

## Elimination of an intraradicular biofilm composed of *Enterococcus faecalis* and *Candida albicans* using a negative apical pressure irrigation system.

Eliminación de una biopelícula intraradicular compuesta por *Enterococcus faecalis* y *Candida albicans* utilizando un sistema de irrigación de presión apical negativa.

Verónica Méndez-González.<sup>1</sup>  
Marlen Vitales-Noyola.<sup>1</sup>  
Ana González-Amaro.<sup>1</sup>  
Carlos Muñoz-Viveros.<sup>2</sup>  
Francisco Ducoing-Hernández.<sup>1</sup>

### Affiliations:

<sup>1</sup>Endodontics Postgraduate Program, Faculty of Dentistry, The Autonomous University of San Luis Potosí, SLP, Mexico.

<sup>2</sup>Kavo Kerr group, Orange County, California, United States.

**Corresponding author:** Francisco Ducoing-Hernández. Manuel Nava 2, 78290, San Luis Potosí, SLP, Mexico.  
**E-mail:** [duco\\_ing@hotmail.com](mailto:duco_ing@hotmail.com)

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**Abstract:** Endodontic treatment consists of the cleaning and disinfecting the root canal system, which is achieved using adequate mechanical instrumentation and chemical irrigation. Endodontic microorganisms are present in root canals in the form of a biofilm, and their elimination ensures the success of endodontic treatment. Irrigation is a key factor contributing to the elimination of this intraconduct biofilm, and different irrigator agents and irrigation techniques, such as irrigation with negative apical pressure, a novel automated irrigation mechanism based on suction intraconduct, have been used. In this study, we evaluated the ability of a negative apical pressure system with different concentrations of sodium hypochlorite and durations to reduce the microbial load. **Materials and Methods:** An intraradicular biofilm composed of *Enterococcus faecalis* and *Candida albicans* was generated during twenty-one days of static culture on one hundred mesio-vestibular roots of upper molars with complex curvatures greater than 30°C, and the roots were classified in six groups with different concentrations and contact times of sodium hypochlorite. Subsequently, the reduction in the microbial load was measured with McFarland scale and the enumeration of colony forming units and was evaluated with scanning electronic microscopy. **Results:** We observed a significant difference in the reduction of the microbial load prior to instrumentation compared with postinstrumentation between the groups treated with 2.25% and 5.25% NaOCl for 30, 60 and 90 seconds of contact time ( $p < 0.05$ ), but we did not observe differences in the reduction of microbial load between different contact times and concentrations of sodium hypochlorite employed ( $p > 0.05$ ). **Conclusion:** Negative apical pressure is a good option for irrigation in endodontics, as it allows the passage of the irrigation fluid along the total length of the root canal and produces a better antimicrobial effect.

**Keywords:** Irrigation, negative apical pressure, biofilm, sodium hypochlorite.

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**Resumen:** El tratamiento de endodoncia consiste en la limpieza y desinfección del sistema de conducto radicular, lo que se logra utilizando instrumentación mecánica adecuada y riego químico. Los microorganismos endodónticos están presentes en los conductos radiculares en forma de una biopelícula, y su eliminación asegura el éxito del tratamiento endodóntico. La irrigación es un factor clave que contribuye a la eliminación de esta biopelícula intraconductora, y se han utilizado diferentes agentes irrigadores y técnicas de irrigación, como la irrigación con presión apical negativa, un nuevo mecanismo automatizado de irrigación basado en la intraconducción de succión. En este estudio, evaluamos la capacidad de un sistema de presión apical negativa con diferentes concentraciones de hipoclorito

de sodio y duraciones para reducir la carga microbiana. **Material y Métodos:** Se generó una biopelícula intraradicular compuesta por *Enterococcus faecalis* y *Candida albicans* durante veintiún días de cultivo estático en cien raíces mesio-vestibulares de molares superiores con curvaturas complejas superiores a 30°C, y las raíces se clasificaron en seis grupos con diferentes concentraciones y tiempos de contacto de hipoclorito de sodio. Posteriormente, la reducción en la carga microbiana se midió con la escala de McFarland y la enumeración de las unidades formadoras de colonias y se evaluó con microscopía electrónica de barrido. **Resultado:** Observamos una diferencia significativa en la reducción de la carga microbiana antes de la instrumentación

