**A laboratory study of root canal and isthmus disinfection in extracted teeth using various activation methods with a mixture of sodium hypochlorite and etidronic acid**

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**ABSTRACT**

Aim To evaluate in a laboratory setting the antibiofilm activity of chemomechanical preparation of several irrigating protocols including conventional irrigation, ultrasonic activation and XP-endo Finisher, with a mixture of sodium hypochlorite and etidronic acid in infected isthmuses and root canals of extracted human teeth.

Methodology Fifty-six mesial roots of mandibular molars, half of them with a continuous isthmus from the cervical to the apical third between the two root canals (type 1), and the other half with a continuous isthmus from the cervical to the middle third and one canal in the apical third (type 2), were included. The root canals were contaminated for seven days with an Enterococcus faecalis suspension. There were 3 experimental groups plus a control group (n = 7 per type of root canal anatomy). All the root canals, except for the control group that was not treated, were chemo-mechanically prepared and then assigned to one of the experimental groups according to the final adjunctive procedure: conventional irrigation, ultrasonic activation, or XP-endo Finisher activation. The irrigating solution used was a combination of 2.5% sodium hypochlorite and 9% etidronic acid, and the final protocols were applied for three cycles of 30 seconds with a 3 mL volume. The antibiofilm activity was evaluated at each location (root canal and isthmus) and third (cervical, middle and apical) using confocal laser scanning microscopy and the live/dead technique. Statistical analysis was performed by means of SPSS (descriptive statistics) and SUDAAN (p-value calculations).

Results Root canals had significantly lower biovolume values than the isthmuses (p<0.05). The biovolume in the root canals was significantly reduced in all the experimental groups in all the thirds except for conventional irrigation in the apical third (p>0.05). In the cervical and middle thirds, ultrasonic activation was associated with the lowest biovolumes (p<0.05), followed by XP-endo Finisher. In the isthmus, disinfection was similar in all the thirds for all the protocols. Conventional irrigation was associated with intermediate values with no significant differences from the control group or from the activated protocols (p>0.05), although the latter were significantly different from the control group (p<0.05). No differences were found between ultrasonic activation and XP-endo Finisher in the middle and apical thirds (p>0.05) in the isthmuses.

Conclusions The isthmus was more difficult to disinfect than root canals. In the root canals, ultrasonic activation and XP-endo Finisher had a greater effectiveness than conventional irrigation. In the isthmuses, no differences were observed between the two activation techniques and conventional irrigation.

**Introduction**

The outcome of root canal treatment depends on the elimination of bacterial biofilm from the root canal system. This goal is difficult to achieve because of anatomical irregularities such as lateral canals, isthmuses and apical deltas that are inaccessible to instruments (de Gregorio *et al*. 2010, Markvart *et al*. 2012). Studies using micro-computed tomographic imaging technology have reported that 9.6%-48% of the main root canal walls remain unprepared after instrumentation (Paque *et al*. 2011, Markvart *et al*. 2012, Gergi *et al*. 2015, Peters *et al*. 2015). An isthmus is a narrow communication between two root canals (Weller *et al*. 1995, Sahar-Helft *et al*. 2015) that accumulates bacterial biofilms and dentine debris produced during root canal instrumentation (Nair *et al*. 2005). Proper cleaning and disinfection of the isthmus area is known to be challenging (Adcock *et al*. 2011, Endal *et al*. 2011, Markvart *et al*. 2012, Neelakantan *et al*. 2016, [Rôças](https://pubmed.ncbi.nlm.nih.gov/?term=R%C3%B4%C3%A7as+IN&cauthor_id=27641947) *et al*. 2016).

Irrigation plays a key role in root canal preparation because it enhances the debridement and disinfection of areas untouched by instruments (Gulabivala *et al*. 2005, Haapasalo *et al*. 2005). The most accepted irrigation protocol includes the application of sodium hypochlorite (NaOCl) during root canal instrumentation followed by a final flush with EDTA. An alternative is to use a combination of NaOCl and the weak chelating agent etidronic acid (HEDP) as the sole irrigating solution, because it maintains the properties of both individual solutions (Zehnder *et al.* 2005, Lottanti *et al.* 2009, Paqué *et al.* 2012, Arias-Moliz *et al.* 2014, Arias-Moliz *et al.* 2015, Tartari *et al.* 2015), which appear to resemble the ones provided by NaOCl followed by EDTA (Tartari *et al.* 2015, Morago *et al.* 2019).

