Nanomed. J. 5(2): 102-110, Spring 2018

RESEARCH PAPER

A comparative study of antibacterial effects of mouthwashes containing Ag/ZnO or ZnO nanoparticles with chlorhexidine and investigation of their cytotoxicity

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ABSTRACT

Objective(s): Chlorhexidine 0.2% mouthwash is commonly used in orthodontic patients for plaque control. But it has some side effects. Metal oxide nanoparticles have been recently used in mouthwashes in reports. So we aimed to evaluate antibacterial effect of ZnO and Ag/ZnO nanoparticles against Streptococcus mutans and compare them with chlorhexidine 0.2%, sodium fluoride 0.05% and some of their compositions.

Materials and Methods: ZnO and Ag/ZnO NPs were synthesized and sixteen groups of mouthwashes were prepared. We used Zone of Inhibition (ZOI) test to evaluation of antibacterial effects of as-prepared mouthwashes, against S. mutans. The cytotoxicity of the ZnO and Ag/ZnO NPs were investigated in the A549 cell line.

Results: Among the study groups, the maximum ZOI (16.60±0.49 mm) pertained to Ag/ZnO, 10 mg NPs plus 100 ml base material, (Ag/ZnO b 10). The results indicate that no significant harmful effect is imposed to the cells up to 0.2 mg/ml of ZnO and Ag/ZnO NPs.

Conclusion: Results showed that mouthwash containing Ag/ZnO b 10 has the highest antibacterial properties against S .mutans amoung study groups and because in this concentration it is safe for cells, so it can be served as an alternative mouthwash in plaque control instead of chlorhexidine 0.2% after in vivo studies.

Keywords: *Ag/ZnO nanoparticle, Antibacterial, MTT Assay, Mouthwash, ZnO nanoparticle*

How to cite this article

Kachoei M, Divband B, Dabaghi Tabriz F, Norouzzadeh Helali Z, Esmailzadeh M. A comparative study of antibacterial effects of mouthwashes containing Ag/ZnO or ZnO nanoparticles with chlorhexidine and investigation of their cytotoxicity. Nanomed J. 2018; 5(2): 102-110. DOI: 10.22038/nmj.2018.005.007

INTRODUCTION

One of the most common side effects of fixed orthodontic appliances is developing of white spot lesions around orthodontic bands and brackets [1]. Most of patients cannot remove microbial plaque effectively from tooth surfaces by means of mechanical methods such as tooth brushing. So the use of chemical methods such as chlorhexidine and fluoride mouthwashes is effective for plaque control [2].

Chlorhexidine has been defined as highly effective mouthwash in reduction of pathogenic microorganisms including *Streptococcus mutans*. So chlorhexidine is considered as gold standard. And in many studies about efficacy of antimicrobial mouthwashes, chlorhexidine is used as positive control [3-6]. But its disadvantages including enamel staining, unpleasant taste, and dryness and burning sensation in the mouth; discourage patients to use this material [7].

Damage of oral cavity microfrola is a long-lasting effect of chlorhexidine [8]. Some routine mouthwashes contain cationic, anionic and nonionic active components that alter the function of bacterial membrane. Cationic ingredients involve chlorhexidine, Cu^{+2}, Zn^{+2} and Sn^{+2} are most widely used [9]. From centuries ago, gold,

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Note. This manuscript was submitted on December 30, 2018; approved on February 15, 2018

silver and zinc have been used for their bacterial properties [10-14].

Bacterial cell membrane function and also enzymatic activity can be modified by metal ions [15].

Modifications of zinc salts and their derivatives can be used in plaque control and the antibacterial activity of zinc chloride mouthwashes against streptococcus bacteria and zinc oxide nano particles against Escherichia coli has been proved [16-19].

So nowadays they are introduced to the field of dental materials [20-24]. Ag has antimicrobial activity as an antiseptic against a wide range of gram-positive and gram-negative bacteria and even against species resistant to vancomycin [25-28] however, it has higher toxicity [29, 30] compared to compounds containing Zn. ZnO nanoparticles are used as nutritional additives and protective material against UVA, UVB in sunscreen creams. Silver and zinc nanoparticles are proved to have antibacterial effect against S.mutans and almost safe [31, 32].

