

Bacterial Microleakage of a Resin-Based Endodontic Sealer Loaded with Chitosan Nanoparticles: An Ex Vivo Study

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ABSTRACT

Aim: The aim of this study was to analyze ex vivo the sealing properties of the resin-based endodontic sealer AH Plus modified by incorporating chitosan nanoparticles (AHCNp) compared with AH Plus without nanoparticles (AHPs). The materials were tested using a bacterial microleakage test method.

Methods: Twenty-two ($n = 22$) human-extracted lower premolars were randomly assigned to two experimental groups of 10 teeth ($n = 10$) each, while the remaining two teeth served as positive ($n = 1$) and negative ($n = 1$) controls. The teeth were auto-claved, and the root canals were chemomechanically prepared under sterile conditions. In Group 1, the canals were obturated with AHCNp and matched-taper single gutta-percha cones. In Group 2, the canals were obturated as in Group 1, except that AHPs was used as the sealer. All teeth were subjected to microleakage testing with an *E. faecalis* culture using a dual-chamber leakage model. The broth in the lower chamber was checked daily for turbidity for up to 90 days. Statistical significance was determined at $P < 0.05$.

Results: In Group 1, the root canals leaked significantly less ($P < 0.001$). In this group, three samples exhibited bacterial leakage, whereas in Group 2, eight samples leaked. The median survival time for both groups could not be calculated because it exceeded 90 days.

Conclusions: After 90 days, AHCNp was significantly more effective than AHPs in resisting coronal bacterial leakage. Long-term experimental and clinical studies are recommended to confirm these findings.

Keywords: Bacterial leakage; *E. faecalis*; endodontics; root canal sealers.

Clinical significance

The findings of this study demonstrated that AH Plus sealer modified by incorporating chitosan nanoparticles showed significantly greater resistance to bacterial leakage than the unmodified AH Plus sealer during a 90-day observation period.

Introduction

It is widely accepted that after instrumentation and disinfection of the root canal, proper obturation of the canal space is essential for successful endodontic therapy [1]. Although gutta-percha is commonly used as the primary core obturating material, it by

itself cannot fill the entire root canal space. In this context, endodontic sealers are necessary to fill the remaining space between gutta-percha and the dentinal walls, thus achieving effective obturation of the root canal system [2]. However, missing or failed coronal restorations can provide a route for microorganisms to access the periapical tissues [2,3], jeopardizing the long-term success of the treatment. To address this problem, various sealer formulations have been tested for root canal obturation [4-6]. Among commercially available materials, the epoxy resin-based sealer AH Plus (AHPs; Dentsply, Tulsa Dental, Tulsa, OK) is considered the gold standard for root canal obturation because of its excellent physicochemical and biological properties [7-9]. However, AHPs does not bond to gutta-percha, allowing bacterial microleakage at the interface between the two materials [10]. Patil et al. [8] speculated that the sealer reacts with other exposed amino groups in dentinal collagen, forming covalent bonds that lead to gap formation and further increase leakage. In the last decade, there has been increased interest in improving the antibacterial and physicochemical properties of endodontic sealers by incorporating natural or metallic nanomaterials into their original formulations [10-15]. Among the wide variety of nanomaterials, chitosan (CHTs), a hydrophilic, non-toxic polysaccharide with proven efficacy for disinfection and obturation of the root canal system, has been widely investigated [11,13,16]. CHTs is derived from the deacetylation of chitin, a natural substance present in crustacean shells and the exoskeletons of various insects [17]. CHTs has inherent antibacterial properties and strong adhesion to dentin, which is further enhanced by forming CHTs nanoparticles (CHNp) with high surface area-to-mass ratios, high chemical reactivity, and sizes ranging from 1 to 100 nm [11,14]. To date, the literature evaluating the sealing properties of AH Plus modified by incorporating CHNp is scarce [18,19]. Therefore, the aim of this study was to compare, using a bacterial microleakage test, the sealing properties of AH Plus loaded with CHNp (AHCNp) and AH Plus without CHNp (AHPs). The null hypothesis was two-fold: (i) there is no significant difference in the frequency of leakage between AHCNp and AHPs, and (ii) there is no significant difference between the two materials with respect to their effectiveness in preventing bacterial leakage.

