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Antibacterial effect of a hyperosmotic solution containing sorbate and ethanol on *Enterococcus faecalis* in planktonic form and as biofilm: an *in vitro* study.

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Key words: biofilm; hyperosmolarity; *E. faecalis;* irrigant; endodontics.

Abstract. The antibacterial effect of a hyperosmotic solution containing sorbate and ethanol on *E. faecalis* in planktonic state and in biofilm was evaluated. Three hyperosmotic solutions (HS-A, HS-B y HS-C) were obtained from different formulations of potassium sorbate and sodium chloride, which were tested as antimicrobials against planktonic forms of *E. faecalis,* in McFarland standards from 0.5 to 7, using the sedimentation technique and colony forming units (CFU) count. Afterwards an *E. faecalis* biofilm was produced in the palatal roots of upper first molars, by a static method in 21 days; subsequently they were prepared biomechanically by the Universal Protaper system, using the hyperosmotic solution B as an irrigant to evaluate the bacterial load reduction. One pre-instrumentation sample and one post-instrumentation sample were taken, and then were processed and cultivated to count CFU. Consecutively, roots were observed by scanning electron microscopy. The hyperosmotic solution had an important antibacterial effect when used against *E. faecalis* in planktonic state; solutions HS-A and HS-B were effective in eliminating *E. faecalis* up to 7 McFarland, while a statistical difference ($p<0.001$) was observed in reducing the bacterial load in the biofilm, based on the $\log_{10}$ CFU count. The final solution tested seemed not to harm the dentinal structure and was capable of causing morphological changes to the bacterial cell consistent with a hyperosmotic shock. Thus, the solutions tested could be an option to be considered as irrigating agents; nonetheless further research is required regarding its biocompatibility.

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Efecto antibacteriano de una solución hiperosmótica a base de sorbato y etanol sobre *Enterococcus faecalis* en forma planctónica y como biofilm: estudio *in vitro*.

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Palabras clave: biopelícula; hiperosmolaridad; *E. faecalis;* irrigante; endodoncia.

Resumen. Se evaluó el efecto de una solución hiperosmótica sobre *E. faecalis* en forma planctónica y en forma de biofilm. A partir de formulaciones de sorbato de potasio y cloruro de sodio se obtuvieron tres soluciones hiperosmóticas (HS-A, HS-B y HS-C) con ellas se realizaron pruebas antimicrobianas contra *E. faecalis* en forma planctónica en concentraciones desde 0,5 hasta 7 McFarland mediante técnica de sedimentación y cuenta de UFC. Posteriormente se formó biofilm de E. faecalis por método estático por 21 días en raíces palatinas de primeros molares superiores, las cuales se prepararon biomecánicamente mediante el sistema Protaper Universal usando la solución hiperosmótica HS-B como irrigante para luego evaluar la disminución de carga bacteriana, comparando el conteo de UFC de muestras pre-instrumentación con muestras post-instrumentación. Consecutivamente las raíces fueron observadas al microscopio electrónico de barrido. En las pruebas contra microorganismos planctónicos las soluciones HS-A y HS-B fueron efectivas en eliminar *E. faecalis* hasta un Mc Farland de 7. En la reducción de carga bacteriana mediante conteo de UFC de los microorganismos embebidos en biofilm, se identificó una diferencia estadística ($p<0,001$). La solución hiperosmótica tiene un importante efecto antibacteriano contra *E. faecalis* en forma planctónica y en forma de biofilm, no parece dañar la estructura dentinaria y es capaz de provocar cambios morfológicos a la célula bacteriana consistentes con shock hiperosmótico. De manera que la solución hiperosmótica parecería ser una opción a ser considerada como agente irrigante o coadyuvante de la irrigación; sin embargo, se requiere investigar más profundamente con respecto a su biocompatibilidad.

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INTRODUCTION

An effective chemo-mechanical instrumentation of the root canal system is crucial for the long-term success of the endodontic treatment. Modern endodontic therapy involves the combination of mechanical debridement of dentine with chemical agents for irrigation and disinfection, with the goal being the removal of all microorganisms from the root canal system (1).

Since the anatomy of the root canal system is complex and its access is limited, microorganisms can remain in the dentinal tubules and other irregular spaces. When these microorganisms find a favorable environment; they can proliferate and reinfect the entire root canal system, and thus infected dentinal tubules and the presence of biofilms make disinfection much more difficult. Biofilms are microbial communities embedded within an extracellular matrix, forming a highly organized structure associated with a surface such as the root canal wall. There is convincing evidence that microorganisms organized in this way are much less suscepti-

ble to antimicrobial agents than their planktonic counterparts (2).

Decades of research have clearly demonstrated that pathogens have developed an arsenal of mechanisms to enhance the virulence potential of the biofilm, the structural and biochemical properties of the matrix provide the emergent properties of biofilms that include surface adhesion, spatial and chemical heterogeneities, synergistic/competitive interactions and increased tolerance to antimicrobials. Despite this, how pathogens modify the spatial–temporal organization and communal behavior to create localized pathological niches remains still widely unknown (3).

