The Effect of Zinc Oxide Nanoparticles on *Streptococcus mutans* of Human Saliva (*In Vitro* Study)

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ABSTRACT

Background: Nanotechnology represents a new science that promises to provide a broad range of uses and improved technologies for biological and biomedical applications. One of the reasons behind the intense interest is that nanotechnology permits synthesis of materials that have structure is less than 100 nanometers. The present work revealed the effect of zinc oxide nanoparticles (ZnO NPs) on *Streptococcus mutans* of Human Saliva in comparison to de-ionized water.

Materials and methods: *Streptococcus mutans* were isolated from saliva of forty eight volunteers of both sexes their age range between 18-22 years and then purified and diagnosed according to morphological characteristic and biochemical tests. Different concentrations of ZnO NPs were prepared from the stock solution; all the experiments were conducted *in vitro*. Disk diffusion method was used to study the sensitivity of *Streptococcus mutans* to different concentrations of zinc oxide nanoparticles in comparison to effect of de-ionized water.

Results: *Streptococcus mutans* were sensitive to concentrations (0.05, 0.1, 0.5, 1, 3 and 5.8) mg / ml of the zinc oxide nanoparticles solution in comparison to de-ionized water, revealing a highly significant difference in all concentrations except for concentration (0.01) mg / ml which was showed no significant difference in comparison to de-ionized water.

Conclusion: This study revealed that zinc oxide nanoparticles were effective against *Streptococcus mutans*.

Key words: *Streptococcus mutans*, ZnO NPs, Human saliva, Dental caries. (J Bagh Coll Dentistry 2016; 28(2):158-164).

INTRODUCTION

Nanoparticles have many different effects on human health relative to bulk material from which they are produced [1]. Increase the biological activity of nanoparticles can be beneficial, detrimental or both [2,3]. Nanotechnology has attracted global attention because nanoparticles (NPs) have properties unique from their bulk equivalents. NPs of silver, cupric oxide and zinc oxide are being used industrially for several purposes including amendments to textiles, cosmetics, sprays, plastics and paints [4]. A common feature of these NPs is their antimicrobial activity [5]. The antimicrobial activity of NPs largely has been studied with human pathogenic bacteria, mainly *Escherichia coli* and *Staphylococcus aureus* [6].

The considerable antimicrobial activities of inorganic metal oxide nanoparticles such as zinc oxide, magnesium oxide, titanium oxide, silicon oxide and their selective toxicity to biological systems suggest their potential application as therapeutics, diagnostics, surgical devices and nano medicine based antimicrobial agents [7]. The advantages of using these inorganic oxides nanoparticles as antimicrobial agents are their greater effectiveness on resistant strains of microbial pathogens, less toxicity and heat resistance [8].

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ZnO NPs has been reported to have extremely good safety profile and no toxicity observed when taken at different nano sizes of the zinc particles [9].

Dental caries is one of the most common infectious diseases in human oral cavity [10]. It continues to be major oral health problems affecting children, adolescents, adults as well as elderly [11]. *Steptococcus mutans,* a member of the oral micro flora, is considered to be the primary causative agent of dental caries (or tooth decay) and is one of the best known biofilm forming bacterium [12]. The emerging infectious diseases and the development of drug resistance in the pathogenic bacteria and fungi at an alarming rate is a matter of serious concern. Despite the increased knowledge of microbial pathogenesis and application of modern therapeutics, the morbidity and mortality associated with the microbial infections still remains high [13].

Zinc oxide nanoparticles have been shown to be useful antibacterial and antifungal agents when used as a surface coating on materials and textiles [14]. The aim of this study was to show effect of zinc oxide nanoparticles on *streptococcus mutans* of human saliva.

MATERIALS AND METHODS

Collection of Saliva Samples

Collection of stimulated salivary samples from forty eight apparently healthy college students from Baghdad University /Collage of Dentistry

their age range between (18-22 years) of both sexes. The collection of stimulated salivary samples was performed under standard condition following instruction cited by Tenovou and Lagerlof [15].

