AJPS, 2014, Vol. 14, No.2

Date of acceptance: 22-9-2013

Antibacterial and Antibiofilm Activity of Zinc Oxide Nanoparticles on *Pseudomonas aeruginosa* Isolates from Diabetic Foot Infections Grade 15

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Abstract:

The aim of this study was to investigate antibacterial activities and disruption of biofilm structure by zinc oxide nanoparticles (ZnO NP). *Pseudomonas aeruginosa* was chosen as an indicator of pathogenic because its natural resistance to antibiotics and its ability to form biofilm on surfaces makes the cells impervious to therapeutic concentrations.

Twenty one isolate were taken from patients suffering from diabetic foot ulcer grads 2 infections who attended from AL-Kindy Teaching Hospital in Iraq. Different concentrations of zinc oxide (NP) had been used (25-20000) µg/ml.

The results showed that the high concentrations (500-20000) µg /ml were lethal to bacteria also the minimum inhibitory concentration (MIC) and sub (MIC) of zinc oxide np was determined in this study. Some of the isolates were inhibit in concentration 100 µg/ml and others inhibit in concentration 75 µg/ml and according to these result the sub–MIC were 75 µg/ml to some isolates and 50 µg/ml to others, these concentration were inhibitor biofilm production.

Our study indicates that zinc oxide nanoparticles could potentially be an antibacterial reagent to treat diseases caused by bacteria.

Keywords: *Zinc oxide nanoparticle (NP), biofilm distribution, pseudomonas biofilm.*

الفعالية المضادة للبكتريا ولتكوين الغشاء الحيوي لدقائق أوكسيد الزنك النانوي على بكتريا الزوائف الزنجارية المعزولة من إصابات القدم السكري من الدرجة الثانية

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الخلاصة:

الهدف من هذه الدراسة هو للتحقيق من فعالية اوكسيد الزنك النانوي وتاثيره على بكتريا *Pseudomonas aeruginosa* وعلى قابليتها لانتاج الغشاء الحيوي. تم اختيار *Pseudomonas aeruginosa* بسبب قابليتها العالية لمقاومة المضادات الحيوية وكذلك قدرتها العالية على انتاج الغشاء الحيوي.

جمعت إحدى وعشرون عزلة من مرضى قسم السكري نوع الثاني من مستشفى الكندي التعليمي في العراق، حيث استخدمت تركيزات مختلفة من أوكسيد الزنك النانوي والتي كانت (25-20000) ميكروغرام/مل.

أظهرت النتائج أن التراكيز العالية (500-20000) ميكروغرام/مل كانت قاتلة للبكتيريا وكان الحد الأدنى للتراكيز المثبطة (MIC) من أوكسيد الزنك النانوي لبعض العزلات 100 ميكروغرام/مل و 75 ميكروغرام/مل للاخرى. أما نتائج sub-MIC كانت 75 ميكروغرام/مل لبعض العزلات و 50 ميكروغرام/مل للاخرى، حيث كانت هذه التراكيز مثبطة لانتاج الغشاء الحيوي. تشير هذه الدراسة إلى أن جزيئات أوكسيد الزنك النانوي من المحتمل أن تستخدم كمضاد حيوي وكذلك تستخدم لمعالجة الأمراض التي تسببها البكتيريا.

Date of acceptance: 22-9-2013

Introduction:

Diabetic foot infections (DFIs) are a common and serious problem in diabetes patients; typically begin in a wound, most often a neuropathic ulceration. While all wounds are colonized with micro-organisms, the presence of infection is defined by≥2 classic findings of inflammation or purulence. Infections are then classified into mild (superficial and limited in size and depth), moderate (deeper or more extensive), or severe (accompanied by systemic signs or metabolic perturbations).

This classification system, along with a vascular assessment, helps determine which patients should be hospitalized, which may require special imaging procedures or surgical interventions, and which will require amputation. Most DFIs are polymicrobial, with aerobic Gram-positive cocci (GPC) and Aerobic Gram-negative bacilli are frequently copathogens in infections that are chronic or follow antibiotic treatment, and obligate anaerobes may be copa-thogens in ischemic or necrotic wounds[1]. The frequency of bacterial isolates from diabetic foot ulcers (Dfu) like; *Escherichia coli*, *Psedomonas aeruginosa* and *Klebsiellaoxytoca* and *Klebsiella pneumonia*, *Acinetobacter sp.*, *Proteus vulgaris*, *Proteus mirabilis* and *Morganellamorganii*[2].