At present it is known that clinical endodontic and oral *Enterococcus faecalis* strains, in contrast to strains from other clinical sources (e.g. particular endocarditis strains), have lower inherent capacity to form biofilms (4). Thus the conditions under which biofilms might occur in infected root canals in vivo are not yet well understood.

E. faecalis has been related to oral diseases such as endodontic infections, periodontitis, and peri-implantitis. It has been frequently implicated in failure of the endodontic treatment, due to its high resistance and ability to produce recalcitrant biofilms both in treated and untreated root canals (5).

Recently an innovative approach has been attempted to inactivate bacterial biofilms through hypertonic saline solutions. These salts are used to prevent bacterial growth in food or cosmetic products that when used at high concentrations; a synergistic effect is induced by hyperosmotic stress (6).

The rationale of the antibacterial effect of hyperosmotic solutions is based on the fact that hyperosmotic stress causes loss of cellular water and cellular contraction, elevates the intracellular ionic force, and generates macromolecular overcrowding and protein denaturation; thus inducing cellular plasmolysis that results in inhibition of a variety of physiological processes, from nutrient absorption to DNA replication. Certain agents such as inorganic ions (e.g. K^+, Na^+, and Cl^-) have the ability to destabilize the secondary structure of proteins and disturb enzymatic activity (7).

Van der Waal *et al*. tested the survival of 48 h *E. faecalis* biofilm expressed as log colony forming units (log UFC) after challenge with a series of combinations of potassium sorbate (KS) and sodium chloride (NaCl) solutions; getting good results with a combination named modified salt solution, formed with 3 M NaCl and 1 M KS (8).

Recently our group carried out an evaluation of the effect of a hyperosmotic aqueous solution composed of 1.5 M NaCl, 1 M KS, 0.051 M HCl, and 38.4% ethylic alcohol against a combination of *Enterococcus faecalis* and *Candida albicans* in planktonic state; showing a potent anti-microbial effect for both endodontic microorganisms (9).

The aim of this study was to evaluate the antibacterial effect of a hyperosmotic solution against *E. faecalis,* in planktonic suspension and embedded in a biofilm, in terms of bacterial load reduction.

MATERIALS AND METHODS

Formulation of hyperosmotic solutions. Three different hyperosmotic solutions: hyperosmotic solution A (HS-A), hyperosmotic solution B (HS-B) and hyperosmotic solution C (HS-C) were evaluated against *E. faecalis* in planktonic state. Based on the results the most promising solution was chosen to carry out tests against the *E. faecalis* biofilm.

Initially we were not able to prepare the hyperosmotic solution reported by Van der Waal *et al.* (3 M NaCl and 1 M KS) due to incomplete dissolution of the salts, thus the concentration of NaCl was decreased to 1.5 M for full solubilization; despite reducing the osmotic value. Looking to compensate this situation, the speciation of sorbate was favored towards the protonated species (sorbic acid) to have a synergetic effect in

the solutions tested. This was accomplished by simply adding HCl to solutions HS-A and HS-B. Given the low solubility of sorbic acid in aqueous solutions, ethanol was added as co-solvent and vehicle to favor the entrance of sorbic acid to the bacterial cell. Solution HS-C was composed of NaCl, ethanol, and sorbic acid the composition of solutions HS-A, HS-B and, HS-C is shown in Table I.

Antimicrobial capacity tests of the hyperosmotic solutions against planktonic microorganisms. This study was registered and approved by the Institutional Ethics Committee of the Faculty of Stomatology at San Luis Potosi Autonomous University (approval code no. CEIFE-057-015) and the patient signed an informed consent form.

A strain of *Enterococcus faecalis* from a patient with persistent apical periodontitis was identified by the conventional API 20 STREP® Test (Biomerieux, France) and maintained in trypticase soy agar (BD Sparks, MD, USA) until its use. It was reactivated in brain heart infusion broth (BHI); the purity of the culture was verified regularly by gram staining.

Centrifugation sedimentation technique. An inoculum of *E. faecalis* in a tube with BHI broth was made; afterwards it was incubated for 24 h until reaching a concentration of 7.5 McFarland, allowing this previous incubation time to carry out the experiment in the stationary phase, from which the corresponding volume was withdrawn to achieve suspensions of microorganisms in the 0.5 to 7 McFarland concentrations range. Each suspension was centrifuged for 20 min at 2500 rpm (Solvat J-600, México). Subsequently, the supernatant of each tube was discarded and the microbial pellet combined with 10 mL of the corresponding hyperosmotic test solution (HS-A, HS-B, or HS-C); each tube was stirred and kept at room temperature for periods of 5, 15, and 25 min. Finally, 100 μL samples from of each solution were spread on BHI agar plates sowing by surface dissemination and incubating at 35 ± 2°C during 24-48 h. The grade of microbial inhibition was based on the count of microbial colonies (CFU). All these experiments were performed in triplicate using a purifier class II Biosafety Cabinet (Labconco Corp. Kansas City, MO, USA).

