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Effect of Zinc Oxide Nanoparticles on *Candida albicans* of Human Saliva (*in vitro* study)

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Abstract

The potential use of zinc oxide and other metal oxide nanoparticles in biomedicine are gaining interest in the scientific and medical communities, largely due to the physical and chemical properties of these nanoparticles, therefore there is an urgent need to develop new classes of antimicrobial agents, and recent studies demonstrate that hold a considerable promises. *Candida albicans* 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 *Candida albicans* to different concentrations of zinc oxide nanoparticles in comparison to effect of de-ionized water. *Candida albicans* were sensitive to all cocentrations (0.01, 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. This study revealed that zinc oxide nanoparticles were effective against *Candida albicans*.

Keywords: *Candida albicans*, ZnO NPs, Human saliva.

Introduction

Nanotechnology is referred to the emerging technology involving fabrication or application of nanosized structures or materials [1]. Nanoparticles are commonly defined as particles with the size of at least one dimension ranging from 1 to 100 nm, which serve as a bridge between bulk materials and atoms/molecules [2]. Zinc oxide nanoparticles [ZnO NPs], is an inorganic white powder insoluble in water [3]. Zinc oxide nanoparticles have been shown to be useful antibacterial and antifungal agents when used as a surface coating on materials and textiles [4]. ZnO nanorod arrays diminished the growth of *Candida albicans* with stable action for two months [5]. *Candida albicans* is a commensal fungal species commonly colonizing human mucosal surfaces [6]. Candida infections can be seen in various parts of the body, including the skin, oral cavity, gastrointestinal tract and vagina. In the oral cavity Candida infection is sometimes visible as thrush, white/yellowish cream like patches on the oral mucosa and the tongue [7]. The incidence of these organisms appears to increase with the age. These fungi can cause characteristic infections that may be quite serious and

even life threatening [8]. Some studies have shown a significant association between *C. albicans* and dental caries in children and young adults [9]. The findings from *in vitro* and animal studies attributing a role for *C. albicans* in caries development and/or progression were solidified by data from a clinical study where the occurrence of caries in children was positively correlated with the frequency of oral candidal carriage [10]. ZnO NPs have shown to have a photo-catalytic effect, which is related their effectiveness as inhibitor of bacteria and fungi [11].

Material and methods

Collection of Saliva Samples

Collection of stimulated salivary samples were taken 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 [12]. Each individual was asked to chew a piece of Arabic gum (0.5-0.7 g) for 1 min, then remove all saliva by expectoration, after that chewing was continued for ten minutes with the same piece of gum and saliva collected in a sterile screw capped bottle. After disappearance of salivary foam, 0.1 ml of saliva is transferred to 0.9 ml of sterile phosphate buffer saline of pH 7.0-7.2 for microbiological analysis.

Preparation of Culture Media

Sabouraud Dextrose Agar [SDA]

The medium which is selective one for cultivation and isolation of *Candida albicans* was prepared and sterilized according to manufacturer's leftlet; 65 g-were suspended in 1000 distilled water. Sterilization was done by autoclaving at 121°C at 15 pounds per square inch for 15 minutes, left to cool to 45-50°C and then chloramphenicol antibiotic [2 g for each 1000 ml of media] was added and poured into petri dishes, left to solidify then put them in incubator at 37°C for 24 hours then stored in refrigerator until being used.

Brain Heart Infusion Broth [BHI]

Preparation of the media was according to the manufacturer's instruction which involved the suspension of 37 g in one liter of de-ionized water. After being completely dissolved, the pH adjusted to 7.2. The media was sterilized in autoclave at 121°C at 15 pounds per square inch for 15 min. then left to cool down to room temperature and thereafter kept in the refrigerator until use.

Mueller Hinton Agar [MHA]

These were prepared according to manufacturer's instruction which involved the suspension of 38 g in 1 L of de-ionized water, after being completely dissolved with boiling, it was sterilized in autoclave, then left to cool at 45-50°C, poured and left to solidify then put them in incubator at 37°C for 24 hrs then stored in refrigerator until being used.

Isolation of *C. albicans*

After mixing of saliva as mentioned previously, ten- folds dilution was performed, from dilution (10^{-1}, 10^{-2}) of salivary samples then 0.1 ml was taken and spread on sabouraud dextrose agar [SDA], and the plates were incubated aerobically for 48 hr at 37°C [13].