The conventional method of irrigation with a syringe and needle often fails to deliver and distribute irrigating solutions adequately within the complex root canal system, especially in the apical third and isthmuses (Thomas *et al*. 2014, Deleu *et al*. 2015, Versiani *et al*. 2015). To overcome this limitation, adjunctive activation methods have been proposed (Virdee *et al*. 2018). Ultrasonic activation entails the activation of an irrigant by a non-cutting, ultrasonically oscillating file. The cleaning action is attributed to cavitation and acoustic streaming (Van der Sluis *et al*. 2007). Laboratory and clinical studies have reported ultrasonic activation to be more effective than conventional irrigation in removing pulp tissue and hard tissue debris; and although a number of studies report a greater antimicrobial activity (Cherian *et al*. 2016, Pladisai *et al*. 2016, Bao *et al*. 2017), the findings are still inconclusive (Paiva *et al*. 2013, Căpută *et al*. 2019).

The XP-endo Finisher (FKG Dentaire, La Chaux-de-Fonds, Switzerland) was introduced to improve root canal cleaning and disinfection. It is a non-tapered size 25 instrument made of nickel-titanium MaxWire alloy (FKG Dentaire SA). At room temperature the file is straight in its martensite phase, and it changes to the austenite phase when exposed to body temperature, developing a sickle shape (Debelian & Trope 2015) that helps the instrument touch the canal walls and distribute the irrigating solution throughout the canal system (Paiva *et al*. 2013, Debelian & Trope 2015). Better solution distribution with XP-endo Finisher as compared to ultrasonic activation throughout the mesial canal of mandibular molars, especially in the apical canal third has been reported (Pacheco-Yanes *et al*. 2019). However, other study found no difference between the two activation systems (Leoni *et al*. 2017). Former studies on the antimicrobial activity of XP-endo Finisher have concluded it to be more effective than ultrasonic activation in reducing the bacterial load on the main root canal walls and inside dentinal tubules (Alves *et al*. 2016, Azim *et al*. 2016, Bao *et al*. 2017). However, its cleaning and antimicrobial activity within root canal irregularities such as the isthmus remain a matter of debate (Alves *et al*. 2016, Bao *et al*. 2017, Pacheco-Yanes *et al*. 2019).

The aim of this study was therefore to evaluate the antibiofilm activity of chemomechanical preparation by means of different irrigating protocols including conventional irrigation, ultrasonic activation and XP-endo Finisher, with the mixture of sodium hypochlorite and etidronic acid, in infected isthmuses and root canals. The null hypotheses were: none of the irrigation protocols reduces the biofilm in the isthmuses and in the root canals significantly; there is no difference between the different experimental protocols in isthmus and root canal disinfection.

**Materials and Methods**

Freshly extracted, mature, non-carious, human mandibular molars were selected. The teeth were stored in 0.1% thymol solution at 4°C until use. The Ethics Committee for the use of human extracted teeth of the institution where the experiments were conducted approved the protocol performed (UGR-438).

The teeth were decoronated using an Accuton-50 machine (Struers, Copenhagen, Denmark). The mesial root was sectioned and maintained and the rest of the root was discarded. The roots were scanned using cone beam computed tomography (Planmeca ProMax 3D; Planmeca, Helsinki, Finland) and the presence of an isthmus was checked in the different sections: axial, sagittal and coronal. The roots selected were those that presented a type V isthmus under the classification of Hsu & Kim (1997), that is, with complete communication or a corridor between the two canals. Based on this classification, specimens having two types of root canal anatomies were included: two root canals with a continuous isthmus from the cervical to the apical third (type 1); and two root canals with a continuous isthmus from the cervical to the middle third and one canal in the apical third (type 2). A total of fifty-six specimens met the criteria, 28 being type 1 and 28 type 2.

Each root canal was enlarged using stainless K-files up to size 25 (Dentsply Sirona, Ballaigues, Switzerland) to the working length (WL), which was established as 1 mm short of the patency length, with distilled water as the irrigant. To allow for handling of the roots during the experiment and avoid contamination, a customized model of each was fabricated with polyvinyl siloxane impression material (Zhermack, Rovigo, Italy). The roots were then sectioned horizontally into three thirds from the coronal part using a low-speed handpiece with a diamond disk (355514220 HP; Edenta AG, Au/St. Gallen, Switzerland) (Figure 1). The root canal anatomy was confirmed under a stereo microscope (Olympus, SZ-PT, Japan). The smear layer formed during the preparation of the specimens was removed with 17% EDTA in an ultrasonic bath, for 5 minutes. The specimens were washed with distilled water, and finally they and their corresponding customized models were sterilized by autoclave.

