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Antibacterial effectiveness of diluted preparations of intracanal medicaments used in regenerative endodontic treatment on dentin infected by bacterial biofilm: An *ex vivo* investigation

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ABSTRACT

Background: Conventional drug mixtures used in regenerative endodontic procedures have a toxic effect and no consensus has been reached about their best composition and concentration. Therefore, the aim of this study was to determine minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum biofilm inhibitory concentration (MBIC) of the antimicrobial preparations and to compare their antimicrobial efficacy on bovine dentin infected by *Enterococcus faecalis*.

Materials and Methods: For this original *ex vivo* investigation, prepared concentrations (MIC, MBC, and MBIC) of triple antibiotic paste (TAP), double antibiotic paste (DAP), modified triple antibiotic paste (MTAP)-1, MTAP2, co-amoxiclav, and calcium hydroxide (CH) were added to the prepared bovine dentin blocks (which incubated in *E. faecalis* suspension previously) and incubated for 3 days. The samples were subsequently prepared for culture and CFU counts. Statistical analysis of data was carried out using one-way analysis of variance and *post hoc* tests. The statistical power was set at $P < 0.05$.

Results: All medicament groups significantly showed an antimicrobial efficacy compared with negative control (without antibiotic) ($P < 0.001$). TAP, DAP, co-amoxiclav, and CH (at its MBC value) were significantly capable of eliminating *E. faecalis* biofilm and showed no significant difference in comparison with positive control (complete biofilm removal) ($P < 0.05$).

Conclusion: TAP, DAP, co-amoxiclav, and CH (at its MBC value) could effectively eliminate biofilm bacteria on the dentin surface. Antimicrobial efficacy of other medicaments containing cefaclor or clindamycin was limited.

Key Words: Biofilms, *Enterococcus faecalis*, regenerative endodontics

INTRODUCTION

Pulpal necrosis and infection of the root canal system are among common complications following traumatic injuries to immature permanent teeth. Successful management of traumatized immature teeth with pulpal necrosis is contemplated a considerable clinical challenge due to the presence of thin and diverging dentinal walls and open apices.

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Recently, regenerative endodontic procedures (REPs) have drawn attention for treating infected immature necrotic teeth, and in some cases, it is considered as the most favorable treatment option. Contrary to previous treatments (long-term intracanal use of calcium hydroxide (CH)[1] and single-visit treatment using apical plug of MTA),[2] continued root development can take place through creating a sterile environment and promoting pulpal tissue regeneration, resulting in improved prognosis in the management of necrotic immature teeth.[3]

Because of the weakness of dentinal walls in such teeth, mechanical debridement is of limited value and more emphasis is placed on the use of chemical irrigants and intracanal medicaments. Although CH had historically been used in REPs, use of antibiotics was taken into consideration as an alternative because of two major CH limitations, including its negative effect on biomechanical properties of dentin[4] and lack of favorable effect on recalcitrant infections.[5]

Because root canal infections are commonly polymicrobial in nature, a mixture of some antibiotics is used to remove intracanal microorganisms. In many of the studies, this antibiotic mixture has been used as a 1000 mg/ml paste. However, a number of studies declared that medicaments with high concentrations might have toxic effects on stem cells compromising regeneration process.[6,7] According to the pivotal role of stem cells in success of REPs, establishing a balance between the concentration of the drug used for adequate disinfection of the root canal space and its cellular toxicity is crucial. According to AAE recommendations, 1–5 mg/ml concentration of the triple antibiotic paste (TAP) is considered favorable.[8] No need to mention that in the scope of regenerative endodontics, the available recommendations are not supported by a high level of evidence and the protocols have been constantly revised based on the knowledge gained mainly through preclinical or not controlled clinical studies.

