IOSR Journal of Applied Chemistry (IOSR-JAC)
e-ISSN: 2278-5736.Volume 9, Issue 4 Ver. II (Apr. 2016), PP 57-63
www.iosrjournals.org

Effect of Zno Nanoparticles on Salivary Peroxidase Activity In Chronic Periodontitis Patients (In Vitro Study)

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Abstract: *The structural and optical properties of Zinc oxide nanoparticles (ZnO NPs) have been investigatedusing (UV-Vis) spectrophotometer and SEM. The nanoparticles show a peak around 375 nm. The nanoparticles have spherical shape with an average particle size <80 nm. The effect of ZnO NPs was studied on the activity of total peroxidase in saliva of 60patients with chronic periodontitis in comparison to 20 healthy subjects with average age about 30- 60 years. The results indicated that salivary peroxidase activity higher in chronic periodontitis patients compare to control group but statistically there was no significant differencebetween chronic periodontitis patients and control group on other hand the correlation between peroxidase activity and clinical periodontal parameter was no significant difference .While total salivaryperoxidase activity inhibited significantly (p=0.011) by ZnO nanoparticles in same patientes.*
Keywords: *ZnO nanoparticles, Peroxidase activity,Saliva, and chronic periodontitis*

I. Introduction

Periodontitis is a chronic infection concerning destruction of the tooth-supporting apparatus, including theperiodontal ligament and alveolar socket support of the teeth. Gingivitis may or may not progress to periodontitis, which is related with accessoryand alveolar bone loss. Periodontal disease is introduced by a local accumulation of bacteria (i.e., dental plaque adjacent to the tooth) and theirmetabolic products (e.g., endotoxin), that stimulate the junctional epithelium to proliferate and produce tissue-destructive proteinases that degradethe basement membrane and allow for the apical immigration of the junctional epithelium alongside the root surface of the tooth, thus extending thegingival crevice to create periodontal pockets and associated attachment loss, which is the hallmark injury of periodontal disease[1].

Nanoparticles can be definide as particles that have one dimension that measures less than 100 µm. The physical properties of materials are change when they converted to nanoparticales, because nanoparticles have a greater surface area per weight in compared to the large particles. This property renders the result nanoparticles more active powder[2].

Applications of nanomaterials in biology or medicine are including, Fluorescent biological labels, Drug and gene delivery, Bio detection of pathogens , Detection of proteins, Probing of DNA structure , Tissue engineering, Tumour destruction via heating (hyperthermia), Separation and purification of biological molecules and cells[3-4]. Zinc oxide nanoparticles are found to be nontoxic, biosafe, and biocompatible and have been used as drug carriers, cosmetics, and fillers in medical materials. On the other hand, most ZnO nano-particles that used commercially have some advantages, compared to silver nano-particle, such as lower cost and white appearance [5].Many material products including plastics, ceramics, glass, cement, lubricants, paints, ointments, adhesives, sealants, pigments, batteries, ferrites, and fire retardants contain zinc oxide powder as an additive substance [6]. The peroxidase enzymes are a large family of enzymes that catalyzed the biochemical reactions of peroxides in presence of electron donor substance. Hydrogen peroxide considers an optimal substrate for most of these enzymes, while other forms are found to be more active with the organic hydroperoxide (as in lipid peroxide). The nature of electron donor is associated with the peroxidase enzyme structure. Peroxidasesare playing an important role in defending against pathogens [7].

Salivary, mammary and mucosal glands can be secreting peroxidase enzyme which functions to convert the reactive oxygen species (H_2O_2) to harmless substance (H_2O) and protect red blood cell membranes from damage .Peroxidases present in liver, spleen, salivary glands, stomach wall, leucocytes, intestinal mucosa ….etc. They have a wide range of biological functions such as a host defensive role or cellular defense against of oxidative damage by removal of hydrogen peroxide [8-9].

There is no study about effect of ZnO NPs on salivary peroxidase in chronic periodontitis patients , so this study was done to evaluate the effect on ZnO NPs on the activity of salivary peroxidase .

