Antibiofilm Activity of Diclofenac and Antibiotic Solutions in Endodontic Therapy

Carmen María Ferrer-Luque, DDS, MD, PhD, Pilar Baca, DDS, MD, PhD,* Carmen Solana, DDS, PhD,* Alberto Rodríguez-Archilla, DDS, PhD,* María Teresa Arias-Moliz, DDS, PhD,† and Matilde Ruiz-Linares, DDS, PhD**

SIGNIFICANCE

Solutions of 5% and 2.5% diclofenac can be considered a valid alternative in control infection teeth with apical periodontitis.

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 (DCSs) against *Enterococcus faecalis* biofilm. **Methods:** Eighty-four sterile radicular dentin blocks were used as biofilm substrate for 3 weeks. The study groups were as follows: (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 laser scanning microscopy. The contact time for the antimicrobial tests was 5 minutes. Bacterial counts were expressed as the reduction percentage of colony-forming units; for the confocal laser scanning microscopic evaluation, the $\log_{10}$ total biovolume and percentage of green population (live cells) were calculated. **Results:** The colony-forming unit reduction percentage ranged between 62.98 and 98.62, respectively, for TAS and 5% DCS. The DCS showed a concentration-dependent effect.For the confocal laser scanning microscopy, the $\log_{10}$ total biovolume in all groups was very similar and showed a scarce (1.39–1.02) but significant reduction with respect to the control; 5% and 2.5% DCSs gave the lowest viable cell percentage. The TAS and DAS groups showed intermediate values without significant differences between them. **Conclusions:** DCSs 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. *(J Endod 2021;47:1138–1143.)*

KEY WORDS

Antibiotic solutions; antibiofilm activity; diclofenac; endodontics; *Enterococcus faecalis*

Root canal disinfection plays a decisive role in the successful outcome of the treatment of teeth with apical periodontitis. To reduce the intracanal bacterial population, mechanical instrumentation and root canal irrigants with antimicrobial properties are needed[1]. Interappointment medication has also been recommended to favor the elimination of residual microorganisms after root canal preparation[2], and calcium hydroxide ($Ca[OH]_2$) pastes are widely used[3].

Regenerative endodontic procedures are related to healing apical periodontitis by thickening and/or lengthening root walls and apical closures[4]. In such cases, minimal or no instrumentation is advised[5], 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 procedures[6]. Triple antibiotic paste (TAP), with a combination of metronidazole, ciprofloxacin, and minocycline, has proven effective *in vivo*[7] in necrotic immature and mature teeth[8,9]. However, dental staining is a drawback because of its minocycline content[10]. To avoid this problem, double antibiotic paste without minocycline has been recommended[10,11].

A number of studies[12–14] reported that some nonsteroidal 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 bacteria[15–17]. Diclofenac and ibuprofen have also exerted significantly greater antibacterial activity against *Enterococcus faecalis* compared with $Ca(OH)_2$[18], and the association of both NSAIDs with the paste increased their antimicrobial action against *E. faecalis* biofilm[19].

From the Departments of *Stomatology and †Microbiology, School of Dentistry, University of Granada, Granada, Spain

Address requests for reprints to Dr Carmen Solana, Department of Stomatology, School of Dentistry, Campus de Cartuja, Colegio Máximo s/n, E-18071 Granada, Spain.
E-mail address: cmsolana4@gmail.com
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Recently, a triple antibiotic solution (TAS) with minocycline, metronidazole, and ciprofloxacin used at 1 mg/mL demonstrated efficacy comparable with a calcium hydroxide/chlorhexidine paste as interappointment medication to control the infection of teeth with apical periodontitis[20]. At our knowledge, the antimicrobial activity of a double antibiotic solution (DAS) (metronidazole and ciprofloxacin) or diclofenac solutions (DCSs) at different concentrations still remains unknown. Therefore, the aim of this study was to compare the antibiofilm effects of TAS; DAS; and 5%, 2.5% and 1.25% DCSs against *E. faecalis* biofilm.