Specimen infection

Once sterile, the three thirds of each root were re-approximated using utility wax with the aid of tweezers to avoid any leaking of the irrigating solution. The apical foramina of each root was also sealed to create a closed-end system. The assembled specimen was then placed in the customized model (Figure 1). For the specimens´ infection, an *Enterococcus faecalis* ATCC 29212 suspension in BHI broth (Scharlau Chemie SA, Barcelona, Spain) of approximately 1×107 colony-forming units per millilitre was prepared. Afterwards, 10 µL of this suspension was added to each root canal and they were incubated for one week under aerobic conditions at 37°C, with reinoculation performed every 24 hours.

Antimicrobial test

Seven specimens of each type, 1 and 2, were randomly assigned to each of the four study groups (three experimental groups plus the control). The irrigating solution used was a combination containing NaOCl at 2.5% (Panreac Química SA, Castellar del Vallés, Spain) and HEDP at 9% (Cublen K8514 GR; Zschimmer & Schwarz, Mohsdorf, Germany). For the 2.5% NaOCl/9% HEBP association, both irrigants were prepared at double concentration and mixed in a 1:1 ratio. Each root canal was instrumented using Protaper Next X1 and X2 files (Dentsply Sirona) to the WL and irrigated with 3 mL of the irrigating solution before each file and after the last one, for a total of 9 mL per canal. Once instrumented, root canals were subjected to one of the following final procedures:

Conventional irrigation group. Syringe delivery of 1 mL of 2.5% NaOCl/9% HEDP was performed in each canal three times for 30 seconds each, for a total volume of irrigating solution of 3 mL and 90 seconds. The needle was inserted 1 mm short of the WL.

Ultrasonic activation group. The irrigant activation was performed with an ultrasonic device (DTE D7, Guangxi, China) and an Irrisafe size 20 ultrasound tip (Saletec, Bordeaux, France) at a power setting of 3 and placed 1 mm short of the WL. One millilitre of 2.5% NaOCl/9% HEDP was applied in each canal for 10 seconds, and then the solution was activated in each for 20 seconds with a slight rotation of the handpiece around the canal axis. This 30-second cycle was repeated three times for a total volume of irrigating solution of 3 mL and 90 seconds.

XP-endo Finisher group. The file was placed in a contra-angle handpiece (Sirona Dental Systems GmbH, Bensheim, Germany), inserted into the canal to WL, then activated at 800 rpm and 1-Ncm torque using slow up-and-down 7- to 8-mm-long movements to contact the full length of the canal, as suggested by the manufacturer. One millilitre of 2.5% NaOCl/9% HEDP was applied in each canal for 10 seconds, after which the solution was activated for 20 seconds. This 30-second cycle was repeated three times, for a total irrigant volume of 3 mL and 90 seconds per root canal.

During the chemomechanical preparation, irrigation was delivered with a 30 G open-ended side-vented needle attached to a 3-mL Luer lock syringe (Monoject, Covidien, Mansfield, MA, USA) taken up to 1 mm short of the WL. During irrigant delivery the needle was moved in and out along the root canal and also along its axis, without exceeding the desired insertion depth, in order to improve cleaning of the canal and to distribute the irrigant homogeneously around the tip of the needle (Boutsioukis *et al*. 2010). All these procedures were performed inside a cabinet (model Bio-II-B; Telstar SA, Terrasa, Spain) and the temperature was maintained at 37 °C by a heater (model B-I; Falc Instruments srl, Treviglio, Italy). Once prepared, the root canals were flushed with 3 mL of 5% sodium thiosulfate for 5 minutes to inactivate the NaOCl, followed by 3 mL of saline solution. A control group consisting of seven specimens of each root canal type that were infected but not treated was also included. Afterwards, the roots were prepared to be observed under a Confocal Laser Scanning Microscope (CLSM).