For the assessment of the effectiveness of antibacterial preparations, biofilm inhibition capability is considered an important criterion. Biofilm formation is one of the most important factors in bacterial resistance of *Enterococcus faecalis* species and because the planktonic form of this microorganism is not able to reproduce the clinical conditions of infected root canals, *E. faecalis* biofilms have been more focused in recent studies.[9]

To date, limited studies have focused on antimicrobial potency of commonly used preparations in REPs using minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum biofilm inhibitory concentration (MBIC) values in equalized clinical conditions.[10-12] As it is likely that high concentrations of drug compounds become highly diluted after exposure to tissue fluids, one of the objectives of this study was to investigate whether these low dilutions (near MIC, MBC, and MBIC) are still effective and whether they can be useful for manufacturing new slow-release drug-containing scaffolds.

Therefore, the aim of this study was to evaluate the antibiofilm effectiveness of all commonly used and proposed drugs at all three concentrations against *E. faecalis* as a resistant bacterial species on bovine dentin.

MATERIALS AND METHODS

Bacterial species and culture medium

In this original *ex vivo* study, a standard species of *E. faecalis* (ATCC29212) was used. Initially, a 24-h culture was prepared on a brain–heart infusion (BHI) agar plate (Merck, Darmstadt, Germany) to pass the bacteria from the stationary state. After 24 h, 0.5 McFarland standard of the bacteria was prepared in Mueller-Hinton Broth (MHB) medium (Merck, Darmstadt, Germany). The 0.5 McFarland turbidity (containing 10^8 bacteria) was confirmed at the wavelength of 625 nm (between 0.08 and 0.11). Afterward, the 0.5 McFarland was diluted to a 1:100 ratio to determine the concentrations used for the study.

Preparation of the drug primary stock solutions

Antibiotic stock solutions were prepared by dissolving equal portions of United States Pharmacopeia (USP)-grade antibiotic powders (Sigma-Aldrich Chemie GmbH, Germany) in distilled water. CH solution was prepared by stirring CH powder (Golchai Co., Iran) in distilled water [Table 1]. More detailed explanations are available in the Supplementary File 1.

Stage I: Determination of minimum inhibitory concentration, minimum bactericidal concentration, and minimum biofilm inhibitory concentration values of the antimicrobial preparations

The method used for the determination of MIC, MBC, and MBIC values was the same used by Sabrah *et al*.[12]

Table 1: Test medicaments' compositions and concentrations

Medicament name	Compositions (equal portions incorporated)	Primary stock solution concentration
TAP	Metronidazole*, ciprofloxacin*, minocycline*	$\frac{10\,mg}{1\,ml}$
DAP	Metronidazole*, ciprofloxacin*	$\frac{10\,mg}{1\,ml}$
MTAP1	Metronidazole*, ciprofloxacin*, cefaclor*	$\frac{10\,mg}{1\,ml}$
MTAP2	Metronidazole*, ciprofloxacin*, clindamycin*	$\frac{10\,mg}{1\,ml}$
Co-amoxiclav	Co-amoxiclav*	$\frac{10\,mg}{1\,ml}$
Calcium hydroxide	Calcium hydroxide**	$\frac{16\,mg}{1\,ml}$

*Sigma-Aldrich Chemie GmbH, Germany, **Golchai Co., Iran. TAP: Triple antibiotic paste; DAP: Double antibiotic paste; MTAP1: Modified TAP-1; MTAP2: Modified TAP-2

Minimum inhibitory concentration determination

After preparation of the drug primary stock solutions, MIC determination was carried out through 2-fold dilution method in a triplicate fashion. In summary, *E. faecalis* culture was placed in 96-well plates (SPL, SPL Life Science Co., Korea) containing MHB culture medium, exposed to 1:10, 1:20, 1:40, 1:80, 1:160, etc., dilutions of drug stock solutions and incubated for 24 h at 37°C. After 24 h, the plates were read at the wavelength of 540 nm by ELISA reader (Anthos 2020, Biochrom Co., UK). MIC was determined as the lowest possible concentration that could cause turbidity of ≤0.05 [Table 2].[13]

