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Antimicrobial Activity of Nano-Magnesium Hydroxide Against Oral Bacteria and Application in Root Canal Sealer

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Data Interpretation D
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Background: The goal of the present work was to assess the antibacterial activity of nano-magnesium hydroxide (NMH) against *Streptococcus mutans* (*S. mutans*) and to explore the antimicrobial function of AH Plus™ sealer incorporating NMH.

Material/Methods: The antimicrobial behavior of NMH against *S. mutans* was evaluated with bactericidal tests. A modified direct contact test was used to assess the antimicrobial activity of unset AH Plus containing NMH after 5 minutes, 20 minutes, and 60 minutes of contact with bacteria. The antimicrobial effects and the amount of surface-adhering bacteria of the solidified materials were explored by SEM and confocal laser scanning microscopy, respectively.

Results: NMH powder presented excellent antimicrobial activity against *S. mutans*. Mg^{2+} and OH^- were not the main factors resulting in bacterial death. Approximately 93.1% and 98% of the *S. mutans* were killed in the AH Plus+7% NMH group after incubation for 5 minutes and 20 minutes, respectively. AH Plus with 5% or 7% NMH were more potent against *S. mutans* compared with AH Plus alone ($P<0.05$). Moreover, the antibacterial function of AH Plus was lost after setting. NMH enabled the solidified AH Plus to still have antibacterial properties on the seventh day.

Conclusions: NMH can be used to modify AH Plus sealer to eradicate residual bacteria and prevent reinfection.

MeSH Keywords: Magnesium Hydroxide • Root Canal Therapy • *Streptococcus mutans*

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Background

Microbial infection is the primary etiologic factor of pulpitis and periradicular and is associated with treatment failure [1–5]. Eliminating microorganisms from the infected in the endodontium is undoubtedly critical to the success of endodontic therapy [6]. Mechanical debridement in combination with chemical antimicrobial can significantly reduce the infective substances. However, bacterial residues are inevitable as a result of the complexity of the root canal anatomy and biofilms resistance to disinfecting drugs, even when using the strategies described above [5–7].

Endodontic sealers with ideal obturation function can control infection by burying residual microbes and preventing the supply of nutrients. In addition, sealers with antimicrobial activity can help further reduce residual bacteria or provide an environment that discourages microbial growth [8]. AH Plus, an epoxy-based endodontic sealer, is regarded as the criterion standard owing to its excellent physicochemical properties [9,10]. However, AH Plus showed weaker antibacterial activity against Gram-positive bacteria [11]. Therefore, AH Plus sealer has been continuously improved to be a better antimicrobial material to kill microbes during endodontic treatment.

Nano-magnesium hydroxide (NMH) has been widely used in biomedical fields due to its broad antibacterial spectrum and biocompatibility. For example, incorporating NMH into synthetic polymer scaffolds can support cartilage regeneration [12]. NMH can enhance the therapeutic effect of sericin on corneal wounds [13]. Moreover, it was observed that NMH, unlike nanosilver, has a non-toxic effect, indicating that it has great potential for biomedical applications [14]. These examples provide insight into the use of NMH as antibacterial dental materials against *Streptococcus mutans* (*S. mutans*).

The microbial flora of endodontic treatment failures is polymicrobial [15]. The most frequent route of infection of pulpitis and periradicular is caries [16]. Bacteria in caries are considered to be the first to invade the pulp and cause root canal infections. *S. mutans* has a high detection rate in dental caries of irreversible pulpitis [17]. Previous evidence suggested that *S. mutans* adheres and grows in infected root canals by recognizing collagen type I in dentin tubule [18], which may provide an environment conducive to colonization by other root canal bacterial populations [1]. Moreover, *S. mutans* was also found in the root canals of patients with periapical disease [19].

Therefore, the present work aimed to determine the antibacterial properties of NMH against *S. mutans* and to investigate related mechanisms of NMH action. We also assessed the antibacterial property of the composite material composed of NMH and AH Plus sealer.

Material and Methods

Material preparation

NMH used in this experiment was obtained by a solid-liquid chemical deposition method using a specific concentration of chloride ion solution and pure magnesium. It was supplied by the Institute of Metal Research, Chinese Academy of Sciences. The modified endodontic sealers were prepared by mixing AH Plus sealer and NMH powder (0 wt.%, 3 wt.%, 5 wt.%, 7 wt.%). The mixture was molded into a diameter of 10 mm and a thickness of 2 mm using a silicone mold. The samples were kept in an incubator for 1 day at 37°C to make them set completely and were sterilized with ultraviolet light for 2 hours. The samples produced by the above method were observed using scanning electron microscopy (SEM, Merlin, Zeiss) and confocal laser scanning microscopy (CLSM, Model C2 Plus, Nikon).

Characterization of NMH

The phase of powders was investigated using an X-ray diffractometer (XRD, Philips PW1700) using CuK_{α}. The structure and morphology of NMH were investigated by SEM (Hitachi S-3400N) and transmission electron microscope (TEM, FEI T20).