Materials and Methods

Specimen selection and preparation

The protocol of this study was approved by the Institutional Research Ethics Committee of the Argentine Dental Association (Approval No. #0121-2). The sample size was calculated using the F Family, ANOVA, and G*Power 3.1 software [20], indicating a minimum of 22 teeth for the present study. Twenty-two ($n = 22$) human-extracted mandibular premolars with single, straight, oval-shaped root canals and a cross-sectional diameter ratio of $\geq 2:1$, stored at 4°C in 0.1% thymol solution, were selected from the tooth bank of the School of Dentistry of the University of Córdoba, Argentina. Mesiodistal and buccolingual digital radiographs confirmed these characteristics. Teeth with root caries, cracks, resorptive defects, or prior endodontic therapy were excluded. The length of the teeth was standardized by cutting the crown perpendicular to the long axis at 18 mm from the apex, using a water-cooled, slow-speed diamond saw (Isomet, Buehler,

Lake Bluff, IL). The working length (WL) was established by advancing a size 10 K-file into the canal until just visible at the apex under a stereomicroscope, followed by subtracting 1 mm. Twenty teeth were assigned to two experimental groups of 10 ($n = 10$) teeth each, while the remaining two teeth were used as positive ($n = 1$) and negative ($n = 1$) controls. To ensure that groups had teeth of similar average dimensions, a stratified sampling method (Minitab 10.1, Minitab Inc., State College, PA, USA) was used. The teeth were mounted on a fixed custom attachment simulating a standardized clinical position, and the canals were instrumented with Reciproc Blue #R25 (25/.08) instruments (RCPB; VDW, Munich, Germany) operated at 350 rpm in reciprocating motion in an X-Smart IQ motor (Dentsply, Sirona) using the predetermined programs according to the manufacturer's instructions. Briefly, the instrument was gently inserted into the canal with an in-and-out pecking motion and light apical pressure until the WL was reached without interference, while maintaining apical patency. After three in-and-out movements, the instrument was removed and cleaned. Each file was used to prepare three root canals and then discarded. Throughout biomechanical preparation, the canals were irrigated with 5 mL of 5.25% NaOCl using a 30-G NaviTip needle (Ultradent Products Inc., South Jordan, UT) placed 1 mm short of the WL without binding. The final irrigation protocol was performed with 5 mL of 17% EDTA followed by 5 mL of 5.25% NaOCl (1 min each), with permanent activation/agitation of the irrigating solutions using XP-Endo Finisher instruments (FKG Dentaire SA, La-Chaux-de-Fonds, Switzerland) with slow up-and-down movements of approximately 5 mm in length. After copious flushing with saline, the canals were dried with paper points. Patency was confirmed with a size 10 K-file. All teeth were steam-autoclaved and stored under sterile conditions at 37°C and 100% humidity until further use.

Specimen obturation

The obturation procedures and further bacterial testing were conducted in a microbiologic laboratory under strict sterile conditions, using a laminar flow cabinet. All teeth were filled to the WL using the single-cone obturation technique with sealer to maintain consistency between groups and according to the following protocol:

Group 1: AHCNp ($n = 10$): The root canals were trial-fitted with a matched-taper single gutta-percha cone sized and tapered to match the final shaping #R25/.08 instrument. The cone was inserted to the WL using visual and tug-back control. After removal of the cone, the canal was flushed again with saline and dried with sterile paper points. AHCNp sealer was prepared and used according to the procedures described by Khoshbin et al. [21]. Briefly, the sealer was modified by incorporating 200 ppm of CHNp powder (Nairotech SA, BA, Argentina), containing nanoparticles averaging 100 nm, at a 1:1 volume ratio to the base paste of the sealer, and then mixed on a sterile glass slab with a plastic spatula. This mixture was then blended with the catalyst paste strictly according to the manufacturers' instructions. A size #20 K-file was used to gently apply the sealer to the canal walls, and the cone was then dipped in the sealer and firmly inserted into the root canal to the WL. Additional accessory gutta-percha cones dipped in the sealer

were also used and laterally compacted until no further placement was possible beyond 3 mm. Immediately after obturation, excess material was removed flush with the canal orifice using a heated instrument, and the gutta-percha was vertically condensed with a plugger. All samples were properly identified and stored in an oven at 37°C and 100% humidity for 7 days to ensure complete setting of the sealer.