Toole *et al.* who observed that, the bacteria are not free floating but grow upon submerged surface. The basic architecture of biofilms show that the microcolony is actually the basic structural unit of the biofilm[3]. Biofilms are defined as multicellular aggregates of sessile cells that are irreversibly attached to a substratum or interface or to each other, encased in a self-produced extracellular matrix of polysaccharides, proteins and nucleic acids and exhibit an altered phenotype in terms of growth rate and gene expression as compared with planktonic bacteria [4].

The formation of biofilms contributes to the high resistance of *Pseudomonas aeruginosa*to antibiotics making the treatment of biofilm infections more difficult. In addition, bacteria in biofilm were demonstrated to show elevated resistance to the host immune system clearance[5,6]. Various factors including defects in host defense mechanisms are responsible for this increase in infection rates.

Wound infection is known toimpair wound healing in both acute and chronic DFUs. Although the numbers and type of bacteria in a wound are critical for infection to occur; recently a new concept of bacterial biofilms has emerged as a potential way to better understand how bacteria deter healing. Changing the perspective chronic infection disease to include biofilm enables two important insights, first, it opens new methods for detection and treatment, and second, it provides a global reconceptualization of many chronic infections disease as resulting form a biofilm, allowing biofilm principles to be shared across disciplines. Recent studies have investigated new methods for detecting the components of a biofilm [7]. Nano-ZnO has known to have strong inhibitory and antibacterial effects as well as a broad spectrum of anti-microbial activities. The availability of a wide range of nano structures makes ZnO an ideal material for nanoscaleopto-electronics and piezoelectric nano-generators as well as an efficient material for biotechnology. Furthermore, ZnO appears to be strongly resisted to microorganisms, and nano-ZnO are now widely used as antibacterial[8].This study aimed to Determination the efficacy of ZnO-NP in disinfecting and disrupting biofilm , determine the MIC and sub-MIC to the ZnO-NP.

Date of acceptance: 22-9-2013

Materials and Methods:

Microorganisms:

Pseudomonas aeruginosa isolated from Diabetic foot ulcer grads 2 infections who attended from center for Endocrinology and Diabetes in AL- Kindy Teaching Hospital (the diagnosis done by vitek 2 system). The samples obtained as clinical swabs were collected according to standard roles. The sample obtained during a period between November 2012 to January 2013.

Minimum Inhibitory Concentration (MIC):

Different concentrations of zinc oxide np had been used (25-20000) µg/ml to determine the minimum inhibitory concentration (MIC). Zinc oxide np will take from Nanobeast (Poland) in size 40 nm and used as solutions, this solution was prepared by dissolving 2 gram of zinc oxideNP in100 ml of sterilized D.W. The minimum inhibitory concentration (MIC) were determined by a method recommended in[9], with some modifications. Briefly, the sterile tubes were incubated aerobically in Shaker incubator at 37°C for 24 h, which contained 5 ml Trypton soy broth (TSB) and glucose 1% with approximate 5×10^8CFU bacterial cells and 0 µg/ml (the control group), (25, 50, 75, 100, 500,1000,5000,10000 and 20000) µg/ml of zinc oxide nanoparticles. Then tested this tubes by using spectrophotometer and after that grow the isolate on nutrient agar to found the viable cell, the concentration of tube without visible growth of the bacterial cells was the MIC[10].

Biofilm Assay-Tissue Culture Plate (TCP):

The biofilm assay described by Mathur*et al.* with some modifications: Stated briefly, 10 ml of trypticase soy broth (TSB) with 1% glucose was inoculated with a loopful of test organism from overnight culture on nutrient agar[11]. The flat bottom tissue culture plates (96 wells) were filled with 200µl of diluted cultures individually. uninoculated sterile broth served as blank. Similarly, control organisms were also diluted and incubated. The culture plates were incubated at 37˚C for 24 hours. After incubation, gentle tapping of the plates was done. The wells were washed with 200 µl of Normal saline four times to remove free-floating bacteria. Biofilms which remained adherent to the walls and the bottoms of the wells stained with 0.1% crystal violet for 10 min.