en comparación con la postinstrumentación entre los grupos tratados con NaOCl 2.25% y 5.25% durante 30, 60 y 90 segundos de tiempo de contacto ( $p < 0.05$ ), pero lo hicimos. No se observan diferencias en la reducción de la carga microbiana entre los diferentes tiempos de contacto y las concentraciones de hipoclorito de sodio empleado ( $p > 0.05$ ). **Conclusión:** La presión apical negativa es una buena opción para el riego en endodoncia, ya que permite el paso del líquido de riego a lo largo de todo el conducto radicular y produce un mejor efecto antimicrobiano.

**Palabras Clave:** Irrigación, presión apical negativa, biopelícula, hipoclorito de sodio.

## INTRODUCTION.

The main objectives of endodontic treatment are cleaning, disinfection and tridimensional sealing of the root canal system.<sup>1</sup> Mechanical instrumentation and chemical irrigation are required to achieve efficient cleaning and disinfection of the root canals,<sup>2,3</sup> this procedure eliminates the smear layer, accumulated debris inside root canals, necrotic and vital pulp tissue, microorganisms and their products, such as toxins and synthetic products.<sup>4-7</sup>

Microorganisms that are frequently isolated inside root canals are facultative anaerobic bacteria, mainly *Streptococcus* and *Enterococcus* species.<sup>8</sup> *Enterococcus faecalis* is facultative, is a gram-positive coccus, and is isolated up to 35% of root canals, mainly in endodontic secondary infections.<sup>9,10</sup> Fungi have been reported inside the root canals as well, mainly yeast of the *Candida* genus, principally *Candida albicans*. This microorganism has been reported in up to 40% of patients with drug-resistant apical periodontitis.<sup>9,10</sup>

However, the microbiota of root canals is diverse. The main endodontic pathogens that cause the primary intraradicular infections are gram-negative anaerobic bacilli, including genera such as *Prevotella*, *Porphyromonas*, *Bacteroides*, *Dialister*, and *Fusobacterium*, gram-positive anaerobic bacilli, such as *Pseudoramibacter alactolyticus*, *Actinomyces* spp, *Propionibacterium propionicum*, and *Eubacterium* spp.

Gram-positive cocci that are present in primary infections include *Streptococcus* and *Enterococcus*.<sup>11</sup> Some microorganisms are resistant to antimicrobial treatment and can survive in the root canals after biomechanical preparation; the most common species are *Fusobacterium nucleatum*, *Prevotella* spp, *Campylobacter rectus*, and gram-positive bacteria, including *Streptococcus*, *Enterococcus*,

*Actinomyces* spp, *Eubacterium* spp.<sup>11</sup>

Chemical irrigation plays a key role in the elimination of these endodontic pathogens from the root canals during and after mechanical instrumentation (final irrigation protocol). The irrigation agents clean and disinfect sites that are inaccessible to rotary or manual files.<sup>4,6</sup> Endodontic irrigation has multiple functions, as it represents an essential step in endodontic treatment; however, no irrigation agent is capable of fulfilling all the characteristics alone.

The principal functions of the irrigates are to dissolve the inorganic and organic material, achieve good penetration inside the root canal system, lubricate the root canals, reduce friction, control the temperature, fail to induce toxicity in the periapical tissues, fail to produce an allergy, prevent weakening of the dentin, and react positively or neutrally with other dental materials, among others. Therefore, the simultaneous use of different irrigator agents is necessary for the optimal cleaning and disinfection of root canals. Direct contact with the dentinal surfaces is necessary for the irrigation solutions to fulfill these functions, which is only achieved through the optimization of the supply of the irrigate.<sup>5-7</sup>

