

In vitro Investigation of the Antimicrobial Activity of Mouth Washes Incorporating Zein-Coated Magnesium Oxide Nanoparticles

Ghada H Naguib^{1,2}

Gamal S Abd El-Aziz³

Hisham A Mously⁴

Wafaa A Alhazmi⁵

Abeer M Alnowaiser⁶

Ali H Hassan⁷

Mohamed T Hamed^{4,8}

¹Department of Restorative Dentistry, Faculty of Dentistry, King Abdulaziz University, Jeddah, Saudi Arabia;

²Department of Oral Biology, Faculty of Dentistry, Cairo University, Cairo, Egypt;

³Department of Anatomy, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia; ⁴Department of Oral and Maxillofacial Prosthodontics, Faculty of Dentistry, King Abdulaziz University, Jeddah, Saudi Arabia;

⁵Department of Medical Laboratory Sciences, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia; ⁶Department of Pediatric Dentistry, Faculty of Dentistry, King Abdulaziz University, Jeddah, Saudi Arabia; ⁷Department of Orthodontics, Faculty of Dentistry, King Abdulaziz University, Jeddah, Saudi Arabia;

⁸Department of Fixed Prosthodontics, Faculty of Dentistry, Cairo University, Cairo, Egypt

Purpose: This in vitro study was undertaken to investigate the antimicrobial effect of distinctive oral mouth washes after the addition of zein-coated (Magnesium oxide) MgO nanoparticles on exemplify of some oral microorganisms.

Materials and Methods: Three hundred and twelve samples were used in this study. A set of five concentrations of MgO nanoparticles with zein and without zein-coating were incorporated into three oral mouth washes: Listerine zero, Listerine total control and Oral B in the mass percentages of 0.3%, 0.5%, 1%, 2%, 5% and 10%, in addition to controls with no MgO nanoparticles. The antimicrobial effect of three mouth washes with variable concentrations of MgO was tested against the following organisms: *Staphylococcus aureus*, *Streptococcus mutans*, *Enterococcus faecalis* and *Candida albicans* using the disc diffusion test (DDT) and direct contact test (DCT). Data were analyzed using one-way ANOVA statistical test.

Results: The tested mouthwashes with zein-coated MgO nanoparticles showed significant differences of antimicrobial activity on *S. mutans*, *S. aureus*, *E. faecalis*, and *C. albicans* in the disc diffusion test. While in the DCT, all tested mouthwashes with MgO nanoparticles with and without zein coating showed antimicrobial activity on all tested microorganisms.

Conclusion: Zein-coated MgO nanoparticles may be considered as a potential antimicrobial agent when added to oral mouthwashes. Future analysis, including in vivo studies, is required in order to incorporate zein/MgO nanoparticles into oral mouthwashes that may improve its antibacterial property.

Keywords: MgO, nanoparticles, zein polymer, antibacterial, mouthwash

Introduction

Oral diseases such as caries, periodontal inflammation and microbial abscesses are the most common diseases affecting the oral environment. The main causative agents are bacteria, such as *Streptococcus mutans*, *Porphyromonas gingivalis*¹ and fungi as *Candida albicans*.² Mouthwashes have been used for the prevention of plaque accumulation to decrease the incidence of these diseases.³

The metal oxide nanoparticles have been used in medicine for their bactericidal and bacteriostatic properties.⁴⁻⁶ These nanoparticles are considered as promising novel antibacterial agents, being harmless to mammalian cells and the oral environment.⁷

As the antibacterial properties of metal ions depend on their surface contact area, the use of nanoparticles leads to increase surface areas and thus increased interaction with organic and inorganic molecules. ZnO and TiO₂ were recently introduced in the