Introducing of Ag^{+} into nano ZnO structure, will cause synergistically effect [33- 35].

Based on our knowledge there are only few studies that have determined antibacterial effect of nanoparticle - containing mouthwashes and no studies have been reported about mouth rinses containing Ag/ZnO nanoparticles, on oral bacteria. So we aimed to investigate antibacterial effect of mouthrinses containing Ag/ZnO and ZnO nanopaticles against *S. mutans* and comparing their results with chlorhexidine 0.2 % and sodium fluoride 0.05% mouthwashes and some of their combinations.

Also the cytotoxicity of ZnO nanoparticles with and without Ag doping was studied by using A549 alveolar adenocarcinoma cells.

MATERIAL AND METHODS

Materials

In this study we purchased mouthwashes including chlorhexidine 0.2% (group1), sodium fluoride 0.05% (group2), a substance without antimicrobial agent (base material) (group3) and mixture of sodium fluoride 0.05% plus chlorhexidine 0.2% (group 4) from Behsa Co.(Iran, Tehran).

Table 1. Descriptives data of all study groups

Group Nomber	Group name	Sample size	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
						Lower Bound	Upper Bound		
2	Fluoride 0.05%	40	8.5	0.50637	0.08006	8.3381	8.6619	8	9
3	Base of mouth wash	40	0	0	0	0	0	0	0
4	Chlorhexidine + Fluoride 0.05%	40	14.8	0.4051	0.06405	14.6704	14.9296	14	15
5	ZnO 5	40	0	0	0	0	0	0	0
6	ZnO 10	40	12.3	0.4641	0.07338	12.1516	12.4484	12	13
7	ZnO 15	40	12.9	0.30382	0.04804	12.8028	12.9972	12	13
8	ZnO 20	40	13.7	0.4641	0.07338	13.5516	13.8484	13	14
9	Ag/ZnO 5	40	9.3	0.4641	0.07338	9.1516	9.4484	9	10
10	Ag/ZnO 10	40	16.6	0.49614	0.07845	16.4413	16.7587	16	17
11	Ag/ZnO 15	40	14.5	0.50637	0.08006	14.3381	14.6619	14	15
12	Ag/ZnO 20	40	13.8	0.4051	0.06405	13.6704	13.9296	13	14
13	ZnO 10 + Chlorhexidine 0.2%	40	14.3	0.4641	0.07338	14.1516	14.4484	14	15
14	ZnO 10 + Fluoride 0.05%	40	10.7	0.4641	0.07338	10.5516	10.8484	10	11
15	Ag/ZnO 10 + Chlorhexidine 0.2%	40	15.3	0.4641	0.07338	15.1516	15.4484	15	16
16	Ag/ZnO 0+ Fluoride 0.05%	40	15.7	0.4641	0.07338	15.5516	15.8484	15	16
	Total	640	11.6688	4.92666	0.19474	11.2863	12.0512	0	17

Zinc acetylacetonate, $AgNO_3$, Polyethylene Glycol (PEG) and ethanol (absolute) were obtained from Merck. Freezed *S. mutans* was prepared from research center of science and technology (Tehran, Iran). Nutrient broth, meuller hinton agar plates and blank disk (from Padtan Teb co. Tehran, Iran) were used for bacterial studies.

Preparation of mouthwashes containing Ag/ZnO and ZnO nanoparticles

Ag/ZnO and ZnO nanoparticles were synthesized by photo reduction technique and polymer pyrolysis method respectively [16]. Despite of our previous work, here we use Zn $(acac)_2$ and the rest of preparation steps are the same. After preparation of nanoparticles they were coated with PEG (20 ppm) under reflux for 12 h and different percent of Ag/ZnO and ZnO coated nanoparticles (5, 10, 15, 20 mg per 100ml of base mouthwash solution) were prepared and grouped as presented in table 1. For example for preparing mouthwashes containing 5 of nanoparticles, 5 mg of nanoparticle was added to 100 ml of mouthwashes (50 ppm).

Measurements

X-ray diffraction patterns (XRD) were collected using a Siemens D500 diffractometer with Cu kα radiation (= 1.5418 A and 2= 4–80◦) at room temperature. Scanning electron microscope (Philips XL30) equipped with energy dispersive X-ray (EDX) facility was used to capture SEM images and to perform elemental analysis. The SEM sample was gold coated prior to examination and SEM was operated at 5 kV while EDX analysis was performed at 15 kV. TEM study was carried out on a Zeiss LEO 912 Omega instrument, operating at 100 kV.