Conventional irrigation is performed with syringe and needle (apical positive pressure). However, this technique does not allow the dispersion of the irrigate solution along the root canal,<sup>5</sup> and thus more efficient irrigation systems that optimize the administration and agitation of irrigator solutions to ensure the contact of solutions with dentinal walls and allow the constant exchange of the solution have been developed.<sup>2-8</sup> The multiple systems that are currently used are classified into two principal groups: manual and automated.<sup>8</sup> Automated irrigation systems improve the quality of the cleaning and disinfection of

root canals using passive ultrasonic irrigation and negative apical pressure. Negative apical pressure is an automated irrigation mechanism based on aspiration or suction within the root canal system and provides a constant flow and generous supply of disinfectant agent. This disinfectant is transported to the entire working length due to the effect of aspiration exerted by this device, and therefore the irrigator solution exerts its effects on this zone and avoids dispersion at periradicular tissues.<sup>12-14</sup>

A few negative apical pressure irrigation systems have been used in endodontics, such as EndoVac Pure, a new version of the EndoVac system. EndoVac Pure is an irrigation system that was recently introduced to the market, its irrigation tip supplies the irrigator agent at a rate of 3-5 milliliters per minute, and a microcannula with a variable length avoids the effect of "steam blockage".<sup>12</sup>

In this study, we employ an automated negative apical pressure irrigation system with a final irrigation protocol with different concentrations and contact times of sodium hypochlorite to evaluate the disinfection of root canals in the presence of mixed biofilm of the endodontic pathogens *Enterococcus faecalis* and *Candida albicans*.

## MATERIALS AND METHODS.

### Samples

One hundred molars extracted from patients with dental caries and/or periodontal disease were included in this study; however we only used the dental roots. All patients visited the oral surgery clinic, answered questions about their clinical history, and signed an informed consent form. This study was approved by ethical and investigation committee of the Dentistry Faculty. An oral review was performed and panoramic and/or periapical radiographs were taken to establish the clinical diagnosis for tooth extraction.

All teeth were radiographed and with the software AutoCad (AUTODESK AUTOCAD 2015 for Mac, San Rafael, CA) and the angle of curvature of roots was calculated using the Schneider method.<sup>15</sup> The mesio-vestibular roots were downcrowned and longitudinally sectioned at a thickness of 16mm. Next, the dental pieces were patented and instrumented manually until file K number 20 was used (Denstsply Millefer, Ballaigues, Switzerland); finally, the cleaning and disinfection protocol was performed using 5.25% sodium hypochlorite, 17% EDTA disodium salt and distilled water<sup>16</sup> in an ultrasonic bath (BioSonic UC50, Whaledent Ink, Mahwah, NJ). Samples were sterilized with moist heat for 15 minutes

at 125°C with 0.15mPa of pressure (Kitlab, Chicago, IL).

### *In vitro* formation of intraradicular biofilm.

*Enterococcus faecalis* and *Candida albicans* were isolated from the root canals of patients who visited the Endodontics Clinic for the treatment of secondary endodontic infections, as clinically isolated microorganisms and their virulence factors can differ from ATCC strains. The microorganisms were identified with biochemical tests using API 20 Strep and API 20 C AUX systems (bioMérieux sa, Marcy l'Etoile, France).

The microorganisms were cultured together in Brain Heart Infusion (BHI) medium (BD Bioxon, Edo. México, México) and Dextrose Sabouraud (DS) (BD Bioxon, Edo. México, México) in a 75%/25% w/v ratio, respectively. The static form mixed biofilm of these microorganisms was prepared on assay tubes (15x20mm), and five teeth were placed in each tube with fifteen milliliters BHI/DS medium and 100µl culture medium with both microorganisms at a 0.5 McFarland scale.

This procedure was performed in purified class II biosafety cabinet (Labconco, Kansas City, MO). Subsequently, the samples were incubated at 37±2°C for 21 days to obtain the mixed biofilm. The culture medium was replaced every 2 days, and 100µl of microorganisms at a 0.5 McFarland scale were added. In some cases, fetal bovine serum was added for the optimum growth of *Candida albicans*. Gram staining (HYCEL, Jalisco, México) was performed at every change of the culture medium to evaluate the purity of the resulting biofilm.<sup>17</sup>

### Mechanical instrumentation and chemical irrigation

After twenty-one days of biofilm formation *in vitro*, the teeth were collected in a special base for instrumentation, supported in speedex putty, a silicone-based impression material (Coltène/Whaledent AG, Altstätten, Switzerland), and completely isolated with a rubber dam and bow of Young. The operative field was cleaned and disinfected with 5.25% sodium hypochlorite, 30% hydrogen peroxide and 10% sodium thiosulfate<sup>16</sup> for 1 minute of contact time, and a sample was collected from the operative field and incubated in blood agar for 24 hours to evaluate the disinfection of the field.