Preparation of Culture Media

1-Mitis-Salivarius Bacitracin Agar (MSB Agar)

This selective media for cultivation of *Streptococcus mutans* was prepared according to the manufacturer's instruction by dissolving 90 gm of MSA in one liter of distilled water; sucrose was added to obtain a concentration of 150 gm/L before sterilization of the agar medium [16], and sterilized by autoclave at 121°C 15 pounds per square inch for 15 minutes, and left to cool till 45 ºC, then bacitracin antibiotic solution was added under aseptic condition. The Bacitracin solution was sterilized by Millipore filter (0.4 µm), then the media was poured into plates, left to solidify then put them in incubator at 37°C for 24 hours then stored in refrigerator until use.

2- Mueller Hinton Agar (MHA)

These were prepared according to manufacturer's instruction which involved the suspension of 38 gm in one liter of de-ionized water, after being completely dissolved with boiling, it was sterilized by autoclave, then left to cool at 45- 50°C, poured and left to solidify then put them in incubator at 37°C for 24 hours then stored in refrigerator until being used.

Isolation of *Streptococcus mutans*:

Salivary samples were dispersed using vortex mixer for 1 minute. Ten folds dilutions were performed by transferring 0.1 ml of each suspension to 0.9 ml of sterile normal saline. From dilution 10^{-3} of salivary samples, 0.1 ml was taken and spread in duplicate on the selective media MSB agar for isolation of *Streptococcus mutans*. The plates were incubated anaerobically using an anaerobic jar for 48 hr at 37°C, then aerobically for 24 hrs at room temperature [17]. The colonies of *Streptococcus mutans* were determined according to the following:-

1 - Morphological characteristic:

Streptococcus mutans were examined directly and under dissecting microscope (magnification x15) and diagnosed according to their morphological characteristics on the MSB agar plates, and according to the description cited by Edwardsson [18].

2-Gram's stain:

A colony was picked up from MSB agar plates separately under sterilized conditions and subjected to Gram's stain according to Koneman *et al.* [19].

3- Motility Examination of the Microbial Cells:

The motility ability of microbial cells was examined under microscope by direct smear and without staining [20].

4-Biochemical Tests:

A- Catalase production Test:

An inoculum from a pure culture of *Streptococcus mutans* from MSB agar was transferred using a sterile loop to the surface of a clean dry glass slide. Few drops of 3% hydrogen peroxide (H_2O_2) immediately were placed on to a portion of a broth on the slide. The evolution of bubbles of gas indicated a positive test [21].

B- Carbohydrate fermentation test:

Cystine Trypticase agar CTA- mannitol medium had been used to test the ability of *Streptococcus mutans* to ferment the mannitol [22].

C- Identification of *Streptococcus mutans* by API 20 strep

API 20 strep was a standardized system containing 20 biochemical tests that offer wide spread capabilities. The strip consists of 20 micro tubes containing dehydrated substrates for the demonstration of enzymatic activity or fermentation of sugars.

Preparation of the strip and inoculums of the *S. mutans* isolates was done according to procedure of biomeriux and the enzymatic tests were inoculated with a dense suspension of organisms made from pure culture, which used to reconstitute the enzymatic substrates.

The reaction was read according to the reading table and the identification was obtained by referring to the Analytical Profile Index or using the identification software.

Characterization of zinc oxide nanoparticles

Zinc oxide nanoparticles provided from Iraqi ministry of sciences and technology, with the concentration 5.8 mg/ml for stock solution and the particles size >50 nm papered by sol gel method. We make different concentration from the stock solution by using dilution low ($N_1V_1=N_2V_2$). To confirm the activity of zinc oxide nanoparticles solution we make the UV-Vis spectra of ZnO NPs which was shown in Figure (1). The absorption peak of the prepared ZnO NPs was found at around 400-500 nm.

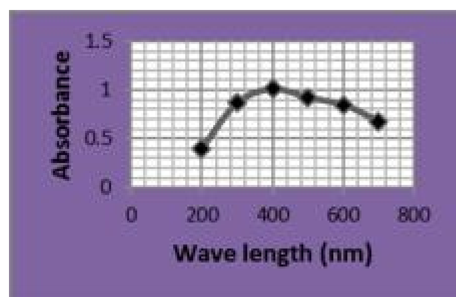

Figure 1: UV-Vis spectra of the ZnO NPs.