CLSM analysis

The specimens were removed from the customized models, disassembled and stained with Syto-9/Propidium iodide (PI) fluorocromes (Live/Dead, Baclight, Invitrogen, Eugene, OR, USA). Syto-9 is a green-fluorescent stain that labels microorganisms with intact membranes, considered as live microorganisms. PI is a red-fluorescent nucleic acid stain and penetrates only the cells with damaged membranes, considered as dead microorganisms. The coronal side of each third was scanned using a CLSM (TCS-SP5 II, Leica, Wetzlar, Germany). Two microscopic confocal volumes were randomly obtained per each location (right and left root canal and isthmus) of each third (cervical, middle and apical) (Chávez de Paz 2009). The CLSM technician, who did not participate in sample preparation, selected at random the two volumes: from the root canals, one in the upper part and the other in the lower part; in the case of the isthmuses, one at the entrance and the other in the middle of the isthmus. The mean of the two volumes scanned per location-third was calculated. Table 1 shows the n values per location-third.

The operative fields were taken using a 40 × oil immersion objective, 2 µm step-size and a format of 512 × 512 pixels. Each picture represented an area of 387.5 × 387.5 µm. For quantification purposes, bioimage\_L (http://www.bioimagel.com) software was used (Chávez de Paz 2009). The parameters evaluated in each group were the total biovolume and the percentage of red population (dead cells). After the study, Sample Power 2.0 (IBM Inc., Chicago, IL, USA) was used for post hoc estimation of the statistical power when comparing our four study groups by pairs. Using the *t*-test for independent groups, with a significance level of alpha=0.05 and a power of 80%, the standardized differences (d) that can be detected are 0.75 when n=28, 0.9 when n=21, 1.1 when n=14 and 1.6 when n=7. The interpretation of d, according to Cohen's scale (Cohen 1988) expanded by Sawilowsky (2009), can be as follows: Medium (0.5 to 0.8), Large (0.8 to 1.2) and Very large (1.2 to 2.0).

Statistical analysis

The descriptive analysis was performed by means of SPSS 15.0 (SPSS Inc.), and the *p*-value calculations by means of SUDAAN 7.0 (RTI, RTP, NC) with the method expressed in Table 1 footnotes. This software allows for clustering, hence multiple sites per tooth. It has a completely factorial design, with two independent factors: location (root canal and isthmus) and third (cervical, middle and apical). A detailed stratified analysis was carried out. The original biofilm biovolume data were not normally distributed, for which reason we proceeded to a base 10 logarithmic transformation. Normality (for each set of data, location-third and group) was checked with the Kolmogorov-Smirnov test (results not shown).

**Results**

The control group had similar biofilm biovolumes in the three thirds of the root canals (*p*=0.055) and isthmuses (*p*=0.616) (Table 1).

Overall, disinfection of the root canals by the different irrigation protocols gave lower biofilm values than the isthmuses. The biovolume in the root canals was significantly reduced by all the experimental groups in all the thirds (*p*<0.05) exceptfor the protocol that included conventional irrigation in the apical third, which did not show statistical differences with respect to the control group (*p*>0.05). In the cervical and middle thirds, a significant gradient of effectiveness between the experimental groups was observed, the protocol with ultrasonic activation being the one that gave the lowest biovolumes (*p*<0.05), followed by XP-endo Finisher. In the apical third, no significant differences between these activated groups were obtained (*p*>0.05).

Isthmus disinfection varied depending on the protocol. The conventional irrigation protocol had intermediate values with no significant differences with respect to the control group or the two activated protocols (*p*>0.05); however, the two activated protocols were significantly different from the control group (*p*<0.05). No significant differences were obtained between ultrasonic activation and XP-endo Finisher in the middle and apical thirds (*p*>0.05).

Considering the activity of the different protocols per location-third, in the root canals the antibiofilm activity was greater in the cervical than in the apical third, with the exception of the protocol with XP-endo Finisher, which was not associated with significant differences within the three thirds (*p*=0.784). In turn, in the isthmuses no significant differences were obtained between the biovolumes of the three thirds in each irrigating protocol (*p*=0.396 for conventional irrigation, *p*=0.088 for ultrasonic activation and *p*=0.489 for XP-endo Finisher).

Globally, although the percentages of dead cells were similar for all groups (including the control group) in the different location-thirds (*p*>0.05), the greatest percentage corresponded to the cervical root canal of the protocol with ultrasonic activation (29.3%), and the lowest to the isthmus of the cervical third with XP-endo Finisher (8.2%) (data not shown). Representative CLSM images of the root canals and isthmuses of the apical third treated with the different irrigating protocols are displayed in Figure 2.