Minimum bactericidal concentration determination

After reading MIC values, a 10 µL sample was taken from the wells of the MIC row as well as those of the upper rows to be cultured in BHI agar medium. All plates were incubated at 37°C for another 24 h. Afterward, BHI agar culture plates were removed from the incubator and were evaluated for growth/nongrowth of bacterial colonies. The plate with nongrowth or elimination of ≥99.9% of bacteria was considered as the antibiotic concentration with bactericidal effect [Table 2].[14]

Minimum biofilm inhibitory concentration determination

To determine the MBIC values, 1:100 suspension of 0.5 McFarland was prepared. Using flat-bottom 96-well plates (SPL, SPL Life Science Co., Korea). *E. faecalis* culture was exposed to 1:10, 1:20, 1:40, 1:80, 1:60, etc., dilutions of the drug stock solutions in wells containing MHB culture medium for 24 h.

Table 2: Minimum inhibitory concentration, minimum bactericidal concentration, and minimum biofilm inhibitory concentration values (mg/mL) of test drugs

Test drugs	Concentrations (mg/mL)		
	MIC	MBC	MBIC
TAP	0.00006	0.00195	0.00195
DAP	0.007	0.031	0.156
MTAP1	0.00195	0.00195	0.0039
MTAP2	0.000976	0.00781	0.25
Co-amoxiclav	0.000976	0.00195	0.00195
$Ca(OH)_2$	0.2	16	Not defined

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; MBIC: Minimum biofilm inhibitory concentration; TAP: Triple antibiotic paste; DAP: Double antibiotic paste; MTAP1: Modified TAP-1; MTAP2: Modified TAP-2

Then, the wells were initially rinsed twice with sterile saline and subsequently fixed with 10% formaldehyde solution. After two more times of sterile saline rinse, they were stained with 0.5% filtered crystal violet for 30 min. Following three times of sterile saline rinse, 200 µL 2-propanol was added and the plates were diluted to 1:5 and read by ELISA reader at 490 nm wavelength after 1 h. The MBIC values were determined through comparison of the resultant turbidity with control. Because, in CH samples, biofilm formation was observed in all rows, MBIC was not defined [Table 2].

Preparation of dentin samples

A total of 126 bovine teeth were washed, and the soft tissue remains was removed. After crown sectioning using a diamond disk, the teeth were sectioned along the buccolingual plane, obtaining two halves for

dentin specimen (4 mm × 4 mm × 1 mm) preparation. The cementum was removed, and the specimens were wet-finished with SiC papers. The samples were initially placed in 2.5% sodium hypochlorite ultrasonic bath followed by 17% ethylenediaminetetraacetic acid, each for 3 min to remove the smear layer. The samples were then rinsed with sterile saline solution for 10 min before being sterilized by autoclave.[15] Afterward, the dentin samples were placed in 24-well plates with their predentin facing upward. 500 µL of bacterial suspension and 500 µL of BHI medium were added and the plates were placed in a shaker incubator for 3 days. During this period, the medium was not changed to ensure biofilm formation and prevent its probable overgrowth.

The samples were subsequently rinsed twice with 0.1 M phosphate-buffered saline (PBS) (pH = 7.4) for 1 min each time. The samples were then placed in another 24-well plates each containing 1 mL PBS with antibiotics in their MIC, MBC, and MBIC dilutions. Six dentin samples were allocated to each antibiotic concentration. Positive (n = 6) and negative control (n = 6) groups were also considered. The plates were incubated for 3 days and then rinsed twice with sterile saline for 1 min each time. Each sample was entered in a vial containing 1 mL of 0.9% saline solution, sonicated for 20s, and vortexed for 30s. Eventually, the contents of each vial were diluted to 1:10, and 20 µL was added to BHI blood agar for colony counts. After 24-h incubation, colony counts and calculation of CFU was carried out.