Identification of *C. albicans*

A. Colony morphology

Colonies of *C. albicans* appeared smooth creamy in color with a yeast odor and typically medium size 1.5-2 mm diameter which later developed into high convex, off-white larger colonies after about 2 days [14].

B. Gram stain

The same procedure as described for *Streptococcus mutans* was used, *C. albicans* appeared as Gram- positive small oval or budding yeast cell**.**

C. Germ tubes formation

Very small inoculums from isolated colonies were suspended in 0.5 ml of normal human serum. The inoculated tubes were incubated at 37°C for 3 hr. After incubation, a drop of yeast suspension was placed on a clean microscopic slide covered with a cover slip and examined under low power magnification for presence of germ tube. Production of germ tubes is characteristic of *C. albicans* [15].

D. Identification of *C. albicans* by Rapid Yeast Plus System

Rapid Yeast Plus System has several reaction cavities molded into the periphery of plastic disposable tray. Reaction cavities contain dehydrated reactions and the tray allows the simultaneous inoculation of each cavity with a predetermined amount of inoculum [16].

Purification and Maintenance of *C.albicans*

A single colony from *C. albicans* was transferred to 10 ml sterile brain heart infusion [BHI] broth and then incubated for 24 hours aerobically at 37°C. The purity of isolates was checked by re-inoculation of 0.1 ml of culture broth BHI on SDA. The plates were incubated aerobically for 48 hr at 37°C, then one colony from each isolates was transferred to 10 ml of sterile BHI broth and then incubated for 24 hrs aerobically at 37°C [17].

Activation of *C. albicans*

Inoculums of *C. albicans* were activated by the addition of 0.1ml of pure broth culture to 10 ml of BHI broth followed by incubation for 18 hr at 37°C [18].

Characterization of Zinc Oxide nanoparticles

Zinc oxide nanoparticles provided from 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 shown in Figure 1. The absorption peak of the prepared ZnO NPs was found at around 400-500nm.

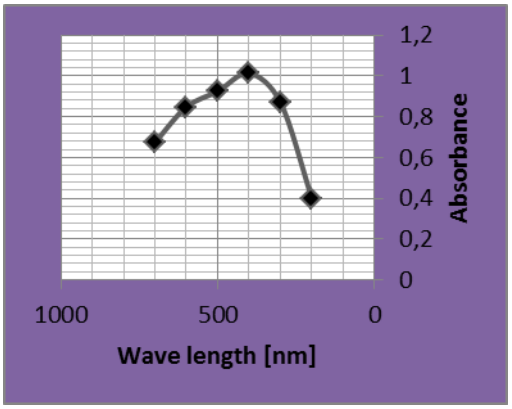

Figure 1. UV-Vis spectra of the ZnO NPs

Determining the Sensitivity of *C. albicans* to Different Concentrations of ZnO NPs and de-ionized water

Fungal inoculums used was prepared by adding a few pure colonies of *C. albicans* to 10 ml of sterile brain heart infusion broth (pH 7.0), then incubated aerobically for 18 hrs at 37°C. The disk diffusion method was used as antifungal susceptibility test. 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. These experiments were conducted on 48 isolates of *C. albicans*.

The sensitivity of *Streptococcus mutans* and *C. albicans* to ZnO NPs

1. A volume of 25 ml of MHA (pH 7.0) was poured into sterile Petri dishes then left at room temperature for 24 hours.

2. To each plate 0.1 ml of activated *C. albicans* inoculum was spread, left at room temperature for 20 mins.

3. Eight filter papers (wattman no.1) of equal size (7 mm in diameter) were prepared; each filter was impregnated with 40 µl of ZnO NPs with different concentrations (0.01, 0.05, 0.1, 0.5, 1.0, 3.0, 5.8 mg/ml) and de-ionized water respectively, and put in each agar plate.

4. Plates were left at room temperature for 1 hour then incubated aerobically for *C. albicans* 24 hour at 37°C. Zone of inhibitions which appears as a clear zone of inhibition around disk were measured across the diameter of each filter paper by using a ruler, no inhibition zone indicated a complete resistance of *C. albicans* to the agents.

Results

Identification of *C. albicans*

Colony morphology

Colony of *C. albicans* appeared smooth, creamy in color with yeast odor and typically medium sized **(**1.5-2 mm**)** diameter within 2 days, they develop into high convex, off- white large colonies (Figure 2).

Figure 2. *C. albicans* colonies on SDA

Microscopic examination

The slide was examined under light microscope; the rounded or oval yeast cells were Gram-positive (Figure 3).