**Discussion**

This study evaluated antibiofilm activity in root canals and isthmuses under three different irrigation protocols. The results revealed that the protocols including activation methods, ultrasonic and XP-endo Finisher, were associated with a greater antibiofilm activity than did conventional irrigation in root canals but not in the isthmuses where this activity was similar for all the experimental groups. None of the activation systems were capable of rendering the isthmuses or the root canals, particularly in the apical third, free of bacteria. Thus, the null hypothesis was partially accepted.

Mesial roots of human mandibular molars were selected because they have a high incidence of isthmus, ranging from 54%-100% (de Pablo *et al*. 2010, Harris *et al*. 2013, Karunakaran *et al*. 2019). As the frequency of this isthmus diminishes towards the apical third (Karunakaran *et al*. 2019), the present study included roots with an isthmus connecting the whole length of the two main canals (type 1), as well as roots with isthmuses only in the cervical and middle thirds (type 2). The data obtained from the isthmus of the apical third is therefore half that from the other thirds, 7 *versus* 14 per group (Table 1). The samples were sectioned before being contaminated for two reasons. First, to confirm the presence of isthmuses, which was previously detected by CBCT (Estrela *et al*. 2015, Neelakantan *et al*. 2016), under a stereo microscopy. CBCT images have shown limited sensibility (65%-75%) in detecting apical isthmuses in mesial roots of mandibular first molars (Tolentino *et al*. 2018, 2020). Second, to avoid sectioning them after the irrigation protocols; doing so could alter the sample because of the action of the diamond disk and the smear layer produced, and consequently alter the evaluation through CLSM. Among the control group, the contamination levels within the root canals and the isthmuses in the three canal thirds were not significantly different, and the standard deviations were not very high, indicating that the contamination method was homogeneous.

The combination containing NaOCl and HEDP was chosen as the single irrigating solution during root canal chemomechanical preparation. This solution induces a continuous chelation that prevents or reduces the formation of smear layer and dentine debris in difficult access areas better than the conventional irrigation protocol of NaOCl followed by EDTA (Lottanti *et al*. 2009, Paqué *et al*. 2012). It also has substantial organic dissolving capacity (Tartari *et al*. 2015, Tejada *et al*. 2019) and antimicrobial activity against dentine surface biofilms and inside dentinal tubules (Arias-Moliz *et al*. 2014, 2015, Morago *et al*. 2019), this activity being enhanced when activated by various methods that include ultrasonic activation (Neelakantan *et al*. 2015). The protocol of three irrigating solution activations for 20 seconds was performed instead of continuous irrigation and agitation, as it facilitates the renewal of the solution and provides for better cleaning of the canal apical third and isthmus (van der Sluis *et al*. 2010, Duque *et al*. 2017, Chan *et al*. 2019).

Overall, the biofilm biovolume reduction in the root canals was greater than in the isthmuses. Disinfection in the root canals was third-dependent, being greater in the cervical and middle thirds than in the apical one. While the conventional irrigation protocol gave the largest residual biofilm volumes, a significant reduction was observed in these two coronal thirds with respect to the control group, indicating its capacity to deliver the irrigant adequately in the main canal space (Brito *et al*. 2009, Johnson *et al*. 2012). However, bearing in mind that the needle was placed 1 mm short of the WL and that the solution can generally penetrate up to 1 mm apically to the needle tip, the limited activity in the apical third may be explained by the difficulty of the solution to contact the bacterial biofilm within this location. Activation of irrigants therefore appears to be beneficial for the disinfecting process, in order to potentiate cleaning of the full canal length.

The protocols that included irrigant activation achieved a greater bacterial biofilm reduction in the root canals. Ultrasonic activation resulted in greater reduction percentages than XP-endo Finisher, but no differences were observed in the middle and apical thirds for the two protocols. Although previous studies have demonstrated a substantial antimicrobial activity of ultrasonic activation in the main canal space (Neelakantan *et al*. 2015), comparison of the two activated protocols points to somewhat controversial findings. Whereas one study reported greater activity under the ultrasonic procedure (Sasanakul *et al*. 2019), another observed similar activity with regard to both activation methods (Alves *et al*. 2016), and a third reported greater effectiveness with XP-endo Finisher than with ultrasonics (Bao *et al*. 2017). Such differences may be explained by the diverse experimental designs as well as sampling, microbiological evaluation, the irrigation solution, volume, the activation protocol and root canal anatomy. Yet in general, and in agreement with the present results, the differences found for the two activation methods were minor, especially in the middle and apical thirds (Bao *et al*. 2017, Sasanakul *et al*. 2019), highlighting the capacity of both protocols to achieve better disinfection in easy-to-reach areas.