Test of antimicrobial capacity against *E. faecalis* biofilm. 25 palatal roots of upper first molars adjusted with a carbide disk at a length of 14 mm were used, 15 were used with the hyperosmotic solution irrigation protocol and 10 for the positive and negative controls (5 each). Each canal was widened with a Protaper SX instrument (Dentsply-Maillefer, Ballaigues, Switzerland) at 250 rpm in order to facilitate bacterial colonization. The organic and inorganic rests including the smear layer were removed by ultrasonic bath treatment (BioSonic UC50) with 17% EDTA (J.T. Baker), followed by 5.25% NaOCl for 4 min according to the Saleh protocol (10). Samples were sterilized in an autoclave at 121°C for 20 min.

The biofilm of *E. faecalis* was produced in the roots using a static method. The inoculation system, based on the system proposed by Hockett *et al.* (11), was carried out as follows: from a culture of *E. faecalis* in a plate of BHI agar, an inoculum was produced in a tube with BHI broth (J.T. Baker); followed by incubation at 37°C for 8 h in aerobic condi-

TABLE I
SOLUTIONS FORMULATED TO TEST AGAINST PLANKTONIC *E. FAECALIS*

Solution	Components			
HS-A	1 M KS	1.5 M NaCl	0.128 M HCl	38.4% Ethanol
HS-B	1 M KS	1.5 M NaCl	0.051 M HCl	19.2% Ethanol
HS-C	1 M NaCl		0.02 M Sorbic Acid	19.2% Ethanol

HS: hyperosmotic solution, KS: Potassium sorbate.

tions. Five drops of 8 h cultures were withdrawn and inoculated in 10 mL of BHI broth, followed by incubation at 37°C for 4 h. Afterwards, turbidity was adjusted to 0.5 McFarland. Five drops of bacterial suspension were placed in the cultured tubes containing the samples. The time of biofilm formation was 21 days, within which refills of fresh broth were made every 48 h incubating it at 37°C. The purity of the strain was monitored for 21 days by gram staining every 48 h.

Instrumentation and irrigation of samples. The chemo-mechanical phase was carried out on an adaptor, which contained 2 inserts to place roots; allowing the absolute isolation of each root. Once isolated, the operative field was disinfected with 30% hydrogen peroxide for 1 min, 5.25% sodium hypochlorite for 1 min, and both inactivated with 10% sodium thiosulfate. Before pre-instrumentation sampling, the canal was moistened with a drop of BHI broth. A #35 sterile paper point (Viarden, México) was placed into the root canal for 1 min and subsequently transferred to a tube with 8 mL of BHI broth, the same procedure was performed with two more paper points for each root.

The cleaning and shaping of the roots were made with Protaper Universal instruments (Dentsply Maillefer, Ballaigues, Switzerland) following the sequence recommended by the manufacturer, irrigation was performed with 2 mL of each irrigation solution between each instrument. The hyperosmotic solution B (HS-B) was used in 15 roots, 5.25% sodium hypochlorite in 5 roots as bacterial growth inhibition control, and distilled water in 5 roots as bacterial growth control. Using a total of 12 mL of solution per root. Each instrument was used to prepare no more than 4 canals. The final irrigation was performed inactivating the hyperosmotic and NaOCl solutions with 2 mL of distilled water, 2 mL of 17% EDTA solution for 1 min, and finally 2 mL of distilled water. Post-instrumentation sampling was carried out by moistening the canal with BHI broth, a sterile #35 paper point was placed in the canal for 1 min and transferred to a tube with 8 mL of BHI broth; the same procedure was performed with 2 subsequent paper points.

Evaluation of the antimicrobial effect on biofilm by quantification of colony forming units (CFU). Once the sampling of the root canal was performed, a 100 μL aliquot of the broth containing the paper points was withdrawn; with it two sowings were carried out in blood agar by surface dissemination, the first direct discharge of 100 mL at 15 min of incubation and the second by making a 0.5×10^{-2} dilution in McFarland scale with 24 h of incubation. The microbial inhibition was calculated based on the number of microbial colonies, the CFU values were transferred to $\log_{10}$. The control experiments behaved as expected: countless growth for distilled water and total elimination with the 5.25% NaOCl solution. All these experiments were performed in a purifier class II Biosafety Cabinet (Labconco Corporation, Kansas City, MO, USA).

Scanning electron microscopy. Five of the treated samples were randomly selected and splitted to be observed in the scanning electron microscope (JEOL, JSM-6610LV, Peabody, MA, USA). The samples were fixed with glutaraldehyde/cyan blue, dehydrated with increasing concentrations of alcohol, and subjected to critical point drying with CO_2. Afterwards they were gold-coated and a general scanning of the canal wall was performed. The cervical, middle, and apical thirds of the root canal wall were observed at 30×, 200×, 1000×, and 5000× magnifications.

Statistical analysis. Data were analyzed 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 Shapiro Wilk test. Comparison among the study groups was carried out applying a Student T test and the statistical significance was determined. To reduce the bias of the measurements, the coefficient of reliability (95%) was calculated with a statistical significance of $p<0.05$.

RESULTS

Antimicrobial capacity test against planktonic cells. Table II shows the mean $\log_{10}$ CFU present in the different McFarland concentrations in contact with the test solutions at different exposure periods (5, 15, and 25 min), in which it is observed that solutions HS-A and HS-B were highly effective at eliminating *E. faecalis* in planktonic suspension; unlike solution HS-C, which stopped working at 6 McFarland.

