Effects of ZnO NPS on *Streptococcus pyogenes* in vivo

Luma S Mohammed[1] and Mais E Ahmed[1]*

1. Department of Biology, College of Science, University of Baghdad, Iraq

*Corresponding author: mais.e.mahmood@gmail.com (Ahmed)

Abstract:
In vivo study revealed that ZnO nanoparticles treatment of *Streptococcus SPP* contaminated injured skin showed good prognosis and good healing process include complete regeneration of the epithelial cells of the epidermis and increase of cellulartiy of the dermal content compared with untreated group. In conclusion, treatment of *S. pyogenes* infected skin with Zinc oxide nanoparticles concentration (2 mg/ml) limit the skin damage and localized the lesion to the incision site with good healing process.

Key words: Biofilm, Silver nanoparticle, Streptococcus.

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Introduction:

Streptococcus mutans has dental caries with helping some host factors gave evidence that this species related with teeth infection. There is an accumulation of dental plaque on the surfaces of teeth. Secondary caries occurs under the restorations is increasing the risk of dental plaque. Increase of plaques on resin is related to the surface example roughness of surface, which is depended to filler size, resin type *in vitro* resins to increase number of bacteria or plaques (1). *S. pneumoniae* (pneumococcus) shown that colonize the as biofilms (nasopharynx) and we have phenotype in vitro using nasopharyngeal environment. In the study biofilms formed on human keratinocytes in vitro we demonstrated that GAS colonize the oropharynx in vivo significantly (2). Dental plaque is causes periodontal diseases such as (gingivitis, periodontitis and dental caries) a biofilm in the human body. Many researches inhibit the formation of dental biofilms and to remove mature biofilm. Te major (inhibit or remove dental plaque) is susceptibility to irritation in oral mucosa (3) Most serotype 2 (SS2) of *Streptococcus suis* clinical isolates can form biofilm, which contribute to persistent infection, transmission and difficulties to eradicate infection (4). Nanotechnology is defined as the study of the materials which it's size lies in the nanometer scale, nanotechnology contain utilization the materials with their components such: fibers, particles, grains which has dimensions less than 100 nm(5) Nano zinc oxide (ZnO NPs) is one of the most important nanoparticles of metal oxide, it is a unique and inorganic material, appears in a white powder is not soluble in water, with an energy gap of 3.37 electron volts at room temperature(6) It has been widely used in wide range applications in different areas such as in industry (including: industry of rubber, concrete production (7) and in biological applications as (anti-bacterial, anti-inflammatory.etc. (8).

Material and methods:

Indicator isolates:

These isolate were obtained from College of Science for Women/ Department of Biology (microbiology lab) /University of Baghdad and conference identification according to (9) were.

Preparation of Zinc Oxide Nanoparticles (ZnO NPs):

The used preparation procedure was described by Koutu, V. et al [10] but with the insert of modifications. Zinc acetate dehydrate and Sodium hydroxide were purchased. ZnO NPs were synthesized by precipitation method using zinc acetate dehydrate (as a source of zinc) and sodium hydroxide used as precursors and deionized water that used to dilution. 0.1 mole of zinc acetate dihydrate Zn $(CH_3CO_2)_2.2H_2O$was taken and dissolved in 100 ml of deionized water with stirring using a magnetic bar stirrer for the purpose of completely dissolving zinc acetate dehydrate, forming transparent solution. After making sure that the zinc acetate dehydrate are completely dissolved, added sodium hydroxide NaOH gradually with stirring at different quantities for the purpose of changing the pH value of the material to be prepared (using PH meter to measure the pH required), here is a white solution formed. Left it on the magnetic stirrer for 30 minutes at 75 °C, then removed the solution from stirrer. The solution washed and filtered five times with deionized water with a process called Washing and Filtering, the white precipitate is formed. and then dried in the electrical furnace at 100 ° C, the white precipitate separated into a part at calcined at 500 ° C for 3 hours, and part of without calcined. The resulting material was grinding by a mortar to obtain final product (ZnO nano in powder shape), as shown in fig (1).

Figure. 1: **ZnO nanoparticles powder.**

Characterization techniques:

ZnO NPs synthesized were characterized by:

- UV-vis Spectroscopy (Shimadzu, UV-1601PC).
- Transmission Electron Microscopy (TEM) (Philips, CM10).
- Atomic force microscope (AFM) (Angstrom Advanced Inc. , AA2000, Contact mode).

Activity of ZnO NPs in vitro:

After pouring the MHA (Muller Hinton Agar) into a petri dish, a small swab of *Streptococcus* Spps sample was taken and distributed it evenly on the surface of MHA, using a special sterile tool called cork borer with diameter 5 mm, wells were done on the MHA surface in petri dish. After that, several prepared concentrations of ZnO NPs were taken (0.5, 1 and 2) mg/ml that dissolved with sterile deionized water by using an ultrasonic bath, in addition to Deionized water as control were poured into wells by 0.1 ml (before pouring, the concentrations put in the vortex mixer in order to homogenize it). Finally, the dishes were inserted into the incubator for 72 hrs at a temperature of 37 ° C and then the growth inhibitory zone was examined if it was formed or not. When the growth inhibition zone was formed, then diameter of inhibition measured by the ruler in millimeters [11].

