estudio muestra que la solución hiperosmótica tiene un potente efecto antimicrobiano en contra de microorganismos endodónticos en estado planctónico, por lo que podría ser usado como un irrigante en endodoncia luego de realizar investigación ex-vivo adicional.

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INTRODUCTION

The success of the endodontic therapy depends on many factors, being the decrease or elimination of bacterial infection one of the most important determinants of treatment success. This elimination is achieved by effective mechanical instrumentation and irrigation.

Inside of root canals a high amount and diversity of endodontic microorganisms can exist. A considerable interest has been focused on *Enterococcus faecalis*, a facultative anaerobia Gram-positive coccus frequently isolated as a monoculture in root-filled teeth but rarely identified in untreated root canals. This can additionally adapt to extreme environments as nutrients shortage, alkaline pH and, penetrate at dentinal tubules and form biofilm in root canals (1).

Fungi have been commonly found in root canals of obturated teeth in which treatment has failed. They have also been isolated from periradicular lesions refractory to endodontic treatment (2). Candida spp. have been found in infected root canals with a prevalence ranging from 0.5%–55%. Possess virulence factors that may play a role

in the onset of endodontic pathosis, these yeasts can adapt to a variety of environmental conditions and adhere to many surfaces including dentin and root filling materials; moreover, *C. albicans* can produce hydrolytic enzymes, undergo morphologic transition, form biofilm, and evade and modulate the host defense (3,4).

The removal of these microorganisms of root canals in endodontic treatment depends on adequate mechanical preparation and copious irrigation.

Several studies have observed that sodium hypochlorite (NaOCl) in range from 0.5% to 5.25% is one of the most commonly irrigating solution used for root canal irrigation (5).

NaOCl has broad spectrum antibacterial effect, is able to kill vegetative cells, spore-forming bacteria, fungi, protozoa and viruses, as inhibit the available enzymes for bacterial metabolism through several mechanisms as, saponification, neutralization and chloramination (6). However, NaOCl has several disadvantages such as cytotoxicity in the periapical or soft tissues, even used at low concentrations. In addition, it is not able to eliminate the smear layer in root canals and has been demonstrated in *in vitro* studies that the long-term exposure of dentine at high concentrations of NaOCl presents a harmful effect on dentin elasticity, which makes it more prone to fractures. Others irrigants used in this treatment include 0.12% chlorhexidine, EDTA C, solutions based in iodine, hydrogen peroxide, urea peroxide, etc., but they all have shortcomings that renders them less suitable as root canal irrigant if used alone.

All this has led to the search of new irrigants that meet most of the characteristics of an ideal irrigant and whose antimicrobial effect can be similar to that of NaOCl.

Osmotic pressure is one of the most significant physical parameters with which bacteria must contend; osmotic stress can consist of either "hyperosmotic shock" due to an increase in external osmolarity, or the opposite, "hypo-osmotic shock" (7).

The use of hyperosmotic solutions has increased in the microbiological area due to their antimicrobial effects and their relatively low processing cost. Hyperosmotic solutions are supersaturated solutions based on salts of potassium and sodium. The hyperosmosis reduces the availability of water of microorganisms and their environment, the stress caused by hyperosmosis inhibits the bacterial growth and finally cause the death of microorganisms (8).

In this study the anti-microbial effect of a hyperosmotic solution, based on sodium chloride and potassium sorbate, on *E. faecalis* and *C. albicans* isolated of root canals was evaluated. Both microorganisms are usually found in the root canal embedded in a biofilm attached to the dentin, and as is known, this can inhibit microbial killing and thus condition the result of the anti-microbial action of any irrigating solution compared to that achieved against it in planktonic state. But our focus considered the planktonic state, as an initial approach to evaluate the effect of the hyperosmotic solution and in a second phase of the work its effect on a multispecies biofilm will be evaluated. The aim of the present study was to evaluate the antimicrobial effect of a hyperosmotic solution based on sodium chloride and potassium sorbate against *E. faecalis* and *C. albicans* in planktonic state.