***In vitro* Experiments**

Determining the Sensitivity of *Streptococcus mutans* to Different Concentrations of ZnO NPs Solution and de-ionized water:

The disk diffusion method was used for testing the antibacterial susceptibility to ZnO NPs. Disposable plates containing Muller-Hinton agar inoculated were applied to study the antibacterial effects of different concentrations of zinc oxide nanoparticles (0.01, 0.05, 0.1, 0.5, 1, 3, and 5.8) mg/ml compared with de-ionized water as negative control on Mueller Hinton Agar (MHA) media. Plates were left at room temperature for 1 hour then incubated anaerobically for 24 hour at 37°C. Zone of inhibitions (no growth of the bacteria) around disk were measured across the diameter of each filter paper by using a ruler, no inhibition zone indicated a complete resistance of *Streprococcus mutans* to the agents.

RESULTS

Identification of *Streptococcus mutans*

Colony Morphology

On the selective MSB agar plates, *Streptococcus mutans* colonies appeared light blue in color about (l-2 mm) in diameter as spherical or ovoid in shape with raised or convex surface, adhered well to the agar surface (Figure 2). Some colonies of *Streptococcus mutans* appeared as irregular colonies with rough or frosted-glass surface appearance (rough colonies), while others appeared with smooth surfaces colonies (smooth colonies) (Figure 3).

Microscopic Examination

Microscopic examination showed that *Streptococcus mutans* cells were Gram positive, spherical or ovoid in shape, arranged in short or medium length non spore forming chains (Figure 4). In addition *Streptococcus mutans* were non-motile when examined their motility under microscope by direct smear.

Figure 2: *Streptococcus mutans* on MSBA.

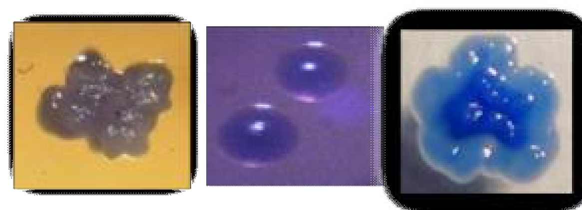

Figure 3: Rough and smooth colony types of *Streptococcus mutans* on MSBA.

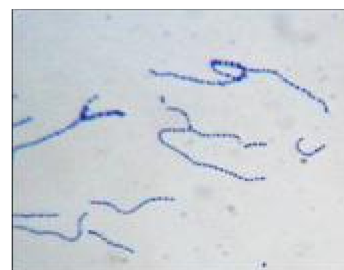

Figure 4: Gram's stain of *Streptococcus mutans* showing the Gram positive stain

Biochemical Tests

Catalase test

Streptococcus mutans colonies were all catalase negative, that's mean there was no gas bubbles produce with H_2O_2.

Mannitol Test

All *Streptococcus mutans* colonies have the ability to ferment mannitol. A positive reaction indicated by change in color from red to yellow by formation of acid after incubation for 48 hours (Figure 5).

Identification of *Streptococcus mutans* by API 20 strep

The reaction read according to the reading table and the identification was obtained by referring to the analytical profile index. The fermentation of carbohydrates was detected by

alteration in pH, the results as shown by (Figure 6).

Figure 5: Mannitol fermentation test of *Streptococcus mutans.*
A : Positive control tube (agar and bacteria without mannitol).
B: Study tube (agar and mannitol inoculated with *Streptococcus mutans*).
C: Negative control tube (agar and mannitol without bacteria).

Figure 6: Biochemical identification of *Streptococus mutans* of API 20 strep.

Sensitivity of *Streptococcus mutans* to Different Concentrations of Zinc Oxide Nanoparticles Solution and De-ionized Water

The diameter of inhibition zones for zinc oxide nanoparticles solution (clear zone of growth inhibition of *Streptococcus mutans* around each filter paper) were found to be increased as the concentration of the solution increased. The stock solution of ZnO NPs which equal to 5.8 mg/ml showed higher zone of inhibition compared to other concentrations. De-ionized water and (0.01) mg/ml conc. showed no zone of inhibition (Figure 7).

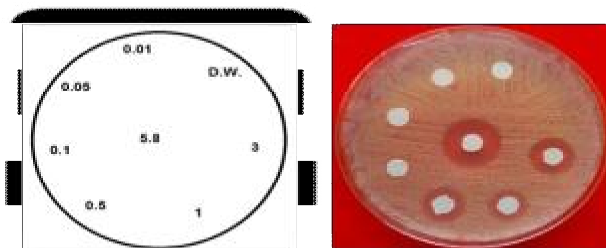

Figure 7: Sensitivity of *Streptococcus mutans* to different concentrations of ZnO NPs.