Bacterial strains and culture medium

The *S. mutans* strain used for antibacterial analysis was ATCC 25175 (Marine Culture Collection of China, Xiamen, China). The strain was proliferated in sterile brain-heart infusion (BHI) broth for 24 hours. The purity of the culture was checked, and a growth curve of *S. mutans* was explored. Two mL EP tubes containing 1.8 mL BHI and 18 µL bacterium suspension were incubated in an anaerobic incubator. The optical density of the bacterial suspension was recorded at a wavelength of 600 nm at intervals of 1 or 2 hours. The growth curve of the bacteria was plotted with absorbance values as the longitudinal coordinate and time as the transverse coordinate.

Antimicrobial efficacy of NMH

The visual plate count method was used to investigate the antimicrobial effects of NMH at various concentrations [20]. The suspensions with 0.5 mL of 1 mg/mL, 2 mg/mL, and 4 mg/mL of NMH were sonicated for 20 minutes, the we added 2 mL to EP tubes containing 0.5 mL bacterial suspension (approximately 2×10^8 CFU/mL). The final concentrations of NMH were 0.5 mg/mL, 1 mg/mL, and 2 mg/mL. We added 0.5 mL physiological saline to 0.5 mL bacterial suspension as the control group. All EP tubes were put in an anaerobic environment and cultured in a shaker at 180 rpm for 4 hours and 8 hours. Then, the 100 µL bacterial suspension was inoculated in BHI agar plates, and colonies of surviving bacteria were counted after 48 hours.

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We added a few drops of hydrochloric acid to 0.5 mg/mL, 1 mg/mL and 2 mg/mL of NMH suspension to adjust pH to 7. The antibacterial activity of NMH against *S. mutans* was studied in the neutral environment of pH 7. Colonies of surviving bacteria were counted at 4 hours as described above.

Antimicrobial efficacy of Mg^{2+}

Magnesium ions suspension in concentrations of 12.5 mM, 25 mM, and 50 mM were prepared by dissolving magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) in sterile physiological saline. *S. mutans* was cultured at 37°C for 4 hours in an anaerobic environment. The survival of *S. mutans* at different levels of magnesium ions was observed by viable plate count [21].

Effect of NMH on protein leakage from *S. mutans*

The suspension with 0.5 mL of 1 mg/mL and 4 mg/mL of NMH was added to 2 mL EP tubes containing 0.5 mL bacterial suspension (approximately 2×10^8 CFU/mL). The final concentrations of NMH were 0.5 mg/mL and 2 mg/mL. We added 0.5 mL physiological saline to 0.5 mL bacterial suspension as the control group. The mixture was put in a shaker at 37°C and 180 rpm for 4 hours, centrifuged at 4500 rpm for 10 minutes, and the supernatant was collected. The protein leakage of *S. mutans* was determined by bicinchoninic acid (BCA) analysis [22].

Proteins adhesion test

Bovine serum albumin (BSA) was added to NMH suspension with a concentration of 2 mg/mL. The mixture was placed on an orbital incubator for 30 minutes at 37°C. Then, the precipitation was harvested by centrifugation and rinsed twice with physiological saline. We added 1% sodium salt (SDS) solution to elute the protein adsorbed on the surface of NMH. SDS without NMH powder served as control. The collected supernatant was used to quantify the protein using BCA assay.

Antibacterial test to planktonic bacteria: modified direct contact test (MDCT)

The antimicrobial effects of the AH Plus sealer loaded with NMH against planktonic bacteria were evaluated by MDCT [23,24]. AH Plus loaded with different mass fractions (0 wt.%, 3 wt.%, 5 wt.%, 7 wt.%) of NMH were developed. An equivalent amount of sealers was added at the bottom of the 96-well microtiter plate, which were kept for 20 minutes at 37°C. We coated 20 µL of *S. mutans* culture with a concentration of 3×10^8 CFU/mL on the surface of samples, and another 20 µL bacterial suspension was transferred into the uncoated wells as control. Freshly mixed sealers were contacted with bacterial suspensions for 5, 20, and 60 minutes at 37°C, and 300 µL PBS was dripped into each well and mixed with a pipette for 1 minute. The bacterial suspension was collected and continuously diluted in PBS. We coated 100 µL bacteria suspension in BHI agar plates for 48 hours. Each material was subjected to 3 parallel experiments. Finally, the antibacterial rate (R) was calculated as:

$$R = (Ncontrol - Nmaterial)/Ncontrol \times 100\%$$

SEM observation of bacteria

The biofilm morphologies on the surface of samples were investigated using SEM (FE-SEM, Ultra-Plus, Zeiss, Germany). The sterile samples were prepared according to the method in the material preparation above and soaked in the 40 mL liquid culture containing 3×10^6 CFU/mL *S. mutans* in an anaerobic environment for 1 day and 7 days, respectively. The samples were rinsed gently with PBS to remove the culture medium and unattached cells [16]. After that, the samples were first immobilized in a 2.5% glutaraldehyde solution above 4°C and dehydrated for 10 minutes in ethanol solutions of 50%, 60%, 70%, 80%, 90%, 95%, and 100% v/v. The samples were dried at room temperature and sprayed with gold film [25].