Group 2: AHPs (n = 10): In this group, the root canals were filled and treated as described for Group 1, except that unmodified AHPs was used as the sealer.

Radiographs were taken from the mesial and buccal aspects of each tooth to assess the quality of the root canal obturation. If voids, bubbles, or unfilled spaces were present, the tooth was excluded from the study and replaced with a new one. After storage, the external surfaces of the teeth were dried with oil-free compressed air and coated with two layers of sterile nail polish varnish, leaving 1 mm around the apical foramen and 1 mm around the coronal access exposed.

Positive control (n = 1): One tooth was obturated as in Group 1, except that it was entirely coated with nail varnish.

Negative control (n = 1): One tooth was not obturated but was varnished, leaving 1 mm around the apical foramen and 1 mm around the coronal access exposed. Both the negative and positive control specimens were tested for leakage using the same procedures as Groups 1 and 2.

Bacterial leakage setup

For bacterial leakage, a modified version of the dual-chamber bacterial leakage model described by Snigdha et al. [13] was used, as shown in Figure 1.

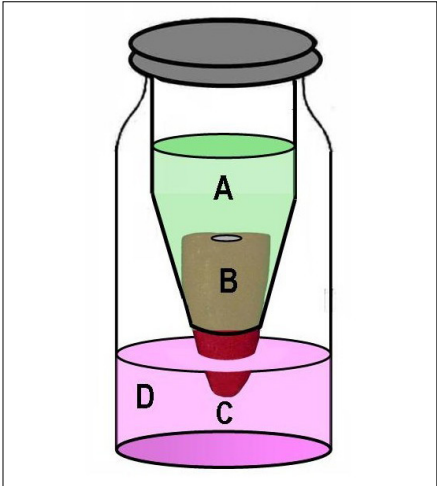


Figure 1: Schematic drawing of the bacterial leakage setup. A: Upper chamber (Eppendorf tube) containing TSB broth with *E. faecalis*. B: Root covered with nail polish. C: Exit for bacteria. D: Lower chamber containing sterile TSB broth.

Briefly, the tip of a 2.5-mL Eppendorf plastic tube (upper chamber) was cut, and the tooth sample was pushed through the opening.

The tubes were placed in glass vials (lower chamber) containing 10 mL of sterile trypticase soy broth (TSB; Difco Laboratory, Detroit, MI) in such a way that approximately 4 mm of the apical root was submerged in the broth. The junction between the tooth and the tube was sealed with three layers of cyanoacrylate (La Gotita, Buenos Aires, Argentina). The junction between the tube and the glass vial was also sealed with three layers of cyanoacrylate. The entire test apparatus was sterilized with ethylene oxide gas for 12 h and then incubated at 37°C for 72 h to ensure sterility. If the TSB broth showed signs of turbidity, the test apparatus was discarded and a new sample was prepared.

Bacterial leakage test

The upper chamber was filled with 1 mL of TSB containing 24 h of growth of *E. faecalis* (ATCC 29212) at 10⁸ colony-forming units/mL. The inoculated apparatus was incubated for 90 days at 37°C. The upper chamber was reinoculated every 7 days with a fresh culture of the microorganism. The TSB broth in the lower chamber was checked daily for turbidity, which, when cloudy, indicated bacterial leakage through the entire length of the root canal. Once turbidity was detected, the day was recorded. Samples from both chambers were then incubated on blood-agar plates to assess bacterial viability by morphological observation and Gram staining. The number of teeth demonstrating bacterial leakage and the days on which leakage occurred were recorded for each group.

Statistical analysis

The time to leakage detection was compared between the groups using Kaplan-Meier survival analysis. Significant differences were assessed using Fisher’s exact test and the log-rank test at P < 0.05.

Results

The results of the study are presented in Table 1 and Figure 2.

Table 1. Leakage frequency.

Groups	Leakage	No leakage	Total
Group 1	3a	7a	10
Group 2	8b	2b	10
Log-rank test P<0,001			

Different lowercase letters indicate significant differences between groups.

