

## Anti-bacterial properties of calcium hydroxide in combination with silver, copper, zinc oxide or magnesium oxide

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### Abstract

Prevention of bacterial growth among root canal treatment sessions is a prerequisite for successful root canal treatment. The most common way to achieve this is to use calcium hydroxide in the treatment sessions. Some studies have shown calcium hydroxide inefficiency in this field. The aim of this study was to investigate and compare the effects of silver, copper, zinc oxide and magnesium oxide nanoparticles on the inhibitory effects of calcium hydroxide based on *Enterococcus faecalis* species. *Enterococcus faecalis* bacteria having 0.5 McFarland concentration were prepared. Plates containing BHI agar medium were prepared. In each plate, four wells were created and the plate was cultured using a sterile swab. Afterwards, calcium hydroxide composition of 1% and 2% concentration from silver, copper, zinc oxide and magnesium oxide nanoparticles were prepared separately, as well as the combination of calcium hydroxide with 1% silver in combination with 1% of copper, zinc oxide and magnesium oxide nanoparticles, which were then transferred to the wells. After 24 hours of incubation, the inhibition zone diameter was measured. Data were analyzed by Mann-Whitney test. At 1% concentration, only the combination of copper nanoparticles with calcium hydroxide could significantly create an inhibition zone larger than calcium hydroxide alone (P value <0.5). At 2% concentration, the combination of copper nanoparticles with calcium hydroxide, and the combination of silver nanoparticles with calcium hydroxide, were significantly higher than calcium hydroxide alone (P value <0.5). The calcium hydroxide composition containing 1% silver nanoparticles in combination with 1% copper, zinc oxide and magnesium oxide nanoparticles significantly increased the growth inhibition zone more than calcium hydroxide alone. (P Value <0.5). Copper nanoparticles showed the best antibacterial properties among silver, copper, magnesium oxide and zinc oxide nanoparticles in combination with calcium hydroxide. Also, the combination of 1% of nanoparticles with each other increases antibacterial properties.

**Key Words:** Copper, silver, zinc oxide, magnesium oxide, growth inhibition zone, *enterococcus faecalis*, calcium hydroxide.

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During the root canal treatment process, bacteria in the root canal play an important role in the development, expansion and stability of root lesions.<sup>1</sup> Considering the complex anatomy of the root canal and the impossibility of mechanical preparation of all root sites to remove bacterial agents, the use of antibacterial agents for root canal removal is recommended.<sup>2</sup> Even by using these methods, the canal is not completely cleared of bacterial agents. According to Bystrom et al., if the canal remains empty between treatment sessions, the bacteria are replicated and the number of bacteria increases even to the initial number at the onset of treatment.<sup>3</sup> To overcome this problem, calcium hydroxide has been used extensively to fill the canal between treatment sessions.<sup>4,5</sup>

Calcium hydroxide has antibacterial properties.<sup>2</sup> Although calcium hydroxide is widely used, its application has been questioned in some studies. Some evidence confirm the inactivation of antibacterial activity of calcium hydroxide by dentin and increased bacteria after treatment with calcium hydroxide.<sup>6,7</sup> Considering the calcium hydroxide weaknesses, efforts have been made to improve the antibacterial properties of calcium hydroxide. Different studies had shown that using Silver or Copper alongside Calcium hydroxide improved Antibacterial properties.<sup>8-12</sup> Metal oxides have been used for centuries as antibacterial agents. Metal oxides like Zinc Oxide and Magnesium Oxide have antibacterial properties in various forms.<sup>13-16</sup> It should be kept in mind that the effectiveness of these elements

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depends on their contact surface. Along with technology advancement and the possibility of reducing metal particle sizes from micro to nanoscale and increasing the contact surface of metal particles, it is possible to increase antibacterial properties.<sup>17</sup> Also, the simultaneous use of antibacterial compounds is a common practice for increasing antibacterial effects. Synergistic effects of antibacterial compounds reduce the need for higher drug doses to limit the side effects.<sup>18-20</sup> According to the above, it seems that the use of calcium hydroxide alone does not eliminate the needs for root canal treatment. On the other hand, considering the antibacterial properties in metallic nanoparticles and the effect of these metals on increasing the antibacterial properties of calcium hydroxide and the lack of comprehensive study to compare the antibacterial properties of these particles in combination with calcium hydroxide, the present study aimed to investigate the antibacterial properties of calcium hydroxide in combination with metal nanoparticles of silver, copper, zinc oxide and magnesium oxide at similar concentrations and in combination with each other compared to calcium hydroxide.

