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Short-term antimicrobial properties of mineral trioxide aggregate with incorporated silver-zeolite

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Abstract – The purpose of this *in vitro* study was to determine whether adding silver-zeolite (SZ) to mineral trioxide aggregate (MTA) would enhance the antimicrobial activity of MTA against *Staphylococcus aureus* (ATCC #25923), *Enterococcus faecalis* (ATCC #29212), *Escherichia coli* (ATCC#25922), *Pseudomonas aeruginosa* (ATCC #27853), *Candida albicans* (ATCC #90028), *Porphyromonas gingivalis* (ATCC #33277), *Actinomyces israelii* (ATCC #12102), and *Prevotella intermedia* (ATCC# 15032). SZ was added at 0.2% and 2% mass fraction concentration to MTA powder. The control group was MTA powder with no SZ. The antimicrobial effect test was accomplished by placing freshly mixed MTA specimens on agar plates inoculated with microorganisms and comparing the zones of inhibition at 24, 48, and 72 h. The amounts of silver ion release from MTA specimens were measured with atomic absorption spectrophotometry at 10-min, 24-, 48-, and 72-h periods. The pH of MTA specimens was measured with a pH meter at 10-min, 24-, 48-, and 72-h periods. MTA with 2% and 0.2% SZ specimens showed inhibitory effects on some microorganisms at all time periods, whereas no antimicrobial activity showed for *P. intermedia* and *A. israelii*. MTA without SZ inhibited *C. albicans*, *E. Coli*, and *P. intermedia*. The highest silver release was detected in 2% SZ MTA at 24 h. The incorporation of SZ may enhance the antimicrobial activity of MTA.

Microorganisms are the main etiological factor in the development and progression of pulpal and periapical disease, and they also play a key role in endodontic treatment failures (1–5). Failure of initial endodontic therapy can be treated by non-surgical or surgical treatment modalities. When non-surgical root canal treatment fails to treat periradicular lesions of endodontic origin or retreatment is not indicated, periradicular surgery may be indicated (6). The placement of root-end fillings during periradicular surgery is a procedure of crucial importance to develop apical seal at the resected root end (7).

Many materials have been used as root-end fillings, including amalgam, zincoxide–eugenol, glass ionomer cement, composite resins, compomers, resin-modified glass ionomers, and mineral trioxide aggregate (MTA) (8–11). The ideal root-end filling material should have good sealing ability and biocompatibility. Besides these properties, the root-end filling material should have some antibacterial activity (12). A number of studies have been conducted on this topic (12–15).

MTA was first described in the dental literature in 1993 and was given approval for endodontic use by the US Food and Drug Administration in 1998 (16, 17). The use of MTA as a root-end filling material was identified because the material is hydrophilic cement that sets in the presence of water (18). Several studies evaluated the effect of MTA on microorganisms (13, 14, 19–22), but these studies have conflicting results. Torabinejad et al.(12) found that MTA was effective against some facultative microorganisms but not against other bacterial strains, including *Enterococcus faecalis*. Similar to these findings, Estrela et al.(19) also could not detect any inhibitory effect of MTA against *E. faecalis*. In other studies, it has been shown that MTA either delayed or inhibited the growth of *E. faecalis* (13, 14, 20, 21). In addition, to enhance the antimicrobial effect of MTA, chlorhexidine (CHX) has been used as a mixing agent instead of sterile water (21).

To enhance the antimicrobial properties of dental materials, several materials have been used. Metallic silver is one of the common elements used in dentistry. Silver is known to possess antibacterial properties. Among metallic ions, ionic silver has the highest antibacterial activity (23, 24). Zeolites are aluminum silicate crystalline structures that present void spaces within the frameworks, 3–10 angstroms in a diameter that are capable of hosting cations, water, or organic molecules. Antimicrobial cations, such as silver and zinc, may be lodged within the void spaces of the zeolites and be exchanged over time with other cations from the environment (25–27). Silver ion–containing zeolite (silver-zeolite, SZ) can provide antibacterial activity to resins, glass ionomer cements, and synthetic fibers by mixing (23, 24, 28–30). Because the zeolite framework does not readily decompose over time, it can serve as a

reservoir of antimicrobial agents. Depending on this property, it has long-lasting antimicrobial effects (31). In addition to this, SZ is considered to have no detrimental effects on tissues (32). Because, silver-zeolite (SZ) has been incorporated into other dental materials, the authors wanted to test the hypothesis that the antimicrobial properties of MTA would be enhanced with addition of silver-zeoilte.

