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Original Article

Prolonged sodium hypochlorite exposure on root canal disinfection: an *in vitro* study

Exposição prolongada ao NaOCl na desinfecção de canais: um estudo *in vitro*

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Resumo

Objetivo: Este estudo avaliou se a exposição prolongada ao hipoclorito de sódio melhora a eficácia antibacteriana em canais radiculares infectados. **Material e método:** 44 pré-molares inferiores humanos contaminados com *Enterococcus faecalis* foram aleatorizados em quatro grupos de acordo com o protocolo de irrigação: solução salina, solução salina com exposição prolongada, hipoclorito de sódio a 2,5% e hipoclorito de sódio com exposição prolongada. Amostras microbiológicas foram coletadas após preparo químico-mecânico, após fase de exposição prolongada e após irrigação ultrassônica passiva. A redução bacteriana foi quantificada pela contagem de unidades formadoras de colônias. A microscopia eletrônica de varredura foi utilizada para a análise qualitativa. Os dados foram analisados pela análise de variância de duas vias e teste de Bonferroni ($\alpha = 0,05$). **Resultado**: O preparo químico-mecânico com hipoclorito de sódio reduziu significativamente as contagens bacterianas comparado à solução salina ($p < 0,05$). A adição de exposição prolongada não resultou em redução bacteriana adicional ($p > 0,05$). A irrigação ultrassônica passiva produziu uma redução

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adicional nas contagens bacterianas nos grupos de hipoclorito de sódio ($p < 0,05$). A análise por microscopia eletrônica de varredura mostrou diminuição da presença microbiana em todos os grupos, embora microrganismos residuais tenham sido observados. **Conclusão**: O prolongamento do tempo de contato do hipoclorito de sódio por meio do protocolo de exposição prolongada não aumentou a eficácia antibacteriana comparado à irrigação convencional. Estratégias de irrigação que incluem hipoclorito de sódio durante o preparo químico-mecânico combinadas com a ativação do irrigante parecem suficientes para alcançar uma redução bacteriana significativa.

Descritores: Hipoclorito de sódio; irrigantes do canal radicular; *Enterococcus faecalis*; biofilme; preparo de canal radicular.

Abstract

Objective: This study evaluated whether prolonged sodium hypochlorite exposure improves antibacterial efficacy in infected root canals. **Material and method:** 44 human mandibular premolars were contaminated with *Enterococcus faecalis* and randomly assigned to four groups according to the irrigation protocol: saline, saline with prolonged exposure, 2.5% sodium hypochlorite, and sodium hypochlorite with prolonged exposure. Microbiological samples were collected after chemomechanical preparation, after the prolonged exposure phase, and after passive ultrasonic irrigation. Bacterial reduction was quantified by colony-forming unit (CFU) counts. Scanning electron microscopy was used for qualitative analysis. Data were analyzed using two-way analysis of variance and the Bonferroni test ($\alpha = 0.05$). **Result:** Chemomechanical preparation with sodium hypochlorite significantly reduced bacterial counts compared with saline ($p < 0.05$). The addition of prolonged exposure did not result in further bacterial reduction ($p > 0.05$). Passive ultrasonic irrigation produced an additional reduction in bacterial counts in the sodium hypochlorite groups ($p < 0.05$). Scanning electron microscopy

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analysis showed decreased microbial presence in all groups, although residual microorganisms were observed. **Conclusion:** Prolonging sodium hypochlorite contact time through a prolonged exposure protocol did not enhance antibacterial efficacy compared with conventional irrigation. Irrigation strategies that include sodium hypochlorite during chemomechanical preparation combined with irrigant activation appear sufficient to achieve significant bacterial reduction.

Descriptors: Sodium hypochlorite; root canal irrigants; *Enterococcus faecalis*; biofilm; root canal preparation.