Excess stain was washed with Normal saline and plates were dried properly then adding 200 µl of the destaining solution (95% ethanol) for 10 min. Finally, 200µl from each well was transferred to a new microtiter plates and measured at 570 nm by microplate reader. The biofilm degree was calculated as follows:Biofilm degree=Mean $OD_{570}$of tested bacteria- Mean OD_{570} of control.

The sub minimum inhibitory concentration (Sub MIC):

Zinc oxide NP effects on biofilm by values of opticaldensity (OD) at 570 nm, the sterile Microtiter plate contained 200 µl of (TSB with glucose 1% contained approximate 5×10^8 CFU bacterial cells and 0 (the control group), (75-50) µg/ml ZnOnp in each wells, this microtiter plate was prepared as in (biofilm assay), and were incubated aerobically at 37°C for 24 h. Then, the microtiter plate were measured the values of OD at 570 nm and bacterial populations. Values of OD at 570 nm were determined using ELISA Reader[12].

Results and Discussion:

Bactericidal Effect of zinc oxide NP:

The result of this study showed that the concentrations (500,1000, 5000, 10000, 20000) µg/ml were lethal to *pseudomonas aeruginosa*, while (100 and 75) µg/ml were inhibitors and the concentrations (50,25) µg/ml were not effective (Figure-1). Concentration and size are two

Date of acceptance: 22-9-2013

important factors affecting antimicrobial properties of ZnO NP[13,14].

A wide variety of synthetic compounds exert antibacterial effect, but just some of them can be used as biocides to develop drugs or coatings. Several antimicrobial mechanisms of zinc oxide were supposed; hydrogen peroxide, which is generated from the surface of zinc oxide, can penetrate through the cell membrane, produce some type of injury, and inhibit the growth of the cells[13,15,16,17]. The affinity between zinc oxide and bacterial cells is an important factor for antibacterial activity [18] of using these inorganic oxides as antimicrobial agents is that they contain environmentally safe mineral elements essential to humans and exhibit strong activity even when administered in small amount[19,20,21,22].

Psedomona saeruginosa: The antimicrobial ability of nano-ZnO might be referred to their small size which is 250 times smaller than a bacterium. This makes them easier to adhere with the cell wall of the microorganisms causing its destruction andleads to the death of the cell; also, metal nano-particles are harmful to bacteria and fungi [22]. They bind closely to the surface of microorganisms causing visible damage to the cells, and demonstrating good self assemblingability. Nano-ZnO posses well-developed surface chemistry, chemical stability which makes them easier to interact with the microorganisms. Also, the particles interact with the building elements of the outer membrane and might cause structural changes, degradation and finally cell death. ZnO, which cause fatal damage to microorganisms [8].

Effect of zinc oxide np on biofilm formation : the result show that the sub-MIC of ZnO-np was 75 µg/ml for isolates P3, P18, P6, P10, P9, P14, P12, P13, P15 and P11. While they were 50 µg/ml for this isolates P1, P5, P8, P7, P16, P19, P21, P20 and P17. This study demonstrated that ZnO-np possessed significant antibiofilm properties and were able to disrupt the multilayered, 3-dimensional biofilm architecture.

This study demonstrated that ZnO-np possessed significant antibiofilm properties and were able to disrupt the multilayered, 3-dimensional biofilm architecture. Here we take 19 isolate not 21 because some of them not biofilm producer (P2 and P4 not producer). ZnO-np when compared with the biofilm bacteria. Direct contact-dependent inhibition of planktonic bacteria might be the main killing mechanism by these nano-particulates, whereas resistance to penetration of the nanoparticulates as a result of negatively charged biofilm EPM could be the cause of higher concentrations and a longer duration of contact required for elimination of biofilm bacteria.In addition, the EPM might also serve as a chemical barrier by adsorbing the harmful ROS from reaching the cell surface, thereby decreasing the effect of ROS. The ROS production by ZnO-np, which was able to diffuse into the biofilm structure. The presence of moist or aqueous environment of the biofilm might augment the production of ROS by ZnO-np. The bacterial biofilm structures demonstrated antimicrobial resistance even with higher concentrations of antimicrobials [25].