The purpose of this study is to determine whether MTA had antimicrobial activity and whether the incorporation of SZ in MTA would enhance this antimicrobial activity.

Materials and methods

Preparation of silver-zeolite and MTA specimens

Zeolite 4A, sodium-type zeolite ($1Na_2O{:}1Al_2O_3{:}2SiO_2$ XH_2O) (X = mole fraction) (Na-Z; Sigma-Aldrich Chemie GmbH, Steinheim, Germany), was used as the host compound. Silver ions were loaded into the zeolite by the ion-exchange method. The ion exchange was carried out by the addition of Na-Z powder (10 g) into a 1 M silver nitrate (Merck KGaA, Darmstadt, Germany) solution (300 cm^3). The suspension was stirred at room temperature in the dark for 24 h, then centrifuged, washed with deionized water, and air-dried.

SZ was added at a 0.2% or 2% mass fraction to MTA powder (Dentsply, DeTrey GmbH, Konstanz, Germany). The control material was MTA powder without SZ.

Test microorganisms and growth conditions

Staphylococcus aureus (ATCC #25923), *E. faecalis* (ATCC #29212), *Escherichia coli* (ATCC#25922), *Pseudomonas aeruginosa* (ATCC #27853), *Candida albicans* (ATCC #90028), *Porphyromonas gingivalis* (ATCC #33277), *Actinomyces israelii* (ATCC #12102), and *Prevotella intermedia* (ATCC# 15032) strains were used in this study. *Staphylococcus aureus* and *E. faecalis* strains were cultured on 5% sheep blood agar (Orbak®, Ankara, Turkey), *E. coli* and *P. aeroginosa* were grown at brain heart infusion agar (Merck®), and *C. albicans* were cultured on Sabourraud dextrose agar (SDA) (Merck®) at 37°C for 24–48 h aerobically. *Porphyromonas gingivalis*, *A. israelii*, and *P. intermedia* were cultured on autoclave-sterilized Schaedler agar (Oxoid, Hampshire, UK) supplemented with sheep blood (50 ml l^{-1}), Vit K (1 μg ml^{-1}) and hemin (5 μg ml^{-1}) at 37°C for 4–5 days in an anaerobic chamber (Electrotek, West Yorkshire, UK). Then, freshly grown bacterial and fungal suspensions were prepared in the sterilized test tubes containing brain heart infusion broth for *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeroginosa*, Sabourraud dextrose broth (Merck®) for *C. albicans*, and Schaedler broth (Oxoid) for *P. gingivalis*, *A. israelii*, and *P. intermedia*. The final concentrations of the bacterial strains were adjusted to 1.5×10^8CFU ml^{-1} and the final concentration of fungal suspension was adjusted to 2.5×10^3CFU ml^{-1} according to the turbidity of 0.5 McFarland test standard. For *S. aureus*, *P. aeroginosa*, and *E. coli* Mueller Hinton agar (Merck®), for *E. faecalis* 5% sheep blood agar (Orbak®), for *C. albicans* SDA, and for *P. gingivalis*, *A. israelii*, and *P. intermedia* Schaedler agar were prepared and poured on the sterilized petri plates at the equal amount of 20 ml. For each material, three holes with a diameter of 2 mm were holed in the agar plates aseptically. Each of strains was spreaded on their specific agar plates. Then, MTA with or without additional SZ was mixed with a sterile spatula on a sterile glass slab according to the manufacturer's instructions by using 1 g of powder for every 0.35 ml of sterile water. The MTA mixtures were placed into wells using sterile amalgam carriers and gently condensed into place by using a non-surgical MTA Manual Carrier (Dentsply/Tulsa, Johnson City, TN, USA). Then, bacterial and fungal strains were incubated at 37°C for 24, 48, and 72 h in the aerobic and anaerobic conditions. At the end of the each incubation time, the diameter of inhibition zones was measured with a digital caliper (Mitutoyo, SP, Brazil) by a blinded, independent observer. The results were subjected to one-way ANOVA, and *Post hoc* comparisons were performed using Tamhane's T2 test at a significance level of 0.05.