### Materials and Methods

*Enterococcus faecalis* bacteria were prepared as biofilm from the Iranian Pasteur Institute with persian type culture collection (PTCC):1394, and then after pouring into a liquid medium of Brain Heart Infusion Broth (Merck, Darmstadt Germany), it was incubated for 48 hours at 37 ° C. It was then cultured on a solid medium of Tryptic Soy Agar (Merck, Darmstadt Germany) for 48 hours to create a colony. Then, using these colonies and with the aid of a physiological serum, a suspension was prepared with a concentration of approximately 0.5 McFarland ( $1.5 \times 10^8$  cells / mL). Pure calcium hydroxide powder (Merck, Darmstadt Germany) and nanosized metal particles with 20 nm dimensions (US Research nanomaterials, Huston, USA) were prepared. For the preparation of calcium hydroxide paste, modified with 1% silver, copper, zinc oxide and magnesium oxide nanoparticles, 990 mg of calcium hydroxide powder was combined with 1 ml of 10 mg / ml solution of the mentioned nanoparticles (Table 1). For the preparation of calcium hydroxide paste, modified with 2% silver, copper, zinc oxide and magnesium oxide nanoparticles, 990 mg of calcium hydroxide powder was combined with 1 ml of 20 mg / ml solution of the mentioned nanoparticles (Table 1). For the preparation of calcium hydroxide paste, modified with zinc oxide nanoparticles, 980 mg of calcium hydroxide powder was combined with 1 ml of 20 mg / ml solution of silver and zinc oxide nanoparticles (10 mg of silver nanoparticles in combination with 10 mg of Zinc oxide nanoparticles) (Table 1). For the preparation of calcium hydroxide paste, modified with silver and copper nanoparticles, 980 mg of calcium hydroxide powder was combined

**Table 1.** Combination groups

Group	Ca(OH) <sub>2</sub>	Nano Particles
Ca(OH) <sub>2</sub> + 1% Ag	990 mg Ca(OH) <sub>2</sub>	1000 ml of 10 mg/ml Nano Ag solution
Ca(OH) <sub>2</sub> + 2% Ag	980 mg Ca(OH) <sub>2</sub>	1000 ml of 20 mg/ml Nano Ag solution
Ca(OH) <sub>2</sub> + 1% Cu	990 mg Ca(OH) <sub>2</sub>	1000 ml of 10 mg/ml Nano Cu solution
Ca(OH) <sub>2</sub> + 2% Cu	980 mg Ca(OH) <sub>2</sub>	1000 ml of 20 mg/ml Nano Cu solution
Ca(OH) <sub>2</sub> + 1% MgO	990 mg Ca(OH) <sub>2</sub>	1000 ml of 10 mg/ml Nano MgO solution
Ca(OH) <sub>2</sub> + 2% MgO	980 mg Ca(OH) <sub>2</sub>	1000 ml of 20 mg/ml Nano MgO solution
Ca(OH) <sub>2</sub> + 1% ZnO	990 mg Ca(OH) <sub>2</sub>	1000 ml of 10 mg/ml Nano ZnO solution
Ca(OH) <sub>2</sub> + 2% ZnO	980 mg Ca(OH) <sub>2</sub>	1000 ml of 20 mg/ml Nano ZnO solution
Ca(OH) <sub>2</sub> + 2% Ag-ZnO	980 mg Ca(OH) <sub>2</sub>	1000 ml of 20 mg/ml Nano Ag-ZnO solution
Ca(OH) <sub>2</sub> + 2% Ag-Cu	980 mg Ca(OH) <sub>2</sub>	1000 ml of 20 mg/ml Nano Ag-Cu solution
Ca(OH) <sub>2</sub> + 2% Ag-MgO	980 mg Ca(OH) <sub>2</sub>	1000 ml of 20 mg/ml Nano Ag-MgO solution
Ca(OH) <sub>2</sub>	1000 mg Ca(OH) <sub>2</sub>	1000 ml distilled water
Positive Control	Chlorhexidine	0
Negative Control	-	-

