**ANTIBIOFILM ACTIVITY OF DICLOFENAC AND ANTIBIOTIC SOLUTIONS IN ENDODONTIC THERAPY**

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**ABSTRACT**

**Introduction:** The aim of this study was to compare the antibiofilm effects of a triple antibiotic solution (TAs), a double antibiotic solution (DAs), and 5%, 2.5% and 1.25% Diclofenac solutions (DCs) against *Enteroccocus faecalis* biofilm.

**Methods:** Eighty-four sterile radicular dentin blocks were used as biofilm substrate for three weeks. The study groups were: 1) 1 mg/mL TAs (minocycline, metronidazole, and ciprofloxacin); 2) 1 mg/mL DAs (metronidazole and ciprofloxacin); 3) 5% DCs; 4) 2.5% DCs; 5) 1.25% DCs, and 6) 0.9% saline solution. The antimicrobial activity was evaluated by bacterial count determinations and confocal scanning laser microscopy (CSLM). The contact time for the antimicrobial tests was 5 minutes. Bacterial counts were expressed as the reduction percentage of colony forming units (CFUs), and for the CSLM evaluation, the Log10 total biovolume and percentage of green population (live cells) were calculated.

**Results:** The CFUs reduction percentage ranged between 62.98 and 98.62, respectively for TAs and 5% DCs. The DCs showed a concentration-dependent effect.

For the CLSM, the Log10 total biovolume in all groups was very similar, and showed a scarce (1.39 to 1.02) but significant reduction with respect to the control. 5% and 2.5% DCs gave the lowest viable cell percentage. TAs and DAs groups showed intermediate values without significant differences between them.

**Conclusions:** Solutions of DCs at 5% and 2.5% have greater antimicrobial effects than TAs and DAs, and may be considered a valid alternative for controlling the infection of teeth with apical periodontitis.

**Key words**: Antibiotic solutions; antibiofilm activity; endodontics; *Enterococcus faecalis*, diclofenac.

**INTRODUCTION**

Root canal disinfection plays a decisive role in the successful outcome of treatment of teeth with apical periodontitis. To reduce the intracanal bacterial population, mechanical instrumentation and root canal irrigants with antimicrobial properties are needed1. Also, inter-appointment medication has been recommended to favor the elimination of residual microorganisms after root canal preparation2 and Calcium hydroxide (Ca(OH)2) pastes are widely used3.

Regenerative endodontic procedures (REPs) are related to healing apical periodontitis, by thickening and/or lengthening root walls and apical closures4. In such cases, minimal or no instrumentation is advised5, whereas irrigation and intracanal medication are needed to achieve disinfection. The combination of antibiotics in a paste is the most common form of intracanal medication in these procedures6. Triple antibiotic paste (TAP), with a combination of metronidazole, ciprofloxacin and minocycline, has proven effective *in vivo7*in necrotic immature and mature teeth8,9. However, dental staining is a drawback, owing to its minocycline content 10. To avoid this problem, double antibiotic paste (DAP) without minocycline has been recommended10,11.

A number of studies12,13,14 report that some non-steroidal anti-inflammatory drugs (NSAIDs)have antibacterial action. Diclofenac sodium, a potent anti-inflammatory medication, has exhibited significant antibacterial effects against both gram-positive and gram-negative bacteria15,16,17. Diclofenac and ibuprofen have also exerted significantly greater antibacterial activity against *Enterococcus faecalis* when compared with Ca(OH)218; and the association of both NSAIDs with this paste increased their antimicrobial action against *E. faecalis* biofilm19.

Recently, a triple antibiotic solution with minocycline, metronidazole, and ciprofloxacin (TAs) used at 1 mg/mL has shown efficacy comparable to a calcium hydroxide/chlorhexidine paste as inter-appointment medication to control the infection of teeth with apical periodontitis20. To the best of our knowledge, however, the antimicrobial activity of a diantibiotic solution —metronidazole and ciprofloxacin (DAs)— or Diclofenac solutions (DCs) at different concentrations remain unknown.

Therefore, the aim of this study was to compare the antibiofilm effects of TAs, DAs, and 5%, 2.5% and 1.25% DCs against *E. faecalis* biofilm.