II. Materials And Methods

1- Nanoparticles

Zinc oxide nanoparticles have been obtained from Nanjing ,china. This product supplies as ZnO Nano powder absorbance spectra of NPs stock solution were measured by UV- VIS spectrophptometer . Structure and nano size measurement of ZnO NPs powder were identified by the Scanning Electron Microscope SEM (Electronic Microscope Centre- College of applied Science , University of Technology , Iraq).

2-Sample Description:

The study sample was consisted of eighty participants with age range of (30-60) years. Sample collection was started on March to June of 2015. The participants recruited for the study were patients who attending the Department of periodontic in the College of Dentistry, University of Baghdad. All participants were informed about the aims of the study orally and by written as a written informed consent was assigned by all participants. All participants were subjected to aquestionnaire (Appendix) about their names ,ages ,full medical history, medication ,if they smoked or not and if there was any previous periodontal treatment. All subjects were presenting at least 20 teeth.Sample of whole unstimulated saliva was taken from each subject. Following this full examination of clinical periodontal parameters (PLI,GI,BOP,PPD and CAL)was done for all subjects.

The participants were divided into two groups; the first group: Study group consisted of sixty participants with chronic periodontitisonly without history of any systemic diseases.(patients with chronicperiodontitis should have at least 4 sites with pocket depths ≥4mm with clinicalattachment loss of (1-2)mm or greater ,this was measured according to[10, 11] .And second group : Control group consisted of twenty participants who were apparently systemically healthyand with clinically healthy periodontium.

3-Saliva Samples Collection

Un-stimulated whole saliva was collected before the clinical examination. A sample was collected after an individual was asked to rinse his mouth thoroughly with water to insure the removal of any possible debris or contaminating materials and waiting for 1-3 min for water clearance. The samples were collected at least 1 h after the last meal. Saliva was collected between 9-11 a.m. Then the collected saliva was separated by centrifuge at 4000 rpm for 10 minutes and then the clear supernatant store at -20˚C (freeze) until biochemical analysis .

4-Salivary Total Peroxidase Assay

Peroxidase activity was determined colorimetrically. Wide variety of hydrogen donors have been utilized in peroxidase assay systems .In this study an improved assay was adopted using 4- aminoantipyrine as hydrogen donor [12]. The activity is determined by measuring the increasein absorbance at λ= 510 nm resulting from the decomposition of hydrogen peroxide per time of incubation. After adding 1.4 ml of (4—aminoantipyrine (2.5 mM) with phenol (0.17 M)) solution to 1.5 ml of (hydrogen peroxide (1.7Mm) in phosphate buffer (0.2 M) pH 7.0) solution ,the reaction was initiated by addition of (100µl) of saliva with mixing .The increasing in the absorbance at 510nm ,was calculated for 5 minutes ,to obtain(ΔA/min). One unit of enzyme activity represent the decompositionof one µmole of hydrogen peroxide per min. at pH = 7.0 underthe specified conditions.

5- Effect Of Zno Nanoparticles On Salivary Total Peroxidase Activity

Stock solution of (300 µg/ml) concentration of ZnO NPs was prepared and then the following concentrations (5, 10, 20, 40, 80, and 100) µg/ml are prepared by diluting with the same solvent . The enzyme activity was measured in human saliva by using 100µl of saliva in the same method with replace 20µl of the solvent (3 :1, water : ethanol) with 20µl of ZnO NPs solution .

The percentage effect on activity was calculated by comparing the activity with and without ZnO NPs and under the same conditions of assay according to the following equations :

٪ inhibition = 100 – 100 x (Activity in the presence of nanoparticles / Activity without the nanoparticles) .

6-Statistical Analysis:

Data were analyzed using SPSS (statistical package of social science) software version 19. Descriptive statistics : including medians, means, standard deviations, minimum and maximum values and statistical tables and figures (including bar charts and scatter diagrams). Inferential statistics: including:
a) Kruskal -Wallis H test: to compare the measured variables among the groups.
b) Mann-Whitney U test: to any statistically significant difference between each two groups.

c) Spearman´s rank correlation coefficient test (r) to test the relation between enzymes measured and periodontal parameters in each group.