Scanning electron microscopic evaluation

Among all dentin samples, 6 blocks were subjected to scanning electron microscopic (SEM) evaluation; 2 samples were evaluated after sterilization to confirm sterility and to evaluate the quality of smear layer removal [Figure 1]; 2 samples were assessed after biofilm formation on the dentin surface to confirm formation of bacterial biofilm [Figure 2]; and the remaining 2 samples were evaluated from positive control group to confirm and evaluate the quality of biofilm removal [Figure 3].

Therefore, the samples were fixed in 2.5% glutaraldehyde for 1 h and then were dehydrated using a series of ethanol concentration (10%, 25%, 50%, 75%, 90%, and 100%) each for 15 min. Then, the samples were gold coated and evaluated by SEM (Zeiss DSM 960A, Germany).

Two-way analysis of variance (ANOVA) was statistically used to evaluate the effects of antibiotic type and concentration (MIC, MBC, and MBIC) against bacterial biofilm. According to the significant interaction of these two factors on each other, the relationship of the three antibiotic concentrations with the drug antibacterial activity as well as the comparison of all antibiotic types in each concentration was carried out using one-way ANOVA and *post hoc* tests. The statistical significance was considered as <0.05 ($P < 0.05$).

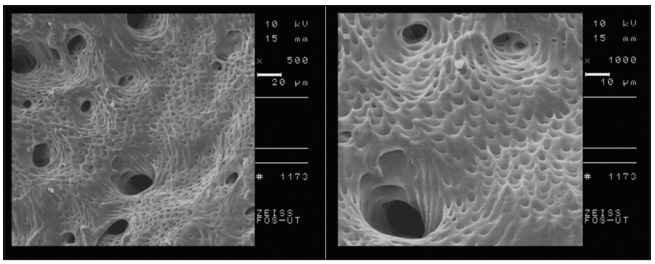

Figure 1: Scanning electron microscopic evaluation of dentin samples to confirm the sterilization and smear layer removal processes.

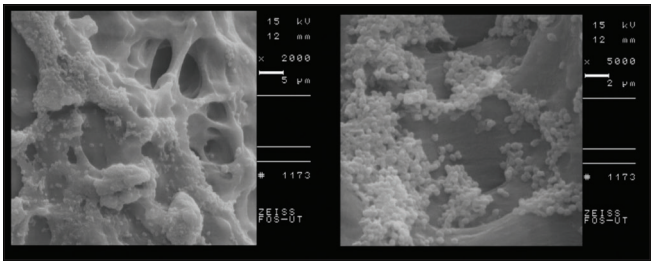

Figure 2: Scanning electron microscopic evaluation of dentin samples to confirm bacterial biofilm formation.

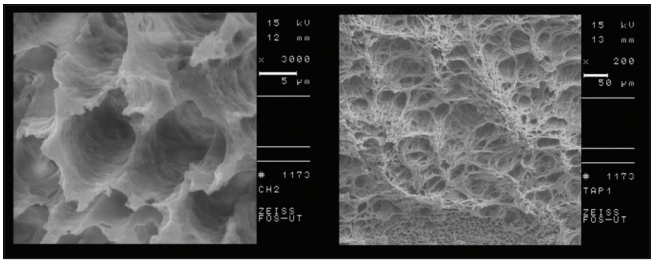

Figure 3: Scanning electron microscopic evaluation of dentin samples in positive control group (10 mg/mL triple antibiotic paste) for confirmation and quality assessment of biofilm removal.

RESULTS

SEM evaluation after sterilization revealed complete removal of microorganisms and smear layer. Electron micrographs after biofilm formation showed the presence of *E. faecalis* biofilm, so that *E. faecalis* cocci were morphologically detected and confirmed

on dentin surface. Eventually, scanning electron micrographs taken from the positive control group revealed effective removal of biofilm at the used concentration (10 mg/mL). Descriptive statistics pertaining to antimicrobial activity of antibiotic preparations at their MIC, MBC, and MBIC are disclosed in Table 3.