**MATERIALS AND METHODS**

This protocol was approved by the Ethics Committee of the University of Granada, Spain (nº 1076 CEIH/2020).

**Bacterial Strain and Antimicrobial Solutions**

The bacteria used in this study was *E. faecalis* American Type Culture Collection 29212, taken from a 4 °C stock culture and streaked out twice on brain-heart infusion (BHI) (Scharlau Chemie SA, Barcelona, Spain) agar plates for 24 hours at 37 °C. From the subculture of *E. faecalis*, a 1 McFarland standard suspension was prepared in BHI broth and then diluted 30-fold to obtain an initial bacterial suspension of 1 x 107 colony-forming units (CFUs) per milliliter.

All of the test solutions were of Spanish Pharmacopeia (SP) grade and master formulation, consisting of a triple antibiotic solution [TAs (minocycline, metronidazole, and ciprofloxacin)] at a concentration of 1 mg/mL; a double antibiotic solution [DAs (metronidazole and ciprofloxacin)] at a concentration of 1 mg/mL; and Diclofenac solutions (DCs) at concentrations of 5%, 2.5% and 1.25% (Table 1).

**Dentin Specimen Preparation and Infection with *E. faecalis***

Sterile radicular dentin blocks were used as the biofilm substrate following a previous protocol21. Briefly, forty-two freshly extracted non-carious single-rooted human teeth were selected and stored at 4 °C until use. Eighty-four dentin specimens were obtained, discarding the crowns and the middle and apical thirds of the roots. Then the coronal portion of the root was divided longitudinally into two halves. The outer cementum of each half was removed, and the inner part of the dentin root was progressively polished with 220- to 800-grit silicon carbide papers to create a flat surface. The size was adjusted by using a caliper to obtain 4 × 4 × 0.7 mm (width × length × height) specimens. The smear layer formed during preparation of the specimens was removed with 17% EDTA for 5 minutes. Afterwards, the samples were washed with distilled water for 10 minutes and sterilized by autoclave for 20 minutes at 121 °C. The sterility of the dentin was checked by incubating each specimen in 5 mL BHI at 37°C for 24 hours, verifying the absence of turbidity in the culture medium.

The wells of 24-well microtiter plates were inoculated with 200 μL of the microbial suspension and 1.8 mL of sterile BHI. The sterile dentin blocks were submerged in the inoculated wells, and they were incubated for 3 weeks at 37°C under aerobic conditions. The BHI was refreshed every 2 days. Four additional dentin blocks were inoculated with sterile BHI as the sterility control throughout the experiments.

**Antimicrobial activity test**

The antimicrobial activity was evaluated by CFUs bacterial determinations and CSLM.

Sixty infected dentin specimens were used for CFUs determination. They were washed with saline solution for 1 minute and randomly divided into 6 groups (n = 10) according to the irrigating solutions (Table 1): group 1, TAs 1mg/mL; group 2, DAs 1mg/mL; group 3, 5% DCs; group 4, 2.5% DCs; group 5, 1.25% DCs, and group 6, 0.9% saline solution (control). The dentin blocks were submerged in 120 µL of the antimicrobial solutions for 5 minutes. After the contact period, the specimens were placed in Eppendorf tubes with 200 μL BHI, stirred in a vortex for 10 seconds, and sonicated for 10 minutes to ensure the recovery of biofilms. For the control group a similar procedure was followed, except that there was no exposure to any antimicrobial.

For bacterial count determination, serial dilutions from 101 to 105 of suspension-recovered biofilms were made, and 10 μL aliquots were seeded onto BHI agar and incubated for 48 hours at 37 ºC. The results were expressed as reduction percentage of CFUs, calculated as follows: 100 – (mean CFUantimicrobial solution× 100/mean CFUcontrol).