Correspondence: Mohamed T Hamed
Department of Oral and Maxillofacial Prosthodontics, Faculty of Dentistry, King Abdulaziz University, P.O.Box 80209, Jeddah, 21589, Saudi Arabia
Email mthamed@kau.edu.sa

field of dental materials. They have been added to resin composite restorations for their antibacterial effects.^{6,8,9} While Mg is an essential metal to the human body, nano-structured MgO has been found to be an essential mineral for optimal human health due to its new physical and chemical characteristics.¹⁰ Nano-MgO was shown to exhibit bactericidal activity directly proportional to particle size and concentration⁸ and to act against both Gram-positive and Gram-negative bacteria.^{8,9,11,12}

Many studies have been conducted to assess MgO nanoparticles due to their properties.⁹ A study done by Makhluaf et al,¹⁰ who synthesized nanocrystalline particles of MgO using microwave radiation in an ethylene glycol solution, has shown that MgO has an antibacterial effect on selected bacteria. After testing the antimicrobial effect of nano metal oxides, some researchers demonstrated that nanoparticles of MgO in solution have a bactericidal effect in opposition to variable bacteria.¹² In contrast, others proved that ZnO behaves more than a growth inhibitor especially on Gram-positive organisms.¹³

Also, studies on MgO nanoparticles' cytotoxicity showed that MgO nanoparticles were safe when used at concentrations lower than 50 µg.mL⁻¹.^{14,15}

Zein is a natural corn polymer that can be used in the nano form to coat the MgO nanoparticles in order to prevent their aggregation.¹⁶ Moreover, it has been reported that it has many unique properties that nominate it to be used in the industry of drug delivery and film coating.¹⁷ Several investigators believed that zein might serve as an inexpensive and effective alternative to synthetic and semi-synthetic film coatings currently used for extrusion coatings.^{18,19}

The addition of antimicrobial agents to mouthwashes and toothpastes preparations inhibited plaque accumulation and bacterial acid production.²⁰ Most of the mouth washes normally contain very low concentrations of zinc that showed antimicrobial activity on oral Streptococci.^{21,22} However, the effect of adding MgO nanoparticles specially coated with zein polymer to the commercial mouthwashes was not studied. Hence, the present study aimed to investigate the antimicrobial effect of the zein-coated MgO nanoparticles when added to distinctive mouthwashes on an exemplary set of different oral organisms.

While our null hypothesis (Ho) was that the addition of zein-coated MgO nanoparticles would not make a significant difference to the antimicrobial activity of mouthwashes, the alternative hypothesis (H_a) was that it would produce a promising antimicrobial dental product.

Materials and Methods

MgO nanoparticles, zein polymer (Sigma Aldrich, Missouri 63103, USA), Listerine Total care mouthwash, Listerine Zero mouthwash (Johnson & Johnson, New Jersey 08933, USA) and Oral B mouthwash (Procter & Gamble, Ohio 45202, USA) were used in this study. Agar plates and the sterile paper discs were purchased from Becton, Dickinson and company (New Jersey 07417, USA).

Synthesis of MgO Nanoparticles

MgO nanoparticles were synthesized by directly reacting magnesium acetate and urea in the microwave hydrothermal technique as follows.²³ A solution of 6.44 g of magnesium acetate in 75 mL of distilled water was stirred magnetically at 25°C for 30 min. Then, drops of a mixture of urea and water (1.2 g/25 mL) were added under vigorous magnetic stirring for 5 min. An autoclave lined with Teflon was charged with the mixture, secured and kept on a 1000 W power microwave at 180°C for 15 min. After cooling the autoclave and resolution of the reaction, the products were centrifuged for 5 min, filtered with distilled water and then dried at 60°C for 24 h. Finally, the white-colored material that resulted was calcinated at 600°C for 1 h.²³

Preparation of MgO-Zein Nanoparticles Using pH-Controlled Nano-Precipitation

The zein polymer was mixed with polyvinyl alcohol (PVA); then MgO nanoparticles were added at a weight ratio of 4:1. The MgO-zein-PVA mélange was then stirred for 30 minutes. The nanoparticles mix of MgO-zein nanoparticles developed by phase separation. After evaporation of the PVA content, the final product was then centrifuged and dehydrated.²⁴

Incorporation of MgO-Zein Nanoparticles in Mouth Wash

MgO nanoparticles and zein-coated MgO nanoparticles were incorporated in three mouthwashes: Listerine Zero, Listerine Total Control (Johnson & Johnson, NJ 08933) and Oral B (P&G, Ohio 45202) in the mass percentages of 0.3%, 0.5% 1%, 2% 5% and 10% based on our previous study,²⁵ on the antimicrobial properties of MgO coated with zein polymer. The solution was kept on a magnetic stirrer at 500 rpm at room temperature for 15 minutes for complete dissolution.