The preinstrumentation samples were root canals after the disinfection of operative field; three sterile paper points were introduced into the root canals for one minute each and placed in BHI/DS medium. Instrumentation was performed with TF Adaptive files (SybronEndo, Glendora, CA) until the ML3 file. After rotatory instrumentation, manual type K #35 files (Denstsply Millefer, Ballaigues,

Switzerland) were used to rectify the apical caliber, and conventional irrigation was performed with sodium hypochlorite. The final irrigation protocol was conducted using the EndoVac Pure system (Kerr Corporation, Orange, CA) with negative apical pressure.<sup>18</sup> The teeth were randomly divided into six groups treated with different concentrations and contact times of sodium hypochlorite:

- Group A: 2.25% sodium hypochlorite for 30 seconds;
- Group B: 2.25% sodium hypochlorite for 60 seconds;
- Group C: 2.25% sodium hypochlorite for 90 seconds;
- Group D: 5.25% sodium hypochlorite for 30 seconds;
- Group E: 5.25% sodium hypochlorite for 60 seconds;
- and Group F: 5.25% sodium hypochlorite for 90 seconds.

At the end of the irrigation protocol, 10% sodium thiosulfate was employed to inactivate the irrigator solutions inside the root canals and post instrumentation samples were collected with sterile paper points.

The tubes containing the pre instrumentation and post instrumentation samples were incubated at  $37\pm 2^{\circ}\text{C}$  for 24 hours (Felisa, Jalisco, México).

#### Evaluation of the reduction in the microbial load

After 24 hours of incubation, the concentrations of microorganisms in pre- and postinstrumentation samples were evaluated using a McFarland densitometer (DENSIMAT, BIOMÉRIUX FI, Italy).

The colony forming units (CFU) were counted by employing the serial dilution method, spreading bacteria with an "L" shape loop on BHI/DS agar and incubating the plates for 24 hours. Counts was performed using semiautomatic counter (Felisa, Jalisco, México). The following formula was employed to calculate the CFUs per milliliter:  $\text{CFU/ml} = [(\text{number of CFU})(\text{serial dilution})] / \text{dilution factor in ml}$ .<sup>19</sup>

#### Evaluation using scanning electronic microscopy

The elimination of biofilm from teeth was evaluated using a scanning electron microscope (SEM) (Jeol JSM-6610LV, Peabody, MA). First, the teeth from the different groups were longitudinally sections in the lingual-vestibular direction and dehydrated with different concentrations of ethanol (10% to absolute alcohol) for ten minutes in each concentration. Next, the teeth were incubated with a mixture of 1% alcian blue and 2% glutaraldehyde for 24 hours at  $4^{\circ}\text{C}$ . Finally the teeth were completely dehydrated in critical point dryer (Leica EM CPD030, Wien, Austria). Subsequently, all dental roots were coated with gold using an automated fine coater (JEOL JFC-1100, Jeol USA, Inc., Peabody, MA)

and images of the samples were captured at different magnifications using the SEM. The images of the apical third of root canals were analyzed for the elimination of the intraradicular biofilm by two evaluators using the modified Rome scale.<sup>20</sup>

#### Statistical analysis

Data are presented as the arithmetic means and standard deviations or as medians and interquartile ranges according to the presence or absence of a normal distribution, respectively. The normality of data was determined using the Kolmogorov-Smirnov test.

Comparisons among groups were performed using the Student's t-test, the Mann-Whitney U test, the Kruskal-Wallis test and one-way analysis of variance, as required, and post hoc tests. Data were analyzed using GraphPad Prism software version 5.0 (GraphPad, San Diego, CA).  $p < 0.05$  was considered significantly different.