Bacterial biofilm observation CLSM

The structures of *S. mutans* biofilm on the samples were investigated by CLSM (C2 Plus, Nikon, Japan). The samples were prepared with or without NMH according to the method described in the material preparation section above and soaked in an anaerobic environment for 1 day and 7 days. Then, the samples were rinsed gently with PBS and stained for 20 minutes in the dark with the fluorescent LIVE/DEAD® BacLight Bacterial Viability Kit, which contains SYTO-9 dye and PI dye. Cells were marked by SYTO-9 dye regardless of membrane integrity and were observed at excitation/emission peaks of 488/520 nm. Cells with destroyed cytoplasmic membrane were marked by PI dye and were observed at excitation/emission peaks of 488/630 nm, and the biofilm structures were then viewed under a CLSM [26–28].

Statistics

The protein adhesion test calculated mean values and standard deviation (SD), and we used the t test for statistical analysis. The differences in antibacterial ratio and cell membrane permeability test results were analyzed by one-way analysis of variance (ANOVA) and Tukey multiple comparison test using SPSS software 20.0. The statistical significance level was set at 95%.

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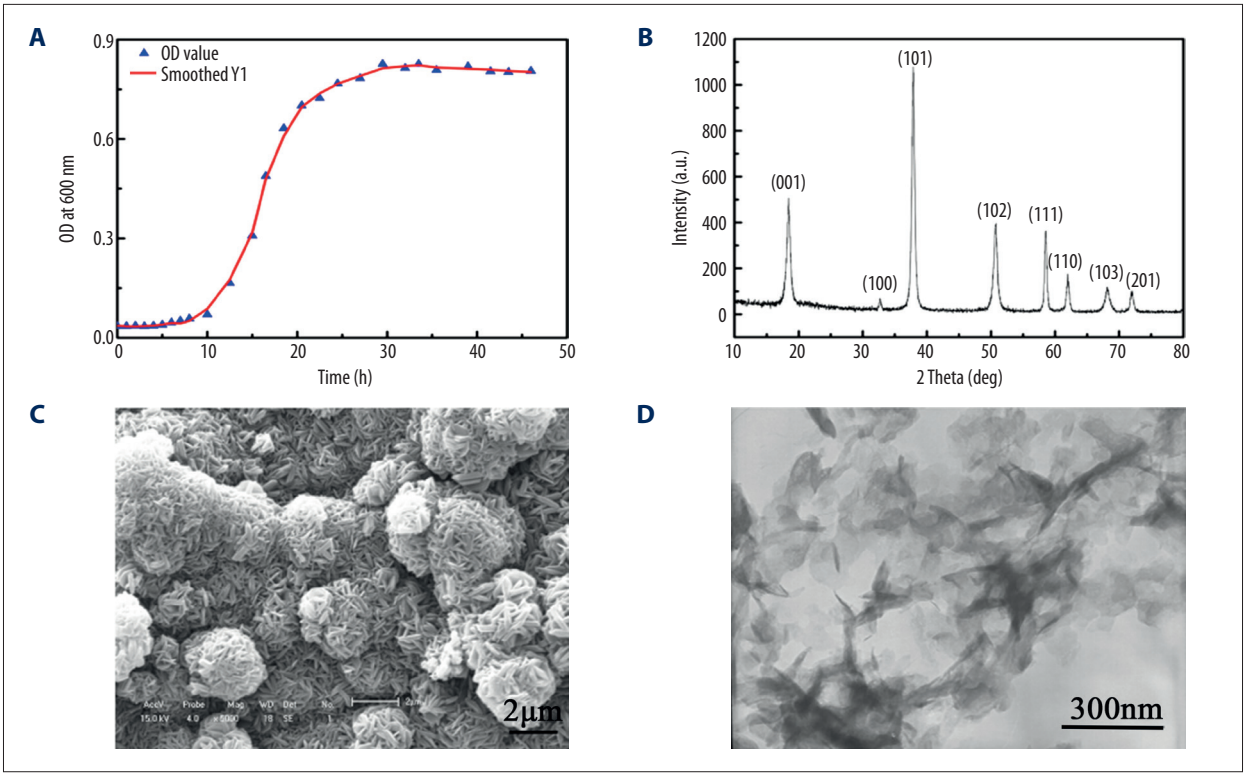

Figure 1. (**A**) Typical growth curves of *Streptococcus mutans*. XRD patterns (**B**), SEM images (**C**), and TEM (**D**) were used to confirm the crystalline structure and morphology of the as-synthesized nano-magnesium hydroxide (NMH), respectively.

Results

Characterization of NMH

The XRD pattern of NMH is illustrated in Figure 1B. The diffraction peaks can be indexed as the hexagonal structure of $Mg(OH)_2$ (JCPDF044-1482 and JCPDS Card No. 7-239) [29]. The pattern exhibited a vigorous intensity of the (101) peak. The sharpness of the diffraction peaks and narrowing of peak width of the nanoparticles showed a high crystallinity of $Mg(OH)_2$. These results are strongly consistent with previous reports [29,30].

As shown in Figure 1C and 1D, the morphologies of the NMH were revealed through SEM and TEM observations. In the TEM image, NMH showed a good dispersity and uniform particle size and mainly exhibited an unusual flake configuration. The particle size of NMH was estimated to be about 30 to 50 nm. Many irregularly arranged NMH flakes formed flower-like structures as imaged by SEM.