The results of this study showed that all drug preparations had a significant antibacterial effect in comparison with negative control ($P < 0.001$). However, only TAP, DAP, co-amoxiclav, and CH (at its MBC value) were effective in the removal of *E. faecalis* biofilm and did not have any significant difference with positive control group at their ($P > 0.05$) [Figure 4]. Although modified triple antibiotic paste (MTAP)-1, MTAP2, and CH (at its MIC value) revealed a significant antibacterial effect, they were unable to completely remove the bacterial biofilm [Figure 4].

Table 3: The means and standard deviations of antibacterial activity of antibiotic preparations at their minimum inhibitory concentration, minimum bactericidal concentration, and minimum biofilm inhibitory concentration

Antibiotic preparation	MIC	MBC	MBIC
TAP	99.83±0.19	99.97±0.08	99.97±0.08
DAP	95.89±3.48	76.14±20.17	94.84±5.14
MTAP1	69.79±9.95	69.79±9.95	85.76±6.73
MTAP2	73.72±4.65	74.13±19.79	86.81±4.90
Co-amoxiclav	84.27±15.11	73.61±22.63	73.61±22.63
Calcium hydroxide	85.59±1.41	100	-

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; MBIC: Minimum biofilm inhibitory concentration; TAP: Triple antibiotic paste; DAP: Double antibiotic paste; MTAP1: Modified TAP-1; MTAP2: Modified TAP-2

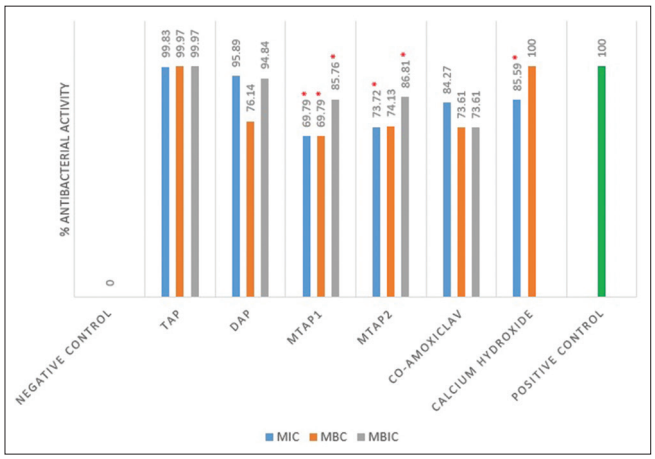

Figure 4: Comparison of antimicrobial activities in different drug preparation at their minimum inhibitory concentration, minimum bactericidal concentration, and minimum biofilm inhibitory concentration with each other and with the positive control group (*$P < 0.05$).

DISCUSSION

Biofilm formation is an outstanding feature of endodontic pathogens, and in fact, apical periodontitis is considered a biofilm-induced disease.[16] During REPs, elimination of intracanal bacteria is relied on chemical disinfection due mainly to thin dentinal walls and mechanical limitations thereof.[17] On the contrary, in conventional root canal treatment (RCT), intracanal debridement is performed chemomechanically, which can serve as a reason why higher success rates are reported in bacterial elimination during conventional RCTs in comparison with REPs.[18]

In recent years, higher concentration of antibacterial agents used in REPs raised a concern of stem cell toxicity.[7] On the other hand, inadequate disinfection of the root canal system can result in a direct stem cell toxicity. Studies have shown that bacterial DNA, lipopolysaccharide, and lipoteichoic acid can activate innate immune reactions,[19,20] resulting in degeneration of the stem cells.[21] Therefore, determination and use of minimal concentrations of drug preparations that can be safe for regional stem cells and are effective in elimination of bacteria from the root canal system cannot be overlooked in successful REPs