with 1 ml of 20 mg / ml solution of silver and copper nanoparticles (10 mg of silver nanoparticles in combination with 10 mg of copper nanoparticles) (Table 1). To prepare the calcium hydroxide paste modified with silver and magnesium oxide nanoparticles, 980 mg of calcium hydroxide powder was combined with 1 ml of 20 mg / ml solution of silver and magnesium oxide nanoparticles (10 mg of silver nanoparticles in combination with 10 mg of magnesium oxide nanoparticles) (Table 1). 2% chlorhexidine was used as positive control group and empty cavity as negative control. To prepare the culture medium, brain heart infusion (BHI) is injected into a sterile disposable plate of 10 cm diameter so that a 5-millimeter thick volume is

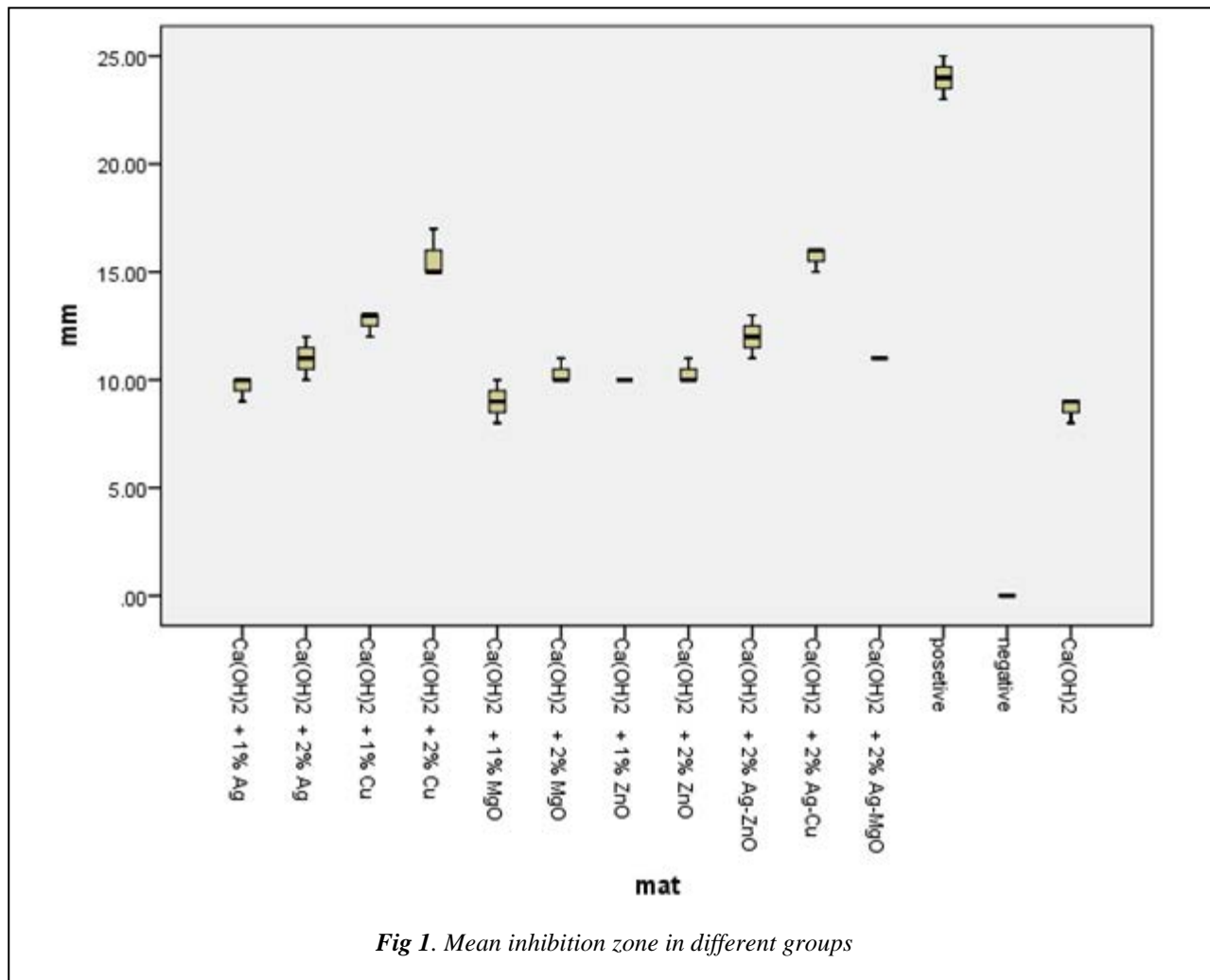


Fig 1. Mean inhibition zone in different groups

created. After cooling the mixture, four cavities with identical intervals of 5 mm diameter and 5 mm depth in agar are created in each container using sterile punch. Then, using sterile swabs, culturing was carried out from the bacteria containing solution. The cavities were then filled with specimens. The plate was incubated for 24 hours. After this time, the diameter of the growth inhibition zone was measured. Data were analyzed by SPSS version 21 software using Kolmogorov-Smirnov and Shapiro-Wilk statistical tests to determine the distribution of normal or abnormal data. Then, based on the test results, in the case of normal distribution, the Tuckey test was used and in the case of abnormal distribution, the Mann-Whitney test was performed.