For CSLM evaluation, 24 infected dentin specimens were randomly divided into six groups (n = 4/group) according to the solutions described above (Table 1). The specimens were washed with saline solution for 1 min and then submerged in the antimicrobial solutions for 5 min. After the contact period, the samples were rinsed again with 0.9% saline solution and stained with the respective dyes: Syto 9/propidium iodide (LIVE/DEAD, BacLight; Invitrogen, Eugene, OR) as previously reported22. After staining the samples with a 1:1 mixture of Syto 9 and PI for 15 min, they were rinsed with saline solution, mounted on a 60 l‐Dish (Ibidi, Martinsried, Germany) with the mounting oil (BacLight; Invitrogen) and directly observed using an inverted CLSM (Leica TCS‐SP5 II, Mannheim, Germany). The respective absorption and emission wave lengths were 494/518 nm for Syto 9 and 536/617 nm for PI. Five microscopic confocal volumes from random areas were acquired from each sample using the 40 x oil lens, 1 μm step size and a format of 512 × 512 pixels. Each picture represented an area of 387 × 387 μm. The scanning was performed from the top of the biofilm to the dentin surface.

For quantification purposes, bioimage\_L software was used23. The variables evaluated in each group were the Log10 total biovolume and percentage of green population (live cells) calculated as follows: green population/ (green population + red population).

**Statistical analysis**

The statistical analysis was performed by means of SPSS 20.0 (SPSS Inc., Chicago, IL). The Log10 total biovolume followed a Gauss distribution by Kolmogorov-Smirnov test. The % reduction of CFUs and the green percentage were normalized by means of the Anscombe transformation. In all variables, the Levene test showed significant differences of variances among groups. Global comparisons were performed using an ANOVA test with Welch´s correction and post–hoc comparison by means of the Games-Howell test.

**RESULTS**

The results in terms of CFU reduction percentage ranged between 62.98 and 98.62 for TAs and 5% DCs, respectively. There were no significant differences between the two antibiotic solutions (TAs and DAs), while the DCs showed a concentration-dependent effect.

For the antimicrobial with CLSM test, a total of 120 operative fields (3D stacks) were evaluated. The Log10 total biovolume in all groups showed a scarce (1.39 to 1.02) but significant reduction with respect to the control, and all the groups gave very similar values (see Table 1). The cell viability of the control group was 98.11%, whereas concentrations of 5% and 2.5% DCs showed the lowest viable cell percentages. Groups TAs and DAs showed intermediate values, with no significant differences between them. Representative images of the biofilms in the different study groups are displayed in Figure 1.

**DISCUSSION**

The persistence of microorganisms that resist disinfection procedures, and/or the recontamination of the root canal system can prove determinant for the healing of teeth with periapical periodontitis24,25. In this work, we tested the antimicrobial activity of soluciones de diclofenaco y las comparamos con soluciones di y triantibióticas to evaluate their potential usefulness as intracanal medication and/or final irrigants in root canal treatment.

To test the antimicrobial activity of the new compounds, was selected a monospecies biofilm. Although a polymicrobial biofilm would be more appropriate to determine their efficacy, due to its clinical reality approach (22), in this first study we used the bacteria that could be consider a reference in this type of works, E. faecalis ATCC 29212, which will allow a more valid and reliable comparison with the results of other works.

On the other hand, a Ca(OH )2 paste was not considered as control, given that the vehicle used to test the compounds was as a solution form, which it allows an exact adjustment of the concentration, easy diffusion and does not require subsequent elimination, as occurs with the form paste presentation. Its known that a triple antibiotic solution, with the same concentration and composition used in this study, have similar effectiveness as a calcium hydroxide paste in root canal disinfection20. In addition, though the inter-appointment medication of choice is Ca(OH)2, given its antimicrobial and biological effects26,27, its use to improve root canal disinfection continues to be controversial20,28,29

To enhance its antimicrobial effectiveness, antibiotics or NSAIDs may be added, showing good results15,18,19,30. An *in vitro* study supports that when NSAIDs, diclofenac and ibuprofen, or the antibiotic ciprofloxacin are incorporated at a 5% concentration to Ca(OH)2, the antimicrobial action of the medication may increase without affecting the pH of the paste19. Diclofenac sodium was found to cause a greater reduction of viable bacteria in biofilm than ibuprofen or ciprofloxacin. In addition, Ca(OH)2 pastes associated with diclofenac, ibuprofen or amoxicillin were not cytotoxic and presented biocompatibility after implantation in rat subcutaneous tissues31.

Meanwhile, a mixture of antibiotics in a paste form (TAP and DAP) is widely used as intracanal medication in REPs5,11. In recent times, the clinically recommended concentration of TAP is 1mg/mL to avoid toxic effects on the stem cells of the apical papilla32.