Antimicrobial capacity test against *E. faecalis* biofilm. The number of CFU developed in the pre-instrumentation and post-instrumentation samples are shown in Table III. Average values were 7.4267 for pre-treatment and 1.9130 for post-treatment, which represents an 80% of reduction in the McFarland lecture.

Once the normality of the variables was analyzed, the Student t test showed that the mean $\log_{10}$ CFU in the post-treatment group was significantly lower ($p<0.001$) than that in the pre-treatment group; as well as for the difference between the pre-treatment and post-treatment number of $\log_{10}$ UFC on plate and total $\log_{10}$ UFC that were statistically significant ($p<0.024$).

Scanning Electronic Microscopy of treated roots. In order to observe the effect of the solution on bacterial cells and the dentinal structure in the root canal wall by means of electronic scanning microscopy; we observed the cervical, medium, and apical thirds to different magnifications (Figs. 1 - 4).

In the cervical third with a 1000× magnification we observed open dentinal tubules without damage in the dentinal structure, as well as low count of microorganisms at a 5000× magnification. In the middle third at 5000× we observed the presence of bacteria with morphology modification, at 1000× the dentinal structure remained without modifications and dentinal tubules were wide open, unlike the apical third where most of dentinal tubules were blocked; the presence of bacteria is also evident.

TABLE II
EFFECT OF THE HYPEROSMOTIC SOLUTIONS HS-A, HS-B AND HS-C ON *E. FAECALIS* IN PLANKTONIC STATE

McFarland	0.5	1	2	3	4	5	6	7
Solution HS-A								
5 min	0	0	0	0	0	0	0	0
15 min	0	0	0	0	0	0	0	0
25 min	0	0	0	0	0	0	0	0
HS-B								
5 min	4	0	0	0	0	0	0	0
15 min	0	0	0	0	0	0	0	0
25 min	13	2	0	0	0	0	0	0
HS-C								
5 min	0	0	11	972	4	1120	1804	
15 min	0	0	0	37	0	2	114	
25 min	0	0	0	0	0	0	15	

The mean of three readings of log10 CFU resulting from the contact between the different concentrations of *E. faecalis* in McFarland scale (0.5 – 7) in contact with the test solutions in the three exposure times (5, 15 and 25 minutes) is shown.

TABLE III
EFFECT OF THE HYPEROSMOTIC SOLUTION HS-B ON *E. FAECALIS* BIOFILM

Variable	Time	n	Mean	Mean error	SD	Min Value	Max Value	Pre-treatment vs. Post-treatment
McFarland	Pre-treatment	15	7.4267	0.0605	0.2344	6.6000	7.50000	p 0.001*
	Post-treatment	15	1.9130	0.5940	2.3020	0.4000	7.30000	
UFC plate	Pre-treatment	15	151.90	59.900	232.00	1.0000	724.000	p 0.024*
	Post-treatment	15	0.2000	0.1450	0.5610	0.0000	2.00000	
UFC total	Pre-treatment	15	15186	59899	231986	1000.0	724000	p 0.024*
	Post-treatment	15	200.00	145.00	561.00	0.0000	2000.00	

Student t test was done to compare pre-treatment and post-treatment log10 CFU count.
* Statistically significant difference ($p<0.05$).