Silver release test

Each composition of the MTA powder was mixed with the recommended liquid according to manufacturer's instruction. Five teflon molds per group with a thickness of 2 mm and a diameter of 4 mm were filled with the freshly mixed MTA. Each specimen was immersed in vials containing 20 ml of deionized water at 37°C. After the deionized water was acidified by adding 1 ml of concentrated HNO_3, the specimens were removed from the solution immediately. The amounts of silver released from the specimen in deionized water were measured with an atomic absorption spectrophotometer (AA-6300; Shimadzu Scientific Instruments, Columbia, MD, USA). The instrument was calibrated using standards containing 1, 2, 3, and 4 ppm silver ion in deionized water. The specimens were removed from the vials, rinsed with deionized water, and reimmersed in fresh deionized water. Silver release was measured at 10 min, 24-, 48-, and 72-h periods. The results were statistically analyzed using ANOVA and Scheffe's test at a significance level of 0.05.

pH measurement

Five specimens of each group, prepared similarly like for the silver release test, were immersed in vials containing 10 ml of deionized water at 37°C. The pH was measured with a pH meter (Expandomatic SS-2; Beckman Instruments, Fullerton, CA, USA) at 10-min, 24-, 48-, and 72-h periods. The results were statistically analyzed using ANOVA at a significance level of 0.05.

Results

Antimicrobial effect

Table 1 shows the antimicrobial activity of MTA groups expressed by means and standard deviation of zones of inhibition in millimeters. In the *E. faecalis*, *S. aureus*,

Table 1. Antibacterial activity, in millimeter inhibition (mean ± SD) zones of 2% and 0.2% incorporated SZ MTA specimens of microorganisms

	MTA specimens								
	24 h			48 h			72 h		
Microorganisms	2% SZ	0.2% SZ	Control	2% SZ	0.2% SZ	Control	2% SZ	0.2% SZ	Control
Enterococcus faecalis	6.03 ± 0.32	5.21 ± 0.53	0.00 ± 0.00	6.92 ± 0.20	5.93 ± 0.53	0.00 ± 0.00	7.06 ± 0.36	6.40 ± 0.45	0.00 ± 0.00
Staphylococcus aureus	7.03 ± 0.34	6.27 ± 0.47	0.00 ± 0.00	7.34 ± 0.94	6.96 ± 0.47	0.00 ± 0.00	7.54 ± 0.36	6.98 ± 0.45	0.00 ± 0.00
Candida albicans	11.10 ± 0.96	6.93 ± 0.32	6.54 ± 0.22	10.90 ± 0.69	6.43 ± 0.56	6.22 ± 0.15	10.73 ± 0.54	6.13 ± 0.47	6.04 ± 0.23
Escherichia coli	10.42 ± 0.74	7.64 ± 0.88	6.28 ± 0.32	9.74 ± 0.34	7.02 ± 0.28	6.12 ± 0.12	9.44 ± 0.18	6.12 ± 0.98	6.02 ± 0.22
Pseudomonas aeruginosa	11.51 ± 0.24	8.68 ± 0.65	7.58 ± 0.36	11.20 ± 0.76	8.22 ± 0.54	7.02 ± 0.78	11.00 ± 0.34	7.91 ± 0.11	6.41 ± 0.54
Porphyromonas gingivalis	10.86 ± 0.76	7.72 ± 0.22	0.00 ± 0.00	9.14 ± 0.78	7.12 ± 0.16	0.00 ± 0.00	8.84 ± 0.88	6.56 ± 0.76	0.00 ± 0.00
Actinomyces israelii	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Prevotella intermedia	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

MTA, mineral trioxide aggregate; SZ, silver-zeolite.

P. intermedia, *A. israelii*, and *P. gingivalis* groups MTA without SZ did not demonstrate any antimicrobial activity at all time periods. MTA with 2% and 0.2% SZ specimens had antibacterial effect on some microorganisms at all time periods, whereas no antimicrobial activity was found for *P. intermedia* and *A. israelii*. No significant differences were found between the inhibitory effects of 2% and 0.2% SZ MTA against inhibited strains. In the *C. Albicans* groups, all the MTA specimens showed antimicrobial activity at all time period. At all time period, 2% SZ MTA specimens had significantly higher inhibition than the MTA without SZ ($P < 0.05$).