The isthmus was found to be more difficult to disinfect than the root canal walls. Conventional irrigation failed to reduce the biofilm within this entire location, showing a limited ability to distribute the solution in hard-to-reach areas, as evidenced by its weak action against the bacterial biofilm in the isthmus (Thomas *et al*. 2014, Versiani *et al*. 2015, Deleu *et al*. 2015, Pacheco-Yanes *et al*. 2019). A slightly greater biofilm reduction, though not significantly different from that of conventional irrigation, was obtained by means of the two activated protocols, with similar values in the three thirds of the isthmuses. These results indicate that the physical action provided by the design and helical movement of the XP-endo Finisher, as well as the acoustic streaming and cavitation produced by the ultrasonic tip, may have allowed the irrigant to reach previously untouched areas and displace part of the bacterial biofilms (Debelian & Trope 2015, Yasui 2018, Căpută *et al*. 2019). Still, even though these activated groups significantly reduced the biofilm within the isthmus when compared to the control, the reduction was not significant with respect to the conventional irrigation group; that is, their effectiveness was limited.

The present results are in line with those of the only study that evaluated the disinfectant activity of ultrasonic activation and XP-endo Finisher in the isthmus and recess areas of mandibular molars (Alves *et al*. 2016). It may be that the flow of irrigant generated during delivery by the needle, or through agitation by mechanical or ultrasonic means, is not sufficient to improve penetration and filling of the isthmus area (Pacheco-Yanes *et al*. 2019). On the other hand, the turbulence produced by the activation systems within the root canal could favour an accumulation of debris and bacteria, hindering access of the irrigating solution to hard-to-reach areas (Hsieh *et al*. 2007, van der Sluis *et al*. 2007, Al-Ali *et al*. 2012).

Taking into account the results in light of the absence of differences in the isthmuses, the greater effectiveness of the activation groups (higher than conventional irrigation) in the root canals might be explained by the physical action of the ultrasonic tip and the XP-endo Finisher on the root canal walls, which could have helped remove the bacterial biofilms more effectively. There is no consensus in the literature regarding the antibiofilm activity of conventional irrigation as opposed to ultrasonic activation: recent reviews (Căpută *et al*. 2019, Silva *et al*. 2019) report that half of the laboratory studies found a superior antimicrobial effect with ultrasonic irrigation, and clinical trials were not able to detect improved reduction of the microbial load or clinical success through periapical healing. In addition, it is important to bear in mind that the physical action of ultrasonic activation depends on local conditions of application, such as the file size, file surface properties, root canal geometry and direction of the oscillation (Ahmad *et al*. 1988, Roy *et al*. 1994, Jiang *et al*. 2010). In fact, file oscillation towards the root canal irregularity reportedly cleans the debris from this location more effectively (Jiang *et al*. 2010). Therefore, the slight rotation of the ultrasonic handpiece performed in this study during its application may have influenced the low activity within the isthmuses, hence a greater effectiveness might be expected if the oscillation of the file is directed towards the isthmus. Then again, the chemical action of the solution might be even more relevant (De Meyer *et al*. 2017), which would explain the slight differences we observed between the conventional irrigation group and the activated ones.

The increased difficulty in disinfecting the apical third of root canals and isthmuses encountered in this study could be extrapolated to the lateral anatomy present within this third. Indeed, no activation system, including sonic and ultrasonic activation and XP-endo Finisher file, is known to penetrate and eliminate biofilms within the lateral anatomy (particularly in the apical third), although the latter two techniques are reportedly the most effective overall (de Gregorio *et al*. 2010, Spoorthy *et al*. 2013, Bao *et al*. 2017, Mohmmed *et al*. 2018). This suggests that their use as an adjunctive irrigation method could improve disinfection of the lateral anatomy of the apical third.