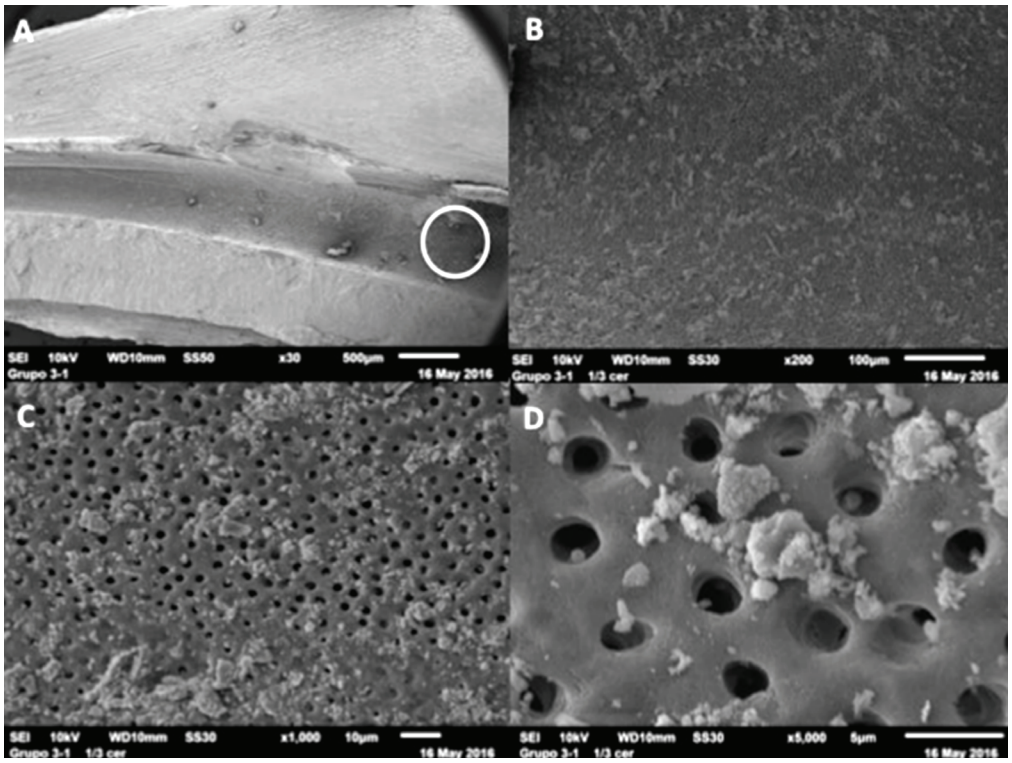

Fig. 1. Photomicrograph of sample #1 where the action of the hyperosmotic solution on biofilm is observed: A. Sample observed to 30x, in the circle the cervical area observed to greater magnifications. B. Area highlighted in cervical third, 200x. C. Area of cervical third showing predominantly open dentinal tubules, 1000x; and D. Cervical third showing scarce presence of microorganisms and no damage to the dentinal structure, 5000x.

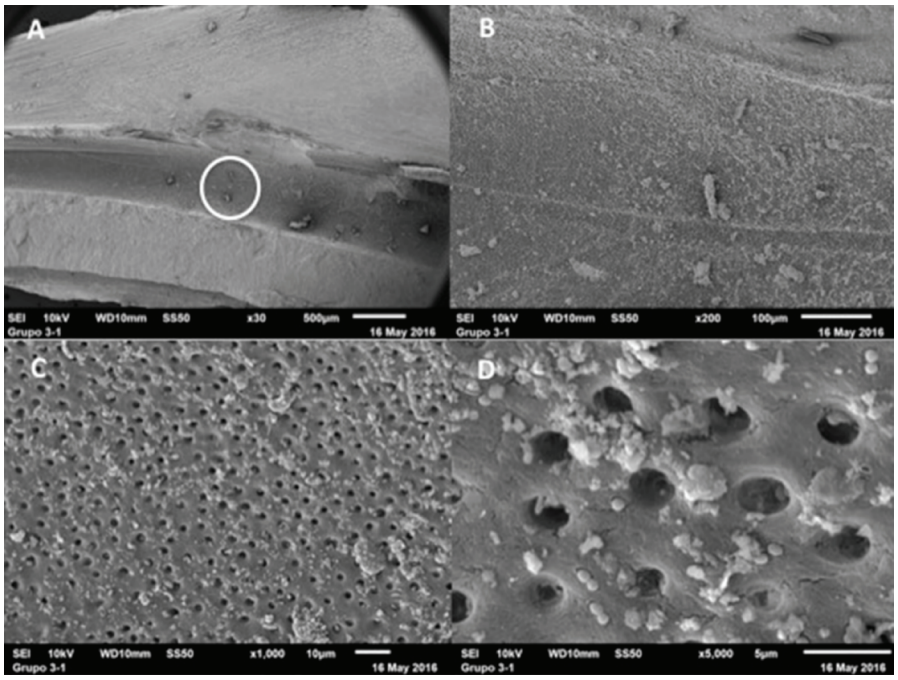

Fig. 2. Photomicrograph of sample #1: A. Observed sample to 30x, in the circle the area of the middle third observed to greater magnifications. B. Highlighted area in middle third to 200x. C. Middle third area showing all open dentine tubules, 1000x and D. Area of middle third where the presence of E. faecalis is observed, some of them present alteration in their morphology; no damage is observed in the dentinal structure, 5000x.