MATERIAL AND METHODS

Samples. The endodontic microorganisms were isolated of root canals of patients attending the clinic of the endodontics postgraduate program. All patients were evaluated by clinical history and oral examination and a sample from the root canal was taken in those patients with periradicular lesions or endodontic failure. Human research ethics approval was granted by the Ethics Committee of Stomatology Faculty of San Luis Potosí (México) and all patients signed an informed consent form. The tooth was isolated with rubber dam, disinfection protocol was

made with 5.25% sodium hypochlorite, 30% hydrogen peroxide and 10% sodium thiosulfate and access to the pulp camera was then gained. Sterile paper points were introduced in root canals and subsequently, placed in thioglycolate medium (BD BBL, Estado de México, México) and incubated in an anaerobic chamber (COY Laboratory Products Inc, Grass Lake, MI, USA) for 48 hours.

Biochemical identification of isolated microorganisms. Samples taken from the root canals were incubated for 48 hours in an anaerobic chamber and later, were grown in CDC anaerobic agar (BD BBL). Colonies with morphological characteristics corresponding to *Enterococcus* and *Candida* were identified through biochemical tests by using API system (BioMérieux, Mercy l'Etoile-France). Gram stain was performed to evaluate microscopic morphology. *Enterococcus faecalis* was identified by using API 20 Strep and *C. albicans* was identified by using API 20 C AUX according to the manufacturer's instructions.

Elaboration of the hyperosmotic solution. The hyperosmotic solution was elaborated with the next reagents: 1M of potassium sorbate, 1M of sodium chloride, 0.051M of hydrochloride acid, 38.4% of ethylic alcohol and deionized water (all by Sigma-Aldrich, St. Louis, Missouri, USA). The hyperosmotic solution was prepared over a hot plate with a magnetic stirrer (Analog stirrer, Labnet International, Edison NJ, USA), and then, the solution was filtered under vacuum with a 2 μm filter. and finally stored in an amber bottle until its use, the pH of the final solution was 6.6.

Reduction of microbial load. *E. faecalis* and *C. albicans* were placed together in a special liquid culture medium that consisted of Brain Heart Infusion (BHI) and Dextrose Sabouraud (DS) (BD Bioxon, México) in a 75:25% ratio respectively to allow for the growth of both microorganisms in 1:1 ratio. The medium with the microbial mixture was incubated at 37 ± 2°C during 24-48 hours, after this time its synergy and balanced growth was verified by gram stain.

From the plate culture with both microorganisms in equilibrium, a suspension was prepared in liquid culture medium at a McFarland 0.5; then, centrifuged for 20 min at 2500 rpm (Solvat J-600, México, D.F.). Subsequently, the supernatant of each tube was discarded combining then the microbial pellet with 10 mL of the hyperosmotic solution. Each tube was stirred and kept at room temperature for periods of 5, 10 and 15 min. Finally, discharges of 100 μL of each solution were spread on BHI/DS (75%/25%) agar plates sowing by surface dissemination and incubating during 24-72 hours. These tests were performed together with a bacterial growth inhibition control (5.25% NaOCl) and a bacterial growth control, (distilled water). The same methodology was performed with a McFarland 3.0 and 7.0 with the aim of evaluate the effect of the hyperosmotic solution by increasing the microbial charge.

The percent of microbial inhibition was calculated based in the count of microbial colonies (CFU). All these experiments were performed in triplicate in a purifier class II Biosafety Cabinet (Labconco Corporation, Kansas City, MO, USA).

Statistical analysis. Data were analyzed by using the statistical software GraphPad Prism v5.0 (GraphPad, San Diego, CA, USA) by a specialist with data blinding. The normality of the variables was analyzed with the Kolmogorov-Smirnov test. Comparison among the study groups was carried out applying an analysis of variance and the statistical significance was determined. A possible correlation between the reduction of microbial load and contact time with the hyperosmotic solution was determined through the Pearson test. To reduce the bias of the measures the coefficient of reliability (95%) was calculated with a statistical significance of $p<0.05$.