Few studies have evaluated the use of antibiotics as irrigating solutions in root canal treatment20,33. Jain *et a*l.33 compared *in vivo* the antimicrobial efficacy of sterile saline, chlorhexidine solution and a triple antibiotic solution of 1% ornidazole, 1% ciprofloxacin and 1% tetracycline. The results showed a similar percentage of microbial reduction for the triple antibiotic and chlorhexidine solutions (66,22% and 73,91%, respectively). The percentage values of bacterial reduction were similar to those found in the present study for TAs (62.98%). A clinical study showed that the application of an inter-appointment medication with TAs (1mg/mL) significantly improved root canal disinfection, giving results comparable to a calcium hydroxide/chlorhexidine paste20. A recent randomized controlled clinical study34 evaluated, in infected root canals, the antimicrobial effectiveness of a Ca(OH)2 paste containing ibuprofen or ciprofloxacin at 5% by weight. The ibuprofen did not significantly increase antibacterial effectiveness when added to the paste, yet it was not tested as an irrigating solution.

Diclofenac is a NSAID widely used in the treatment of pain. Its mechanism of action on inflammation is through inhibition of cyclooxygenase-2, reducing angiogenesis and inducing the process of programmed cell death**35**. However, different options have been suggested for its antibacterial action, between others: inhibition of bacterial DNA synthesis13, impairment of membrane activity15, anti-plasmid activity17, alteration in genes encoding transport/binding proteins and down-regulation of efflux pumps **36.**

The results of the present study showed higher or similar reduction percentages of CFUs with DCs as opposed to TAs and DAs. The greatest reduction was obtained by 5% DCs, followed by 2.5% DCs, while the concentration of 1.25% did not show significant differences with respect to the DAs. The outcomes of CFUs appear to agree with the determination of viable cells (green %). The 5% and 2.5% DCs showed the lowest viability values (5.01 and 11.66%, respectively), these values being significantly different from those of the other experimental groups. It is also important to note that the solutions barely reduced the total biovolume; from a clinical standpoint, this finding is of little relevance, because its use as a temporary medication or final irrigating solution would follow the use of NaOCl during instrumentation.

Taking into account that DCs have greater antimicrobial effects than TAs and DAs, as observed here, it would seem reasonable to consider them a valid alternative for controlling infection of teeth with apical periodontitis. Furthermore, potential use in all cases might lessen the risk of sensitizing patients or causing allergic reactions and resistance to antibiotic formulations37. The anti-inflammatory topical action of NSAIDs could help reduce postoperative pain after endodontic treatment38.

Since promising results have been obtained, future research evaluating, on more complex biofilm, the activity of DCs and other compounds in different vehicles is necessary before it can be routinely recommended in a clinical protocol.

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| Table 1. Reduction percentage of CFUs, Log10 biovolume (µm3) and green percentage (%) after 5 min of contact with irrigating solutions on *E. faecalis* biofilms. Mean (standard deviation) | | | |
| Solutions | % Reduction  CFUs | Total biovolume  Log10 | Green  Percentage |
| Triantibiótic | 62.98 (0,17)a | 3.40 (0,44)a | 61.75 (14,09)a,b |
| Diantibiótic | 68.01 (0,15)a,b | 3.69 (0,70)a,b | 45.20 (27,24)a |
| 5% Diclofenac | 98.62 (0,01)c | 3.72 (0,17)a,b | 5.01(8,06)c |
| 2.5% Diclofenac | 90.42 (0,13)c,d | 3.77 (0,18)b | 11.66 (12,18)c |
| 1.25% Diclofenac | 84.71(0,12)b,d | 3.66 (0,38)b | 76.79 (21,17)b |
| 0.9% Saline solution\* | - | 4.79 (0,27)c | 98.11 (2,18)d |
| \* Values of CFUs: mean (standard deviation):144550 (88237).  Global comparison between groups determined by ANOVA test with Welch´s correction (*p*<0.001). The same superscript letter read vertically indicates differences that were not statistically significant according to the Games-Howell test. | | | |
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| Figure 1. Representative CLSM images of the different study groups. |