MATERIALS AND METHODS

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

Bacterial Strain and Antimicrobial Solutions

The bacteria used in this study was *E. faecalis* ATCC 29212 (American Type Culture Collection, Manassas, VA) 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 1×10^7 colony-forming units (CFUs)/mL.

All of the test solutions were of Spanish Pharmacopeia grade and master formulation consisting of a TAS (minocycline, metronidazole, and ciprofloxacin) at a concentration of 1 mg/mL; a DAS (metronidazole and ciprofloxacin) at a concentration of 1 mg/mL; and DCSs 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 protocol[21]. Briefly, 42 freshly extracted noncarious 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 2 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 $4 \times 4 \times 0.7$ mm (width × length × height) specimens. The smear layer formed during the preparation of the specimens was removed with 17% EDTA for 5 minutes. Afterward, 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 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 CFU bacterial determinations and confocal laser scanning microscopy. Sixty infected dentin specimens were used for CFU 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 1 mg/mL; group 2, DAS 1 mg/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. Then, 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 10^1–10^5 of suspension-recovered biofilms were made, and 10-μL aliquots were seeded onto BHI agar and incubated for 48 hours at 37°C. The results were expressed as the reduction percentage of CFUs calculated as follows: $100 - (\text{mean CFU}_{\text{antimicrobial solution}} \times 100/\text{mean CFU}_{\text{control}})$.

For confocal laser scanning microscopic evaluation, 24 infected dentin specimens were randomly divided into 6 groups ($n = 4$/group) according to the solutions described previously (Table 1). The specimens were washed with saline solution for 1 minute and then submerged in the antimicrobial solutions for 5 minutes. After the contact period, the samples were rinsed again with 0.9% saline solution and stained with the respective dyes (Syto 9/propidium iodide [PI] and LIVE/DEAD BacLight; Invitrogen, Eugene, OR) as previously reported[22]. After staining the samples with a 1:1 mixture of Syto 9 and PI for 15 minutes, they were rinsed with saline solution, mounted on a 60 I-Dish (Ibidi, Martinsried, Germany) with the mounting oil (BacLight, Invitrogen), and directly observed using an inverted confocal laser scanning microscope (Leica TCS-SP5 II; Leica Microsystems, Mannheim, Germany). The respective absorption and emission wavelengths were 494/518 nm for Syto 9 and 536/617 nm for PI. Five microscopic confocal volumes from random areas were acquired

TABLE 1 - The Reduction Percentage of Colony-forming Units (CFUs), Log_{10} Biovolume (μm^3), and Green Percentage (%) after 5 Minutes of Contact with Irrigating Solutions on *Enterococcus faecalis* Biofilms

Solutions	% Reduction CFUs	Total biovolume $\log_{10}$	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 are presented as means (standard deviation). A global comparison between groups determined by the analysis of variance test with the Welch correction ($P < .001$). The same superscript letter read vertically indicates differences that were not statistically significant according to the Games-Howell test.
*Values of CFUs: mean (standard deviation) = 144550 (88237).

from each sample using the 40× 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 (http://www.bioimageL.com/get_bioimage_L) was used[23]. The variables evaluated in each group were the $\log_{10}$ 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 (IBM Corp, Armonk, NY). The $\log_{10}$ total biovolume followed a Gauss distribution using the Kolmogorov-Smirnov test. The percent reduction of CFUs and the green percentage were normalized by means of the Anscombe transformation. For all variables, the Levene test showed significant differences of variances among groups. Global comparisons were performed using an analysis of variance test with the Welch 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 2 antibiotic solutions (TAS and DAS), whereas the DCS showed a concentration-dependent effect.

For the antimicrobial test with confocal laser scanning microscopy, a total of 120 operative fields (3-dimensional stacks) were evaluated. The $\log_{10}$ total biovolume in all groups showed a scarce (1.39–1.02) but significant reduction with respect to the control, and all the groups had very similar values (Table 1). The cell viability of the control group was 98.11%, whereas concentrations of 5% and 2.5% DCSs showed the lowest viable cell percentages. The TAS and DAS groups 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 determinantal for the healing of teeth with periapical periodontitis[24,25].