Antibacterial study

S. mutans was cultured on nutrient broth with streak plate method and was incubated at 37°C (centigrade) for 48 hours in anaerobic environmental condition. After activation of bacteria, we cultured them on 640 MHA plates with spread plate method by sterile swap. Paper discs were treated by 100 microliter of each study group mouthwashes and dried under sterile hood and placed on culture media. After 48 hours of incubation at 37°C in anaerobic environmental condition, widths of inhibition zone which is also named halo were measured.

One way analysis of variance (ANOVA) was run to determine any significant differences in width of inhibitory zone of the study groups, followed by High Significant Difference tukey test (HSD tukey) for pair wise comparisons. The statistical analysis was performed through SPSS (Statistical Package for Social Sciences), version 17, and the significance level was determined at $P<0.05$.

MTT assay for cell viability

A549 alveolar adenocarcinoma cells (7×10^3 cells/well for the both cell lines) were incubated in 96-well plates each containing 200 µL of supplemented cell culture media for 24 hours at 37°C and 5% CO_2.

The cells were divided in 5 groups in triplicates: blank, ZnO and Ag/ZnONPs (different concentrations: 0.05, 0.1, 0.2, 0.4 & 0.6 mg/ml) were treated. After an incubation period of 24 h, the spent media were removed and the plate wells were washed with Phosphate-buffered solution. Briefly, 50 µL of 2 mg/mL MTT (3-(4, 5-dimetylthiazol-2-yl)-2, 5-diphenyl- trazolium bromide) and 150 µL culture medium of was added to each well.

The cells were incubated at 37°C and 5% CO_2 for 4 hours and then the media was discarded and dimethyl sulfoxide and Sorenson buffer was added to each well as solubilizer buffer. Finally, absorbance was read using an ELISA plate reader (BioTeck, Bad Friedrichshall, Germany) at 570 nm wavelength.

RESULTS

Fig 1 shows the powder XRD patterns of as-prepared Ag/ZnO with different Ag loadings. As depicted in Fig. 1(b) after higher Ag loading (≥0.7 wt%) residual phases of Ag were observed. Appropriate position of Ag^+ is in grain boundaries. As seen in Fig. 1(b), lower Ag loading (≤0.5 wt%) causes no additional peaks, so with these amounts of Ag loading, Ag^+ ions are in crystal lattice of ZnO.

Transmission Electron Microscope (TEM) and Scanning Electron Microscope (SEM) techniques were used to investigation of as-prepared nanoparticle products (Fig 2(a,b)). Both TEM and SEM images reveal that Ag/ZnO particles are hexagonal and polydispersed with size varying between 20-50 nm and ZnO particles are spherical with mean particle size of 50-58 nm. With changing the Zn^{2+} source from Zn $(CH_3CHOO)_2\cdot2H_2O$ [24] to Zn $(acac)_2$, the particle size of the composites are decreased from about ~60 nm to ~35 nm.