Antimicrobial Assay

Two antimicrobial activity tests were carried out: the Kirby-Bauer agar diffusion test²⁶ and the direct contact test.²⁷

Cultures of the Microbial Organisms

The organisms used in the study were acquired from the American Type Culture Collection (ATCC, VA 20110, USA). They are as follows: *Streptococcus mutans* (ATCC 25175; gram-positive coccus), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212) and *Candida albicans* (ATCC 10231 fungus). The bacteria and fungi concentrations were prepared from stock and cultured on agar and sabouraud agar plates.²⁸ All cultures were incubated (Thermo Fischer Scientific, Waltham, MA, USA) at 37°C.

Determination of Antimicrobial Sensitivity Test of the Mouthwashes Incorporated with MgO Nanoparticles Disc Diffusion Test (DDT)

The Kirby-Bauer disc diffusion test²⁶ was performed using 156 samples. Freshly prepared inoculums of microorganisms were cultured on blood agar and sabouraud plates. Sterile paper discs of 6 mm in diameter were filled with 100 µL of MgO nanoparticles or zein-coated MgO nanoparticles in a solution of different concentrations. Bacteria and fungi were allowed to grow for one day in the 37°C incubator, and then inhibition zones were measured.¹¹ Antimicrobial inhibition was assessed by measuring the diameter of bacterial inhibition zone (mm), data represents the mean ± SD.⁶

Direct Contact Test

The direct contact test²⁷ was applied on 156 samples. Freshly prepared inoculums of microorganisms were prepared into a 1 Mc Farland dilution. An equal amount of the bacteria solution and the solution of the different concentrations of MgO nanoparticles or zein-coated MgO were mixed. The tubes were incubated at 37°C. Solutions were then examined for bacterial growth by subculture on fresh media and sabouraud agar plates at different time points: 1 hour and 1, 2, 3, 4, 5, 6 and 7 days.

Plate Count

Viable bacterial cell concentrations were estimated by counting colony forming units (CFU) before and after exposure to the nanoparticles solutions. This was

performed by serial dilution in Broth and then removing 10µL of the serially diluted culture and spreading with sterile glass beads (5 mm, Sigma, UK) onto an agar plate (Tryptic Soya Agar) in triplicate. The plates were then incubated at 37°C for 24 hours and CFUs were counted. The growth of tested organisms (*S. mutans*, *S. aureus*, *E. faecalis* and *C. albicans*) was observed for the three mouthwashes after 24 hours, 2, 3, 4, 5, 6 and 7 days.

Statistical Analysis

Data were analyzed using SPSS version 21.0. one-way ANOVA test was performed and the *P* value less than 0.05 (*p* < 0.05) was considered statistically significant.

Results

Results showed a statistical significant increase in antimicrobial activity against tested organisms when zein-coated MgO nanoparticles were added to all tested oral mouthwashes rather than non-zein MgO nanoparticles at (*p* < 0.05).

The Effect of Tested Mouth Washes on *S. mutans*

The addition of different concentrations of zein-coated MgO nanoparticles to Listerine Total care showed a statistical significant increase in the antimicrobial activity against *S. mutans*, (*P*=0.0001) (Figure 1). A similar effect was noticed with Listerine Zero and Oral B mouth washes. There was a significant difference in the antimicrobial activity against *S. mutans* with the addition of different concentrations of zein-coated MgO nanoparticles compared to the control and the regular MgO nanoparticles (*P*=0.0001) (Table 1, Figure 1).