Statistical Analysis Tests for Inhibition Zone of ZnO NPs on *Streptococcus mutans*

Descriptive statistics for inhibition zones are used to examine the differences among different concentrations of ZnO NPs (0.01, 0.05, 0.1, 0.5, 1, 3, 5.8) mg/ml with de-ionized water to make eight groups respectively (Table 1), each group consist of 48 tests for the same concentration and the mean of inhibition zones measured in mm with the filter paper included in measurements, so when there is no inhibition zone we measure the filter paper only which is equal to 7 mm and refer to it in the Table (1) which show mean and SD of inhibition zones for different concentrations of ZnO NPs and de-ionized water on *S. mutans.*

The Kruskal-Wallis H and Mann-Whitney U non-parametric statistical tests are used to analyze (concentrations of ZnO NPs) groups**.** Results of Kruskal-Wallis test showed highly significant differences among all groups $p<0.01$ (Table 2). The results of Mann-Whitney U test for the (0.01 and 0.05) mg/ml with other groups showed no significant differences only between (0.01 and 0.05) mg/ml $P> 0.05$ while the other differences are highly significant between other groups in comparison with (0.01 and 0.05 mg/ml). The other results of Mann-Whitney U test for the (0.1, 0.5, 1, 3 and 5.8 mg/ml) with other groups showed highly significant differences between each groups $P< 0.01$ (Table 3).

DISCUSSION

The results of the quantitative antibacterial assessment by disk diffusion method are reported in Table (1) from which it is observed that the size of the inhibition zone (The antibacterial activity) was found to depend strongly on the concentration. This also was found with Negahdary *et al.* [(23)] who showed complete bacterial inhibition depends upon the concentrations of zinc oxide nanoparticles in their study, and also this agrees with the study of Azam [(24)] that showed that ZnO NPs have greatest antibacterial activity against both Gram-positive and Gram-negative bacteria. It was observed that ZnO NPs have excellent bactericidal potential.

The diameters of the inhibition zones were found to increase when the concentration of the ZnO NPs increased. This may be attributed to the significantly higher surface to volume ratio for more effective ion-exchange. The least concentration of ZnO NPs that affecting antibacterial was 0.05 mg/ml. The de-ionized water and concentration 0.01 mg/ml of ZnO NPs had no effect on the bacteria and this appearing by absence of inhibition zone. Several mechanisms for the antimicrobial action by ZnO NPs have

Table 1: Descriptive Statistics of Inhibition Zones of ZnO NPs and De-ionized Water on *S. mutans*.

Groups	No.	Median	Mean	SD	Min.	Max.	Interquartile range
0.01	48	7.00	7.00	0.00	7.00	7.00	0.00
0.05	48	7.00	7.02	0.14	7.00	8.00	0.00
0.1	48	8.00	7.86	0.57	7.00	9.00	0.50
0.5	48	11.00	11.06	0.86	9.00	12.00	2.00
1	48	14.00	14.13	1.06	12.00	16.00	2.00
3	48	18.00	17.98	1.36	15.00	20.00	2.00
5.8	48	24.00	23.58	1.89	20.00	27.00	3.00
D.W	48	7.00	7.00	0.00	7.00	7.00	0.00

Table 2: Inhibition zones of *Streptococcus mutans* to different concentrations of ZnO NPs.

	Groups	Conc. of ZnO NPs	N	Mean Rank	Kruskal Wallis Test
I. Zones	1	(0.01 mg/ml)	48	77.00	Chi-Square= 372.110 df= 7 P < 0.01 HS
	2	(0.05 mg/ml)	48	78.98	
	3	(0.1 mg/ml)	48	153.07	
	4	(0.5 mg/ml)	48	217.01	
	5	(1 mg/ml)	48	264.63	
	6	(3 mg/ml)	48	312.03	
	7	(5.8 mg/ml)	48	360.28	
	8	D.W	48	77.00	

been proposed. The mechanism of antibacterial activity of ZnO NPs is thought to be via generation of reactive oxygen species, such as hydrogen peroxide (H_2O_2) as the concentrations increase causing increased antibacterial activity of the extract, which could damage cell membranes, causing dysfunction of cellular components, and leading to final cell death [(25)].