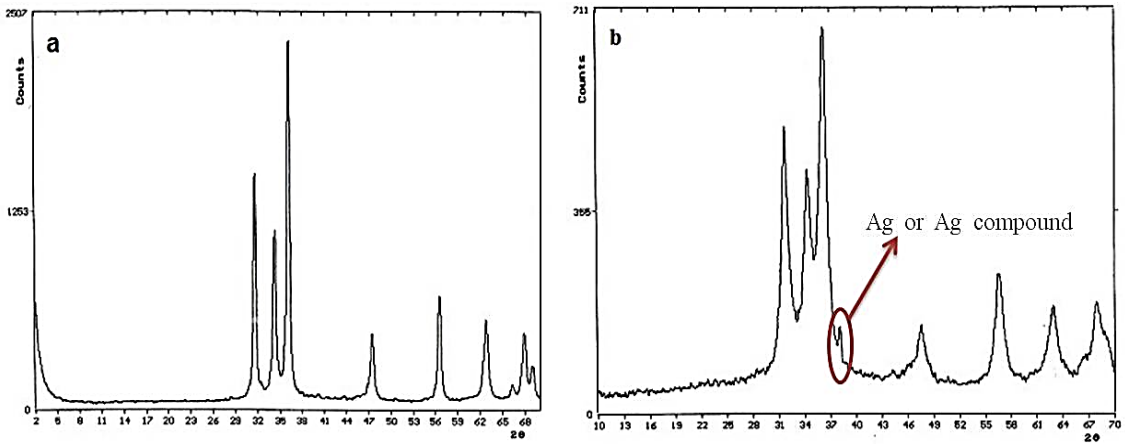

Fig1. XRD patterns of the Ag/ZnO nanoparticle with different Ag loadings

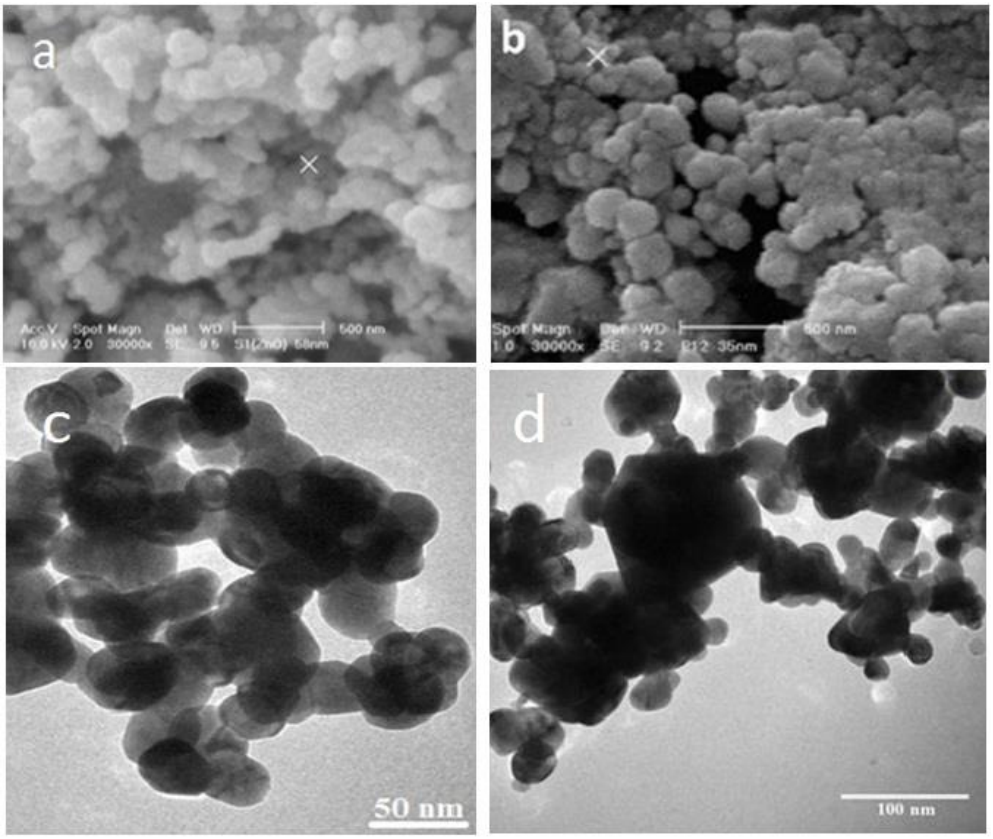

Fig 2. Electron micrographs: (a) and (b) SEM images (scale bar: 500 nm) of ZnO and Ag/ ZnO nanohybrid, respectively, (c) and (d) respective TEM images (scale bar: 100 nm)

Table 1 and 2 presents the means and standard deviation regarding zone of inhibition width and ANOVA of study groups against S.mutans respectively.

As depicted in Fig 3(a,b), Zone Of Inhibition (ZOI) formed by Ag/ZnO 10 NPs plus base material (Ag/ZnO ^{b} 10) is more compared to chlorhexidine 0.2% suggesting that antibacterial effect of Ag/ ZnO ^{b} 10 is more than that for chlorhexidine 0.2%.

The maximum ZOI pertained to Ag/ZnO ^{b} 10 NPs (16.60 mm) and the minimum ZOI is related to ZnO 5 NPs plus base material (ZnO ^{b} 5) and base material (0.00±0.00 mm).