The Effect of Tested Mouth Washes on *S. aureus*

All concentrations added of zein-coated MgO nanoparticles to Listerine Total care showed a statistical significant increase in the antimicrobial activity against *S. aureus* (*P*=0.0001) (Table 1, Figure 2). Furthermore, the addition of all concentrations of zein-coated MgO nanoparticles to Listerine Zero and Oral B showed a statistically significant increase in the antimicrobial activity against *S. aureus*, rather than the control and the regular MgO nanoparticles (*P*=0.0001) (Table 1, Figure 2).

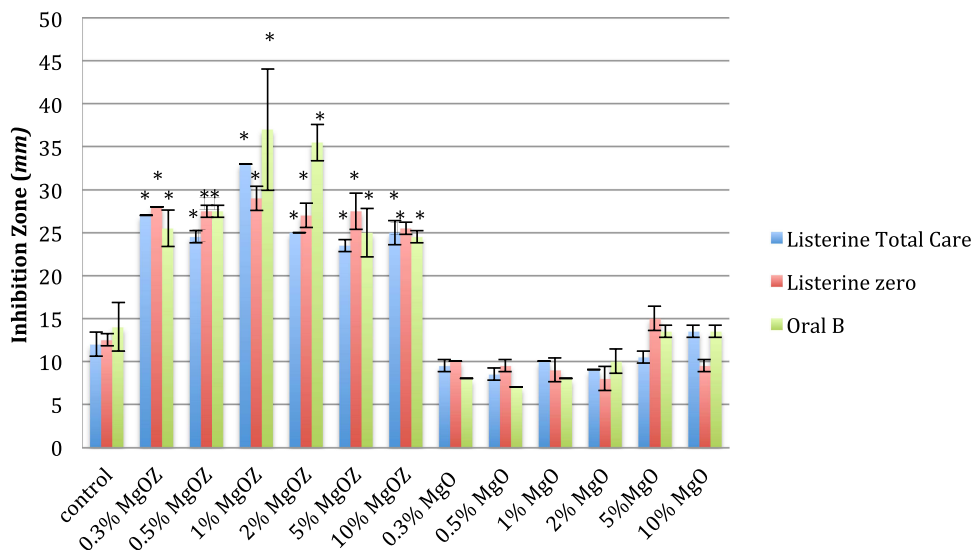
S. mutans

Figure 1 Diameter of inhibition zones (in mm) for Oral B, Listerine Zero and Listerine Total Control with and without zein coated MgO nanoparticles with *S. mutans*. *Significance at $p < 0.05$.

The Effect of Tested Mouth Washes on *E. faecalis*

The antimicrobial effects of all the three mouthwashes: Listerine Total Care, Listerine zero and Oral B after the addition of zein-coated MgO nanoparticles were statistically significant against *E. faecalis* ($P=0.0001$) (Table 1, Figure 3).

The Effect of Tested Mouth Washes on *C. albicans*

The addition of different concentrations of zein-coated MgO nanoparticles to Oral B and Listerine Total Care showed a statistically significant increase in the antimicrobial activity against *C. albicans* compared to the control ($P=0.0001$) (Table 1, Figure 4). However, the 5% and 10% of zein-coated MgO nanoparticles added to Listerine Total care showed indifference in the antimicrobial activity between coated and non-coated MgO nanoparticles.

Meanwhile, there was a statistically significant increase in the antimicrobial activity of Listerine Zero after the addition of 0.3% and 0.5% of zein-coated MgO nanoparticles compared to the control and the MgO nanoparticles ($P=0.002$). However, there was no significant difference in the antimicrobial activity between the effect of adding coated or non-coated MgO nanoparticles in the following concentrations 1%, 2%, 5% and 10% ($p = 0.177$, $p = 0.242$, $p = 0.242$, $p = 1.00$) (Figure 4).