RESULTS

Reduction of microbial load employing hyperosmotic solution. To evaluate the reduction of the microbial load employing the hyperosmotic solution, we added the hyperosmotic solution at a mixture of microbial culture during 5, 10 and 15 minutes of contact time and with different concentrations of microorganisms (0.5, 3 and 7 McFarland scale). We observed a significant difference in the reduction of bacterial load expressed as log colony forming units (CFU) during 5 minutes of contact time with the hyperosmotic solution ($p<0.05$), using a mixture of microorganisms at a concentration of McFarland 0.5 (Fig. 1A), similar results were observed with 10 and 15 minutes of contact time ($p<0.05$) (Fig. 1B, C). In addition, we evaluated higher concentrations of the microbial mixture and we observed a significant difference in the reduction of the bacterial load with a concentration of McFarland 3 and 7 with 5, 10 and 15 minutes of contact time with the hyperosmotic solution ($p<0.05$) (Fig. 1A-C).

Correlation analysis between contact time with hyperosmotic solution and its antimicrobial effect. The correlation analysis between the different contact times (5, 10 and 15 minutes) employing the hyperosmotic solution and the number of colony forming units expressed as log, was performed and no correlation was observed, ($r= -0.13$, $p>0.05$) (Fig. 2A).

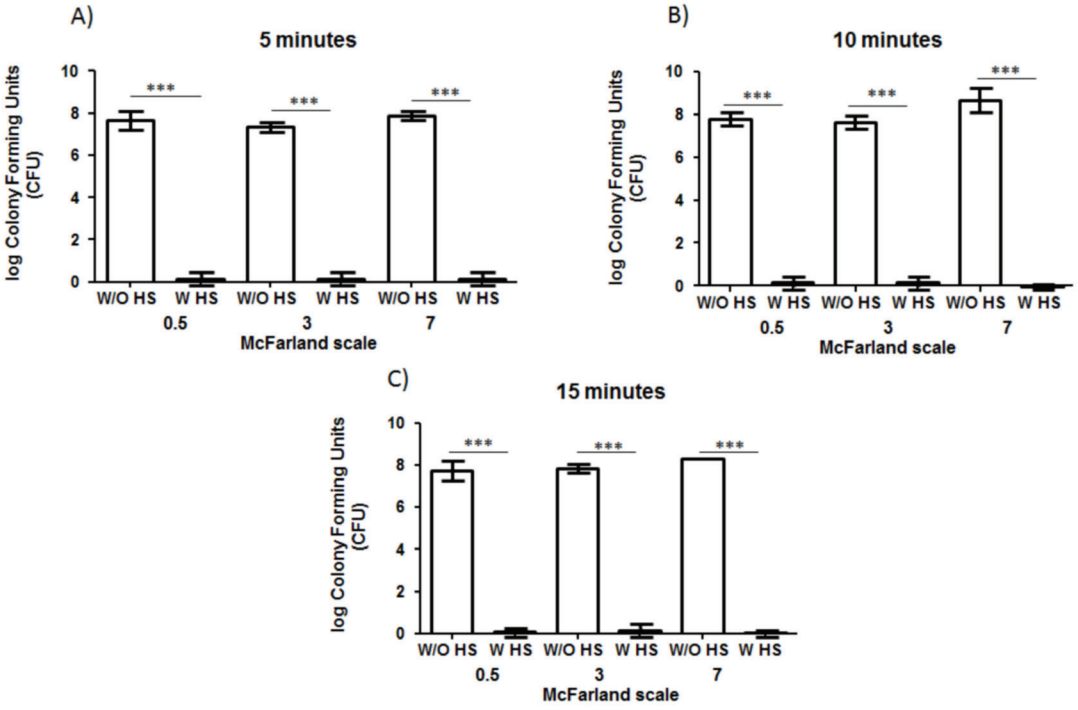