**Results and Discussion**

The mean diameter of the inhibition zone is shown in Figure 1. According to the results, all groups have a higher inhibition zone than calcium hydroxide alone. The statistical results of Kolmogorov-Smirnov and Shapiro-Wilk showed an abnormal data distribution. Therefore, data were analyzed using the Mann-Whitney test.

Based on the statistical results, In 1% nano metal concentration, inhibition zone diameter for Ca(OH)<sub>2</sub> + 1% Cu was significantly different with calcium hydroxide alone (P value <0.05). based on the statistical analysis for 2% concentration of Nano metal, there were a significant difference between inhibition zone of Ca(OH)<sub>2</sub> + 2% Ag and Ca(OH)<sub>2</sub> + 2% Cu compare to calcium hydroxide alone (P value <0.05). In the combination of calcium hydroxide with silver nanoparticles and copper nanoparticles, zinc oxide and magnesium oxide, the inhibition zone diameter of all groups (Ca(OH)<sub>2</sub> + 2% Ag-ZnO, Ca(OH)<sub>2</sub> + 2% Ag-Cu, Ca(OH)<sub>2</sub> + 2% Ag-MgO) had a significant difference with calcium hydroxide alone (P value <0.05). (Table 2). Statistical analysis was performed among groups that had a significantly larger inhibition zone than calcium hydroxide alone. The inhibition zone diameter for Ca(OH)<sub>2</sub> + 2% Ag, Ca(OH)<sub>2</sub> + 1% Cu, Ca(OH)<sub>2</sub> + 2% Ag-MgO and Ca(OH)<sub>2</sub> + 2% Ag-ZnO, statistically had no significant difference. The inhibition zone diameter was similar between Ca(OH)<sub>2</sub> + 2% Ag-Cu and Ca(OH)<sub>2</sub> + 2% Cu groups and did not show any significant difference (P Value <0.05). Also, based on the results, the largest inhibition zone was related to

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**Table 2.** P-values of Ca(OH)<sub>2</sub> group compared to other groups