INTRODUCTION

Endodontic therapy aims to eliminate microorganisms from the root canal system and prevent reinfection, thereby promoting healing of periapical tissues.[1,2] The success of root canal treatment is directly associated with effective chemomechanical preparation, which combines mechanical instrumentation and chemical irrigation to reduce the intracanal microbial load.[3] However, complete microbial elimination remains challenging because of the anatomical complexity of the root canal system. Structures such as isthmuses, lateral canals, apical ramifications, and dentinal tubules may harbor persistent bacteria and biofilms that are not reached by instruments alone.[4]

Sodium hypochlorite (NaOCl) is widely regarded as the irrigant of choice because of its broad-spectrum antimicrobial activity and unique ability to dissolve organic tissue.[5–7] Nevertheless, its effectiveness in disinfecting the root canal system depends on factors such as the volume, renewal, and contact time of the irrigating solution within the root canal.[8,9] With technological advancements, single-file instrumentation has shortened preparation procedures, potentially reducing the time available for irrigant contact and renewal.[10] Although this has improved clinical efficiency, shorter exposure to NaOCl may compromise its antimicrobial effectiveness, particularly in areas not directly reached by instrumentation.[11,12] Strategies aimed at prolonging the presence of NaOCl within the root canal system may therefore enhance

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disinfection by increasing contact time and facilitating its action within anatomical complexities.[13]

Enterococcus faecalis has been widely used in experimental endodontic biofilm models because of its ability to persist under adverse conditions and its association with persistent endodontic infections.[4,10,14] Although endodontic infections are polymicrobial and the inclusion of other microorganisms could provide a broader representation of the clinical microbiota, *E. faecalis* provides a well-established and standardized model for assessing the antimicrobial effectiveness of endodontic disinfection protocols.[15,16.]

The literature lacks sufficient evidence regarding the influence of prolonged NaOCl contact with dentinal walls and organic tissues on its antimicrobial effectiveness compared with conventional irrigation protocols. Therefore, the present study aimed to evaluate whether an intra-appointment prolonged exposure protocol with NaOCl improves antibacterial efficacy compared with standard irrigation approaches. The null hypothesis was that there would be no difference in antimicrobial effectiveness among the irrigation protocols.

MATERIAL AND METHOD

Sample Design

This study employed an *in vitro* design to investigate the antibacterial performance of a 2.5% sodium hypochlorite irrigating solution during its resting phase, after shaping the main canal in teeth contaminated with *Enterococcus faecalis (*ATCC 51299). The research protocol was duly approved by the Research Ethics Committee of the Federal University of Santa Maria (74542023.7.0000.5346). The determination of the number of teeth per group was based on previous studies that followed a similar methodology.[14]

Selection and preparation of samples

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The methodological approach employed followed the model proposed by Hartmann et al.[14], with specific adaptations. In conducting the research, forty-four mandibular premolars extracted for periodontal reasons were used, each presenting a canal classified as Vertucci type I.[17] These specimens underwent a rigorous cleaning process for the removal of tissue residues, and before the beginning of the laboratory stage, two digital radiographs were taken for each tooth: one in the buccal-lingual direction and one in the mesial-distal direction, in order to determine the root canal anatomy, identify the radiographic apex, measure the root canal curvature, and confirm the presence of a single canal. Only roots that showed moderate curvatures (<10°) and a single rounded canal (buccal-lingual/mesial-distal radiographic ratio < 2:1) were included.[18]

The crowns were sectioned close to the cementoenamel junction, resulting in root segments of 15 mm. The canals were explored with size 15 K-type hand files (Dentsply Sirona, Ballaigues, Switzerland) and a Logic 40.05 rotary file (Easy Equipamentos Odontológicos, Belo Horizonte, Brazil) powered by an X Smart Plus endodontic motor (Dentsply Sirona, Ballaigues, Switzerland), with a working length standardized at 14 mm. Teeth with an apical diameter greater than a size 15 K-file were replaced. Then, the teeth were embedded in a silicone-based putty material (DuoSil Putty Set; Bukwang, Busan, Korea) to prevent visualization of the foramen during root canal instrumentation and to simulate the presence of surrounding tissues. Teflon tape was used to protect the root apex during this procedure. Irrigation was performed with 2.5% sodium hypochlorite solution. Besides standardizing canal diameter, this anatomical enlargement aimed to facilitate root canal contamination after sterilization.

Sterilization control

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To ensure the effectiveness of sterilization, all teeth were subjected to a cycle in a properly calibrated autoclave. One tooth was randomly selected from each group. During this procedure, the canals were filled with sterile 0.9% saline solution, followed by insertion of a paper point into the root canal for 30 seconds, agitating it circumferentially with gentle contact against the canal walls. Each sample was then transferred to a plastic microtube containing 1 mL of saline solution, homogenized, and a 10 µL aliquot was cultured in Mueller-Hinton medium. The samples were incubated at 37°C for 24 hours to assess bacterial growth. The results showed no bacterial growth in the tested samples, corroborating the effectiveness of the sterilization process.