Antimicrobial efficacy of NMH

Figure 2A shows that the bactericidal function of NMH depends on the co-culture duration and the concentration of NMH. After 4 hours of incubation, the number of bacteria decreased significantly with the increase of NMH concentration. *S. mutans* interacting with 1 mg/mL or 2 mg/mL of NMH for 8 hours could be entirely killed. Moreover, NMH still had antibacterial effects even at a pH of 7 (Figure 2B). To quantify the contributions of released Mg^{2+}, the different concentrations of Mg^{2+} were used to interact with bacteria, showing that the number of live colonies did not significantly change with increased Mg^{2+} concentration (Figure 2C).

Cell wall permeability of *S. mutans*

Quantification of protein leakage from *S. mutans* exposed to NMH was determined (Figure 3A). The standard equation between the absorbance values at 470 nm and the concentrations of protein is $y=1.4533x+0.1623$ ($R_2=0.9977$). After *S. mutans* were treated with concentrations of 0.5 mg/mL and 2 mg/mL NMH, the protein leakage reached 4.4 μg/mL and 21.4 μg/mL, respectively. Both concentrations of NMH resulted in a significant difference in the permeability of the cell membrane of *S. mutans* compared with 0 mg/mL NMH ($F=18.896$, $P<0.05$, by ANOVA). The amount of protein leakage increased significantly as the concentration of NMH increased.

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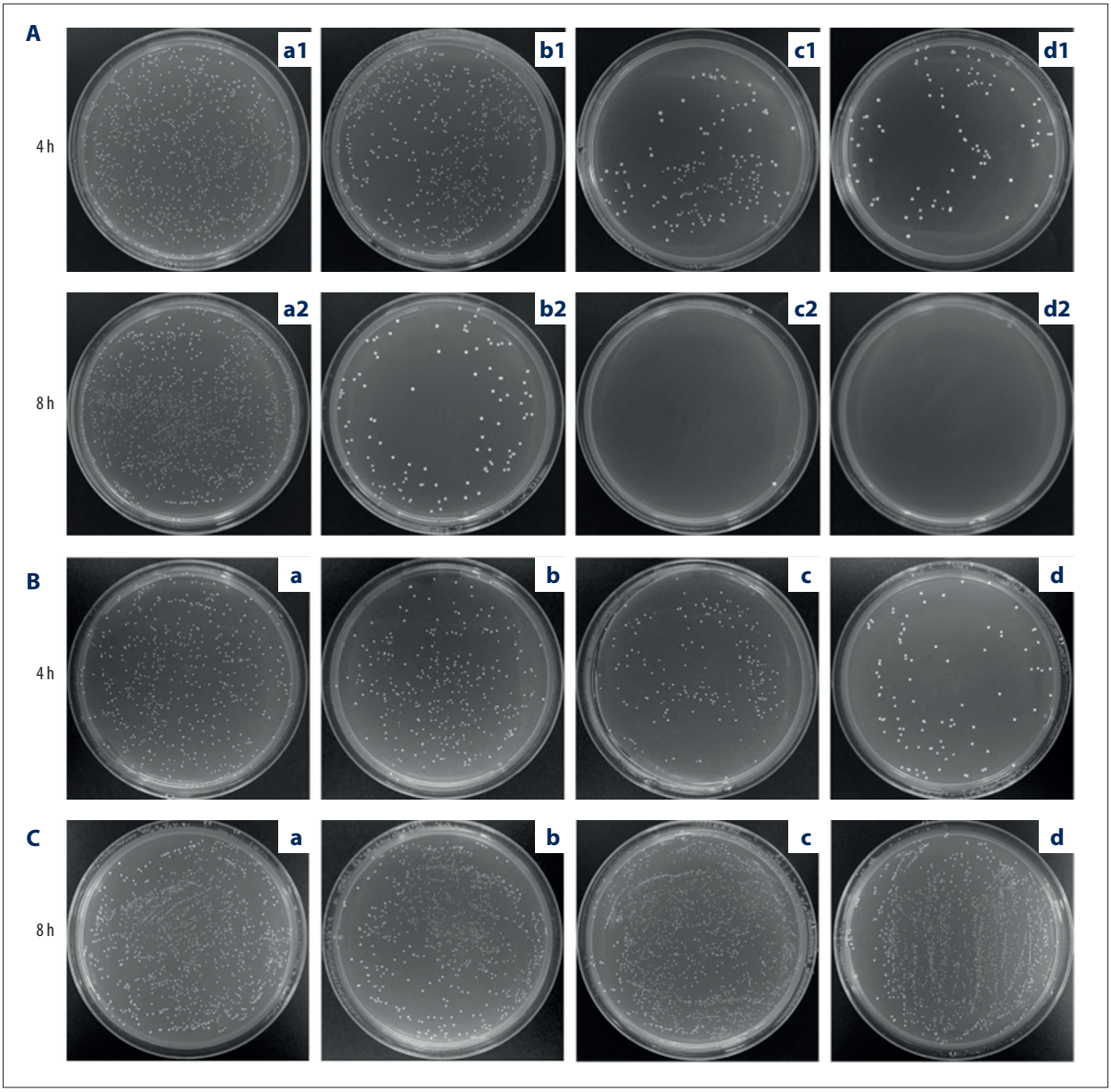