Bacteria are permanently in contact with reactive oxygen species (ROS), both over the course of their life cycle as well that present in their environment. These species cause damage to proteins, lipids, and nucleotides, negatively impacting the organism. To detect these ROS molecules and to stimulate the expression of proteins involved in antioxidative stress response, bacteria use a number of different protein-based regulatory and sensory systems ROS-based stress detection, mechanisms induce posttranslational modificati-ons, resulting in overall conformational and structural changes within sensory proteins. The subsequent structural rearrangements result in changes of protein activity, which

Date of acceptance: 22-9-2013

lead to regulated and appropriate response on the transcriptional level. Many bacterial enzymes and regulatory proteins possess a conserved signature, the zinc- containing redox centre Cys-X-X-Cys in which a disulfide bridge is formed upon oxidative stress. Other metal - dependent oxidative modifications amino acid side-chains (dityrosines, 2-oxo-histidines, or carbonylation) also modulate the activity of redox-sensitive proteins[26]. In summary, the present study highlighted the efficacy of ZnO-np to reduce biofilm bacteria and disrupt biofilm structure as in Fig. (2), Table (3).

Table-1: Minimum inhibitory concentration of zinc oxide np on the bacterial *Pseudomonas aeuroginosa* 100 &75(µg/ml).

Isolate type	MIC (µg/ml)	Isolate type	MIC (µg/ml)
P1	75	P12	100
P2	75	P13	100
P3	100	P14	100
P4	75	P15	100
P5	75	P16	75
P6	100	P17	75
P7	75	P18	100
P8	75	P19	75
P9	100	P20	75
P10	100	P21	75
P11	100		

Table-2: Biofilm reader with and without zinc oxide np.

Isolate type	Without Zno-np	With Zno-np	Isolate type	Without Zno-np	With Zno-np
P1	0.157	0.0216	P14	0.166667	0.0063
P3	0.594367	0.0225	P15	0.273	0.0065
P5	0.392667	0.0235	P16	0.47	0.0065
P6	1.133	0.0248	P17	0.225333	0.0066
P7	0.952	0.0262	P18	0.691	0.007
P8	0.327	0.0275	P19	0.692367	0.0072
P9	0.622	0.0057	P20	0.2217	0.009
P10	0.752	0.0058	P21	0.868667	0.0125
P11	0.267133	0.006	P14	0.166667	0.0063
P12	0.276233	0.0058	P15	0.273	0.0065
P13	0.357333	0.0062			

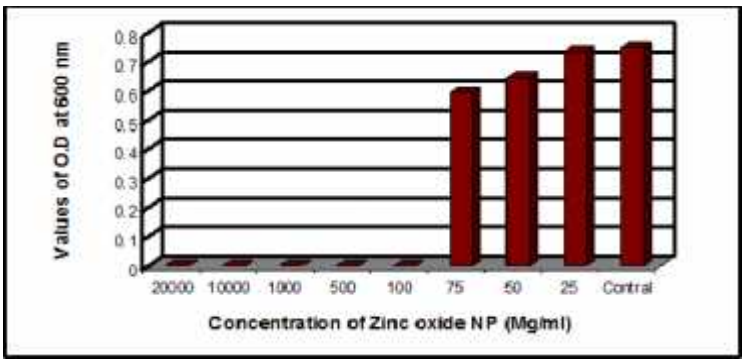

Figure-1: Effects of zinc oxide nanoparticles on values of OD at 600 nm of *Pseudomonas aeuroginosa* (P18).

Date of acceptance: 22-9-2013

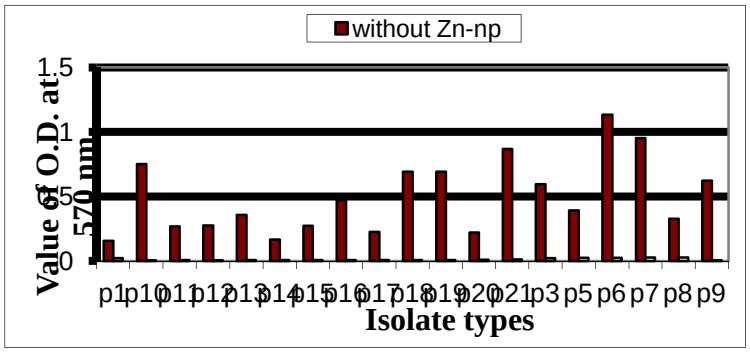

Figure-2: Biofilm after and before treatment with zinc oxide np.

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Date of acceptance: 22-9-2013

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