Bacterial Culture and Inoculum Preparation

In this study, the strain used was *E. faecalis* (ATCC 51299), a vancomycin-resistant strain from the American Type Culture Collection. A 24-hour culture was suspended in 0.9% saline solution, generating an inoculum of approximately 10^6 CFU/mL (0.5 on the McFarland scale). Fifteen microliters of the bacterial suspension were inoculated into the root canals, supplemented with sterile BHI to completely fill the canal. The specimens were maintained at 37°C for 21 days, with medium renewal every 48 hours to promote bacterial growth. All procedures were carried out under aseptic conditions in a laminar flow hood, with strict infection control measures.

Treatment Group Classification

After the incubation period, the specimens were randomly assigned, using the website random.org, to one of four groups: Group 1 (G1): protocol with saline solution (SS); Group 2 (G2): protocol with saline solution and a prolonged exposure (SS+PE); Group 3 (G3): conventional treatment protocol with 2.5% sodium hypochlorite irrigating solution (SH); Group

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4 (G4): treatment protocol with a prolonged exposure of the 2.5% sodium hypochlorite irrigating solution (SH+PE).

Chemomechanical preparation protocol in Group 1 (SS)

The canals, previously enlarged to a Logic 40.05 file (Easy Equipamentos Odontológicos, Belo Horizonte, MG, Brazil) and contaminated, were prepared using a Reciproc Blue R50 file (VDW, Munich, Germany) driven by an X Smart Plus endodontic motor (Dentsply Sirona, Ballaigues, Switzerland) in the "Reciproc All" program, followed by refinement with a K-55 hand file (Dentsply Sirona, Ballaigues, Switzerland). The file was passively placed in the canal until its engagement point. At this point, the file was activated and five pecking movements (up and down) were performed, and the file was removed from the canal. The root canal was then irrigated with 5 mL of sterile 0.9% saline solution for 20 s using a 30-gauge endodontic needle (MK Life, Porto Alegre, RS, Brazil). The canal preparation procedure was performed by a single experienced operator. Two teeth per group were selected for quantification of viable colonies after the chemomechanical process, and the collection methodology followed the same protocol described above. This protocol followed Plotino et al.[18], with modifications.

Chemomechanical preparation protocol in Group 2 (SS+PE)

The canals in this group were instrumented and irrigated as in G1, but the prolonged exposure protocol was performed. The shaped canals were filled with saline solution, which remained passively inside the canal for 5 minutes. Using a 30-gauge endodontic needle (MK Life, Porto Alegre, RS, Brazil) positioned 1 mm short of the working length, the canal was irrigated with the corresponding solution for 30 seconds to renew the liquid and then refilled with the solution. These steps were repeated 4 times, totaling 20 mL of saline solution, which

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remained in the canal for a total period of 20 minutes. Two teeth per group were selected for quantification of viable colonies after the chemomechanical procedure, following the same protocol described above.

Chemomechanical preparation protocol in Group 3 (SH)

The canals in this group were instrumented following the same protocol described for G1 (SS), but 2.5% sodium hypochlorite was used as the irrigating solution.

Chemomechanical preparation protocol in Group 4 (SH+PE)

The canals in this group were instrumented, irrigated, and activated as in G2 (SS+PE), but with 2.5% sodium hypochlorite.

Agitation protocol

After completion of chemomechanical preparation in G1 and G3, and execution of the prolonged exposure protocol in G2 and G4, passive ultrasonic irrigation was performed using the corresponding irrigating solution. Through a syringe and a 30-gauge endodontic needle (MK Life, Porto Alegre, RS, Brazil), the irrigating solution was introduced into the canal and agitated for 20 seconds. Subsequently, the solution was renewed, and the cycle was repeated three times. After irrigation procedures, all samples underwent a final rinse with saline solution to remove the test irrigants. Three teeth per group were selected for quantification of viable colonies after the chemomechanical procedure, following the same protocol described above.