Figure 2. Representative plate images of viable *Streptococcus mutans* strain after antibacterial tests. (**A**) Antibacterial effect of different concentrations of NMH (**a1**, **a2**, control; **b1**, **b2**, 0.5 mg/mL; **c1, c2**, 1 mg/mL; **d1, d2**, 2 mg/mL) at different culture times. (**B**) The antibacterial effect of different concentrations of NMH (**a**, control; **b**, 0.5 mg/mL; **c**, 1 mg/mL; **d**, 2 mg/mL) co-cultured with *Streptococcus mutans* for 4 hours at pH of 7. (**C**) Antibacterial effect of different concentrations of Mg^{2+} (**a**, control; **b**, 12.5 mM Mg^{2+}; **c**, 25 mM Mg^{2+}; **d**, 50 mM Mg^{2+}) co-cultured with *Streptococcus mutans* for 4 hours.

Proteins adhesion determination

The results showed that the control group without NMH could not adhere to proteins (Figure 3B). There was a statistically significant (t=14.002, $P<0.01$, by *t* test) difference in the amount of protein leakage between the control group and the group containing 2 mg/mL NMH.

Antibacterial test of planktonic bacteria

The antibacterial ratios of the endodontic sealer unmodified or containing NMH are depicted in Figure 4. The antibacterial ratios of the AH Plus group was 33.16% after co-culture for 5 minutes whereas AH Plus+3% NMH, AH Plus+5% NMH, and AH Plus+7% NMH were 48.33%, 69.35%, and 93.10%, respectively. AH Plus+5% NMH ($P<0.05$) and AH Plus+7% NMH ($P<0.001$) had stronger antimicrobial activity against *S. mutans* than the AH Plus

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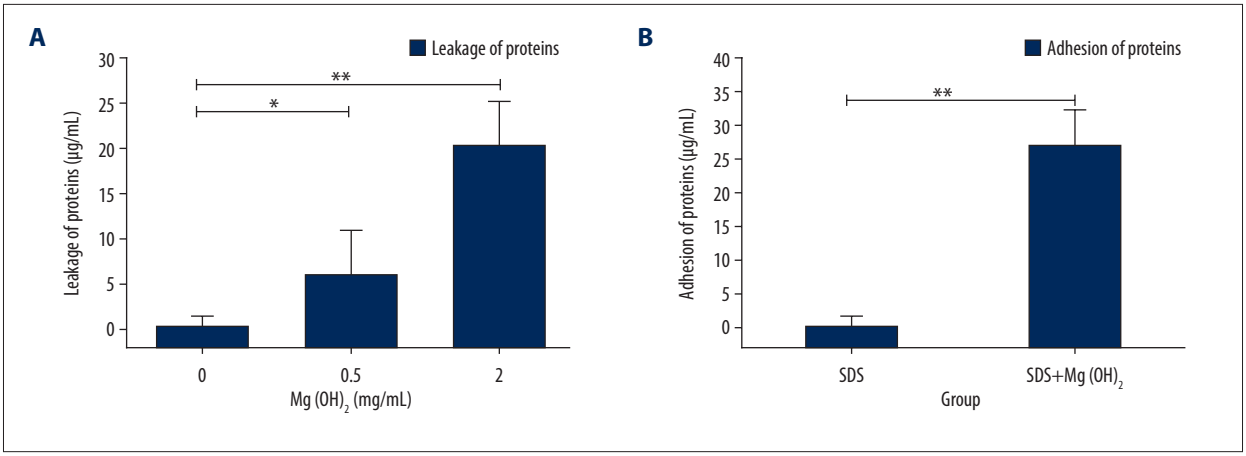

Figure 3. (**A**) Leakage of proteins from *Streptococcus mutans* incubated in NMH using BCA assay. (**B**) BCA assay was used to detect the ability of NMH to adsorb protein. Data=mean±standard deviation, n=4; * *P*<0.05; ** *P*<0.01.

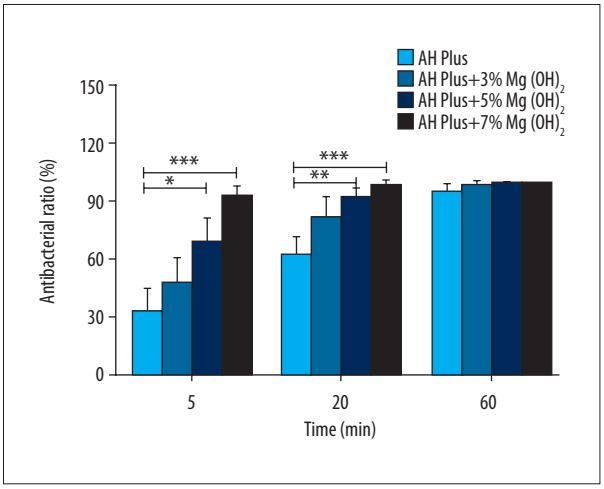

Figure 4. Antibacterial ratios of the AH Plus sealers with different concentrations of NMH against *Streptococcus mutans*. The data points are the means±SD of 3 independent experiments, each performed in triplicate. Asterisks (*) indicates statistically significant differences between the bacterial cells treated with NMH and AH Plus group (* *P*<0.05; ** *P*<0.01; *** *P*<0.001). Error bars indicate standard errors of the means.

group (F=18.163, by ANOVA). AH Plus+5% NMH (P<0.01) and AH Plus+7% NMH (P<0.001) significantly reduced the numbers of viable bacteria within 20 minutes of contact with *S. mutans* when compared with the AH Plus group (F=14.050, by ANOVA). AH Plus+7% NMH eradicated all bacteria within 60 minutes of contact. The MDCT showed that more than 5% NMH powder improved the antibacterial activity of AH Plus in the fresh state.