Ca(OH) <sub>2</sub> + 1% Ag	0.90
Ca(OH) <sub>2</sub> + 2% Ag	0.02
Ca(OH) <sub>2</sub> + 1% Cu	0.00
Ca(OH) <sub>2</sub> + 2% Cu	0.00
Ca(OH) <sub>2</sub> + 1% MgO	1.00
Ca(OH) <sub>2</sub> + 2% MgO	0.27
Ca(OH) <sub>2</sub> + 1% ZnO	0.59
Ca(OH) <sub>2</sub> + 2% ZnO	0.27
Ca(OH) <sub>2</sub> + 2% Ag-ZnO	0.00
Ca(OH) <sub>2</sub> + 2% Ag-Cu	0.00
Ca(OH) <sub>2</sub> + 2% Ag-MgO	0.00
Posetive Control	0.00
Negative Control	0.00

Ca(OH)<sub>2</sub> + 2% Cu and Ca(OH)<sub>2</sub> + 2% Ag-Cu, which statistically had significantly larger inhibition zone than other study groups (P Value <0.05). According to the results of the present study for the inhibition zone diameter of 1% concentration of different metal nanoparticles with calcium hydroxide, the diameter of the inhibition zone of calcium hydroxide alone had a significant difference with the Ca(OH)<sub>2</sub> + 1% Cu group. Based on the statistical analysis of the inhibition zone diameter in 2% concentration of metal nanoparticles in combination with calcium hydroxide, calcium hydroxide alone had a significant difference with Ca(OH)<sub>2</sub> + 2% Ag and Ca(OH)<sub>2</sub> + 2% Cu. According to Afkhami et al., The combination of calcium hydroxide paste and silver nanoparticles with 100PPM concentration, significantly improved the antibacterial properties in contrast to calcium hydroxide alone.<sup>8</sup> According to Samii et al., the use of mineral trioxide aggregate (MTA) containing 1% silver nanoparticles significantly inhibits the growth of *E. faecalis*, *C. albicans*, and *P. aeruginosa* compared to MTA alone.<sup>9</sup> Based on the study of Javadi et al., The combination of 10% calcium hydroxide and 200PPM silver significantly improved the growth inhibition of *E.*

*faecalis* compared to calcium hydroxide alone in the infected teeth canal.<sup>10</sup> The results of studies by Fuzz et al. and Knappwost et al. showed that the antibacterial properties of calcium hydroxide and copper combination was superior to calcium hydroxide alone.<sup>11,12</sup> Based on the results of this study, there was no difference between the Ca(OH)<sub>2</sub> + 1% MgO, Ca(OH)<sub>2</sub> + 2% MgO, Ca(OH)<sub>2</sub> + 1% ZnO, Ca(OH)<sub>2</sub> + % ZnO group and calcium hydroxide alone in case of the inhibition zone diameter. According to Sawai et al., 100 mg / ml magnesium oxide concentrations have antibacterial effects against *Escherisia coli* and *Stafilocus aureus*.<sup>13</sup> According to the Jin and He study, nano particles of magnesium oxide at 8 mg / ml concentration inhibit the growth of *Escherichia coli*.<sup>14</sup> According to the Sawai study, MgO and ZnO metal particles have anti-bacterial properties against *Staphylococcus aureus* and *Escherichia coli*.<sup>15</sup> According to a study by Aydin Sevinç et al., the addition of 10% zinc oxide nanoparticles to the composite reduced the growth of *Streptococcus sobrinus* to 80% and the minimum inhibitory concentration of zinc oxide nanoparticles was determined at 50 µg / ml.<sup>16</sup> There are many differences between the effective concentration and the efficacy of different compounds against microorganisms among different studies. This difference may be due to the use of different methods to investigate the alteration of antibacterial properties of materials by nanoparticles. For example, in the present study, the inhibition zone diameter of *Entrococcus faecalis* for calcium hydroxide containing 1% silver nanoparticles, was significantly different from calcium hydroxide alone. While in another study, the use of a solution containing 10% calcium hydroxide and 200 PPM of silver in the canal infected with *Entrococcus faecalis* had better antibacterial properties than calcium hydroxide solution.<sup>10</sup> The effect of nanoparticles on the alteration of antibacterial properties of substances may also be affected by the accompanying material used. For example, in the present study, the inhibition zone diameter of *Entrococcus faecalis* in calcium hydroxide containing 1% silver did not differ significantly from calcium hydroxide alone. While in another study, the MTA composition containing 1% silver nanoparticles significantly improved the *Entrococu faecalis* growth inhibition,<sup>10,21-23</sup> better than MTA alone. Also another possible cause for differences in the antibacterial nature is particle size. Different studies have shown an increase in antibacterial properties by reducing particle size.<sup>24,25</sup> In the present study, for the combination of 1% nano particles with calcium hydroxide, copper, zinc oxide, silver and magnesium oxide had the highest to lowest inhibition zone diameter, respectively. In the combination of 2% nano particles with calcium hydroxide, copper, silver and then zinc oxide equal with magnesium oxide, had the highest to lowest inhibition zone diameter, respectively. In the study of Top et al., the results of antimicrobial properties of copper, silver