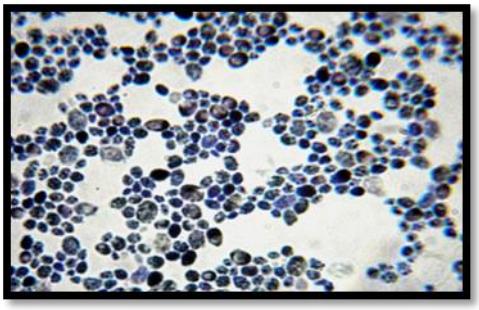

Figure 3. Gram's stain of *C. albicans*

Germ Tube Formation

All isolates of *C. albicans* under light microscope (100×magnification) show the presence of germ tubes which is a characteristic feature of *C. albicans* (Fig. 4).

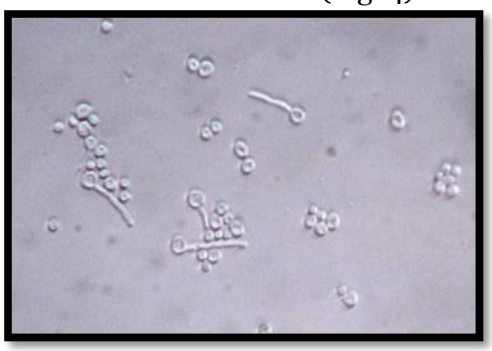

Figure 4. Different shapes of germ tube of *C. albicans*

RapID Yeast Plus System for identification of *C. albicans*:

The RapID Yeast Plus System Differential Chart illustrates the expected results for *C. albicans*. Table 3-8 show the results after incubation at 30°C in incubator for 4 hours.

Table 1: The Results of RapID Yeast Plus System for Identification of *C. albicans*

Test	**Abbreviations**	**Positive result of *C.albicans***
Glucose	GLU	+
Maltose	MAL	+
Sucrose	SUC	_
Trehalose	TRE	_
Raffinose	RAF	_
Fatty acid ester	LIP	_
ρ-Nitrophenyle-N-acetyle-β,D-galactosaminide	NAGA	+
ρ-Nitrophenyl-α, D-glucoside	αGLU	+
ρ-Nitrophenyl-β, D-glucoside	βGLU	_
ρ-Nitrophenyl-β, D-galactoside	ONPG	_
ρ-Nitrophenyl-α,D-galactoside	αGAL	_
ρ-Nitrophenyl-β, D-fucoside	FUCβ	_
ρ-Nitrophenyl phosphate	PHS	**V**
ρ-Nitrophenyl phosphorylcholine	PCHO	_
Urea	URE	_
Proline-β-naphthylamide	PRO	+
Histidine β- naphthylamide	HiST	**V**
Leucyl-glycine naphthylamide	LGY	**V**

+, positive; -, negative; V, variable

Sensitivity of *C. albicans* to Different Concentrations of ZnO NPs Solution and De-ionized Water

The diameter of inhibition zones for zinc oxide nanoparticles solution (clear zone of no growth for *C. albicans* around each filter paper) as found to be increased as the concentration of the solution increased. The stock solution of ZnO NPs which equal 5.8 mg/ml showed higher zone of inhibition compared to other concentrations. De-ionized water showed no zone of inhibition (Figure 5).

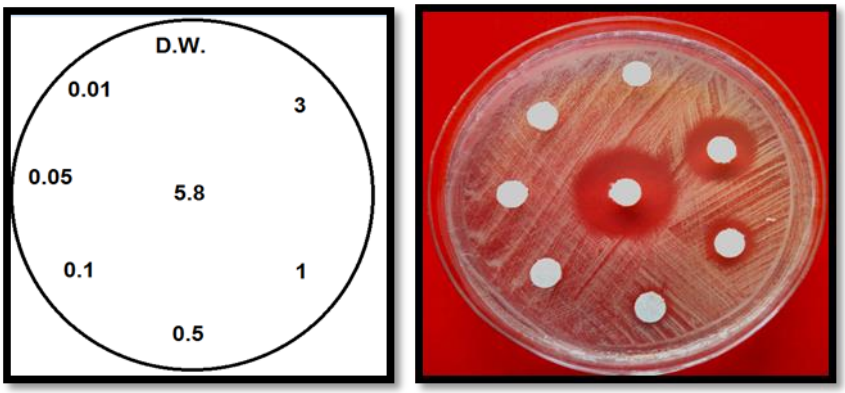

Figure 5. Sensitivity of *C. albicans* to different concentrations of ZnO NPs

Statistical Analysis Tests for Inhibition Zone of ZnO NPs on *C. albicans*

All data for the inhibition zone for all groups concentrations of ZnO NPs (except 5.8) is not normally distributed (sig. < 0.05) by Kolmogorov-Smirnov test for test of normality (Table 2). Therefore the tests used are non-parametric.