Observation by SEM

The number and morphologies of *S. mutans* were observed using SEM (Figure 5). After 1 day of culture, we could see numerous bacteria on AH Plus samples split into intact coccoid chain structure with a smooth surface, and the bacterial chain was consecutive (Figure 5a1), indicating that the growth of microorganisms had not been affected. The AH Plus sealer solidified for 1 day did not show excellent bactericidal performance. Compared to the AH Plus samples, only a few spherical bacteria were found on the AH Plus+5% NMH samples, and the bacterial chain was non-existent (Figure 5c1). Bacterial debris was widely found when adding 7% NMH powder (Figure 5d1). When the immersion time extended to 7 days, more bacteria were observed in the AH Plus specimens and many areas presented as fused colonies (Figure 5a2). In contrast, the AH Plus+5%/7% NMH samples had almost no live bacteria and displayed much bacterial debris.

Observation by CLSM

Representative live/dead images of the bacterial biofilm on the samples are shown in Figure 6. The CLSM image showed that the biofilm of *S. mutans* was thinner after 1 day of incubation than that of thicker and denser biofilms after 7 days. After 1 day of culture, the number of live bacteria stained as green was significantly higher than dead cells on the AH Plus and AH Plus+3% NMH samples. On the contrary, AH Plus with 7% NMH showed the most dead bacteria (red), followed by a concentration of 5%. The above results indicated that the antibacterial effect against *s. mutans* was significantly enhanced when the concentration of NMH exceeded 5%. After 7 days of cultivation, the bacterial number of AH Plus group increased, and very few dead bacteria were observed in the bacterial biofilm. In contrast, dead bacteria were visualized on the surface of AH Plus mixed with NMH. The AH Plus+5%/7% NMH group showed a yellow color, indicating that the red fluorescence of dead bacterial was covered by the green fluorescence of the surviving bacteria within the biofilm structure.

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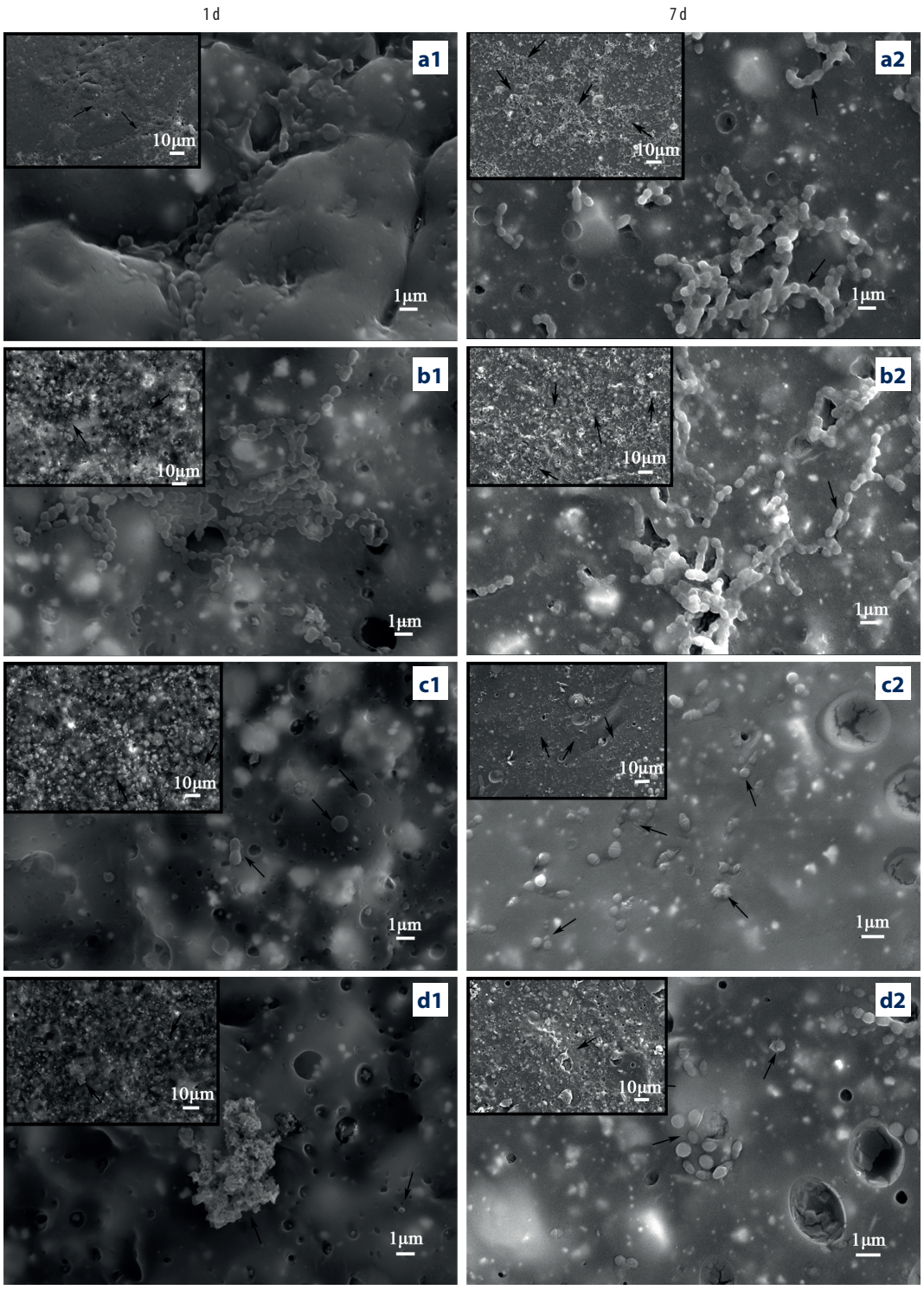