Silver release test

The release rates of silver from the MTA specimens are summarized in Table 2. Two percentage of SZ MTA produced the highest amount of silver within each of the four times. The amount of silver release increased with the increase in the ratios of incorporated SZ ($P < 0.05$). The highest silver release was detected in 2% SZ MTA at 24 h.

pH measurement

Figure 1 shows changes in pH of the MTA specimens. Adding SZ to MTA specimens decreased the initial pH of SZ MTA specimens. Twenty-four hours later, the pH of SZ MTA specimens increased to a pH of 11.0 and stabilized for all time period.

Table 2. Silver releases from 2% MTA SZ and 0.2% MTA SZ

	MTA Specimens		
	2% SZ	0.2% SZ	Control
10 min	0.59 ± 0.08	0.47 ± 0.16	0.00 ± 0.00
24 h	0.86 ± 0.20	0.47 ± 0.36	0.00 ± 0.00
48 h	0.28 ± 0.08	0.27 ± 0.84	0.00 ± 0.00
72 h	0.23 ± 0.02	0.12 ± 0.04	0.00 ± 0.00

Mean values expressed in ppm (Mean ± SD).
MTA, mineral trioxide aggregate; SZ, silver-zeolite.

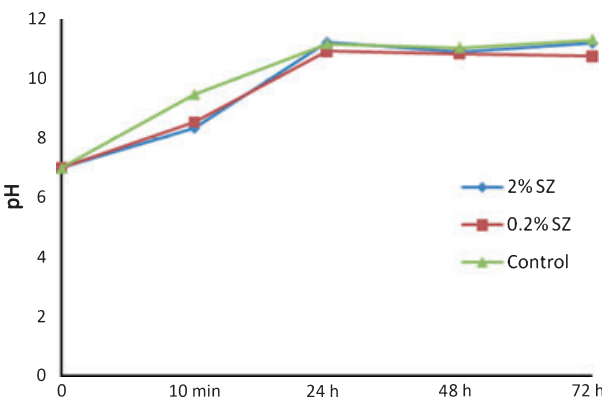

Fig. 1. The pH values of mineral trioxide aggregate specimens at different time intervals.

Discussion

The antimicrobial activity of endodontic sealers can be evaluated *in vitro* by the agar diffusion method (33–35). Agar diffusion, despite limitations, such as lack of standardization of inoculum density, adequate culture, size and number of specimens per plate, and time and temperature of incubation (36), is still the most widely used *in vitro* method of evaluation of antimicrobial activity. The advantage of the agar diffusion test is that it allows direct comparisons of materials against the test microorganisms. A great disadvantage of this method is that it does not distinguish between microbiostatic and microbicidal properties of the materials (33). In addition, the result of agar diffusion test does not depend only on the toxicity of the material for the particular microorganism, but is also highly influenced by the diffusibility

of the material across the medium (37). A material that diffuses more easily will probably provide larger zones of microbial growth inhibition (38, 39).

The microorganisms in this study were selected to represent aerobes, anaerobes, and a yeast found in infected canals. These microorganisms commonly found in infected root canals and that are usually resistant to routinely used endodontic intracanal medicaments (34, 40).

MTA with SZ inhibited growth of some microorganisms tested, and no antimicrobial activity was demonstrated for *P. intermedia* and *A. israelii* in this study. The incorporation of SZ enhanced the antimicrobial activity of MTA. In particulary, the inhibition of *E. faecalis* is very important because this bacteria is the most frequently isolated microorganism recovered from endodontically treated cases (41). In the present study, it has been shown that MTA without SZ did not have an inhibitory effect against *E. faecalis* and *S. aureus*. Similar to our findings, Torabinejad et al.(12) reported that MTA did not have an inhibitory effect against *E. faecalis* and *S. aureus*. In addition, Estrela et al. (19) also could not detect any inhibitory effect of MTA against *E. faecalis*.