The emergence of antibiotic resistance is a major concern, which has led to the search for new alternative approaches to disinfection of the root canal, including the use of NSAIDs. In this context, we tested the antimicrobial activity of DCSs and compared them with DASs and TASs to evaluate their potential usefulness as intracanal medication and/or final irrigants in root canal treatment.

To test the antibiofilm activity of the new solutions, a monospecies biofilm was selected. Although a polymicrobial biofilm would be more appropriate to determine their efficacy

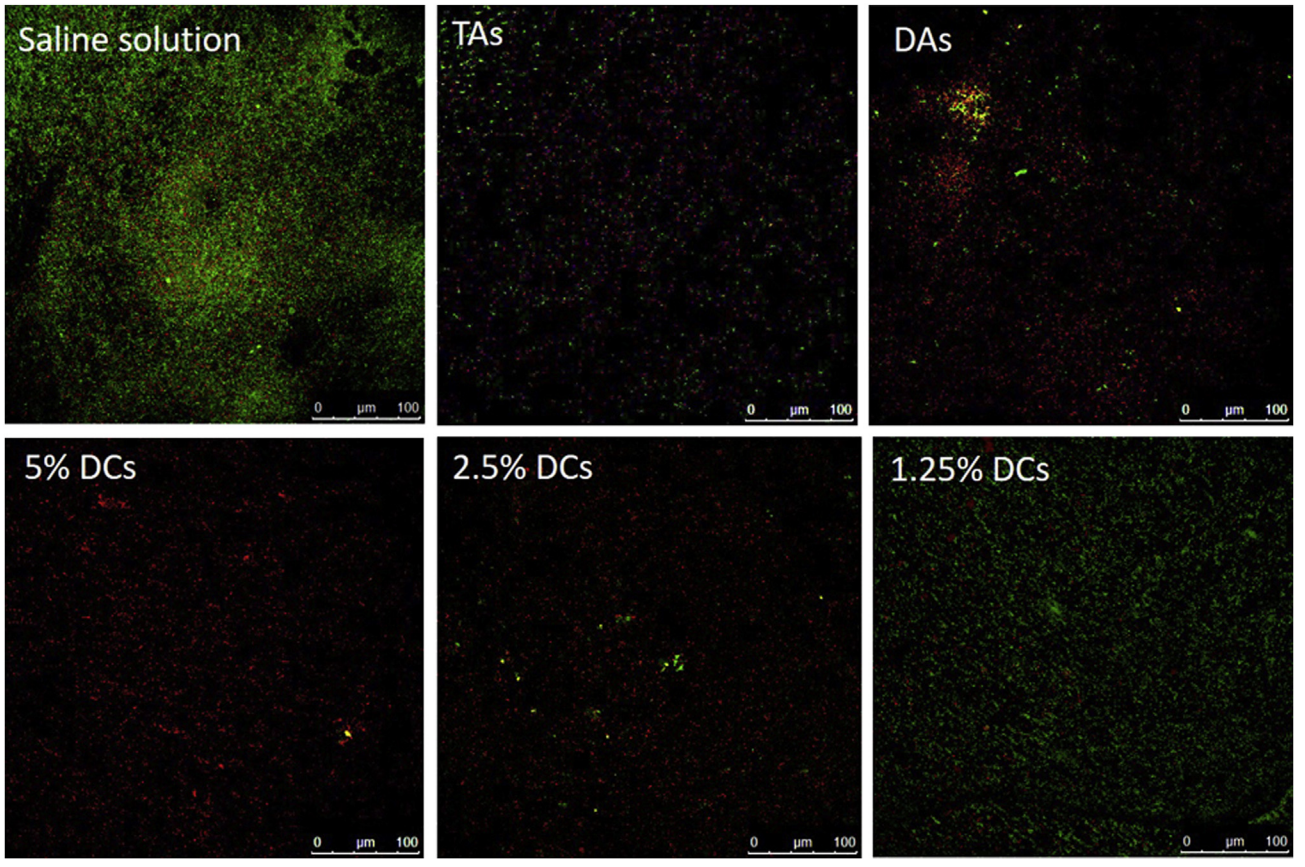

FIGURE 1 – Representative confocal laser scanning microscopic images of the different study groups.