Figure 5. *Streptococcus mutans* cultured on AH Plus (**a1, a2**), AH Plus+3% NMH (**b1, b2**), AH Plus+5% NMH (**c1, c2**), and AH Plus+7% NMH (**d1, d2**) for 1 day and 7 days. Black arrows indicate *Streptococcus mutans*.

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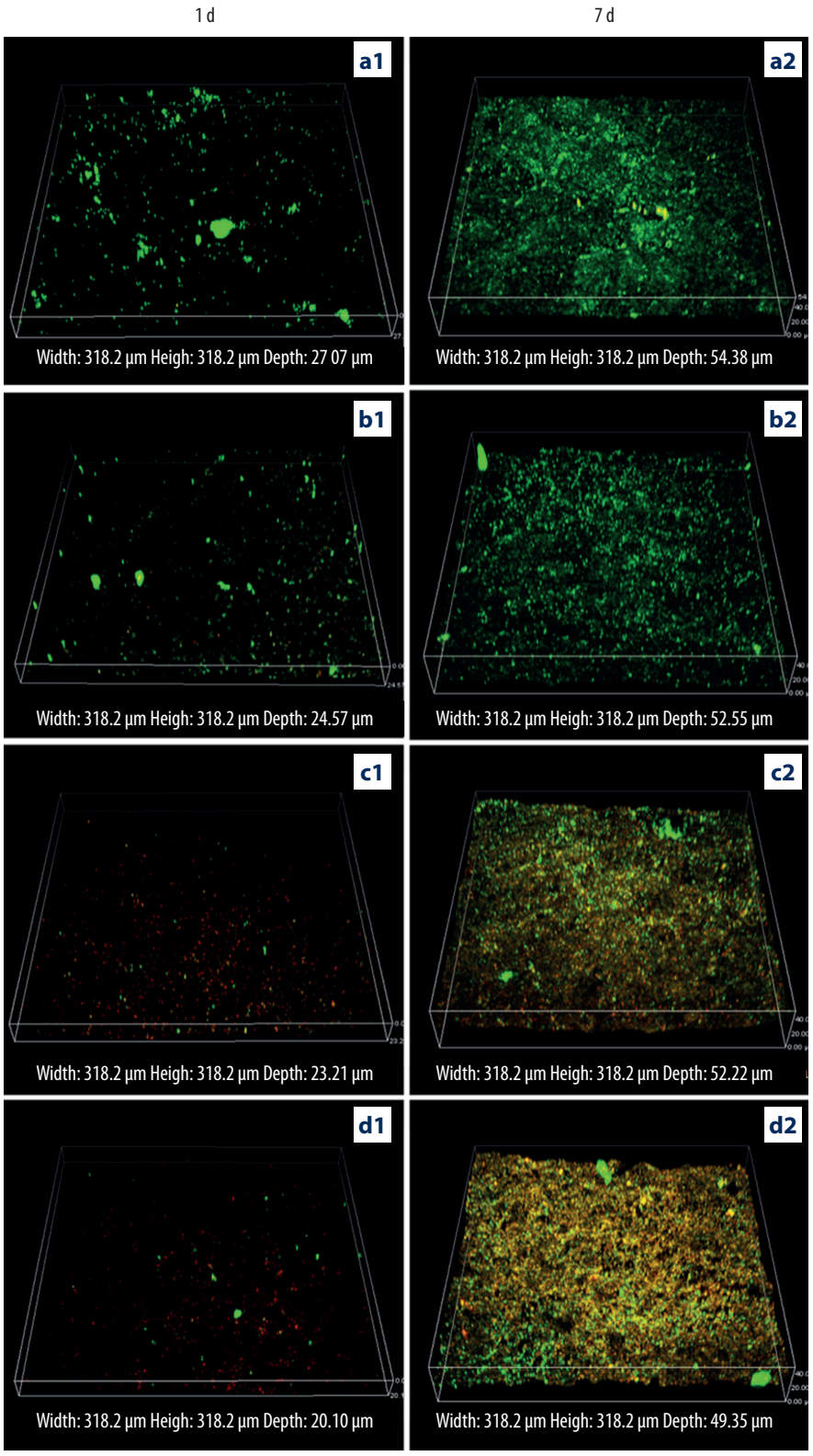

Figure 6. The samples of AH Plus (**a1, a2**), AH Plus+3% NMH (**b1, b2**), AH Plus+5%NMH (**c1, c2**), and AH Plus+7% NMH (**d1, d2**) were immersed in the culture media inoculated with *Streptococcus mutans* for 1 day and 7 days.