Scanning Electron Microscopy (SEM)

Specimen preparation and the imaging technique for SEM-based microbial visualization followed the protocol described by Plotino et al.[16] After the intracanal procedures,

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the teeth were removed from the silicone base and sectioned longitudinally into two halves by creating two grooves with a diamond-coated high-speed bur. Care was taken to avoid penetrating into the canal. The roots were then split with a hammer and chisel, resulting in mesial and distal halves of the root canals. Both halves were prepared for SEM investigation, and the root canal walls of all root sections were observed under SEM to evaluate the cleanliness of the canal walls in the coronal, middle, and apical thirds. The area with the largest amounts of smear layer and debris was photographed for each third of the canal (12 fields per tooth) at ×1000 magnification. The samples were then sputter-coated with gold (Spray Coater 108A; Cressington Scientific Instruments Inc., Watford, UK). The JSM-6360 scanning electron microscope (JEOL, Tokyo, Japan) was used.

Statistical analysis

CFU data were log-transformed and analyzed for statistical variance using two-way analysis of variance (ANOVA), followed by the Bonferroni post-hoc test, with GraphPad Prism software, version 8.0.

RESULT

In the prolonged exposure groups (SS+PE and NaOCl+PE), chemomechanical preparation with sodium hypochlorite resulted in significantly lower bacterial counts compared with saline ($p < 0.05$). However, no significant differences were observed between protocols with and without prolonged exposure for the same irrigant ($p > 0.05$) (Figure 1).

In the groups without prolonged exposure (SS and NaOCl), sodium hypochlorite produced a significantly greater reduction in bacterial load compared with saline after chemomechanical preparation ($p < 0.05$). Passive ultrasonic irrigation resulted in an additional

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significant reduction in bacterial counts in the NaOCl group ($p < 0.05$), whereas no significant change was observed in the saline group ($p > 0.05$).

SEM analysis showed reduced microbial presence across all groups and root canal thirds. However, residual microorganisms were detected in all experimental conditions. Representative images are shown in Figure 2.

DISCUSSION

The present study investigated whether prolonging the intracanal contact time of sodium hypochlorite (NaOCl) through a prolonged exposure protocol enhances antibacterial efficacy. The findings demonstrated that although NaOCl significantly reduced bacterial load compared with saline, extending its contact time did not result in additional antimicrobial benefit. Therefore, the null hypothesis was not rejected.

The antimicrobial effectiveness of NaOCl is well established and is attributed to its ability to release hypochlorous acid and free chlorine, which oxidize and degrade essential bacterial components.[5,6] However, its activity is not solely dependent on contact time. Factors such as concentration, volume, ताप turnover, and especially fluid dynamics play a decisive role in determining its effectiveness within the complex root canal system.[19,20]

In the present study, the absence of additional antibacterial reduction after prolonged exposure may be explained by the rapid consumption of available chlorine upon contact with organic matter. Previous investigations have demonstrated that NaOCl loses its chemical activity over time when not replenished, particularly in the presence of dentin and biofilm substrates.[21,22] Thus, maintaining the irrigant statically inside the canal, even for extended periods, may not sustain its antimicrobial potential. This supports the concept that irrigant refreshment is more relevant than passive retention time, as also highlighted in recent reviews.[8,9]

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Another important aspect relates to the timing of the prolonged exposure phase. In this study, it was performed after chemomechanical preparation, a step known to drastically reduce bacterial load.[10] Consequently, the remaining microbial population may have been located in protected anatomical areas such as dentinal tubules and isthmuses, where irrigant penetration is limited. In such cases, simply increasing contact time may not overcome the physical barriers to disinfection, reinforcing the importance of strategies that improve irrigant penetration rather than duration alone.[23,24]

The additional bacterial reduction observed after passive ultrasonic irrigation corroborates this rationale. Ultrasonic activation enhances acoustic streaming and cavitation, promoting better irrigant penetration into inaccessible areas and disrupting biofilm structure.[20,25] Systematic reviews have consistently shown that activation techniques improve the efficacy of NaOCl compared with syringe irrigation alone.[9,26] Therefore, the present findings reinforce that activation methods are more impactful than prolonged static exposure.

The discrepancy between the present results and those reported by Silva et al.[12], who observed improved disinfection with extended NaOCl exposure, may be attributed to methodological differences. Variations in NaOCl concentration, substrate type (bovine vs. human dentin), and the timing of irrigant application relative to instrumentation can significantly influence outcomes. Additionally, continuous replenishment protocols, as opposed to passive soaking, may maintain higher levels of active chlorine, thereby enhancing antimicrobial activity.