Discussion

The mouthwashes often contain several antimicrobial agents. They also contain methylparaben (methyl p-hydroxybenzoic acid methyl ester) as a preservative that has an antimicrobial activity.²⁹ Listerine mouth washes contain essential oils that may enhance the antimicrobial activity.³⁰ However, our in vitro studies showed that the coating of MgO nanoparticles with zein polymer had no chemical interaction but improved the MgO nanoparticles stability and prevented their aggregation.²⁴ It was also shown that the zein coating by preventing the aggregation of the MgO nanoparticles enhanced their antimicrobial effect against different oral microorganisms.²⁵

Several investigations have added bactericidal agents to oral hygiene products to prevent the plaque accumulation and reduce the bacterial acids.^{21,31,32} Researchers have added copper and zinc in order to enhance the antimicrobial activity of mouthwashes,^{20,22,33} where their studies have reported that mouthwashes with concentration less than 1% of bactericidal agent can exhibit potential antibacterial effects.^{22,33}

In the present study, we added different concentrations of zein-coated and non-coated MgO nanoparticles to the three most common mouthwashes, in order to examine their potential antimicrobial effect against the most common oral pathogens including *S. mutans*, *S. aureus*, *E. faecalis* and *C. albicans*.

Table 1 Means (mm) and Standard Deviations of Inhibition Zones of Different Microorganisms Around Different Mouth Washes Mixed with Different Magnesium Oxide (MgO) Nanoparticles Concentrations

Microorganism	Mouth Wash	Inhibition Zone (mm) at MgO Concentration												p-value	
0%		0.30%		0.50%		1.00%		2%		5%		10%			
		MgOZ	MgO	MgOZ	MgO	MgOZ	MgO	MgOZ	MgO	MgOZ	MgO	MgOZ	MgO	MgOZ	MgO
<i>S. mutans</i>	LT	12 (1.4)	27 (0)	9.5 (0.7)	24.5 (0.7)	8.5 (0.7)	33 (0)	10 (0)	25 (0)	9 (0)	23.5 (0.7)	10.5 (0.7)	25 (1.4)	13.5 (0.7)	0.0001*
	LZ	12.5(0.7)	28(0)	10(0)	27.5(0.7)	9.5(0.7)	29 (1.4)	9 (1.4)	27 (1.4)	8 (1.4)	27.5 (2.1)	15 (1.4)	25.5 (0.7)	9.5 (0.7)	0.0001*
	OB	14 (2.8)	25.5 (2.1)	8 (0)	27.5 (0.7)	7 (0)	37 (7)	8 (0)	35.5 (2.1)	10 (1.4)	25 (2.8)	13.5 (0.7)	24.5 (0.7)	13.5 (0.7)	0.0001*
<i>S. aureus</i>	LT	18 (0)	26 (0)	0 (0)	27.5 (3.5)	0(0)	29 (1.4)	0 (0)	25.5 (0.7)	0 (0)	26.5 (0.7)	8 (0)	27 (0)	8 (0)	0.0001*
	LZ	0 (0)	28.5 (2.1)	0 (0)	26.5 (0.7)	0 (0)	30.5 (0.7)	0 (0)	29 (2.8)	0 (0)	29.5 (0.7)	7.5 (0.7)	28 (0)	0 (0)	0.0001*
	OB	13 (1.4)	26 (1.4)	11.5 (0.7)	23.5 (0.7)	10.5 (0.7)	27.5 (0.7)	11.5 (0.7)	25.5 (0.7)	13.5 (0.7)	27 (1.4)	11.5 (0.7)	26.5 (0.7)	11 (0)	0.0001*
<i>E. faecalis</i>	LT	7.5 (0.7)	19.5 (2.1)	8.5 (0.7)	20.5 (3.5)	8.5 (0.7)	28 (2.8)	8.5 (0.7)	21 (2.8)	9.5 (0.7)	23.5 (0.7)	9.5 (0.7)	22 (0)	0 (0)	0.0001*
	LZ	8 (0)	22 (1.4)	8.5 (0.7)	20.5 (0.7)	7.5 (0.7)	22.5 (0.7)	7.5 (0.7)	23 (1.4)	0 (0)	25 (1.4)	0 (0)	23 (0)	0 (0)	0.0001*
	OB	10.5 (0.7)	19.5 (0.7)	12.5 (0.7)	18.5 (0.7)	12.5 (2.1)	20.5 (0.7)	11.5 (0.7)	20.5 (0.7)	11.5 (0.7)	22 (0)	11 (0)	22 (0)	11 (0)	0.0001*
<i>C. albicans</i>	LT	7.5 (0.7)	12.5 (0.7)	7.5 (0.7)	13.5 (0.7)	8 (0)	15.5 (2.1)	10 (1.4)	11.5 (0.7)	9.5 (0.7)	13 (0)	16 (0)	12.5 (0.7)	13.5 (0.7)	0.0001*
	LZ	7 (0)	19.5 (6.3)	10 (1.4)	20 (2.8)	8.5 (0.7)	15 (1.4)	15 (4.2)	13.5 (2.1)	10.5 (0.7)	10.5 (0.7)	13.5 (0.7)	11.5 (0.7)	11.5 (0.7)	0.02*
	OB	9 (1.4)	16 (1.4)	9.5 (0.7)	16 (1.4)	8.5 (0.7)	17 (0)	10.5 (0.7)	19.5 (0.7)	14 (1.4)	19 (1.4)	11 (0)	19 (0)	11 (1.4)	0.0001*