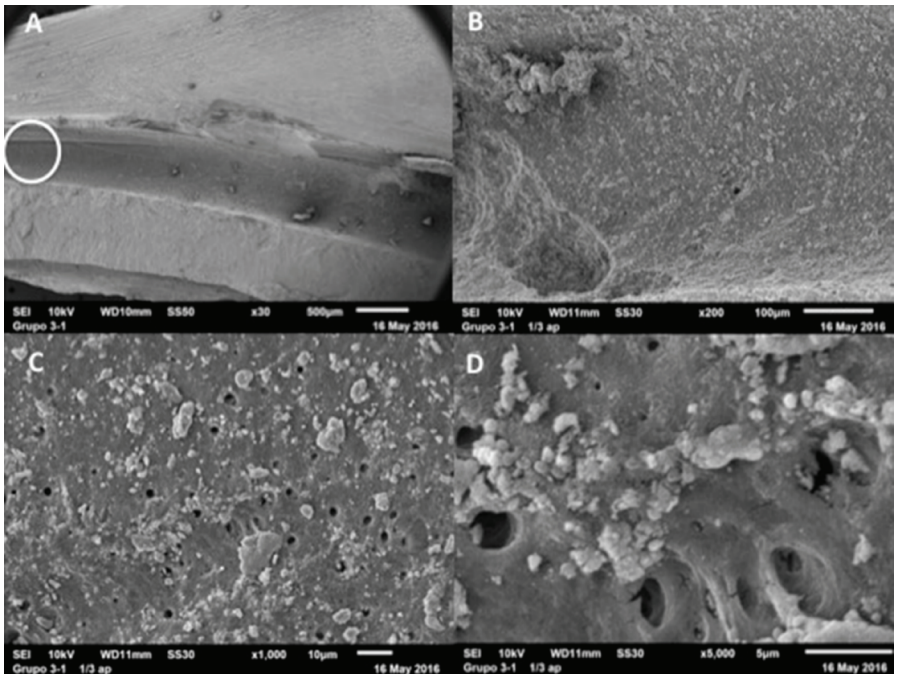

Fig. 3. Photomicrograph of sample #1. A. Panoramic view of the root canal and in the circle the apical area to be observed at greater magnifications, 30x. B. Root canal wall in apical third, 200x. C. Apical third showing a large majority of blocked dentin tubules, 1000x, and D. It is observed the presence of microorganisms in the apical third and some disruptions in the dentinal structure, in form of micro-cracks, 5000x.

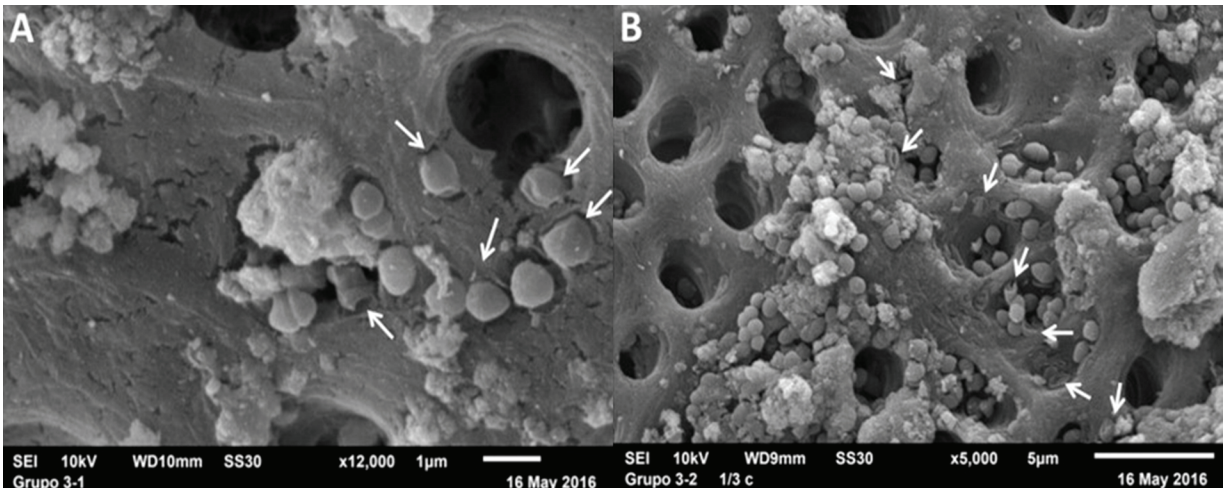

Fig. 4. Photomicrograph showing in A. Dentinal wall in the middle third of sample #1 where the irregular morphology of *E. faecalis* is observed (arrows), 12,000x. B. Middle third of sample #2 where a bacterial agglomeration is observed, showing the irregular morphology of bacterial cells (arrows).

DISCUSSION

Biofilms are defined as structured communities of microorganisms that are attached to a surface and enmeshed in an extracellular polymeric matrix (12).

The fight against bacterial biofilms has led us to investigate several methods to eliminate or control them. In this last regard biological agents have been tested. In periodontology, Patini *et al.* (13) used the genus *Bdellovibrio* with predatory selectivity against Gram-negative bacteria; they obtained a favorable response from this microorganism versus periodontopatogenic bacteria, which promotes research in this arena to find something similar in other areas of dentistry.

In the endodontic area mechanical methods have been implemented to remove biofilms inside the root canal during the irrigation protocol using sonic and ultrasonic devices. Actually, the gold standard is the PUI (passive ultrasonic irrigation), which has been compared to conventional needle irrigation (CNI) and passive sonic irrigation (PSI). Eneide *et al.* (14) found no difference between PUI and PSI in terms of CFU reduction, while CNI was less effective.

A final irrigation protocol was carried out, which includes the use of EDTA for the smear layer removal; allowing the permeability of dentinal tubules. This procedure does not influence our results of antimicrobial efficacy since EDTA with mild agitation or CNI do not show important antimicrobial properties (15).

It is vital to consider bacterial biofilm models as essential models for in vitro microbiological investigations that assess different disinfectants and disinfection strategies in endodontics.

The methodology to generate biofilm in the present study was a monoculture under static growth conditions as reported by Paranjpe *et al.* (16).

Although some authors privilege the relevance of a polymicrobial biofilm over a mono-species biofilm; it has been reported that biofilms produced in pure cultures of bacteria under laboratory conditions and the mixed-species biofilms produced in natural ecosystems show similar basic organization (17). Thus the *E. faecalis* biofilm produced in this work, with a degree of maturity of 21 days, can be considered as suitable model to test the efficacy of the hyperosmotic solution in vitro.