under a clinical reality approach[22], in this initial study we used a bacteria held to be the reference in this type of work (*E. faecalis* ATCC 29212) so that comparison with the results of other authors would be more valid and reliable.

In turn, a $Ca(OH)_2$ paste was not considered for control given that the vehicle used to test the compounds was in the form of a solution, allowing for exact adjustment of the concentration and easy diffusion while not requiring subsequent elimination, as would be the case with a paste presentation. It is known that a TAS having the same concentration and composition as used in this study shows effectiveness similar to that of a calcium hydroxide paste in root canal disinfection[20]. In addition, although the interappointment medication of choice is $Ca(OH)_2$, given its antimicrobial and biological effects[26,27], its use to improve root canal disinfection continues to be controversial[20,28,29].

To enhance antimicrobial effectiveness, antibiotics or NSAIDs may be added; these have shown good results[15,17–19]. 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 paste[19]. 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 tissues[30].

Meanwhile, a mixture of antibiotics in a paste form (TAP and DAP) is widely used as intracanal medication in regenerative endodontic procedures[5,11]. In recent times, a TAP concentration of 1 mg/mL has been clinically recommended to avoid toxic effects on the stem cells of the apical papilla[31].

Few studies have evaluated the use of antibiotics as irrigating solutions in root canal treatment[20,32]. Jain et al[32] compared *in vivo* the antimicrobial efficacy of sterile saline; chlorhexidine solution; and a TAS of 1% ornidazole, 1% ciprofloxacin, and 1% tetracycline. The results showed similar percentages 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%). One clinical study showed that the application of an interappointment medication with TAS (1 mg/mL) significantly improved root canal disinfection, providing results comparable with a calcium hydroxide/chlorhexidine paste[20]. A recent randomized controlled clinical study[33] 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 widely used NSAID for the treatment of pain and inflammation. Its mechanism of action is through the inhibition of cyclooxygenase-2, reducing angiogenesis and inducing the process of programmed cell death[34]. However, different options have been suggested for its antibacterial action, including the inhibition of bacterial DNA synthesis[13], impairment of membrane activity[15], antiplasmid activity[17], alteration in gene-encoding transport/binding proteins, and down-regulation of efflux pumps[35].

The results of the present study showed higher or similar reduction percentages of CFUs with DCSs as opposed to TAS and DAS. The greatest reduction was obtained by 5% DCS followed by 2.5% DCS, whereas 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% DCSs showed the lowest viability values (5.01 and 11.66%, respectively); these values were 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 sodium hypochlorite during instrumentation.

Taking into account that DCSs have greater antimicrobial effects than TASs and DASs, as observed in the present study, it would seem reasonable to consider them a valid alternative for controlling the 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 formulations[36]. The anti-inflammatory topical action of NSAIDs could help reduce postoperative pain after endodontic treatment[37].

Given that promising results were obtained here, future research involving more complex biofilm should be conducted to evaluate the activity of DCs and other compounds in different vehicles before they can be recommended in a clinical protocol.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Carmen María Ferrer-Luque: Conceptualization, Methodology, Writing - review & editing. **Pilar Baca:** Formal analysis, Supervision. **Carmen Solana:** Investigation, Writing - original draft. **Alberto Rodríguez-Archilla:** Software, Validation. **María Teresa Arias-Moliz:** Software, Validation. **Matilde Ruiz-Linares:** Conceptualization, Methodology, Writing- review & editing.

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The authors deny any conflicts of interest related to this study.

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