Notes: *Means Significance p-value <0.05.

Abbreviations: LT, Listerine Total; LZ, Listerine Zero; OB, Oral B.

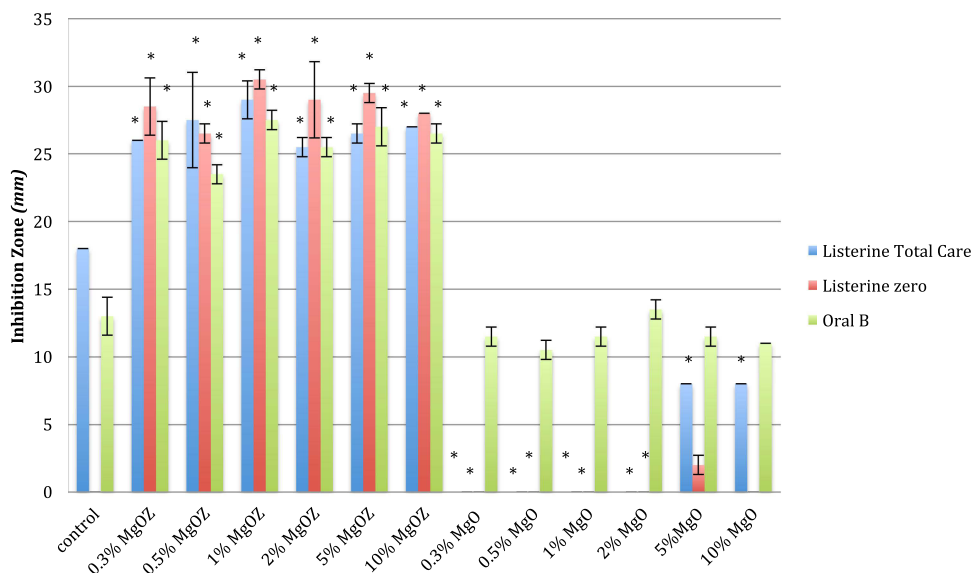
S. aureus

Figure 2 Diameter of inhibition zones (in mm) for Oral B, Listerine Zero and Listerine Total Control with and without zein coated MgO nanoparticles with *S. aureus*. *Significance at $p < 0.05$.