## RESULTS.

### Reduction of the microbial load

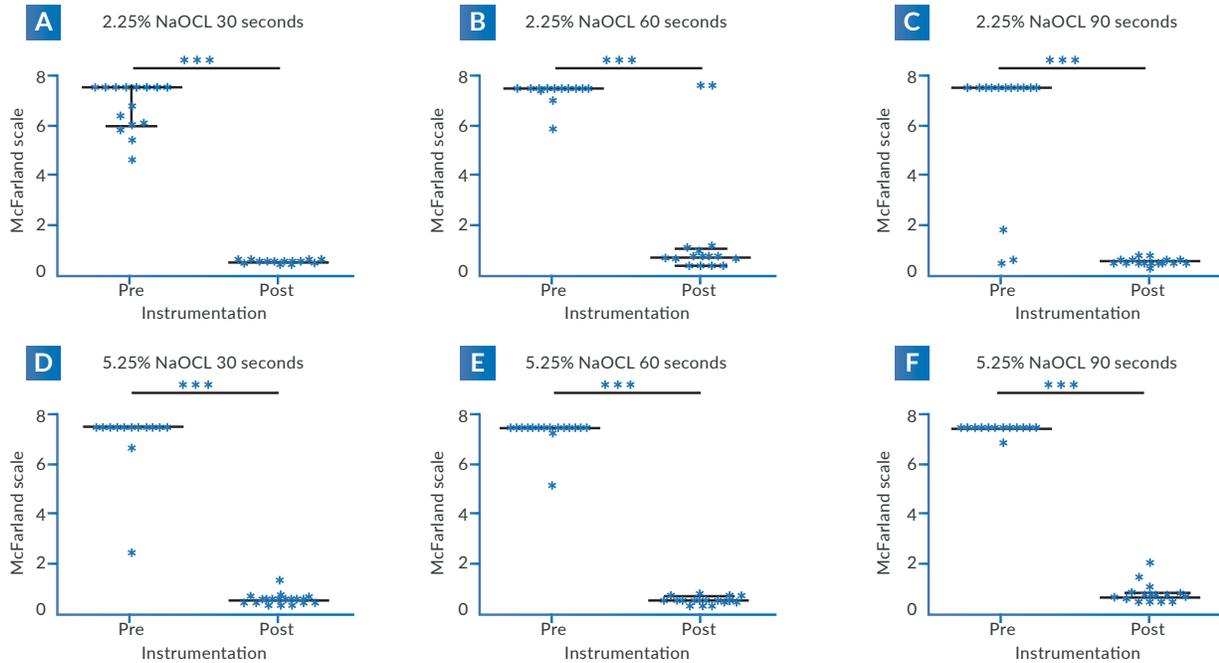
We observed a significant difference in the reduction of microbial load evaluated with McFarland scale after instrumentation compared to before instrumentation in the group irrigated with 2.25% sodium hypochlorite for 30 seconds of contact time ( $p < 0.0001$ ) (Figure 1A). Similar results were observed in the following groups: 2.25% sodium hypochlorite for 60 and 90 seconds of contact time ( $p < 0.0001$ ) (Figure 1B and Figure C), and 5.25% sodium hypochlorite for 30, 60 and 90 seconds of contact time ( $p < 0.0001$ ) (Figure 1D-F).

Additionally, the reduction of the microbial load was evaluated by assessing the number of colony forming units (CFUs), and we observed a significant difference after instrumentation in all groups compared to the values obtained before instrumentation ( $p < 0.0001$ ) (Figure 2A and Figure 2F). SEM images were evaluated to determine the elimination of the intraradicular biofilm and we observed significant differences in the all samples after instrumentation compared to before instrumentation for each group evaluated ( $p < 0.0001$ ) (Figure 3A-D).

