



ANTIMICROBIAL EFFICACY OF SODIUM HYPOCHLORITE ALONE AND COMBINED WITH DUAL RINSE HEDP AGAINST *ENTEROCOCCUS FAECALIS*: AN *IN VITRO* STUDY

Aleksandra Pecheva¹, Kostadin Georgiev¹, Emiliya Simeonova¹, Elena Boyadzhieva¹, Iliyan Dobrev²

1) Department of Operative dentistry and endodontics, Faculty of Dental Medicine, Medical University of Plovdiv, Bulgaria.

2) Department of Microbiology and Biotechnologies, Faculty of Technology, University of Food Technologies, Plovdiv, Bulgaria.

SUMMARY

Purpose: This *in vitro* study evaluated the antimicrobial effectiveness of 5.25% sodium hypochlorite (NaOCl) alone and combined with the commercially available Dual Rinse HEDP (Medcem, Switzerland), which contains etidronic acid (1-hydroxyethylidene-1,1-diphosphonic acid, HEDP) in powder form, against *Enterococcus faecalis*.

Materials and methods: The study involved preparing bacterial cultures of *Enterococcus faecalis*, which were grown on esculin base agar and Luria-Bertani (LB) agar, then suspended in sterile saline to a concentration of approximately 8×10^8 CFU/ml. These cultures were spread in Petri dishes, followed by punching wells, adding test solutions (Group I – 5.25% NaOCl; Group II – Dual Rinse HEDP dissolved in 5.25% NaOCl; Group III – Dual Rinse HEDP dissolved in ultra-pure water; Group IV – ultra-pure water, control), and incubating at 37°C for 48 hours to assess antimicrobial activity by measuring inhibition zones. The experiment was repeated in six independent experiments ($n = 6$ per group), and the data were analyzed with SPSS using the Shapiro–Wilk test, one-way ANOVA and Tukey's post hoc test.

Results: The largest inhibition zones were recorded for 5.25% NaOCl (17.8 ± 0.9 mm), followed by Dual Rinse HEDP in NaOCl (16.3 ± 1.1 mm) and Dual Rinse HEDP in ultra-pure water (8.5 ± 0.6 mm); no inhibition was observed in the control group. The difference between NaOCl alone and NaOCl combined with HEDP was small (1.5 mm) but statistically significant ($p = 0.027$), and both NaOCl-containing solutions produced significantly larger zones than HEDP in water ($p < 0.001$).

Conclusion: Under the conditions of this agar diffusion model, the addition of HEDP to 5.25% NaOCl resulted in a small but statistically significant reduction of the inhibition zone against *E. faecalis*, while the mixture retained high antimicrobial activity. HEDP alone showed limited antimicrobial activity.

Keywords: Sodium hypochlorite, etidronic acid, HEDP, *Enterococcus faecalis*, antimicrobial activity, endodontic irrigants

INTRODUCTION

Successful root canal treatment depends on the effective eradication of microbes within the complex root system [1]. Apical periodontitis is primarily a polymicrobial infection involving various anaerobic and facultative bacteria. Microorganisms form biofilms within the root canal system, contributing to the development of persistent infections and lesions. One of the most frequently isolated pathogens is *Enterococcus faecalis*—which can survive in nutrient-deprived, high pH, and antimicrobial environments. It is often associated with persistent and secondary endodontic infections, especially in cases of failed root canal therapy [2].

Sodium hypochlorite (NaOCl) remains the gold standard due to its broad-spectrum antimicrobial and tissue-dissolving properties [3]. However, its full potential is only realized when the root canal walls are clean and free of smear layer, which increases the need for a chelator. The most common chelator is EDTA. The major disadvantage of EDTA, however, is significant demineralization and erosion of dentin. It cannot be combined with sodium hypochlorite; in contact, they neutralize each other [4]. This necessitates irrigation with a neutral solution like distilled water or saline between these irrigants, which prolongs the procedure and precludes continuous chelation.

Etidronic acid (1-hydroxyethylidene-1,1-diphosphonic acid, HEDP) is a weak chelator proposed as an alternative to EDTA. According to the manufacturer's instructions (Dual Rinse HEDP, Medcem, Switzerland), it can be mixed with NaOCl, resulting in a stable, biocompatible irrigant solution. The mixture has been proposed to combine antimicrobial activity with smear layer removal

[5]. Some available studies are skeptical about the simultaneous application of both chemicals, as the working lifespan of NaOCl may decrease, resulting in a reduction of its antibiofilm efficacy over time [6].

AIM

The study aimed to experimentally assess the antimicrobial effectiveness of Dual Rinse HEDP with sodium hypochlorite compared to that of sodium hypochlorite alone against a standard laboratory strain of *E. faecalis*.

MATERIALS AND METHODS

Enterococcus faecalis (ATCC 12984), a standard laboratory strain, was chosen as the test microorganism.