Fig. 1. Reduction of microbial load employing hyperosmotic solution with different concentrations of the microbial mixture. The count of colony forming units (CFU) was made in all culture plates with hyperosmotic solution (W HS) and without hyperosmotic solution (W/O HS) and transforming it at log. Was employed a low, a medium and a high concentration of the microbial mixture (0.5, 3 and 7 in McFarland scale). **A)** Log colony forming units without hyperosmotic solution vs with hyperosmotic solution with contact time of five minutes. **B)** Log colony forming units without hyperosmotic solution vs with hyperosmotic solution with contact time of ten minutes. **C)** log colony forming units without hyperosmotic solution vs with hyperosmotic solution with contact time of fifteen minutes. A-C mean are represented, ***$p<0.05$. W/O HS= without hyperosmotic solution, W HS= with hyperosmotic solution.

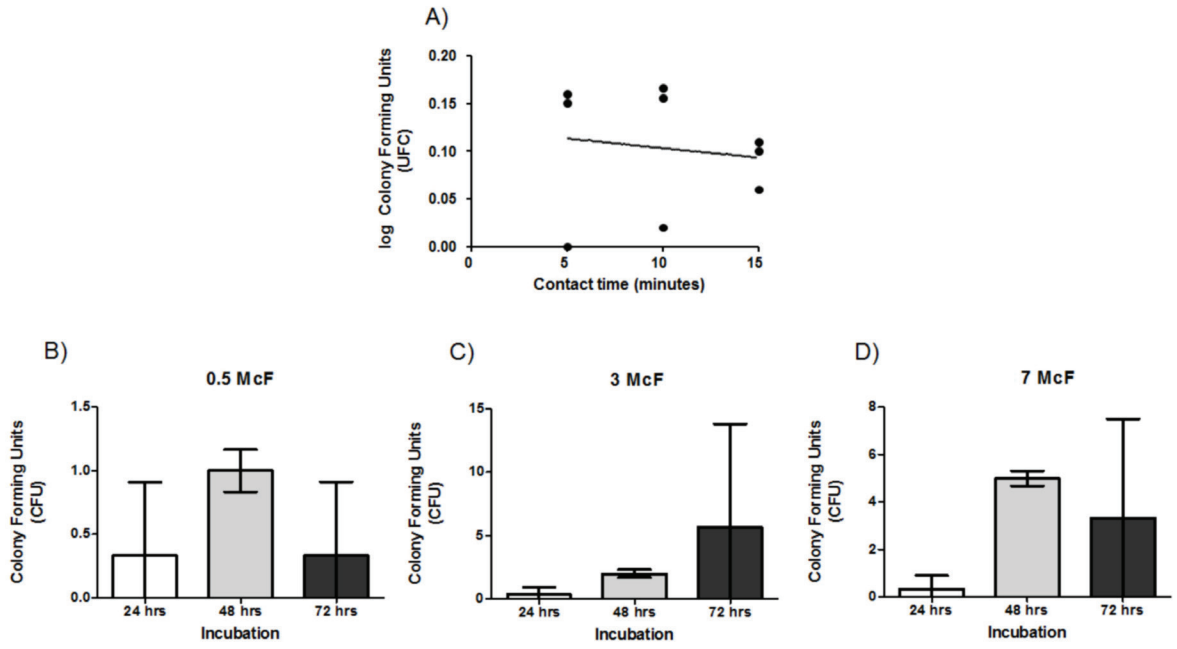

Fig. 2. Correlation between antimicrobial effect of hyperosmotic solution and contact time and evaluation during different periods of incubation. In order to assess the correlation between the contact time with the hyperosmotic solution and its antimicrobial effect, a correlation test between the tested times (5, 10 and 15 minutes) and the number of colony forming units (CFU) expressed as log was performed. **A)** Analysis of correlation between the log of colony forming units and contact time with hyperosmotic solution. **B)** CFU at 24, 48 and 72 hours of incubation at concentration of 0.5 McFarland. **C)** CFU at 24, 48 and 72 hours of incubation at concentration of 3 McFarland. **D)** CFU at 24, 48 and 72 hours of incubation at concentration of 7 McFarland. B-D mean are represented, McF= McFarland scale.