Van der Waal *et al.* developed an aqueous solution based on 3 M NaCl + 1 M KS that proved to be extremely effective at inactivating a 48 h *E. faecalis* biofilm within 1 h of contact (8), while the hyperosmotic solution tested here showed an accentuated bacterial reduction when used as irrigating agent for a shorter period of time and with a small volume (12 mL) per root.

Analyzing the significant antibacterial effect that took place in this evaluation for both solutions, HS-A and HS-B, against bacteria in planktonic state and the effect shown by solution B against the *E. faecalis* biofilm, it can be considered that a synergy is occurring between the hyperosmolarity of the solution and the presence or sorbic acid molecules at low pH. The antimicrobial properties of potassium sorbate are observed at a pH near or less than 4 (18,19); where the protonated species dominates (sorbic acid). Sorbic acid is a monoprotic acid with a pKa equal to 4.76; thus, in solutions HS-A and HS-B only 2.8 and 1.4%, respectively, of the dissolved sorbate molecules exist as sorbic acid. Sorbic acid, being more hydrophobic than the sorbate ion, can penetrate the lipid membrane of the bacterial cell and act as inhibitor of various enzymes involved in the carbohydrate metabolism and the citric acid cycle. Despite having low percentages or sorbic acid molecules in solutions HS-A and HS-B, once they are contacted with *E. faecalis*; sorbic acid molecules will enter the cells promoting that more sorbate ions, outside the bacterial cell, are converted to sorbic acid. To favor the existence of solubilized sorbic acid, looking to guarantee entrance to the bacterial cell, a co-solvent (ethanol) was added to the solutions tested. Besides being a co-solvent, ethanol is a solute that contributes to the increase in hyperosmolarity of the solutions tested and can help killing bacteria per se. In this last regard ethanol has been evaluated as antimicrobial in both planktonic and biofilm-producing microorganisms. Using a solution containing 25% of ethanol, Suchomel *et al.* found a 96.5% reduction of live *E. faecalis* cells in planktonic form after 5 min (20). Nonetheless when a 95% ethanol solution was tested against an *E. faecalis* biofilm, 34% of live cells were recorded 72 h after 5 min of irrigation (21). Similarly using a multispecies biofilm Duarte et al. reported 27% of live cells using 95% ethanol after 10 min of irrigation (22). In the present work, solution HS-A contained 19.2% of ethanol, while solution HS-B had 38.4%; both solutions were able to completely kill planktonic *E. faecalis*, even at 7 McFarland. Nevertheless, when using solution HS-C (which contained 19.2% of ethanol), live cells were found starting at 2 McFarland. Thus, we can establish that ethanol is not completely responsible of the antimicrobial effect of the solutions tested. But acts synergically with the hyperosmolarity of the solutions and the presence or sorbic acid.

The ability to enter the cell and inhibit vital functions for survival was demonstrated in the present study by observing the antibacterial effect shown by the solutions tested on bacterial cells both in planktonic form and in the form of biofilm. In the latter, SEM images evidence modification of cellular morphology as the literature indicates; since the shocking effect on the cell causes water outflow, thus reducing the cytoplasmic volume and inducing cell plasmolysis (23,24). This is why it was possible to observe deformed, concave, and flattened cells, not all of them but most. Based on the results of the microbiological tests, we can conclude that solution HS-B is effective at reducing bacterial load of a root canal with a biofilm of *E. faecalis*. The hyperosmotic solutions elaborated on the basis of sodium chloride, potassium sorbate, and ethanol were effective at eliminating *E. faecalis* in planktonic form in the 0.5 to 7 McFarland range. In addition, solution HS-B was effective at reducing bacterial load in infected canals with a mature biofilm of *E. faecalis*.

It seems unclear whether the bacterial reduction was caused by killing, detachment

of biofilm cells from the substrate, or both. The hyperosmotic solution appears to be able to disperse biofilms, based on some of the photomicrographs and it may also aid at cleaning the root canal system.

Along with the ability to provoke morphological changes consistent with hyperosmotic stress damage to bacterial cells. The contact of the hyperosmotic solution with the dentin surface does not cause structural changes, however further studies are needed to clarify the origin of some micro-cracks observed.

It can be concluded that the hyperosmotic solution (HS-B) showed considerable antibacterial capacity against *E. faecalis* in both planktonic and biofilm form; with the ability to generate changes in the morphology of bacterial cells consistent with hyperosmotic shock. The need to conduct cytotoxicity tests is highlighted before it is possible to propose solution HS-B as irrigation agent or irrigation adjuvant in endodontics.