The study utilized various media and solutions to facilitate the experiments. These included esculin base agar, Luria Bertani (LB) agar, sterile saline solution (0.9% NaCl), and ultra-pure water filtered through a 0.03 μm membrane filter. The test solutions examined were 5.25% sodium hypochlorite (NaOCl), Dual Rinse HEDP (Medcem, Switzerland) dissolved in 10 ml of 5.25% NaOCl, Dual Rinse HEDP dissolved in 10 ml of ultra-pure water, and ultra-pure water serving as a control group. Both Dual Rinse HEDP solutions were prepared according to the manufacturer's instructions by dissolving the content of one capsule (0.9 g HEDP powder) in 10 ml of the respective liquid (approximately 9% w/v HEDP) and mixing until complete dissolution. The solutions were freshly prepared at room temperature immediately before application and were not stored. For the procedures, sterile Petri dishes approximately 9 cm in diameter were used, along with a digital caliper for measuring diameters. The experiments were conducted within a laminar flow hood or sterile worktop to maintain aseptic conditions. Incubation was carried out at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. Additionally, sterile pipettes, tips, and micropipettes were employed for accurate liquid handling.

The *Enterococcus faecalis* strain was activated by plating from a stock culture onto esculin base agar and LB agar, then incubated at 37°C for 24–48 hours. After growth, the bacteria were maintained in agar slants. To prepare the bacterial suspension, 5 ml of sterile saline was added to each tube, and the bacterial biomass was washed off using sterile technique. The biomass from 2–3 tubes was collected in a sterile central vessel, and the suspension was adjusted to approximately 8×10^8 CFU/ml.

For the preparation of agar Petri dishes, 100 μl of the bacterial suspension was added to sterile dishes with a diameter of 9 cm. This was followed by pouring 18 ml of molten LB agar, cooled to about $45\text{--}50^{\circ}\text{C}$ to prevent

killing the bacteria, while gently rotating the dish to ensure even distribution. The dishes were then incubated at 37°C for 2 hours to facilitate adhesion and uniform microbial distribution. Six cylindrical wells with a diameter of 6 mm were punched into each plate using a sterile punch.

In the application phase, four Petri dishes were used in each experiment, each designated for one of the test solutions. The dishes were labelled according to the test groups: Group I = 5.25% NaOCl, Group II = Dual Rinse HEDP + 5.25% NaOCl, Group III = Dual Rinse HEDP + ultra-pure water, and Group IV = ultra-pure water (control). Six microliters of each test solution were carefully dropped into the six wells of the respective dishes, ensuring the liquid was added centrally without disturbing the well walls. The Petri dishes were then incubated at 37°C for 48 hours (± 2 hours) in a humid environment to prevent drying of the agar.

After incubation, the antimicrobial activity was assessed by measuring the diameter of the inhibition zones (in millimeters) around each well using a digital caliper. For each well, two perpendicular measurements were taken and averaged. The 6-mm diameter of the well itself was not included; thus, the reported values represent only the zone of inhibition surrounding the well, and wells without a clear zone were recorded as 0 mm. The entire experiment was performed six times as independent experiments. Within each experiment, the six wells of each dish were considered technical replicates, and their mean value was used as a single observation. Thus, the statistical unit was the independent experiment ($n = 6$ per group). If present, zones without bacterial growth and any "fusion" effects between zones at close distances were also noted and considered during analysis.

All obtained data were entered into a table and processed with SPSS statistical software (v.25.0, IBM Corp., Armonk, NY, USA). Initially, a test for normality of distribution (Shapiro–Wilk test) was performed. Since no inhibition was observed in any well of the control group (Group IV; zero variance), this group was reported descriptively and was not included in the inferential analysis. When confirming the normal distribution, one-way analysis of variance (One-way ANOVA) was applied to assess the differences between Groups I–III. If a statistically significant difference was found ($p < 0.05$), a post hoc analysis according to Tukey was performed for multiple comparisons between the individual groups. The results are presented as mean $\pm$ standard deviation (SD). Exact p-values are reported, except for values below 0.001. The level of statistical significance was set at $p < 0.05$.

RESULTS

Representative plates from each group are shown in Figures 1–4.

Fig. 1. Sample of 5.25% NaOCl applied – large inhibition zone.



Fig. 2. Sample of Dual Rinse HEDP + 5.25% NaOCl applied – large inhibition zone.



Fig. 3. Sample of Dual Rinse HEDP + ultra-pure water applied – small inhibition zone



Fig. 4. Sample of ultra-pure water applied – no inhibition zone



After 48 hours of incubation at 37°C, clear zones of inhibition were observed around the wells containing the tested substances, except for the control group, where no antimicrobial activity was detected. One-way ANOVA revealed a statistically significant difference among Groups I–III ($F(2,15) = 188.5, p < 0.001$). The largest diameters of inhibition were observed with 5.25% sodium hypochlorite, which demonstrated the most pronounced antimicrobial

activity against *E. faecalis*. The combination of Dual Rinse HEDP and NaOCl produced slightly smaller zones (mean difference 1.5 mm; 95% CI 0.2–2.8 mm), and this difference was statistically significant (Tukey's test, $p = 0.027$) (Table 1).