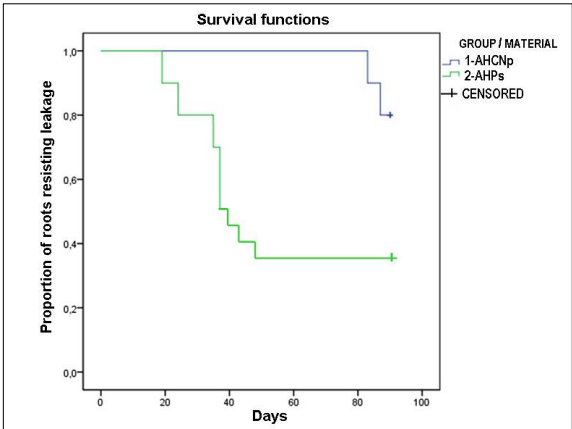


Figure 2: Kaplan-Meier survival curves for AHCNp and AHPs.

In Group 1, leakage was observed at 83 days (one sample) and 87 days (two samples), while seven samples did not show leakage. In Group 2, leakage was observed at 21 days (one sample), 24 days (one sample), 35 days (one sample), 37 days (two samples), 41 days (one sample), 45 days (one sample), and 51 days (one sample), while two samples did not show leakage. The median survival time (MST) for both groups could not be determined because it exceeded 90 days, the duration of the experiment. The log-rank test revealed significant differences in microleakage frequency between Groups 1 and 2 ($P < 0.001$). Fisher's exact test also showed significant differences between groups with respect to their capacity to prevent bacterial microleakage ($P < 0.001$). Therefore, the first and second null hypotheses were rejected. The positive control did not show leakage during the entire experiment (90 days), while the negative control demonstrated leakage after 7 hours. In all samples that showed leakage, bacteriological testing of the contents of the lower chamber revealed viable *E. faecalis*.

Discussion

It is well established that the presence of microorganisms within the root canal space or at the interface between the obturating material and the root canal walls is one of the most important factors contributing to endodontic failure [22-25]. Sealers used to fill the root canal space must establish a tight seal at the sealer/gutta-percha and sealer/root canal wall interfaces [26-28]. In this ex vivo study, we analyzed the coronal resistance to bacterial leakage in root canals filled with gutta-percha and AHCNp or AHPs. We assessed the number of samples exhibiting coronal leakage of *E. faecalis* and the time required for the microorganisms to reach the lower chamber of the test apparatus. Controversy still exists in the literature regarding whether ex vivo and in vivo microleakage testing correlate and whether results from ex vivo experiments can be correlated with the clinical situation [29]. Although the present assay is a laboratory study, it does provide useful information about the incorporation of CHNp to enhance the sealing properties of AH Plus.

E. faecalis was chosen as the marker because it is present in the human oral flora and is frequently implicated in the etiology of secondary endodontic infections, along with other aerobes and facultative anaerobes [21,22,30-32]. As per protocol, the parameters used for leakage evaluation were qualitative, i.e., the presence or absence of turbidity in the lower chamber, not quantitative, since the number of bacteria that caused turbidity was not calculated. Although a 90-day bacterial leakage test under ex vivo conditions, as reported here, may not exactly mimic events in a clinical environment, it still allows for a comparison of material behavior under strictly controlled conditions. After 90 days, bacteria may still be present in the canals of teeth that have not shown turbidity in the lower chamber. Therefore, longer observation periods are expected to eventually result in more samples clouding the lower chamber, an issue that still needs further investigation. The results of the study showed that root canals in which AHCNp was used as the sealer provided a significantly more effective seal than the canals filled with AHPs. The improved sealing ability of AHCNp may be attributed to the small size and hydrophilic nature of CHNp, which

enhance its ability to make close contact with root canal walls, thus increasing the penetration of the sealer into the dentinal tubules [19,33]. The superior sealing properties demonstrated by AHCNp may also be attributed to the pronounced antibacterial activity of CHNp, which inhibits further bacterial growth [12,16,21,34]. Although promising, the results of incorporating CHNp into the original formulation of AH Plus sealer should be interpreted with caution, and no direct correlation to the clinical situation is claimed until further studies and clinical trials are conducted.

Conclusions

Within this study's scope, the root canals filled with gutta-percha and AHCNp exhibited significantly higher resistance to *E. faecalis* bacterial microleakage compared to canals in which AHPs was used as the sealer. It is believed that adding CHNp to the original AH Plus formulation significantly contributed to the sealing effectiveness observed.

Conflict of Interest and Funding

The author declares no conflict of interest and received no specific funding for this work.

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