Table 2. One way analysis of variance (ANOVA) regarding all study groups

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	15396.575	15	1026.438	5658.105	0.000
Within Groups	113.200	624	0.181		
Total	15509.775	639			

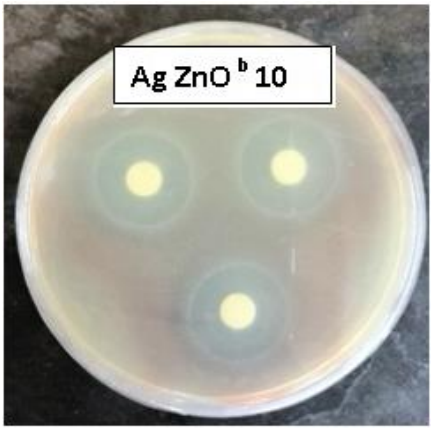

Fig 3. (a,b) Photograph showing zone of inhibition formed. Clear zone formed against the growth of S. mutans

Among study groups which had antibacterial effect against *S. mutans*, the minimum ZOI pertained to sodium fluoride 0.05% (mean ZOI = 8.50±0.50 mm).

ANOVA revealed a significant difference in ZOI of study groups.

Between group comparisons by HSD, Tukey test, demonstrated that ZOI widths of all study groups had statistically significant difference with the exception of chlorhexidine 0.2% and ZnO 10 plus chlorhexidine 0.2% (ZnO ^{ch} 10%) that had the same antibacterial effect toward *S. mutans* (p=1.000) and also the difference between ZOI of Ag/ZnO^{b}20 (13.80±0.40 mm) and ZnO ^{b} 20 (13.70±0.46 mm) was not significant (p=0.999).

ZOI of Ag/ZnO ^{b} 15 (14.50±0.50 mm) was not significantly different from chlorhexidine 0.2% (14.30±0.46 mm) (p=0.726) and ZnO ^{ch} 10 (14.30±0.46 mm) too (p=0.726).

Chlorhexidine 0.2% (mean ZOI=14.30±0.46 mm) had more antibacterial effect than that of sodium fluoride 0.05% (means ZOI=8.50±0.50 mm) (p<0.001).

Chlorhexidine 0.2% plus sodium fluoride 0.05% mouthwashes (ZOI mean =14.8±0.40 mm) were more effective than chlorhexidine 0.2% mouthwashes (p<0.001).

There was statistically significant difference between solusions containing different concentrations of ZnO ^{b}.NPs (5, 10, 15, 20) (p<0.001). ZOI in these groups from maximum to minimum was related to ZnO ^{b} 20, ZnO ^{b} 15, ZnO ^{b} 10 and ZnO ^{b} 5, in order (Table 3, 4).

ZOI in Ag/ZnO 10 plus sodium fluoride 0.05% (Ag/ZnO ^{f} 10) (15.7±0.46 mm) was significantly more than that for Ag/ZnO 10 plus chlorhexidine 0.2% (Ag/ZnO ^{ch} 10) group (15.30±0.46 mm), ZnO ^{ch} 10 (14.30±0.46 mm), ZnO 10 plus sodium fluoride 0.05% (ZnO ^{f} 10) (10.70±0.46 mm) and (p<0.001).

Table 3. Descriptives data regarding ZnO-containing mouthwashes

Samples	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
ZnO 5	40	0	0	0	0	0	0	0
ZnO 10	40	12.3	0.4641	0.07338	12.1516	12.4484	12	13
ZnO 15	40	12.9	0.30382	0.04804	12.8028	12.9972	12	13
ZnO 20	40	13.7	0.4641	0.07338	13.5516	13.8484	13	14
Total	160	9.725	5.66569	0.44791	8.8404	10.6096	0	14

Table 4. One way analysis of variance (ANOVA) regarding ZnO-containing mouthwashes

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5083.500	3	1694.500	12957.941	0.000
Within Groups	20.400	156	0.131		
Total	5103.900	159			

Table 5. Descriptives data regarding Ag/ZnO-containing mouthwashes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
Ag/ZnO 5	40	9.3	0.4641	0.07338	9.1516	9.4484	9	10
Ag/ZnO 10	40	16.6	0.49614	0.07845	16.4413	16.7587	16	17
Ag/ZnO 15	40	14.5	0.50637	0.08006	14.3381	14.6619	14	15
Ag/ZnO 20	40	13.8	0.4051	0.06405	13.6704	13.9296	13	14
Total	160	13.55	2.70987	0.21423	13.1269	13.9731	9	17

ZnO ^{ch} 10 group was more effective than ZnO ^{f} 10 ($p<0.001$).