SEM analysis supported the microbiological findings, showing a reduction in microbial presence but not complete elimination. Residual microorganisms were observed in all groups and across all root canal thirds, which is consistent with previous studies demonstrating the persistence of bacteria despite thorough chemomechanical preparation.[15,23] This highlights the

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inherent limitations of current endodontic disinfection protocols and the need for adjunctive strategies.

The use of *E. faecalis* in the present study is also relevant considering its ability to persist under adverse environmental conditions and its recognized association with persistent endodontic infections. This microorganism can survive under nutrient-limited conditions, form biofilms, and persist within the root canal environment, characteristics that may increase its tolerance to antimicrobial procedures.[10,14,27] Therefore, the persistence of viable microorganisms after the tested protocols may also reflect the challenging characteristics of the bacterial model used. Nevertheless, endodontic infections are polymicrobial, and the behavior of *E. faecalis* alone cannot fully represent the microbial diversity and interactions found under clinical conditions.

This study has limitations that should be considered. Although *E. faecalis* provides a standardized and challenging model for evaluating antimicrobial protocols, the use of a mono-species biofilm does not fully reproduce the complexity of polymicrobial endodontic infections, which may exhibit different microbial interactions and susceptibility patterns.[16,27] In addition, the *in vitro* design does not account for host factors, fluid exchange *in vivo*, or immune responses. Future research should focus on multispecies biofilm models, dynamic irrigation systems, and clinically relevant conditions to better understand the role of prolonged NaOCl exposure.

From a clinical perspective, the present findings suggest that increasing NaOCl contact time alone is insufficient to improve disinfection outcomes. Instead, emphasis should be placed on adequate irrigant volume, frequent renewal, and the use of activation techniques to enhance its distribution and effectiveness within the root canal system.

AUTHORS' CONTRIBUTIONS

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Laura Fagundes Dalforno - Conceptualization; Data curation; Investigation; Methodology; Validation; Visualization; Writing - original draft; Writing - review & editing.

Pedro Henrique Fortes Guerim – Conceptualization; Data curation; Investigation; Methodology; Software; Validation; Visualization; Writing - original draft; Writing - review & editing.

Patrícia Kolling Marquezan - Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing - review & editing.

Natália de Freitas Daudt - Methodology; Software; Validation; Writing - review & editing.

Leonardo Quintana Soares Lopes - Methodology; Software; Validation; Writing - review & editing.

Bianca Schlesener Dettmer - Methodology; Conceptualization; Writing - review & editing.

Carlos Alexandre Souza Bier - Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing - review & editing.

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CONFLICTS OF INTERESTS

The authors declare that there is no conflict of interest related to this study.

DATA AVAILABILITY

The contents underlying the research text are included in the manuscript.

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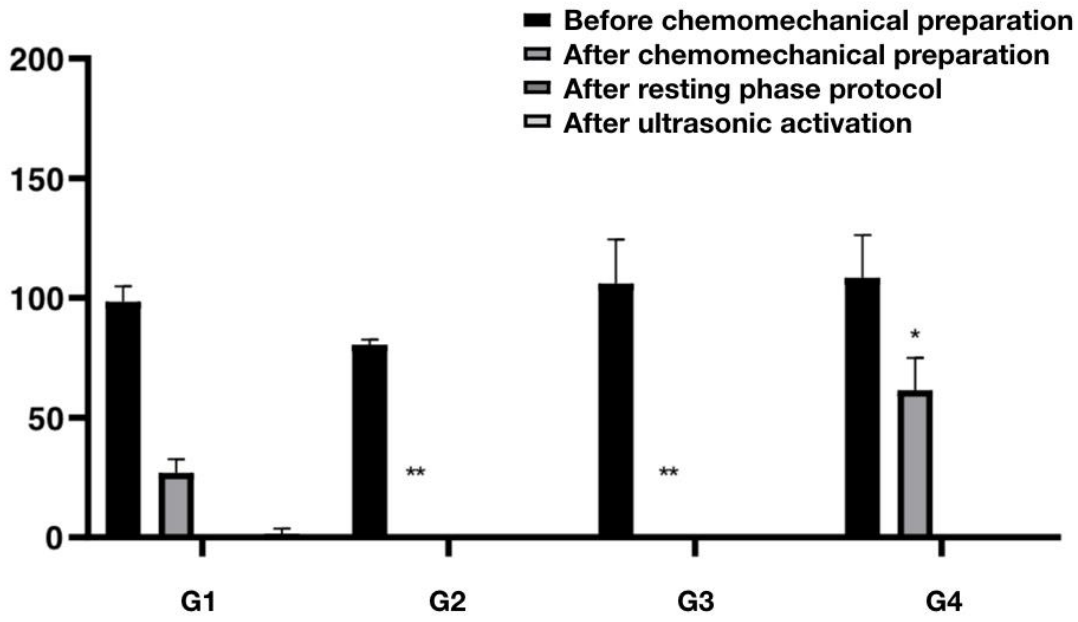