Evaluation of reduction of the microbial load during different periods of incubation. To evaluate the effect of contact time on the antimicrobial effect of the hyperosmotic solution, the different concentrations of the microbial mixture in combination with the hyperosmotic solution were incubated during 24, 48 and 72 hours, without a significant difference in colony forming units (CFU) being observed after those periods of time ($p=0.076$), with a microorganism concentration equals to 0.5 McFarland scale (Fig. 2B). Similar results were observed with higher concentrations of microorganisms, 3 and 7 in McFarland scale ($p>0.05$) (Fig. 2C, D). In addition, we evaluated 10 and 15 minutes of contact time of hyperosmotic solution with the same incubation time (24, 48 and 72 hours) and not significant differences were observed ($p>0.05$).

Comparison of the reduction of microbial load with sodium hypochlorite. We evaluated if the hyperosmotic solution has a similar effect in the percent of microbial inhibition in comparison to 5.25% NaOCl. We do not observe a significant difference in the percent of microbial inhibition of hyperosmotic solution in comparison to 5.25% NaOCl, when using a low concentration of microorganisms, 0.5 McFarland scale (90 vs 99.9%, respectively, $p=0.0765$) (Fig. 3A). Similar results were observed with higher concentrations of microorganisms, 3 and 7 McFarland scale ($p=0.07$) (Fig. 3B, C); however, with the major concentration of microorganisms (7 McFarland) the percent of microbial inhibition of the hyperosmotic

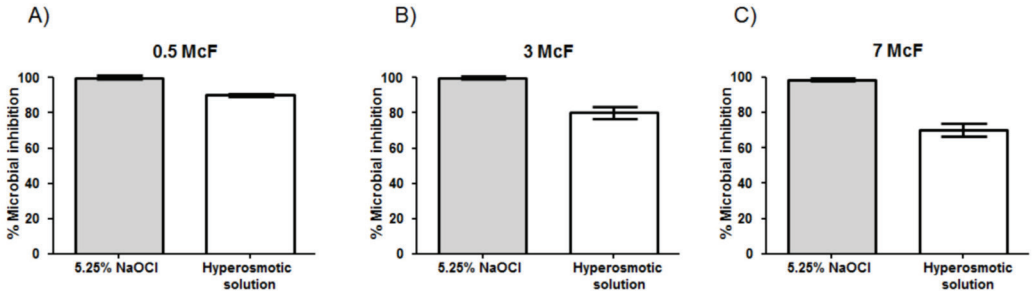

Fig. 3. Comparison of the reduction of microbial load with sodium hypochlorite. The percentage of microbial inhibition of the hyperosmotic solution compared to 5.25% sodium hypochlorite was determined, **A)** Percent of microbial inhibition of 5.25% sodium hypochlorite vs hyperosmotic solution at 0.5 McFarland scale of microbial concentration. **B)** Percent of microbial inhibition of 5.25% sodium hypochlorite vs hyperosmotic solution at 3 McFarland scale of microbial concentration. **C)** Percent of microbial inhibition of 5.25% sodium hypochlorite vs hyperosmotic solution at 7 McFarland scale of microbial concentration. A-C mean are represented, McF= McFarland scale.

solution diminished in comparison to NaOCl (70 vs 99.9%, Fig. 3C), but this decrease in the percent of microbial inhibition was not statistically significant (p=0.07).

DISCUSSION

Periapical and pulpal infections are caused by a diverse community of microorganisms that colonizes the root canals and invade dentinal tubules (9,10). One objective of endodontic treatment is the elimination of these microorganisms inside the root canals by the use of irrigants. The irrigant most commonly used in endodontics is NaOCl at 2.5% or 5.25%, because of its mechanisms of action has a potent antimicrobial effect; however, NaOCl has toxic effects on tissues, thus the search for new irrigants is essential in endodontic area (11).