The difference between ZOI of Ag/ZnO ^{ch} 10 (15.30±0.46 mm) and and that for mouthrinses containing chlorhexidine 0.2% plus sodium fluoride 0.05% (14.80±0.40 mm) was statistically significant ($p<0.001$).

After all according this study, mouthwash containing Ag/ZnO ^{b} 10, was the most effective mouthrinse against S.mutans among study groups.

Fig 4 shows the relative cell viability ($[C_r/C_0]$ 100%) vs. different concentration of ZnONPs, determined by the MTT assay. Here, C_o is the viable cell numbers of the control sample, and C_r is the viable cell numbers treated with the ZnONPs. The error bars are the calculated standard deviation.

Table 6. One way analysis of variance (ANOVA) regarding Ag/ZnO-containing mouthwashes

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1133.200	3	377.733	1712.977	0.000
Within Groups	34.400	156	0.221		
Total	1167.600	159			

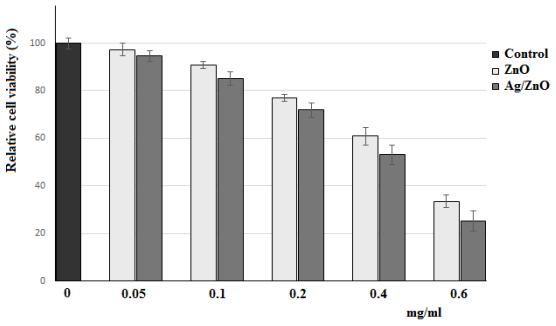

Fig 4. Effect of coated ZnO and Ag/ZnO NPs on A549 alveolar adenocarcinoma cell viability

The relative viability of cells treated with 0.05 mg/ml of ZnONPs is about 97 ± 3%. The relative viabilities (%) of cells treated with higher concentrations of ZnONPs (0.1, 0.2, 0.4 & 0.6 mg/ml) are 90, 77 & 60% respectively after 24-h incubation and for Ag/ZnO nanoparticles (0.05, 0.1, 0.2, 0.4 & 0.6 mg/ml) the relative viabilities (%) are 94, 85, 71 and 53%. The results indicate that no significant harmful effect is imposed to the cells up to 0.1 mg/ml (100 ppm) of ZnO and Ag/ZnO nanoparticles.

DISCUSSION

mutans has been considered as an essential etiologic factor in dental plaque formation and thus dental caries and periodontal problems [35]. It has been documented that this bacterium cannot provide the nutrients necessary for its survival and reproduction. Application of mouthwashes has been considered as a complementary method to mechanical ways of plaque removal. [2]. Many studies have demonstrated that adding antibacterial agents to mouth rinses or toothpastes can be used for inhibition of plaque growth and reduction of bacterial acid formation. [9].The most popular mouthwashes used for prevention of dental caries or periodontal problems, are sodium fluoride and chlorhexidine [2].

Present study was designed to comparison of antibacterial effect of chlorhexidine 0.2% and sodium fluoride 0.05% and ZnO NPs and Ag/ZnO NPs and some of their compositions.

Hernandez-Sierra [37] demonstrated that antibacterial effect of nanosilver against S.mutans is higher than ZnO nanoparticles. Although it has been proved that Ag and ZnO nanoparticles have antibacterial effect against S.mutance, their mechanism is still not fully understood. Proposed

antibacterial mechanism of nanostructures can be classified into two main categories: [26, 27, 32] (1) oxidative stress [17] and (2) physical attack. There are some other mechanisms like (a) interfere in enzymatic activity of bacterial respiratory chain [28] (b) altering bacterial structure [36] and (c) DNA damages [30].