III. Results And Discussion:

The Table (1)showed the mean and stander deviation of Peroxidase for control group (90.062 ± 48.405)and in CP group the mean ± SD (107.650 ± 36.097) , there was non-significant difference between control and chronic periodontitis patients in clinical parameter of control group and CP group shown in table (2)and (3).On other hand, table (4) show a weak non significant correlation was revealed between peroxidase activity and PLI, pocket depth and bleeding of scored (0) , and a weak non significant negative correlation shown in GI, clinical attachment level, bleeding of scored(1) in chronic periodontitis patients.

Table (1): **Descriptive statistics and significant difference of salivary Peroxidase activity in control and chronic periodontitis groups**

Groups	Descriptive statistics						
	N	Median	Mean	S.D.	Min.	Max.	p-value
Activityin control	20	93.01	90.062	48.405	41.95	182.06	0.039 (S)
Activityin CP	60	91.865	107.650	36.097	60.3	183.51	

*Significant NS at (P>0.05) level of significance

Table(2): Comparing the periodontal parameters between the control and CP group

Parameters	Groups	N	Median	Mean	S.D.
PLI	Normal	20	0.85	0.77	0.22
	C.P	60	1.515	1.61	0.46
GI	Normal	20	0.50	0.53	0.12
	C.P	60	1.38	1.37	0.29

*Significant HS at (P≤ 0.01) level of significance

Table(3): Descriptive analysis of Clinical Periodontal parameters in chronic periodontitis patients .

Variables	N	Median	Mean	S.D.	Min.	Max.
CAL	60	2.21	2.37	0.58	1.71	4.13
PP	60	3.22	3.35	0.30	3.05	4.7
BOP% (0)	60	14.45	17.84	12.21	0	72.12
BOP% (1)	60	85.56	82.17	12.21	27.89	100

Table(4):Correlation between salivary Peroxidase activity and periodontal parameters inchronic periodontitis patients .

Peroxidase		PLI	GI	CAL	PPD	BOP% (0)	BOP% (1)
activity without nano (U\L)	r	0.049	-0.055	-0.081	0.033	0.153	-0.153
	p-value	0.711 (NS)	0.676 (NS)	0.538 (NS)	0.801 (NS)	0.242 (NS)	0.242 (NS)

*Significant NS at(P>0.05) level of significance

This result is in agreement with other results (Zeyad & Mahmoud)[13]who found that salivary peroxidase increased in CP patients and its increase is not significant this result is similar to Gurven who noted that salivary peroxidase remained constant within normal range in several diseases such as patiens with insulin – dependent (type-1) diabetes mellitus [14].(Dagar et al. 2015), in agreement with this study , were found significant strong correlation between peroxidase activity and clinical parameters PLI and PD [15].(Rachna et al2014) found that salivary peroxidase activity significantly increases with inflammation and reduces after oral hygiene measures [16].In the same way(Priti & Patil 2011) found that patients with periodontal disease have demonstrated high levels of peroxidase activity in saliva [17].

While another study found that glutathione peroxidase was decreased in saliva level in patients with periodontopathy compare to control group [18].Oral peroxidase plays a role in control the level ofhydrogen peroxide secreted by bacteria and leukocytes present in theoral cavity, and has a specific antibacterial activity, inhibiting themetabolism and proliferation of various bacteria in the oral cavity. And this may be explain the increasing in its activity in saliva of chronic periodontitispatients.ZnONanoparticales solution was absorbed in a peak around(375nm) , as shown in figure (1) which characterized the UV-Vis absorption spectraof ZnO NPs solution.

Figure(1): UV – VIS spectra of the ZnO NPs solution.