### Comparisons between the different times and concentrations of sodium hypochlorite

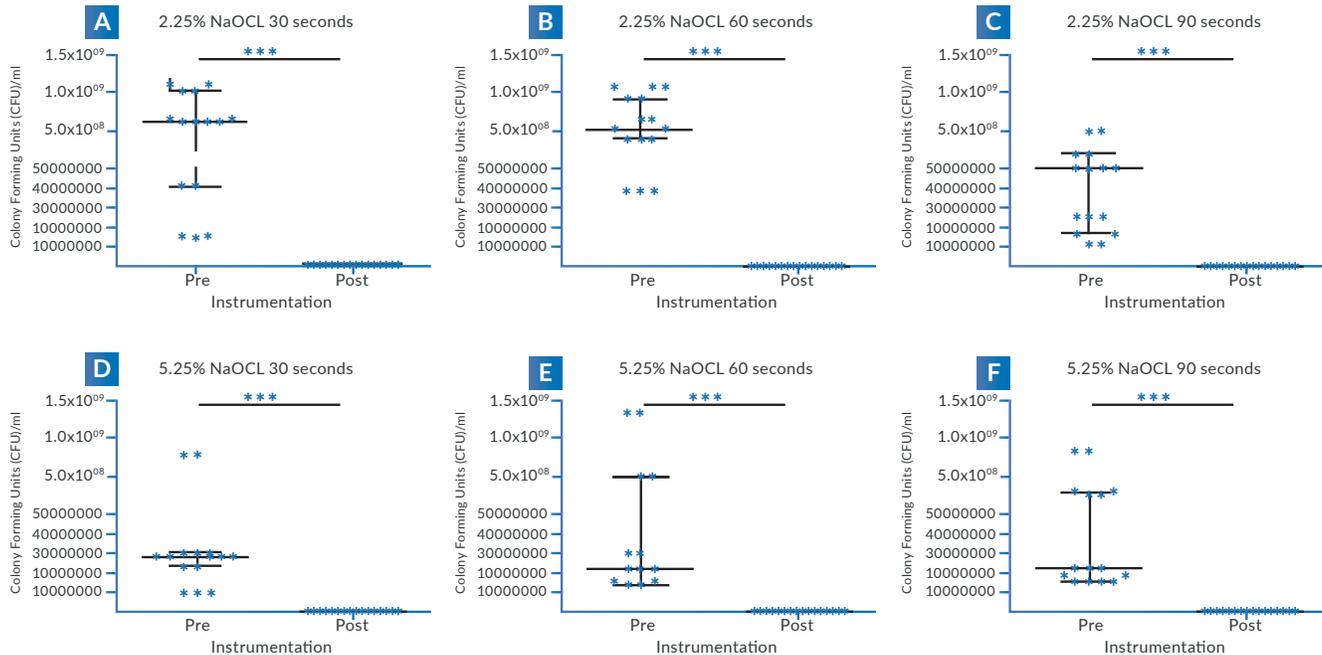
Differences in reductions in the microbial load after the application of different concentrations of sodium hypochlorite for various contact times were not significant ( $p > 0.05$ ), as evaluated by determining the concentration of microorganisms using the McFarland scale and counting the colony forming units (CFU) (Figure 4).

**Table 1.** Reduction in the microbial load before and after instrumentation. The reduction in the microbial load was evaluated using the McFarland scale, as indicated in the materials and methods.



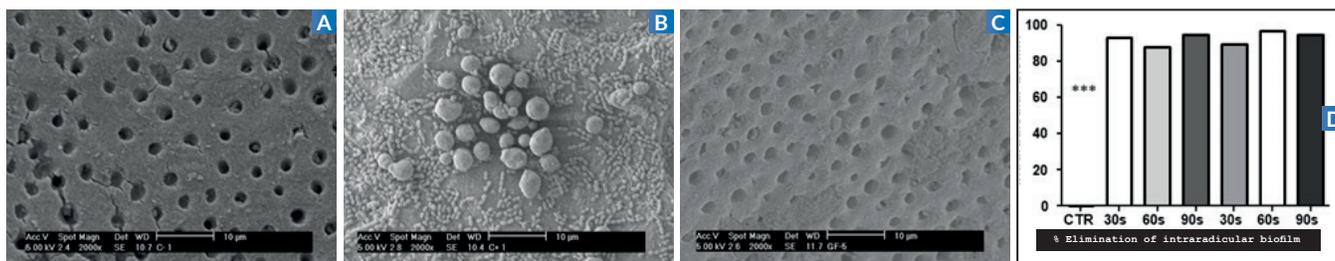
A: The reduction in the microbial load observed before and after instrumentation following irrigation with 2.25% sodium hypochlorite for 30 seconds.  
 B: Reduction in the microbial load observed before and after instrumentation following irrigation with 2.25% sodium hypochlorite for 60 seconds.  
 C: Reduction in the microbial load before and after instrumentation following irrigation with 2.25% sodium hypochlorite for 90 seconds.  
 D: Reduction in the microbial load before and after instrumentation following irrigation with 5.25% sodium hypochlorite for 30 seconds.  
 E: Reduction in the microbial load before and after instrumentation following irrigation with 5.25% sodium hypochlorite for 60 seconds.  
 F: Reduction in the microbial load before and after instrumentation following irrigation with 5.25% sodium hypochlorite for 90 seconds. The data shown in A-F are presented as medians and interquartile ranges.  
 \*\*\*:  $p < 0.0001$ .