and zinc ions in combination with Clinoptilolite showed that the highest antibacterial properties against *Pseudomonas aeruginosa* and *Escherichia coli* were related to the silver compound and the copper was at next place.<sup>26</sup> According to Du et al., the anti-bacterial properties of Chitosan alone and in combination with silver, copper, zinc, magnesium and iron particles, silver, copper, magnesium and zinc had the highest to lowest antibacterial strength, respectively. Combining these particles with Chitosan made it possible to significantly improve the antibacterial properties of chitosan.<sup>27</sup> According to McLean et al., comparing the antibacterial properties of silver and copper combination on different carriers, results showed higher antibacterial effects for copper than silver.<sup>28</sup> Contrary to the results of the present study which copper particles had higher antibacterial properties than silver, in studies of Du et al. and Top et al., silver alone and in combination with other materials had antibacterial properties higher than copper.<sup>26,27</sup> While the results of the study by McLean et al., similar to the present study, showed better antibacterial properties of copper than silver.<sup>28</sup> This difference in the amount and strength of antibacterial particles of similar particles in different compounds can be related to the oxidation-reduction state in the composition of metal particles combined with different materials.<sup>29</sup> Also, the combination of accompanying particles and carrier used may also affect the extent of particle effects.<sup>28,30</sup> According to the results of this study, the inhibition zone diameter of calcium hydroxide composition with 1% silver nanoparticles was not significantly different from calcium hydroxide alone. By adding 1% copper, magnesium oxide or zinc oxide nanoparticles to calcium hydroxide containing 1% silver nanoparticles, the inhibition zone diameter expanded significantly more than calcium hydroxide alone. According to Jin and He, the addition of Nisin to magnesium oxide nano particles increases the antibacterial properties of magnesium oxide. While the addition of zinc oxide nanoparticles to magnesium oxide nanoparticles does not alter material properties against *Escherichia coli* 14. In a study by Jia et al., SiO<sub>2</sub> nanoparticles coated with silver and zinc particles had antibacterial properties higher than silver coated with SiO<sub>2</sub> nanoparticles alone.<sup>31</sup> According to Mclean et al., the combination of silver and copper on different carriers, had higher anti-bacterial properties than copper and silver alone.<sup>28</sup> Similar to the results of this study, other studies also confirm the presence of synergistic properties between metal particles. These effects may be due to application of antibacterial effects in different ways individually by each particle or due to composition obtained from the particle combinations.<sup>26-28,32</sup> In conclusion, the use of metal nanoparticles is effective on the extent of the inhibition zone caused by calcium hydroxide. In the concentrations used, copper nanoparticles had higher properties than silver nanoparticles at similar concentrations. The

combination of silver particles with copper, magnesium oxide, or zinc oxide significantly increased the inhibition zone diameter of silver, indicating synergy among particles.

### List of acronyms

PPM - parts per million  
MTA - mineral trioxide aggregate  
SPSS - Statistical Package for the Social Sciences  
BHI - Brain heart infusion  
PTCC - Persian type culture collection

### Author's contributions

Each author contributed in equal part to the manuscript.

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### Conflict of Interest

The authors declare no conflicts of interests.

### Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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