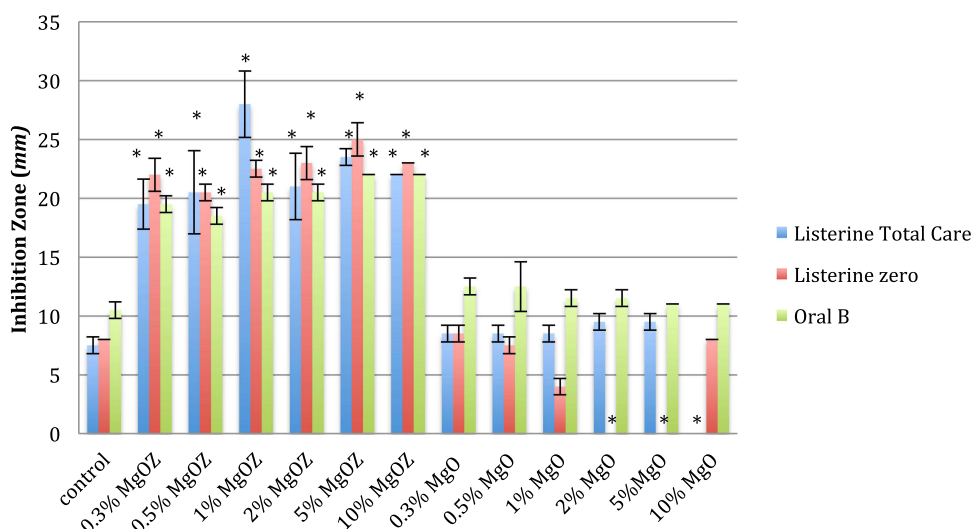
E. faecalis

Figure 3 Diameter of inhibition zones (in mm) for Oral B, Listerine Zero and Listerine Total Control with and without zein coated MgO nanoparticles with *E. faecalis*. *Significance at $p < 0.05$.

Interestingly, the overall results showed that incorporating MgO/zein nanoparticles into the tested mouthwashes could significantly enhance the antimicrobial activity against all tested organisms. Although the MgO nanoparticles have antimicrobial properties, the preparation of these nanoparticles exhibits aggregation issues that can degrade their properties. Therefore, the use of a surfactant was necessary to help make these particles more soluble.^{34,35}

For *S. mutans*, *S. aureus* and *E. faecalis*, all three mouthwashes showed a significant antimicrobial activity results after the addition of 1% of zein-coated MgO nanoparticles. These results showed that the addition of zein-coated MgO nanoparticles had improved the antimicrobial activity of the tested mouthwashes compared to using MgO without zein. Consequently, the advantage of zein to enhance the antimicrobial activity of the MgO was

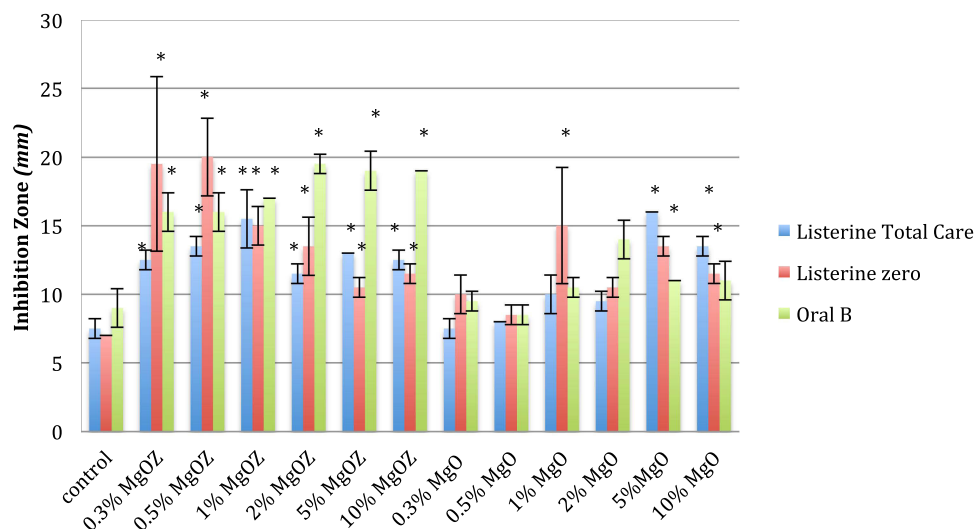
C.albicans

Figure 4 Diameter of inhibition zones (in mm) for Oral B, Listerine Zero and Listerine Total Control with and without zein coated MgO nanoparticles with *C. albicans*. *Significance at $p < 0.05$.