In this study, *E. faecalis* was used, which is the most important bacterial species in therapy-resistant endodontic infections and persistent endodontic disease conditions.[22] REPs have also been proposed as an alternative treatment to manage teeth with persistent endodontic disease showing promising results.[23] Therefore, counteracting resistant microorganism is a considerable challenge in REPs. According to the guidelines by the Clinical and Laboratory Standards Institute and some other investigations,[12,24] *E. faecalis* ATCC29212 was used to determine the MIC values using dilution method.[25]

This investigation aimed at bacterial biofilm elimination of some antimicrobial preparations at their MIC, MBC, and MBIC values in a bovine dentin model. Bovine dentin can be a potential alternative to human dentin in *ex vivo* studies because of its greatest similarity among mammalian species to human dentin[26] as well as the possibility of producing larger and more uniform specimens. Although this investigation was also feasible on polystyrene plates or hydroxyapatite discs,[27] dentin blocks were used for biofilm formation

because of possible influence of dentin on antimicrobial properties of intracanal medicaments and[28] similarity with clinical conditions. Sabrah *et al*.[12] used microtiter plate method to determine minimal concentrations of antimicrobial agents and eventually evaluated the direct effect of drugs on bacterial biofilm. The reason for the numeral difference between the concentrations of the present study and Sabrah's can be related to the difference in the drug powders used specifically about CH. In the current study, drug powders were obtained from Sigma-Aldrich (Chemie GMBH, Germany) for the preparation of the TAP, DAP, MTAP1, MTAP2, and co-amoxiclav, whereas in the study by Sabrah *et al*., drug powders were obtained from CHAMPS Medical Company to prepare TAP and DAP. In the current study, CH (Golchai Co., Iran) was used as the CH preparation, whereas Sabrah *et al*. used UltraCal (UltraCal XS; Ultradent, South Jordan, UT, USA). In addition, Sabrah *et al*. did not evaluate the effects of the resultant drug concentrations on dentin surface biofilms. They also used polystyrene plates because of the complexity of root canal system and possible contaminations while direct sampling from the root canal system.[12] According to our results, although all drug preparations could effectively inhibit and diminish biofilm formation in polystyrene plates, TAP, DAP, co-amoxiclav, and CH (in high concentration of MBC) were significantly effective in counteracting the 3-day biofilm formed on dentin surface.

Contrary to the differences, both studies showed that TAP, DAP, and CH were effective in much lower concentrations in comparison with those proposed by AAE clinical considerations for a regenerative procedure.[8] Similar results have been reported by Hoshino[29] and Chuensombat,[30] although different methods have been used. On the contrary, Tagelsir *et al*.[31] questioned the use of 0.1 mg/ml DAP and disclosed that it had a limited antibiofilm efficacy. This can be attributed to the use of 3-week biofilms used by Tagelsir *et al*.; also, in another study by Sabrah *et al*.,[24] 0.125 mg/ml DAP (close to the concentration recommended by the current study) showed a significant antibacterial effect. However, in a study by Latham *et al*.,[21] inadequate effects of 0.1 mg/ml DAP and TAP were stated. The difference can be related to the use of dental models and 0.1, 1, and 10 mg/ml concentrations for TAP, DAP, and UltraCal XS used as CH. In the current study, all three concentrations of DAP could significantly remove bacteria within the biofilm.

Several studies have focused on the effect of antibiotic-containing scaffolds on bacterial biofilm,[15,32,33] in which drug concentrations were different from one to another. In these studies, certain amounts of drug powders were added to a polymer solution in a certain weight ratio and then nanofibers containing different weight percentage amounts of drugs were prepared. Furthermore, in these studies, there is no agreement on the amount or weight percentage of the antibiotics used and variable drug preparation ratios have been investigated as nanofibers.