The CLSM helped to evaluate *in situ* the viability, location and distribution of the bacterial biofilms within the root canal systems, a novel contribution of this study. Regarding the viability of the residual bacteria, none of the irrigating protocols was effective in killing the bacteria in the root canal walls and the isthmuses; the low death percentages in the different study groups were similar to the control group. This finding, largely unexpected, may not be very relevant, as it reflects the bacterial viability of the residual biovolume alone. At any rate, the results reported here should be considered with some caution, because root canal anatomy can vary among specimens, implying an anatomical influence on the results. Notwithstanding, the fact that the experimental parameters were standardized and controlled, including the irrigating solution, volume, temperature, activation time, needle type, and depth of placement of the needle, lends rigour to the present findings. Further studies with standardized canal systems evaluating the behaviour of the irrigation protocols within the isthmuses, also including conventional irrigating solutions such as NaOCl and EDTA, or combining the activation methods such as ultrasonic activation and XP-endo Finisher, are needed to corroborate the results reported here and find new strategies to enhance disinfection within areas of difficult access.

**Conclusions**

The isthmus proved more difficult to disinfect than the root canals. In the root canals, ultrasonic activation and XP-endo Finisher were more effective than conventional irrigation. In the isthmuses, no differences were observed between the two activation techniques and conventional irrigation. .

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| Table 1. Biofilm biovolume (μm3) in the root canals and isthmuses of the different thirds after treatment with the irrigation protocols. Mean ± standard deviation (sd). | | | | | | | |
|  |  |  | Positive control | Conventional irrigation | Ultrasonic activation | XP-endo Finisher | Global *p*-value |
| Location | Third | n | Mean ± sd | Mean ± sd | Mean ± sd | Mean ± sd |
| Root canal | Cervical | 28 | 5696±61641,a | 2459±47422,a | 306±3343,a | 596±5374,a | <0.001 |
| Middle | 28 | 13079±194771,b | 3775±51052,a,b | 255±2673,a | 703±9714,a | <0.001 |
| Apical | 21 | 21419±352121,b | 11819±163261,b | 697±8492,b | 1479±36582,a | 0.006 |
| Global *p*-value |  | 0.036 | 0.031 | 0.033 | 0.425 |  |
| Isthmus | Cervical | 14 | 7228±97371,a | 4197±48751,2,a | 1030±21702,a | 1836±13282,a | 0.025 |
| Middle | 14 | 6847±54331,a | 8281±88991,a | 2538±45492,a | 3716±32811,2,a | 0.040 |
| Apical | 7 | 7232±39901,a | 9920±114151,a | 6289±148641,a | 4121±65431,a | 0.570 |
| Global *p*-value |  | 0.974 | 0.151 | 0.485 | 0.057 |  |
| Read vertically, the same letters show differences that are not statistically significant in each group in between thirds, either in the root canal or in the isthmus. Read horizontally, the same numbers show differences that are not statistically significant in between groups.  Global *p*-value calculations were performed with REGRESS procedure in SUDAAN to adjust for clustering (multiple locations per tooth); if significant (*p*<0.05), we proceeded with paired comparisons by means of the DESCRIPT procedure in SUDAAN.  n, number of data/group according to the *location – third* as follows:  - Root canal *–* cervical and middle: right + left root canal data in type 1 and 2 root canal configurations.  - Root canal *–* apical: right + left root canal data in type 1 + the only root canal data in type 2.  - Isthmus *–* cervical and middle: isthmus data in type 1 and 2.  - Isthmus *–* apical: isthmus data in type 1. | | | | | | | |

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| Figure 1. Schematic representation of type 1 specimen preparation. A, a customized model of each specimen was fabricated with polyvinyl siloxane impression material; B, the roots were sectioned horizontally into three thirds; C, the three thirds were assembled using utility wax and the specimen was placed in the customized model. |

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| Figure 2. Confocal Laser Scanning Microscope (CLSM) images of apical root canal and isthmus disinfection by different irrigation protocols with the combination of NaOCl and HEDP. A, contaminated root canal; B, contaminated isthmus; C, root canal treated with conventional irrigation protocol; D, isthmus treated with conventional irrigation protocol; E, root canal after activating the solution ultrasonically; F, isthmus after activating the solution ultrasonically; G, root canal after activating the solution with XP-endo Finisher; H, isthmus after activating the solution with XP-endo Finisher. |