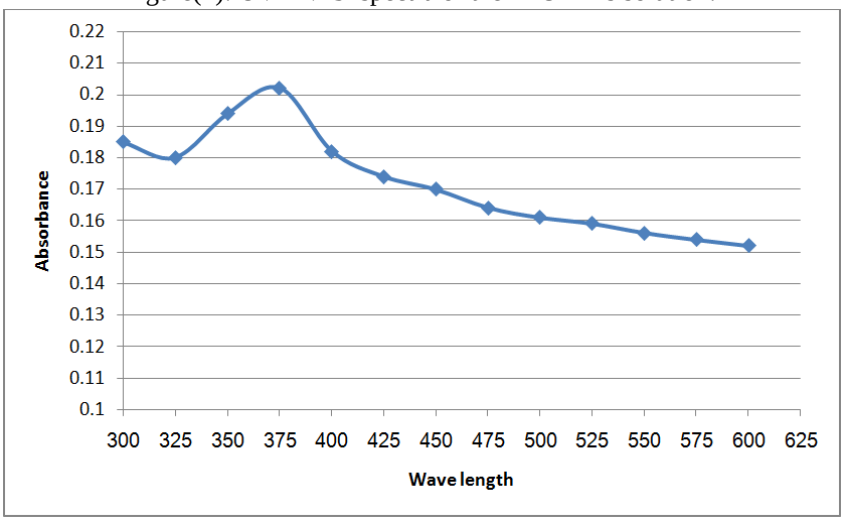
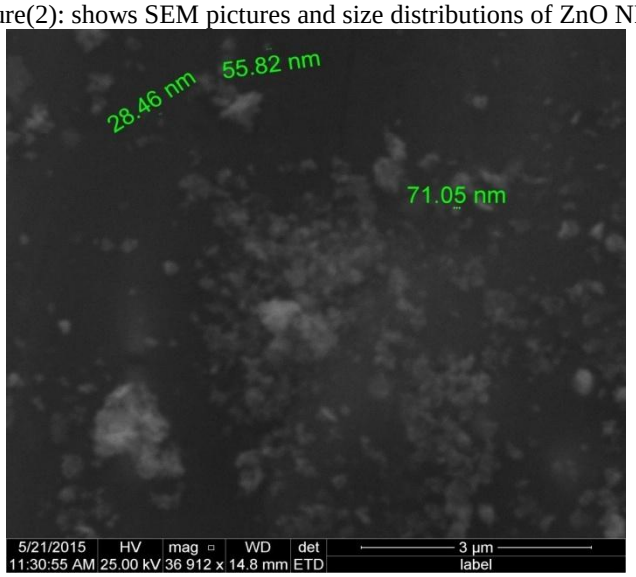
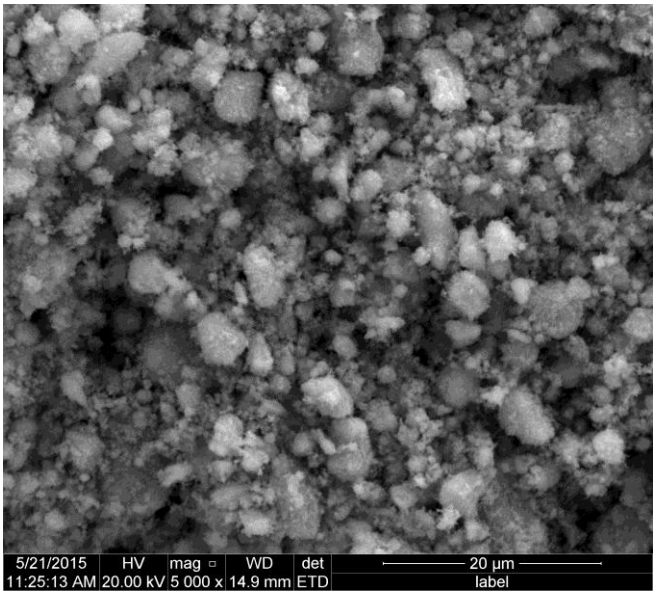


Figure (2) shows SEM pictures and size distributions of ZnO NPs using in this study . The NPs have the average diameters of <80nm

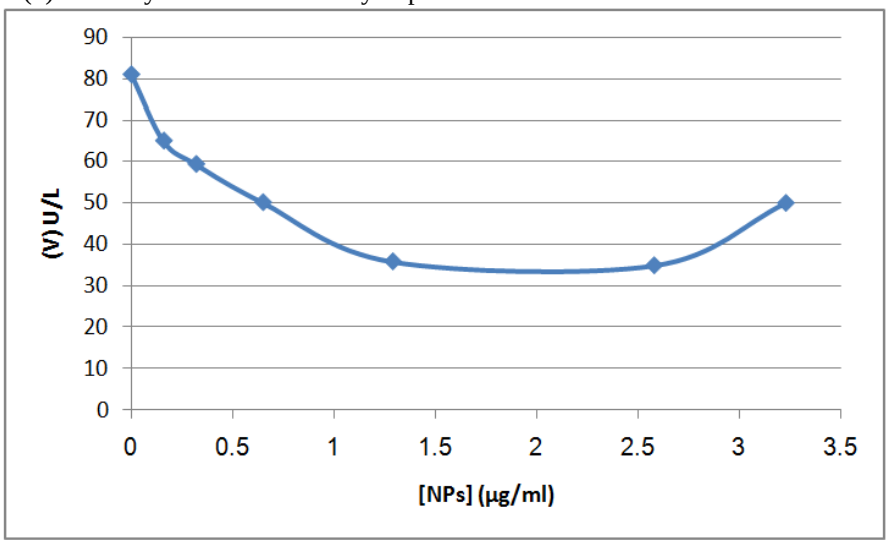
Figure(2): shows SEM pictures and size distributions of ZnO NPs .