Table 2. Test of Normality

Kolmogorov-Smirnov Test		
I.Zones	Groups	Sig.
	0.01	.000
	0.05	.000
	0.1	.001
	0.5	.005
	1	.000
	3	.000
	5.8	.020

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 in (Table 3) each group consist of 48 tests for the same concentration and the mean of inhibition zones measured in mm which included the diameter of filter paper in measurements, So when there is no inhibition zone we measure the diameter of filter paper only which is equal to 7 mm and refer to it in Table 4.

Table 3: Descriptive statistics of inhibition zones of ZnO NPs and de-ionized water on *C. albicans*.

Groups	No.	Median	Mean	SD	Interqua-rtile range
0.01 mg/ml	48	8.00	8.00	0.97	2.00
0.05 mg/ml	48	9.00	8.88	1.48	3.00
0.1 mg/ml	48	10.00	10.25	1.87	4.00
0.5 mg/ml	48	12.00	12.19	2.16	4.00
1 mg/ml	48	14.00	14.69	2.59	3.00
3 mg/ml	48	17.00	19.08	3.65	4.75
5.8 mg/ml	48	25.00	25.69	3.54	4.75
D.W	48	7.00	7.00	0.00	0.00

The Kruskal-Wallis H and Mann-Whitney U non-parametric statistical tests test 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 4).

Table 4: Zones of inhibition of *C. albicans* to different concentrations of ZnO NPs

	Groups	N	Mean Rank	Kruskal-Wallis H Test
I.Zones	1	48	88.53	Chi-Square = 334.877 df= 7 P < 0.01 HS
	2	48	116.74	
	3	48	161.91	
	4	48	210.44	
	5	48	257.63	
	6	48	309.09	
	7	48	354.67	
	8	48	41.00	

Further analysis using a Mann-Whitney U Test was done to determine which of the eight groups of ZnO NPs was different from the other groups. The results of Mann-Whitney U test with each other groups showed highly significant P< 0.01 between all the groups (Table 5).

Table 5: Mann-Whitney U test of inhibition zones of C. albicans

Groups	Median	Mean Rank	U Value	Z Value	P Value	Sig.
0.01	8.00	39.67	728.00	3.22	< 0.01	HS
0.05	9.00	57.33				
0.01	8.00	32.41	379.50	5.80	< 0.01	HS
0.1	10.00	64.59				
0.01	8.00	25.36	41.50	8.22	< 0.01	HS
0.5	12.00	71.64				
0.01	8.00	24.59	4.50	8.49	< 0.01	HS
1	14.00	72.41				
0.01	8.00	24.50	0.00	8.53	< 0.01	HS
3	17.00	72.50				
0.01	8.00	24.50	0.00	8.51	< 0.01	HS
5.8	25.00	72.50				
0.05	9.00	38.23	659.00	3.67	< 0.01	HS
0.1	10.00	58.77				
0.05	9.00	29.32	231.50	6.85	< 0.01	HS
0.5	12.00	67.68				
0.05	9.00	24.85	17.00	8.38	< 0.01	HS
1	14.00	72.15				
0.05	9.00	24.50	0.00	8.51	< 0.01	HS
3	17.00	72.50				
0.05	9.00	24.50	0.00	8.50	< 0.01	HS
5.8	25.00	72.50				
0.1	10.00	37.48	623.00	3.93	< 0.01	HS
0.5	12.00	59.52				
0.1	10.00	27.56	147.00	7.44	< 0.01	HS
1	14.00	69.44				
0.1	10.00	24.50	0.00	8.49	< 0.01	HS
3	17.00	72.50				
0.1	10.00	24.50	0.00	8.47	< 0.01	HS
5.8	25.00	72.50				
0.5	12.00	35.79	542.00	4.52	< 0.01	HS
1	14.00	61.21				
0.5	12.00	25.80	62.50	8.03	< 0.01	HS
3	17.00	71.20				
0.5	12.00	24.51	0.50	8.46	< 0.01	HS
5.8	25.00	72.49				
1	14.00	32.07	363.50	5.81	< 0.01	HS
3	17.00	64.93				
1	14.00	24.85	17.00	8.35	< 0.01	HS
5.8	25.00	72.15				
3	17.00	29.97	262.50	6.56	< 0.01	HS
5.8	25.00	67.03				

The results of Mann-Whitney U test for the de-ionized water with other groups showed highly significant differences between all groups (0.01, 0.05, 0.1, 0.5, 1,3 and 5.8 mg/ml) P< 0.01 (Table 6).