Many studies evaluated the effect of MTA on microorganisms associated with endodontic disease, but these studies have conflicting results (12–14, 20, 21). Ribeiro et al.(42) suggested that these variations might be the results of the methodology used, such as aerobic and anaerobic incubations. It has been shown that MTA in an aerobic atmosphere could generate reactive oxygen species (ROS), which have antimicrobial activity. However, under anaerobic conditions, a decrease in the generation of ROS was observed (43). Ribeiro et al.(44) reported that in an anaerobic atmosphere, MTA was incapable of generating the ROS responsible for the antimicrobial effect on the different bacterial strains. In addition, Torabinejad et al. found that MTA had no antibacterial effect against on any of the strict anaerobic bacteria. Similar to our results, these authors observed no antibacterial activity for *P. intermedia*.

The antifungal activity of MTA was shown in several studies (21, 22, 45, 46). Similar to previous studies, we found good antifungal effect of MTA specimens on the tested *C. albicans*. The incorporation of SZ enhanced the antifungal activity of MTA. Two percentage of SZ MTA specimens had significantly higher inhibition than the other MTA specimens ($P < 0.05$). However, the antifungal activity of MTA specimens, in this study, insignificantly decreased at 72-h periods.

Although silver is known to possess antibacterial properties, the exact mechanism of action is not fully understood. Three possible suggestions could be considered: (i) silver ions destroy the cell wall; (ii) silver interrupts the RNA replication process of the microbe, thereby preventing cell multiplication; (iii) silver ions cause cellular respiration to be blocked, effectively choking the microbe (32). Another possible antibacterial mechanism of silver ions is the interaction with thiol groups in proteins, which induce the inactivation of the bacterial proteins (47). In addition, the catalytic action of silver causes oxygen to change into oxygen radicals by the action of light energy and/or H_2O in the air or water only at polar surfaces and this active oxygen causes structural damage in bacteria (48). These phenomena lead to the damage or even the death of the microorganism.

For the preparation of silver-containing materials, zeolites have been used as the host inorganic compound. Silver ions bind particularly to zeolite, resulting in a gradual, stable, and long-lasting release of silver ions from zeolite (ca.10 ppb into water). Similar to our results, Hotta et al. (27) showed that the increase in the zeolite content increased the release of silver into ambient solution. In the presented study, 2% and 0.2% of SZ MTA did not show any difference for the antimicrobial activity. The current results confirm the study by Hotta et al.(27) in which he stated that the large amounts of silver were not needed to produce an antibacterial effect.

An important consideration with regard to the use of silver as an antimicrobial is the potential for the development of resistance. Silver resistance was found in both Gram-positive and Gram-negative bacteria, mediated by plasmid and transposon mechanisms (49). However, silver sulphadiazine continues to be the antimicrobial agent most often used in burn care facilities.

The MTA is a Portland-type cement that contains calcium oxide, which in contact with tissue fluid or water is converted to calcium hydroxide. The MTA hydration results in the calcium hydroxide dissociating into calcium and hydroxyl ions, increasing the pH and calcium concentrations (50). In the present study, the pH of SZ MTA specimens increased to a pH of 11.0 and stabilized for all time period, and the pH value for MTA specimens did not exhibit any statistical difference. Many investigations explained the antimicrobial action of MTA by its high pH (14, 22, 45, 51). However, it has been shown that the conditions under which several endodontic microorganisms were killed, were not pH mediated (52).

To enhance antimicrobial activity of MTA, some investigations replaced distilled water with other liquids to mix with MTA powder (21). Another study mixed 2% CHX with MTA powders (20). On the basis of these results, it appears that enhancing antibacterial property of MTA by adding various liquids might adversely affect other properties of the material (53). Therefore, MTA powder without SZ was used as control group in this study.

It is important to recognize the limitations of *in vitro* antimicrobial testing *per se* and the difficulty in correlating *in vitro* results with the *in vivo* activity (54). To fully assess the viability of SZ MTA, further studies are needed to gauge the physical properties, e.g., setting and working time. In addition, adding SZ to MTA may affect the material's properties. On the other hand, considering MTA is placed in patients with the expectation that it will remain in place for years, long-term effects of adding SZ to MTA should be investigated.

In summary, adding to SZ to MTA may enhance the antimicrobial activity of MTA *in vitro*. Apparently, inhibition of the growth of some microorganisms is related the existence of silver. However, increases in the silver level did not enhance the antimicrobial properties

of MTA. It can be assumed that incorporating a small amount SZ into MTA was sufficient for enhancing its antimicrobial activity.

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