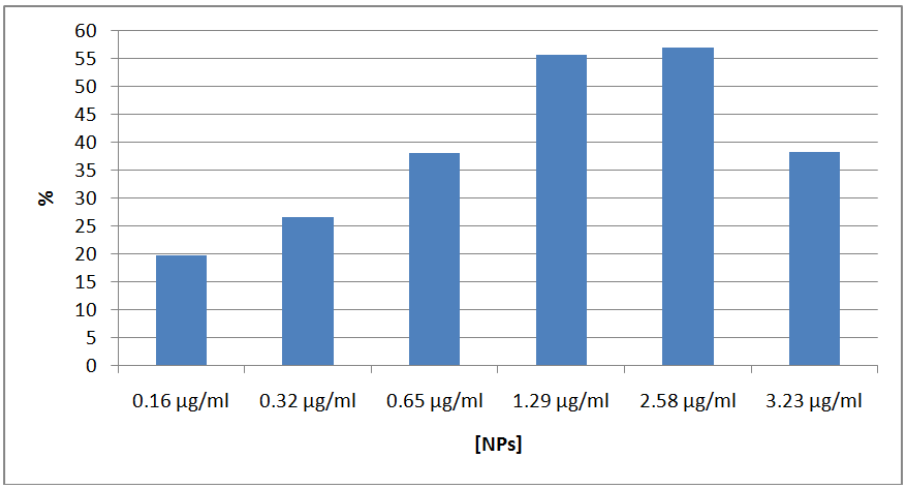
The results in figure(3) shown that ZnO NPs caused inhibition effect on salivary peroxidase activity of chronic periodintitis patients . The greater inhibition of ZnO NPs on enzyme activity was at concentration (2.58µg/ml) in total volume (3100µl) of reaction mixture.

Figure(3): Salivary Peroxidase activity in presence of different concentration of ZnO NPs.



The greater percentage of inhibition of ZnO NPs on enzyme activity was 55.25% at concentration (2.58µg/ml)as a final concentration as seen in figure(4).

Figure(4): percentage inhibition of salivary peroxidase activity in different concentrations of ZnO NPs.



Our results is in agreement to that of Abd which found significant inhibition of the activity of the total salivary peroxidase of human saliva(age between 18-22 years) in the presence ZnO NPs[19] . In a study on kinetic andphysicochemical properties of enzymaticproducts in the presence of silver nanoparticles, results showed that there is inhibition effect on LDHactivity and by florescence spectral assays, the silvernanoparticle was determined to be directly bound to LDHand induced the protein unfolding[20]. Cytotoxicity of ZnO nanoparticles is due to their increasedsolubility. High concentration of metal oxide nanoparticles inthe environment and food chain may affect human health [21]. Enzymatic peroxidationof fatty acids leads to the generation of the reactiveoxygen species [22]. The cells are affected when exposed to a higher concentrationof reactive oxygen species [23]. ZnOnanoparticles are not toxic at low concentration, but at higherconcentration increase ROS through increased MDA content[24]. In addition ,MDA content was altered significantly even at low concentration of ZnO NPs [25].

According to our results , we can suggest that the effect of ZnO NPson oxidative stress may be due to its inhibition to peroxidase activity in the cell. The descriptive statistics of total salivary peroxidase for all groups(control , salivary patients with NPs and without NPs) were shown in Table (5). The Figure (5) shows that median of peroxidase activity (U/ L) was higher in group 2 and 3 compared to group 1.