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REFERENCES

1. **Walsh L, George R.** Activation of alkaline irrigation fluids in endodontics. Materials 2017; 10(10): 1214. *doi: 10.3390/ma10101214.*
2. **Bowen WH, Burne RA, Wu H, Koo H.** Oral biofilms: pathogens, matrix, and polymicrobial interactions in microenvironments. Trends in Microbiology 2018; 26(3): 229–242.
3. **Mira A, Simon-Soro A, Curtis MA.** Role of microbial communities in the pathogenesis of periodontal diseases and caries. J Clin Periodontol 2017; 44 (Suppl 18): S23-S38.
4. **Duggan JM, Sedgley CM**. Biofilm formation of oral and endodontic *Enterococcus faecalis*. J Endod 2007; 33:815– 818.
5. **Komiyama EY, Lepesqueur LSS, Yassuda CG, Samaranayake LP, Parahitiyawa NB, Balducci I.** Enterococcus species in the oral cavity: prevalence, virulence factors and antimicrobial susceptibility. PLoS ONE 2016; 11(9): e0163001.
6. **Rossi-Fedele G, Guastalli AR.** Osmolarity and root canal antiseptics. Int Endod J 2014; 47(4):314–320.
7. **Wood JM.** Bacterial responses to osmotic challenges. J Gen Physiol 2015; 145: 381–388.
8. **Van der Waal SV, Jiang LM, de Soet JJ, van der Sluis LWM, Wesselink PR, Crielaard W.** Sodium chloride and potassium sorbate: a synergistic combination against *Enterococcus faecalis* biofilms: an in vitro study. Eur J Oral Sci 2012; 120(5):452–457.
9. **Galdámez Falla VM, González Amaro AM, González Ortega O, Mariel Cárdenas J, Gutiérrez Cantú FJ, Vitales Noyola M. Oliva Rodríguez R.** Antimicrobial effect of a hyperosmotic solution on endodontic microorganisms in planktonic state. Invest Clin 2019; 60(1):38-46.
10. **Saleh IM, Ruyter IE, Haapasalo M, Òrstavik D.** Survival of *Enterococcus faecalis* in infected dentinal tubules after root canal filling with different root canal sealers in vitro. Int Endod J 2004; (37) 3:193–198.
11. **Hockett JL, Dommisch JK, Johnson JD, Cohenca N.** Antimicrobial efficacy of two irrigation techniques in tapered and nontapered canal preparations: an in vitro study. J Endod 2008;34(11):1374–1377.
12. **Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S**. Biofilms: an emergent form of bacterial life. Nat Rev Microbiol 2016;14(9):563-575.
13. **Patini R, Cattani P, Marchetti S, Isola G, Quaranta G, Gallenzi P.** Evaluation of predation capability of periodontopathogens bacteria by Bdellovibrio Bacteriovorus HD100. An in vitro study. Materials 2019; 12: 2008. *doi: 10.3390/ma12122008.*

14. **Eneide C, Castagnola R, Martini C, Grande NM, Bugli F, Patini R, Cordaro M, Sanguinetti M, Olivi G, Isola G, Marigo L.** Antibiofilm activity of three different irrigation techniques: An in vitro study. Antibiotics 2019; 8: 112. *doi: 10.3390/antibiotics8030112*.
15. **Hartmann RC, Neuvald L, Barth V Jr, de Figuereido JAP, de Oliveira SD, Scarparo RK, Waltrick SB, Rossi-Fedele G.** Antimicrobial efficacy of 0.5% peracetic acid and EDTA with passive ultrasonic or manual agitation in an *Enterococcus faecalis* biofilm model. Aust Endod J 2019;45(1):57-63.
16. **Paranjpe A, De Gregorio C, Gonzalez AM, Gomez A, Silva Herzog D, Piña AA.** Efficacy of the self-adjusting file system on cleaning and shaping oval canals: A microbiological and microscopic evaluation. J Endod 2012;38(2):226–231.
17. **Stoodley P, Sauer K, Davies DG, Costerton JW.** Biofilms as complex differentiated communities. Annu Rev Microbiol 2002: 56:187–209.
18. **Yigit A, Korukluoglu M.** The effect of potassium sorbate, NaCl and pH on the growth of food spoilage fungi. Ann Microbiol 2007; 57(2):209–215.
19. **Simpson CA, Sofos JN.** Antimicrobial Ingredients. In: Tarté R. (eds) Ingredients in Meat Products 2009; Springer, New York, NY.
20. **Suchomel M, Lenhardt A, Kampf G, Grisold A**. *Enterococcus hirae*, *Enterococcus faecium* and *Enterococcus faecalis* show different sensitivities to typical biocidal agents used for disinfection. J Hospit Infect 2019; 103(4):435-440. *doi: 10.1016/j.jhin.2019.08.014*. Epub 2019 Aug 23.
21. **Suman JM, Kuga MC, Abreu Da Rosa R, Santini MF, Grazziotin-Soares R, Montagner F, Só MVR.** Antibacterial activity of chlorhexidine after final irrigation with ethanol: CLSM and culture-based method analysis. Microsc Res Tech 2015; 78(8): 682-687.
22. **Duarte PH, Barcellos P, Abreu R, Montagner F, Húngaro MA, Carlos M, Vinícius M**. Effect of ethanol on the antimicrobial properties of chlorhexidine over oral biofilm. Microsc ResTech 2018; 81(4):408-412. *doi: 10.1002/jemt.22992*. Epub 2018 Jan 17.
23. **Burkewitz K, Choe KP, Lee EC, Deonarine A, Strange K.** Characterization of the proteostasis roles of glycerol accumulation, protein degradation and protein synthesis during osmotic stress in *C. elegans*. PLoS One 2012; 7(3):1-14.
24. **Gutiérrez C, Abee T, Booth IR.** Physiology of the osmotic stress response in microorganisms. Int J Food Microbiol 1995; 28(2):233-244.