Table 6: Mann-Whitney U test of inhibition zones of *C. albicans*

Groups	Median	Mean Rank	U Value	Z Value	P Value	Sig.
0.01	8.00	64.50	384.00	6.76	< 0.01	HS
D.W	7.00	32.50				
0.05	9.00	65.00	360.00	6.88	< 0.01	HS
D.W	7.00	32.00				
0.1	10.00	71.50	48.00	8.76	< 0.01	HS
D.W	7.00	25.50				
0.5	12.00	72.50	0.00	9.04	< 0.01	HS
D.W	7.00	24.50				
1	14.00	72.50	0.00	9.05	< 0.01	HS
D.W	7.00	24.50				
3	17.00	72.50	0.00	9.06	< 0.01	HS
D.W	7.00	24.50				
5.8	25.00	72.50	0.00	9.04	< 0.01	HS
D.W	7.00	24.50				

Discussion

The results of present study showed the significantly antifungal activity against *C.albicans* using the ZnO NPs as low as 0.01 mg/ml. As the concentration of ZnO NPs increased from 0.01 to 5.8 mg/ml the efficacy of ZnO NPs treatment was enhanced. The fungicidal activity of ZnO NPs was due to destroying cell membrane integrity [19]. The results of the quantitative antifungal assessment by disk diffusion method are reported in Table 3 from which it is observed that the size of the inhibition zone (the antifungal activity) was found to depend strongly on the concentration of ZnO NPs, and these results agree with Eman *et al* in 2013 [20] who showed the fungicidal effect of ZnO NPs is concentration dependent and also indicate that the mechanism of the fungicidal action of ZnO NPs involves disrupting the membrane. These results agree with those obtained by Shi *et al* in 2010 and Lipovsky *et al* 2011 [21, 22], who recorded the ability of ZnO NPs to affect the viability of the pathogenic yeast, *C. albicans*, as well as a concentration-dependent effect, but does not agree with present results in that the minimal fungicidal concentration of ZnO NPs was found to be 0.1 mg/ml. This concentration caused an inhibition of over 95% in the growth of *C. albicans*. While the current results observed that the minimal fungicidal concentration of ZnO NPs was found to be 0.01 mg/ml. This may be due to the incubation time which was 5 days in study of Eman *et al* 2013 [20] while in our study it was 24 hours only, or may be due to *C. albicans* that is isolated from the skin in study of Eman *et al* 2013 [20] which differ from *C. albicans* isolate that is isolated from human saliva in that study. The current study found that the least inhibition zone of *C. albicans* was 8 mm in concentration 0.01 mg/ml of ZnO NPs but this result in contrast to the results of Jehad *et al* 2012 [23] who showed that the best inhibition zone of *C. albicans* was 18 mm in concentration 10 µg/ml which is equal to 0.01 mg/ml the least conc. That is used in this study. This may be attributed to the antibacterial technique that used in both study as Jehad *et al* 2012 [23] used Agar Diffusion Technique in which hollows of 10 millimeters diameter wells were cut from the agar, and 0.1 ml of each of the tested solutions were poured into the wells. While in this study the disk diffusion method was used in which the filter paper of 7 mm in diameter was impregnated in 40 µl only the difference in amount of ZnO NPs may cause the difference in inhibition zone diameter for the same concentration.

The study of [21] mentioned that, for almost all fungi, the central core of the cell wall is a branched β-1, 3, 1, 6 glucan that is linked to chitin via β-1, 4 linkages. The binding of the oxides particles on the fungal cell surface through electrostatic interactions could be a possible mechanism.

Conclusion

1. Zinc oxide nanoparticles have inhibition effect in different concentrations on *Candida albicans*, starting from the concentration 0.05 mg/ml.

2. Sensitivity of *Candida albicans*, to ZnO NPs increases with the increase of concentration of ZnO NPs solution in comparison to de-ionized water.

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