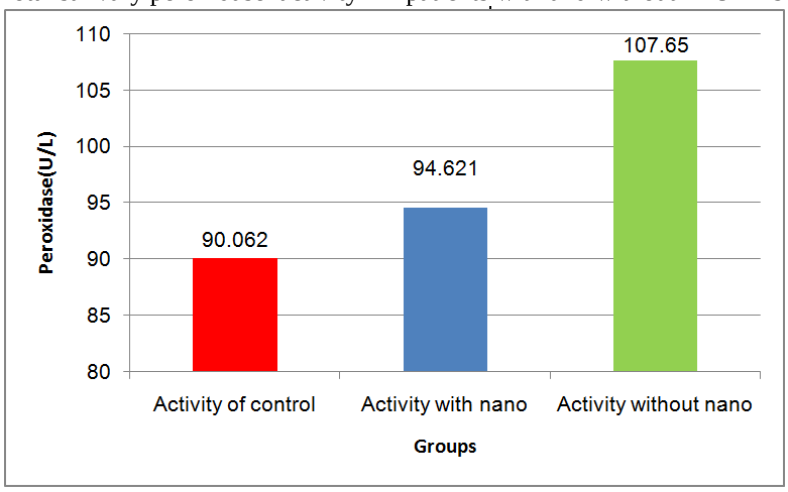
The highest mean of peroxidase activity was found in saliva of chronic periodontitis patients without ZnO NPs (107.650 U/L) and the lowest mean was in control group (90.062 U/L), while the mean of enzyme activity in saliva of chronic periodontitis patients with ZnO NPs was (94.621 U/L). The three groups showed significant(P =0.030) effect .The results in our study indicated that ZnO NPs have an inhibition effect on total salivary peroxidase activity of chronic periodontitis patients.

Table (5): The median, mean and standard deviation for Total salivary peroxidase activity in chronic periodontitis patients with and without ZnO NPs and control group**.**

Groups	Descriptive statistics						
	N	Median	Mean	S.D.	Min.	Max.	p-value
Activityof control	20	93.01	90.062	48.405	41.95	182.06	0.030 (S)
Activitywith nano	60	78.675	94.621	36.393	36.7	182.79	
Activitywithout nano	60	91.865	107.650	36.097	60.3	183.51	

*Significant S at (0.05 ≥P>0.01) level of significance

Figure(5):Mean of Total salivary peroxidase activity in patients with and without ZnO NPs and control group .



Further analysis using a Mann – Whitney U Test was doneas shown intable (6) to determine which pair of the testing groups have the significant indication .Group1 and 2 showed significant effect (p=0.039), but between group 2 and 3 there was a significant effect (P=0.011)and finally group 1,3 showed significant effect(P=0.048)**.**

Table (6):Mann – Whitney U Test among different three groups of control ,saliva of patients with and without ZnO NPs.

	Control vs. with nano	Control vs. without nano	With nano vs. without nano
Mann-Whitney U	542	461	1313.5
p-value	0.048 (S)	0.039 (S)	0.011 (S)

*Significant S at (0.05 ≥P>0.01) level of significance

From these results it is clear that ZnO NPs significantly inhibited total salivary peroxidase activity in patients with chronic perodontitis.This effect may be due to conformational changes of protein structure of the peroxidase.

ZnO NPs have been reported to modify the secondary structure of lysozyme with circular dichroism the content of α helix and β sheet has been determined in presence of ZnO NPs [26].

The interaction of lysozyme with NPs has also been described for TiO2 NPs.

Lysozyme seems to form bridges between the NPs . A changein the conformation of lysozyme was observed in the presence of TiO2 . Indeed the content of α helix decreased while the content of β sheet increased, resulting in a loss of activity [27]. Wang et al. demonstrated the inhibition of both acetylcholinesterase and butyrylcholinesterase by NPs in a dose dependant manner. Moreover this inhibition is due to the adsorption of the enzyme on the NPs [28].Some studies are conducted onthe crucial metabolism enzymes and enzyme dysfunctions, which are related to various pathologies[29,30,31].

In summary ,It is very important to understand how NPs are able to bind proteins, and how this binding can help NP internalization to cell. The NP-induced protein modifications are promising fields for future research. So, we need many studies to explain this interaction.

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