clearly observed. This was in accordance with previous reports that showed the significance of using nanoparticles to improve the antimicrobial activity against certain pathogenic bacteria, suggesting that the use of less than 1% of bactericidal agent can yield a considerable antimicrobial effect.^{22,33} A similar effect was reported that using 1% of Cu-Ti oxide-treated nanotube coatings has significantly decreased the number of oral bacteria suggesting the efficient antimicrobial effect of nanoparticles.³⁶

In this study, various concentrations of MgO/zein were added to mouthwashes and showed distinct outcomes towards *C. albicans*. The addition of 0.5, 1 and 2% of MgO/zein into Listerine zero, Listerine Total care and Oral B, respectively, revealed significant antifungal effects against *C. albicans*. Similarly, previous studies^{24,37} reported that the use of 0.5–1% of nanoparticles could considerably decrease the number of oral microbes. It was previously suggested that the inclusion of metal oxides nanoparticles could result in antifungal activity against oral Candidiasis in vitro. Furthermore, several carbon-based nanomaterials, silica, ZnO, and CuO have been utilized as potential antimicrobial agents for *Candida* species. For example, small amounts of ZnO nanoparticles showed an enhanced antimicrobial activity against *C. albicans* due to the damage of fungal cell membrane.³⁸ This is in accordance with our results in which using only 0.5% of zein-coated MgO nanoparticles has demonstrated a significant antimicrobial effect on *C. albicans*.

The application of nanoparticles can be quite useful to decrease the rate of microorganisms as well as decrease the chance for the microbes to develop resistance against such molecules.^{39,40} The elevated pH (alkaline) of MgO nanoparticles was reported to have a role in their antimicrobial action as this alkaline trait of MgO may help prevent acidogenic organisms causing dental caries.⁴¹

The current research was performed to test the benefits of using zein-coated MgO nanoparticles as antimicrobial agents using simple microbiological methods that are cost-effective and available. However, it has been suggested to extend this project to investigate other properties of MgO nanoparticles, such as anti-biofilm. In addition, one of the limitations of the study is testing the materials against only Gram-positive organisms, and this was due to the difficulties we faced to find and culture *Porphyromonas gingivalis*, which is known as Gram-negative oral pathogen. Nevertheless, it would be recommended to further examine the effect of zein-coated MgO nanoparticles towards *P. gingivalis* in future investigation. Another limitation of the current study is being an in-vitro analysis of the antimicrobial effect of zein-coated MgO nanoparticles. Therefore, further in-vivo studies are required to examine the resulting outcomes of the study.

Overall, the results of this in-vitro study revealed that the addition of zein-coated MgO nanoparticles to mouthwashes can be effective in improving oral hygiene as it showed a promising antimicrobial activity. However,

being an in-vitro study can be considered a limitation, as the clinical results of in-vivo studies are still required to confirm the benefits of addition of antimicrobial MgO/zein nanoparticles. Also, it would be useful to further extend the investigation and examine the effect of these nanoparticles on biofilm infection in the near future to verify the results of this study.

Conclusion

Within the limitations of this study, zein-coated MgO nanoparticles have shown a potent antimicrobial activity when incorporated in oral mouth wash products. The zein-coated MgO nanoparticles can be excellent candidates for further application in the development of oral mouth washes.

Abbreviations

CFU, colony forming units; DDT, Disc Diffusion Test; DCT, Direct Contact Test; PVA, polyvinyl alcohol; KAU, King Abdulaziz University; ANOVA, analysis of variance; LSD, least significant difference.

Data Sharing Statement

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

The present study has been done and followed by the Ethical Guidelines in the Faculty of Dentistry at King Abdulaziz University (reference number #094-10-17).

Consent for Publication

The authors have given their consent for their data to be published in the report.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors certify that they have no affiliation with or involvement in any financial interest (such as honoraria, educational grants, participation in speakers bureau, membership, employment, consultancies, stock ownership, or other equity interest, and expert testimony or patent licensing arrangements) or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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