Combining of Ag NPs with ZnO NPs to prepare Ag/ZnO NPs causes synergistic effect on antibacterial properties [33]. It was noted that in order to have bactericidal and bacterostatic effect against Gram -positive and -negative bacteria by means of Ag/ZnO NPs, less dosage of Ag/ZnO NPs than Ag NPs or ZnO NPs was needed. There have been no explanations of antibacterial mechanism of hybrid systems like Ag/ZnO until Somnath Ghosh [33] gave a plausible mechanism of Ag/ZnO based on TEM and EPR (Electron Paramagnetic Resonance spectroscopy) studies: Ag on Ag/ZnO NPs is positively charged but bacterial surface is electronegative due to carboxylic acid groups on bacterial membrane [38]. These particles enter into bacterial cell and cause cell death. Antibacterial effect of ZnO NPs can be due to free radicals that induce oxidative stress in bacteria causing cell death but antibacterial effect of nanohybrid (Ag/ZnO) against gram positive bacteria like S.mutans is mainly due to physical attack on cell membrane not because of free radicals. Even lower concentrations of Ag nanoparticles (even in hybrid form of Ag/ZnO) can kill S.mutans. So very low concentrations of Ag/ZnO can be used for plaque control [37]. Burguera–Pascu [39] reported that zinc salts have very high antibacterial effect against S.mutans. Mouthwashes containing little amounts of zinc compounds have been demonstrated to have high antimicrobial effect on streptococcus in mouth [19].These results are similar to our study. We proved that ZnO in concentrations above 5%, had statistically significant effect on *S. mutans*. Even its antibacterial effect was more than sodium fluoride 0.05%. Our study demonstrated that antibacterial effect of Ag/ZnO10 nanoparticle against *S. mutans* is more than that of chlorhexidine 0.2%. Other studies showed that antibacterial effect of chlorhexidine 0.2% against *S.mutans* is more than all kind of nanostructures used in their study [21]. In contrast, Sadeghi [40] reported that antibacterial effect of nanoparticle against S.mutans is comparable to that of chlorhexidine 0.2%. These differences can be attributed to the kind of nanoparticle they tested. On the other hand our study concluded that antibacterial effect of chlorhexidine 0.2% is more than that of ZnO NPs Ahrari F [21] have reported similar results too. We showed that mouthwash containing chlorhexidine 0.2% plus sodium fluoride 0.05% is more effective than chlorhexidine 0.2% mouthwash against S.mutans. This result is comparable to those obtained by Marsh [41]. He mentioned that anticarious effect of chlorhexidine 0.2% plus sodium fluoride 0.05% against S.mutans is more than that of chlorhexidine 0.2% or sodium fluoride 0.05%.

The cytotoxicity of the ZnO and Ag/ZnO nanoparticles were investigated in the A549 cell line. The results indicate that no significant harmful effect is imposed to the cells up to 0.1 mg/ml (100 ppm) of ZnO and Ag/ZnO nanoparticles. According to the previous reports, the MTT reduction observed after 24 hours of exposure of ZnO nanorods in A549 cells at the concentrations of 0.01, 0.025, 0.050 and 0.1 mg/ml was 73%, 60%, 49%, and 41%, respectively [42] also the size of nano particles, their surface charge and the type of cell line are important factors [43-45]. But the as synthesized ZnONPs produced in this study exhibit poor cytotoxicity because they are coated with PEG. Therefore to use nanoparticles in medical applications, it is better to coat them with a biocomapatible polymer.

CONCLUSION

The Ag/ZnO ^{b} 10 - containing mouthwash proved to be an effective antibacterial agent. It showed highest antibacterial activities against *S. mutans* compared to those of ZnO 10, chlorhexidine 0.2% and sodium fluoride 0.05%. So after some *in vivo* complementary studies, it can be considered as a good alternative to chlorhexidine 0.2% and sodium fluoride 0.05% mouthwashes in plaque control. But ZnO NP, in different concentrations, showed lower antibacterial properties against *S. mutans* compared to chlorhexidine 0.2%.

Here it was demonstrated that coated ZnO and Ag/ZnO NPs had not toxic effects on mammalian cells, and this effect was dependent on the ZnO concentration and the cell line used. Thus it needs further investigations before it can substitute chlorhexidine 0.2%.

ACKNOWLEDGEMENTS

This study was supported by Tabriz University of Medical Sciences, Iran.

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