Another study showed that the antimicrobial ability of ZnO NPs might be referred to their small size which is 250 times smaller than a bacterium. This makes them easier to adhere to the cell wall of the microorganisms causing its destruction and leads to the death of the cell [(26)]. Another possible mechanism for ZnO NPs antibacterial activity is the release of Zn^{2+}ions which can damage the cell membrane and interact with intracellular contents [(27)].

Electron micrographs have shown that ZnO NPs damage the bacterial cell wall, increase cell membrane permeability, and change the cell morphology [(28-30)], which was assumed to be due to the interactions of ZnO NPs with bacterial membrane (e.g. the cellular internalization of ZnO NPs). This resulted in the membrane dysfunction [(31)] change of membrane permeability, leakage of intracellular substrates [(29,30)] and finally cell death. Nair *et al.* [(31)] attributed this interaction to electrostatic effects due to the opposite surface charges of nanoparticles and cell membrane. While in study Sharma *et al.* [(32)] showed that on contact with bacteria, the cytotoxic behavior of ZnO NPs ruptures the lipid bilayer of bacterium resulting in leakage of cytoplasmic contents. Another possible mechanism for inhibition of *Streptococcus mutans* is that a weak DNA damage was observed in bacteria treated with ZnO NPs [(33)].

It is believed that microorganisms carry a negative charge while metal oxides carry a positive charge. This creates an “electromagnetic” attraction between the microbe and treated surface. Once the contact is made, the microbe was oxidized and dies instantly. Generally, it is believed that nano-materials release ions, which react with the thiol groups (-SH) of the proteins present on the bacterial cell surface [(34)]. Such proteins protrude through the bacterial cell membrane, allowing the transport of nutrients through the cell wall.

As a conclusion; all these findings and results reflect that zinc oxide nanoparticles have an excellent anti-bacterial effect and potential in reducing bacterial growth for practical applications. Zinc oxide nanoparticles have inhibition effect in different concentrations on *Streptococcus mutans*, starting from the concentration 0.05 mg/ml. Sensitivity of *Streptococcus mutans* to ZnO NPs increase with the increase of concentration of ZnO NPs solution in comparison to de-ionized water.

Table 3: Mann-Whitney U test of inhibition zones of *S. mutans* to different cons. of ZnO NPs.

Groups	Median	Mean Rank	U Value	Z Value	P Value	Sig.
0.01	7.00	48.00	1128	1.00	0.317	NS
0.05	7.00	49.00				
0.01	7.00	29.50	240.0	7.69	< 0.01	HS
0.1	8.00	67.50				
0.01	7.00	24.50	0.00	9.10	< 0.01	HS
0.5	11.00	72.50				
0.01	7.00	24.50	0.00	9.08	< 0.01	HS
1	14.00	72.50				
0.01	7.00	24.50	0.00	9.06	< 0.01	HS
3	18.00	72.50				
0.01	7.00	24.50	0.00	9.04	< 0.01	HS
5.8	24.00	72.50				
0.05	7.00	29.98	263.0	7.46	< 0.01	HS
0.1	8.00	67.02				
0.05	7.00	24.50	0.00	9.06	< 0.01	HS
0.5	11.00	72.50				
0.05	7.00	24.50	0.00	9.04	< 0.01	HS
1	14.00	72.50				
0.05	7.00	24.50	0.00	9.02	< 0.01	HS
3	18.00	72.50				
0.05	7.00	24.50	0.00	9.00	< 0.01	HS
5.8	24.00	72.50				
0.1	8.00	24.55	2.50	8.60	< 0.01	HS
0.5	11.00	72.45				
0.1	8.00	24.50	0.00	8.60	< 0.01	HS
1	14.00	72.50				
0.1	8.00	24.50	0.00	8.58	< 0.01	HS
3	18.00	72.50				
0.1	8.00	24.50	0.00	8.60	< 0.01	HS
5.8	24.00	72.50				
0.5	11.00	25.06	27.00	8.36	< 0.01	HS
1	14.00	71.94				
0.5	11.00	24.50	0.00	8.53	< 0.01	HS
3	18.00	72.50				
0.5	11.00	24.50	0.00	8.52	< 0.01	HS
5.8	24.00	72.50				
1	14.00	25.19	33.00	8.29	< 0.01	HS
3	18.00	71.81				
1	14.00	24.50	0.00	8.50	< 0.01	HS
5.8	24.00	72.50				
3	18.00	24.72	10.50	8.41	< 0.01	HS
5.8	24.00	72.28				

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