In this study we evaluated the antimicrobial effect of a hyperosmotic solution made with salts of sodium chloride and potassium sorbate and we found a total reduction or almost total reduction of the mixture of endodontic microorganisms, even at high concentrations of microorganisms. It has been reported that the mechanism of action of this solution is due to hyperosmotic shock altering the cellular membrane of the microorganisms, by reduction of available water, thus causing their death (8). Some authors have proposed the use of hyperosmotic solutions for the elimination of microorganisms inside root canals- Van der Waals et al demonstrated the antibacterial efficacy of a 3M hyperosmotic solution against biofilm of *E. faecalis.* We employed a low concentration of a hyperosmotic solution (1M), obtaining a good antimicrobial efficacy (8,12,13). However, it would be interesting to evaluate the antimicrobial effect of this solution on endodontic biofilm, since it has been demonstrated that microorganisms in biofilm have a greater capacity of resistance to the action of antimicrobials (14,15). However, we decided in this preliminary study to evaluate the action of a hyperosmotic solution on endodontic microorganisms in planktonic state, to know the antimicrobial effect of the proposed solution. In the light of these results we will have to focus on evaluating the effect of the hyperosmotic solution on microbial biofilm.

We employed a mixture of microorganisms of *E. faecalis* and *C. albicans to* evaluate the antimicrobial effect of hyperosmotic solution, both microorganisms are frequently

isolated of root canals. Interestingly, we observed in those cases where there were some colony forming units in culture plates corresponding to *E. faecalis* only, so that *C. albicans* was sensitive to the action of the hyperosmotic solution. In accordance with this, it has been reported that *E. faecalis* is the microorganism mostly isolated of endodontic failures and endodontic infections, this is due to multiple virulence factors as production of enzymes and high adherence capacity to dentinal tubules surface. In addition, it can resist environmental changes, such as increases of pH, thus this microorganism is usually more resistant to the action of antimicrobials (16,17). It should be mentioned that the contact times employed for the hyperosmotic solution with the microorganisms were during 5, 10 and 15 minutes, and we do not observed correlation between the contact times and number of colony forming units (expressed as log); so that, we can conclude that the antimicrobial effect of this solution is not dependent of the contact time, given that since the shorter contact period showed high antimicrobial activity.

To evaluate if the hyperosmotic solution still has antimicrobial effect on cultures of endodontic microorganisms over time, we performed different incubation times, at 24, 48 and 72 hours, and not significant differences were observed between the different periods of time and concentration of microorganisms; thus, the antimicrobial effect of the hyperosmotic solution seems to last sufficiently. This may be due to a potent antimicrobial effect observed for the hyperosmotic solution thanks to the mechanism of action on microorganisms in planktonic state (8). It has been reported that on biofilm the microorganisms have more resistance, so again it should be noted the importance to evaluate the effect of this hyperosmotic solution on microbial biofilm and even more so when it is conformed by two or more microorganisms, as occurs in the root canal (18,19).

The sodium hypochlorite has been used in endodontics for a long time as an irrigation solution and actually, is one of the best antiseptics in the area; with a potent antimicrobial effect, altering essential enzymes for the metabolism of microorganisms. It has several action mechanisms such as saponification, neutralization and chloramination (11). We compared the percent of microbial inhibition of sodium hypochlorite vs. the hyperosmotic solution on the endodontic microorganisms and we found that the hyperosmotic solution has a good antimicrobial effect in comparison to sodium hypochlorite; however, although the antimicrobial ability of this solution is good, it is necessary to perform more studies to evaluate the stability of the solution, its cytotoxic effects and damage or alterations of the dentinal structure.

In conclusion, the hyperosmotic solution has a good antimicrobial effect against endodontic microorganisms in planktonic state and may be used as endodontic irrigant; however, more studies are necessary to evaluate the clinical feasibility of using this solution within the root canal, as biofilms may not be disturbed by this solution.

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