Studies concerning TAP, DAP, and CH are numerous, but the mixture of ciprofloxacin–metronidazole–cefaclor and also ciprofloxacin–metronidazole–clindamycin has scarcely been investigated and the studies are at the level of case reports.[34,35] Recently, Karczewski *et al*.[36] introduced a nanofiber containing a modified triple mixture with (35 wt.%) clindamycin as an alternative to a triple mixture containing minocycline. The mixture had a significant antimicrobial efficacy, no cytotoxicity, or tooth discoloration. In the current study, although the modified mixtures containing cefaclor and clindamycin resulted in a decrease or inhibition of bacterial biofilm, it was considered significant compared with positive control and complete removal of biofilm and showed a limited antibiofilm performance in the range of the investigated concentrations. This difference can be attributed to variable methods used in two studies.

Our results showed that co-amoxiclav significantly eliminated the biofilm bacteria in lower concentrations. This finding is in accordance with Kaur *et al*.[37] and AlSaeed *et al*.[11] However, Ruparel *et al*.[7] reported higher cell toxicity in compositions containing augmentin. Hence, prior to recommendation, the use of a certain antimicrobial agent considering other aspects including safety and stem cell compatibility is mandatory alongside with its antibacterial efficacy.

CONCLUSION

Regarding the results of the current investigation, it can be stated that TAP even in lower concentrations can be considered as an effective antimicrobial preparation in eliminating biofilm bacteria from the dentin surface. DAP was also comparable with TAP in terms of its potency in the elimination of biofilm bacteria. Other investigated drug preparations were

of limited value, especially when REPs are indicated for teeth with failed initial RCTs or in cases of *E. faecalis* biofilm contaminations. More extensive investigations are recommended to evaluate the efficacy of minimum antimicrobial values of different intracanal medicaments on polymicrobial biofilms and their application and influence in antibiotic-releasing scaffolds.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial, or nonfinancial in this article.

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SUPPLEMENTARY FILE 1

PREPARATION OF THE DRUG PRIMARY STOCK SOLUTIONS

1. Triple antibiotic paste (TAP): 16.5 mg of each drug was used to prepare TAP stock solution, i.e., metronidazole, ciprofloxacin, and minocycline (Sigma-Aldrich Chemie GmbH, Germany) were carefully weighed using a technical scale (Sartorius BP211D Analytical Balances, Germany) to prepare a total of 50 mg drug mixture. Then, the mixture was dissolved in 5 mL distilled water to create a $\frac{50\ mg}{5\ ml} = \frac{10\ mg}{1\ ml}$ concentration of the stock solution
2. DAP: 25 mg of each metronidazole and ciprofloxacin (Sigma-Aldrich Chemie GmbH, Germany) was measured and a total of 50 mg drug mixture was dissolved in 5 mL distilled water to make a $\frac{10\ mg}{1\ ml}$ concentration of the drug stock solution
3. Modified triple antibiotic paste 1: This mixture contained metronidazole, ciprofloxacin and cefaclor (Sigma-Aldrich Chemie GmbH, Germany). 16.5 mg of each antibiotic powder was weighed and a total of 50 mg drug mixture was dissolved in 5 mL distilled water to create a $\frac{10\ mg}{1\ ml}$ concentration of the primary stock solution
4. Modified triple antibiotic paste 2: This medicament contained metronidazole, ciprofloxacin and clindamycin (Sigma-Aldrich Chemie GmbH, Germany). 16.5 mg of each drug was weighed and a total of 50 mg drug mixture was dissolved in 5 mL distilled water to create a $\frac{10\ mg}{1\ ml}$ concentration of the primary stock solution
5. Co-amoxiclav: 10 mg of co-amoxiclav (Sigma-Aldrich Chemie GmbH, Germany) was dissolved in 1 mL distilled water to create a $\frac{10\ mg}{1\ ml}$ concentration of the primary stock solution
6. Calcium hydroxide (CH): 16 mg of CH powder (Golchai Co., Iran) was weighed and dissolved in 1 mL distilled water to make a $\frac{16\ mg}{1\ ml}$ concentration of primary stock solution.[1]

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