Only a minimal zone of inhibition was observed in the samples treated with Dual Rinse HEDP dissolved in ul-

tra-pure water (Group III), while no growth inhibition was detected in the control group (Group IV, ultra-pure water). The inhibition zones in Group III were significantly smaller than those in Groups I and II ($p < 0.001$ for both comparisons).

The measured mean diameters of the zones of inhibition (in mm, $\pm$ SD) are presented in Table 1.

Table 1. Diameter of the zone of inhibition of bacterial growth (n = 6 independent experiments per group)

Group	Description	Inhibition zone diameter (mm), mean $\pm$ SD
I	5.25% NaOCl	17.8 $\pm$ 0.9
II	Dual Rinse HEDP + NaOCl (5.25%)	16.3 $\pm$ 1.1
III	Dual Rinse HEDP + ultra-pure water	8.5 $\pm$ 0.6
IV	Control group (ultra-pure water)	0.0 $\pm$ 0.0

Note: Values represent only the zone of inhibition surrounding the well (the 6-mm well diameter is not included). Tukey's post hoc test: I vs II, $p = 0.027$; I vs III and II vs III, $p < 0.001$. Group IV was not included in the inferential analysis because no inhibition was observed in any well.

DISCUSSION

The inhibition zone of the sodium hypochlorite group was the largest, confirming the strong antimicrobial action of this irrigant against *Enterococcus faecalis*. The concentration used in this study was 5.25%, and the results are consistent with those reported in previous studies [7, 8].

The findings of this study underscore the potent antimicrobial efficacy of 5.25% sodium hypochlorite (NaOCl) against *E. faecalis*, as evidenced by the largest inhibition zones observed in this group. These results align with the established understanding of NaOCl as a highly effective endodontic irrigant with substantial antimicrobial properties [7, 8]. When NaOCl was combined with Dual Rinse HEDP, its antimicrobial activity was slightly but statistically significantly reduced (by 1.5 mm, i.e., approximately 8% of the zone diameter). The mixture nevertheless retained high antimicrobial activity, markedly greater than that of HEDP alone. This suggests that the combination may still serve as an effective irrigant, providing both antimicrobial activity and a chelating effect for smear layer removal – the so-called continuous chelation – although the clinical relevance of the small reduction observed in this model remains to be established. Some studies have reported even greater antimicrobial activity with the combination of the two irrigants; notably, these were performed in dentin models, where the removal of the smear layer is essential for disinfecting the dentinal tubules [9].

Conversely, Dual Rinse HEDP dissolved in ultra-pure water exhibited a minimal zone of inhibition, indicating a considerably lower antimicrobial activity compared to NaOCl. As HEDP is a chelator and its function in the chemo-mechanical preparation of the root canal is to

loosen the biofilm attached to the dentinal wall, this result was expected. A 2020 study concluded that Dual Rinse solution was able to inhibit *E. faecalis* growth only when not diluted [10]. In the present study, the dissolved product still produced a small inhibition zone.

Although the difference between NaOCl alone and its combination with HEDP reached statistical significance ($p = 0.027$), its magnitude was small, and the mixture retained a high level of antimicrobial activity under the conditions of this experimental model. This is broadly in line with studies reporting that freshly mixed HEDP does not substantially impair the antimicrobial efficacy of NaOCl [9, 11], whereas a reduction of the antibiofilm efficacy of the mixture over time has also been described [6]. These findings support the potential use of HEDP as a chelating agent combined with NaOCl, provided that the mixture is prepared immediately before use.

This study has several limitations. Only one standard laboratory strain of *Enterococcus faecalis* was tested, and the agar well diffusion method reflects the diffusion of the solutions in agar rather than their activity against biofilms within the root canal system. Therefore, the results should be regarded as preliminary, and further studies using multispecies biofilms, dentin models and clinical isolates are needed.

CONCLUSION

Within the limitations of this *in vitro* study, the addition of Dual Rinse HEDP to 5.25% sodium hypochlorite resulted in a small but statistically significant reduction of the inhibition zone against *Enterococcus faecalis* ($p = 0.027$), while the mixture retained high antimicrobial activity. HEDP alone showed limited antimicrobial activity.

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Address for correspondence:

Aleksandra Pecheva
Department of Operative Dentistry and Endodontics, Faculty of Dental Medicine, Medical University of Plovdiv;
3, Hristo Botev Blvd, Plovdiv, Bulgaria.
E-mail: Aleksandra.pecheva@mu-plovdiv.bg,