**Table 1.** Reduction in the number of colony forming units before and after instrumentation. The reduction in the microbial load was evaluated by counting the number of colony forming units in serial dilutions, as described in the materials and methods.



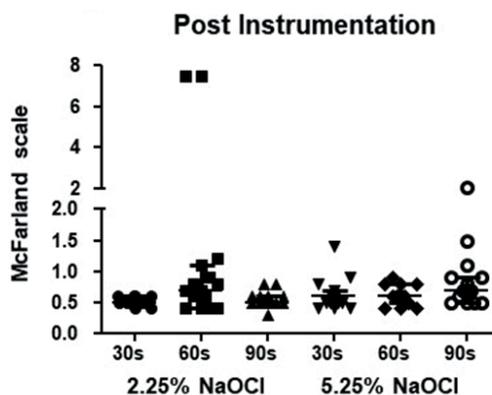
A: The reduction in the microbial load observed before and after instrumentation following irrigation with 2.25% sodium hypochlorite for 30 seconds.  
 B: Reduction in the microbial load observed before and after instrumentation following irrigation with 2.25% sodium hypochlorite for 60 seconds.  
 C: Reduction in the microbial load before and after instrumentation following irrigation with 2.25% sodium hypochlorite for 90 seconds.  
 D: Reduction in the microbial load before and after instrumentation following irrigation with 5.25% sodium hypochlorite for 30 seconds.  
 E: Reduction in the microbial load before and after instrumentation following irrigation with 5.25% sodium hypochlorite for 60 seconds.  
 F: Reduction in the microbial load before and after instrumentation following irrigation with 5.25% sodium hypochlorite for 90 seconds. The data shown in A-F are presented as medians and interquartile ranges.  
 \*\*\*:  $p < 0.0001$ .

**Figure 3.** Elimination of the intraradicular biofilm. The elimination of the intraradicular biofilm of *Enterococcus faecalis* and *Candida albicans* was evaluated using scanning electronic microscopy.



A: Image of a representative sample of the apical third of the negative control without the presence of the intraradicular biofilm, 2000X magnification.  
 B: Representative image of a sample of a root canal without instrumentation as a positive control for the formation of the intraradicular biofilm of *Enterococcus faecalis* and *Candida albicans*, 2000X magnification.  
 C: Representative image of the evaluated group with instrumentation; the sample correspond to irrigation with 5.25% sodium hypochlorite for 90 seconds of contact time, 2000X magnification.  
 D: Percent elimination of the intraradicular biofilm following irrigation with different concentrations of sodium hypochlorite (NaOCl) for various contact times postinstrumentation.

**Figure 4.** Comparison of the postinstrumentation reduction in the microbial load following irrigation with different concentrations of sodium hypochlorite for various contact times. The reduction in the microbial load was evaluated using the McFarland scale.



A: Concentration of microorganisms expressed on the McFarland scale observed after 30, 60 and 90 seconds of irrigation with 2.25% and 5.25% sodium hypochlorite. Mean values are presented.

## DISCUSSION.

Instrumentation and irrigation are essential in endodontic treatment for eliminating the microorganisms causing infections in root canals; multiple systems of instrumentation and several irrigation agents are available. In this study, we evaluated the reduction in the microbial load at different contact times and concentrations of an irrigate agent that is frequently used in endodontics, employing a negative apical pressure system.

We observed a significant reduction in microbial load after instrumentation compared to before instrumentation at different contact times and concentrations of sodium hypochlorite, including lowest concentration (2.25%) of irrigate agent and contact time.