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Discussion

Nanomaterials have been widely investigated for their antimicrobial properties in the past few years [31–33]. The present study showed that NMH had stronger antibacterial effects against *S. mutans*. The effectiveness of NMH was also reported by Dong et al. [21,34,35], who demonstrated that NMH exhibited outstanding antimicrobial property against *E. coli* and Micro-$Mg(OH)_2$ has no antimicrobial activity [35]. The superior antimicrobial effects of NMH, when compared with Micro-$Mg(OH)_2$, may be due to the higher specific pore surface area and smaller size, which facilitates biochemical reactions with microorganisms [36,37].

NMH still had antibacterial action after adding hydrochloric acid to eliminate the bactericidal action of OH^-. This is beneficial to kill root canal microorganisms resistant to high pH [38]. Previous studies have found that the antimicrobial property of nanomaterials is mainly affected by the significant release amount of ions [39,40], whereas Mg^{2+} released to the solution in the present study had no significant antimicrobial effect on NMH (Figure 2C). By comparing the antimicrobial activity of $MgSO_4$ and $Mg(OH)_2$ to *E. Coli*, Dong et al. [21] concluded that Mg^{2+} had no antibacterial effect, which is consistent with the present study.

Improving and prolonging the antibacterial effect of sealers is considered useful as complementary to control infection [23]. Adnan et al. [41] reported that *Streptococcus species* were often detected in infected root canals, and *S. mutans* was present in 15.6% of teeth. Moreover, *S. mutans* in root canals may lead to bacteremia [42]. Therefore, it is meaningful to study the antimicrobial activity of AH Plus on *S. mutans*. The growth curve of *S. mutans* (Figure 1A) showed that cell growth entered into the exponential phase after 9 hours and the strain should be used within 9 to 25 hours.

The MDCT is a reproducible method to quantitatively evaluate the antimicrobial effects of endodontic sealers [23,43]. The present study also evaluated the antibacterial effect of unset AH Plus sealer incorporated with NMH against *S. mutans* by MDCT. AH Plus sealer with more than 5% NMH was more effective in the eradication of planktonic than AH Plus sealer alone (Figure 4). The enhanced antibacterial properties of unset root canal sealers are expected, as they can quickly kill microorganisms in some areas that cannot be treated with chemo-mechanical debridement measures. The AH Plus+7% NMH sealer killed almost all the bacteria when exposed to bacteria for 5 minutes.

The results of SEM and CLSM revealed that the AH Plus without additional NMH (control) lost almost all antibacterial effect after 1 day of solidification. Similar result was found by Zhang et al. [23] and Pizzo et al. [44] who both reported that only unset AH Plus possessed antimicrobial activity, while no significant antibacterial activity was found after 1 day of setting. The reason for this phenomenon is the burst release of formaldehyde during the setting process, which leads to short-term antibacterial activity [44,45]. Adding more than 5%, NMH prolonged the bactericidal effects of AH Plus. It was reported that permanent antibacterial activity can only be achieved through direct contact between the surface of the antibacterial materials and the bacteria [46]. The NMH immobilized within AH Plus sealer can enable AH Plus sealer to produce long-term antibacterial effects. The CLSM images of the AH Plus+5%/7% NMH group exhibited yellow color, meaning that direct contact may be the primary killing mechanism of NMH. Indeed, the antibacterial activity could still be observed even after 7 days after adding NMH to the AH Plus. However, the antibiotic mechanisms of AH Plus loaded with NMH is unclear and needs further study.

Reactive oxygen species (ROS) is often regarded as the most crucial antibacterial mechanism of nanomaterials, and the production of ROS requires O_2 as a precursor [47,48]. However, NMH exhibited an excellent antibacterial activity, even in an anaerobic environment, which ruled out the action of ROS. Although the precise antibacterial mechanism for NMH has not been elucidated, the ROS generation is not as significant an antibacterial factor. The molecular mechanisms were analyzed in both protein adsorption and cell wall permeability experiments. These results showed that NMH could significantly improve the penetrability of *S. mutans* cell membrane, and the protein leakage increased with the increase of NMH concentration. Pan et al. observed that NMH was easily adsorbed on the surface of bacteria but did not penetrate the bacterial cells [20]. Therefore, NMH may cause bacterial death by binding to cell wall proteins and enhancing the penetrability of the membrane, leading to the leakage of cellular contents. In the future we plan to perform experiments to assess the antibacterial effect of the new material against Gram-negative bacteria and to explore the antibiotic mechanisms involved. The effect of adding NMH on physical properties and biocompatibility also need to be evaluated.

Conclusions

In summary, this study confirmed that NMH had antibacterial effects on oral microorganisms, indicating that it can be used in combination with root canal sealer. NMH enhanced the antibacterial function of AH Plus in the fresh state and generated long-lasting antimicrobial effects. These results suggest that AH Plus loaded with NMH has great potential in endodontics to eradicate residual pathogens and prevent reinfection.

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