Figure 1. Comparison among groups. Data are expressed as mean ± standard deviation (SD). Analyses were performed using two-way ANOVA followed by Bonferroni's post hoc test. $p < 0.0001$ was considered statistically significant. $p < 0.05$.

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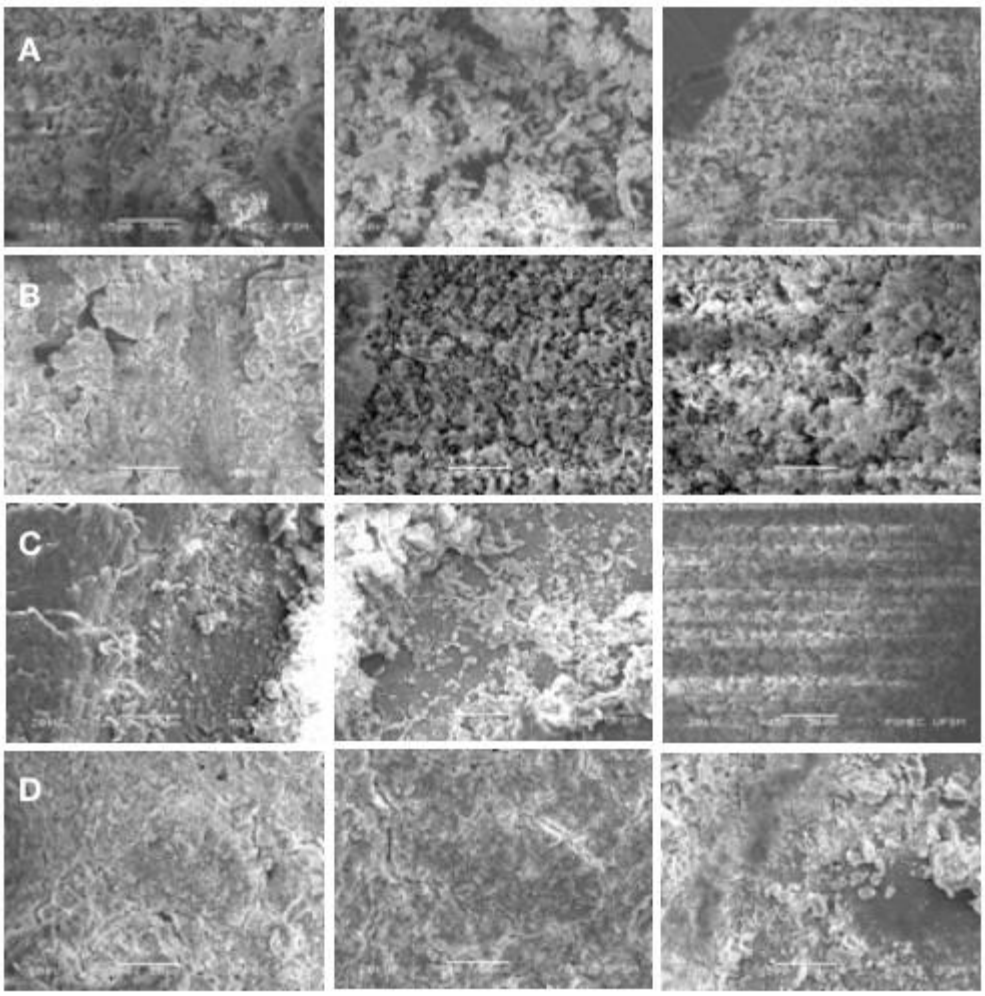


Figure 2. SEM images of the experimental groups in the apical, middle, and cervical thirds. (A) G1: irrigation protocol with saline solution; (B) G2: saline solution with resting phase; (C) G3: conventional irrigation protocol with 2.5% sodium hypochlorite; (D) G4: sodium hypochlorite with resting phase.

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