The negative apical pressure system has a fundamental characteristic of constantly exchanging the irrigator agent, thus allowing the direct contact of the irrigator

with dentinal surfaces to achieve a better antimicrobial effect.<sup>12</sup> In addition, the “steam block” effect observed at the apex is eliminated with this system. According to Bronnec et al, the space between the point and root canal walls is essential to allow the irrigator flow to contact the root canal walls and eliminate the steam block, and the hydrodynamic forces inside root canals optimized the function of irrigator agent.<sup>21</sup>

Other authors reported similar results,<sup>14,22</sup> indicating that a negative apical pressure system provides better cleaning and disinfection by irrigator agents, avoiding the steam block and periapical extrusion of the irrigator. However, disadvantages of this system are the lack of ergonomics, variable supply of irrigator and plugging of the microcannula.

Marion et al observed similar antimicrobial effects of 2.25% and 5.25% sodium hypochlorite.<sup>23</sup> Based on

this finding and an evaluation of the benefits and cost, the use of 2.25% sodium hypochlorite for 30 seconds represents a great advantage in the clinic, as the time of endodontic treatment is reduced with the confidence of obtaining a good antibacterial effect similar to 5.25% sodium hypochlorite applied for 90 seconds. In addition, the 2.25% concentration has greater safety due to low levels of cytotoxicity.

However, controversial results have been reported for the antibacterial capacity of the irrigator agent employed here. Baumgartner *et al.*,<sup>24</sup> described a similar antimicrobial efficacy of 5.25% sodium hypochlorite applied for 30 seconds on *Enterococcus faecalis* biofilm using negative apical pressure (EndoVac) and irrigation with positive apical pressure.

We postulate that the discrepancies with our results are because the authors used the Endovac system with entry and exit movements, which can interfere with the effectiveness of system. Another factor that potentially alters the efficacy of disinfection by NaOCl or other antimicrobial agents is the intrinsic capacity of several strains to form a biofilm, as strains may produce more virulence factors or differently express several genes. In this study, we only tested the biofilm formed by one strain of *Enterococcus faecalis* and *Candida albicans*; however, a study evaluating biofilms formed by different strains and their elimination would be of interest.<sup>25,26</sup>

We observed great effectiveness of the irrigation of root canals with an apical caliber of 0.35mm in the mesial-vestibular roots from upper molars with complex curvatures greater than 30°C. However, a negative apical pressure system required an apical caliber greater than or equal to 0.40 mm for good disinfection in a previous study.<sup>27</sup> De Gregorio *et al* evaluated the irrigation capacity of a negative apical pressure system (EndoVac) in teeth with canals with low curvature (0°C - 10°C), moderate curvature (11°C - 30°C) and severe curvature (31°C - 65°C) and different apical calibers and concluded that the ideal caliber is 0.40mm with a taper of 0.04mm to obtain a greater volume of penetration of the irrigator agent throughout the working length, but the volume of irrigator is reduced, depending of the extent of curvature.<sup>28</sup>

The pressure used for the suction of negative apical pressure system is a fundamental requirement for obtaining highly effective irrigation, because a higher pressure represents a large volume of irrigator solution and a greater antimicrobial effect on the inside surfaces of root

canals. Several authors have established the pressure and suction volume of this negative apical pressure system for different apical calibers and curvatures of roots.<sup>27,28</sup> We employed suction of 3ml per minute with a pressure of 16.24mmHg (0.055 MPa), and we observed good disinfection of the root canals in teeth with curvatures greater than 30°C and a low apical caliber, in contrast to other studies.

A challenge in endodontic treatment is to obtain the complete disinfection of root canal systems, and different solutions with antimicrobial properties have been used to attain this goal, including sodium hypochlorite. However, its use and supply must be optimized. In the present study, negative apical pressure produced good results in the irrigation process, as sodium hypochlorite was able to be used for a short time at a low concentration, avoiding damage to tissues and reducing the time required to complete the procedure.

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**Conflict of interests:** The authors have no conflicts of interest related to this study to declare.

**Ethics approval:** This study was approved by Comité de Ética e Investigación de la Facultad de Estomatología with approval number CEIFE-035-017.

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