Experimental design:

Twenty mice were divided into 4 groups, the mice in the 1^{st}, $2^{nd}$and 3^{rd}groups (n=15 for each group) were anesthetized with an intraperitoneal injection of a mixture of xylazine (5 mg/kg) and ketamine (75 mg/kg), then the hair of the right flank was shaved (3×2 cm) using electrical shaver and the remaining hair was shaved using disposable hand shaver. The shaved area was cleaned by soap and sterile D.W., after drying skin wound was induced using sterile lancet in which 3 parallel line of superficial skin wound was made. The 4^{th} group (n=5) considered as control negative group.The mice in the 1^{st} group were considered as positive control group and the injured skin did not receive any treatment, while the injured skin of mice in the 2^{nd} and 3^{rd} group was contaminated by *Streptococcus spp* using one drop from the bacterial suspension 1×10^{6} cfu/ml (12). The injured skin of mice in the 3^{rd} group was treated locally with ZnO NPs concentration ZnO at (2 mg/ml) by swab after 2 hr post infection and treatment repeated every 12 hr.

Histopathological Study:

All mice were euthanized after 72 hr post infection and samples (1×2 cm) of injured skin were taken and fixed immediately in 10% formalin solution for 48 hrs, then the samples were processed routinely and sectioned by microtome (thickness 4-6 micron) and the slides stained by Hematoxyline and Eosin stain (12).

Result and Discussion:

Isolation and identification of *Streptococcus spp* : a species of Gram-positive bacteria, these bacteria are aero tolerant and made up of non-motile and non-sporing cocci.. Group A streptococci when grown on blood agar (Figure1) typically produces small zones of beta-hemolysis, a complete destruction of red blood cells. (A zone size of 2–3 mm is typical.) It is thus also called group A (beta-hemolytic) streptococcus (13).

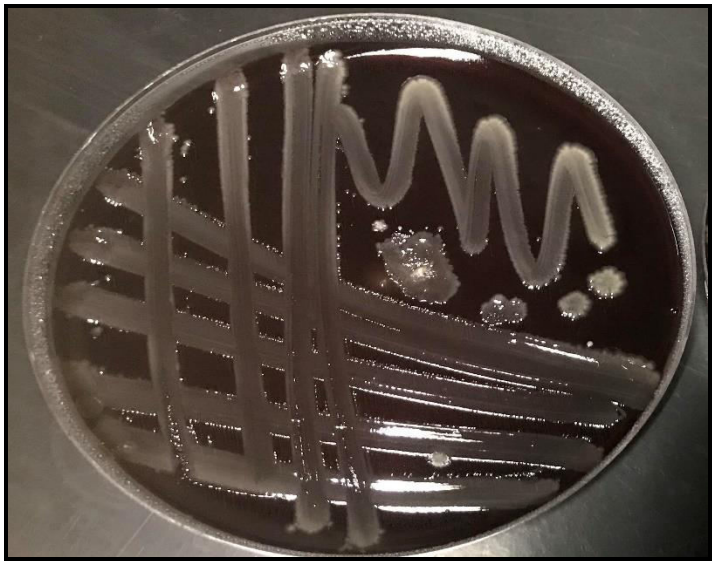

Figure (1): *Streptococcus spp* on Blood agar at 37°C for 24 hrs

Figure 2 showed indicates to the optical absorption of ZnO NPs in range from 0 - 1000 nm at room temperature. It shows no other strong peak was observed in the UV spectrum of ZnO [**14**].

The sharp peak absorption at 420 nm in absorption spectrum of ZnO NPs (at PH= 14) . This result almost compatible with Ghorbani, H. et al which found that the peak absorption of ZnO NPs) was 372 nm [15]

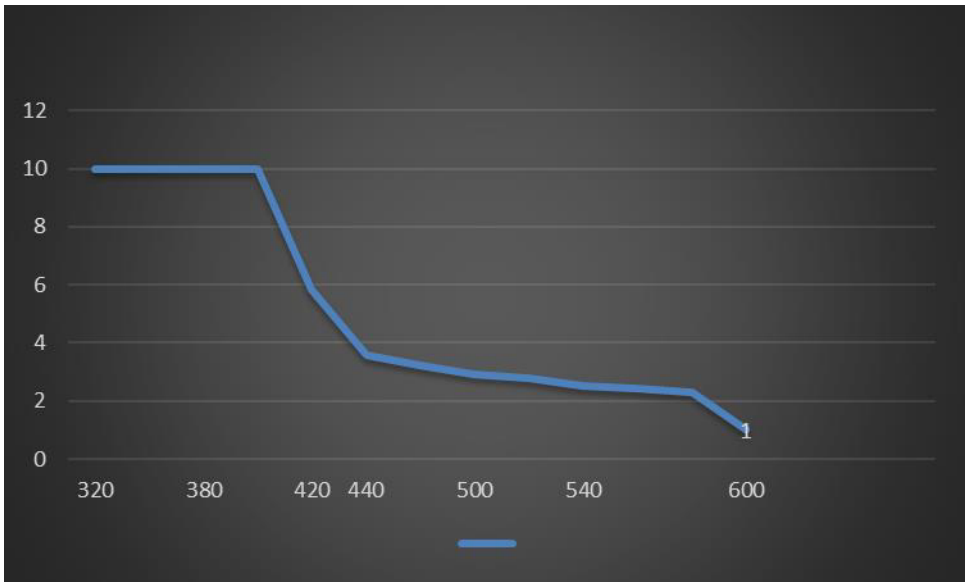

Figure.2 : UV-visible absorption spectra of ZnO nanoparticles synthesized

Transmission Electron Microscopy analysis (TEM) of silver nanoparticles:

Figure **3** indicates the transmission electron microscopy of ZnO NPs. We observe from TEM the morphological characterization of ZnO NPs, the shape is spherical and the sizes are 30 nm. This result is match with Bagheri, S. et al. which found the ZnO NPs after heat treatment (500 ˚C for 3 hours) spherical shape [16].

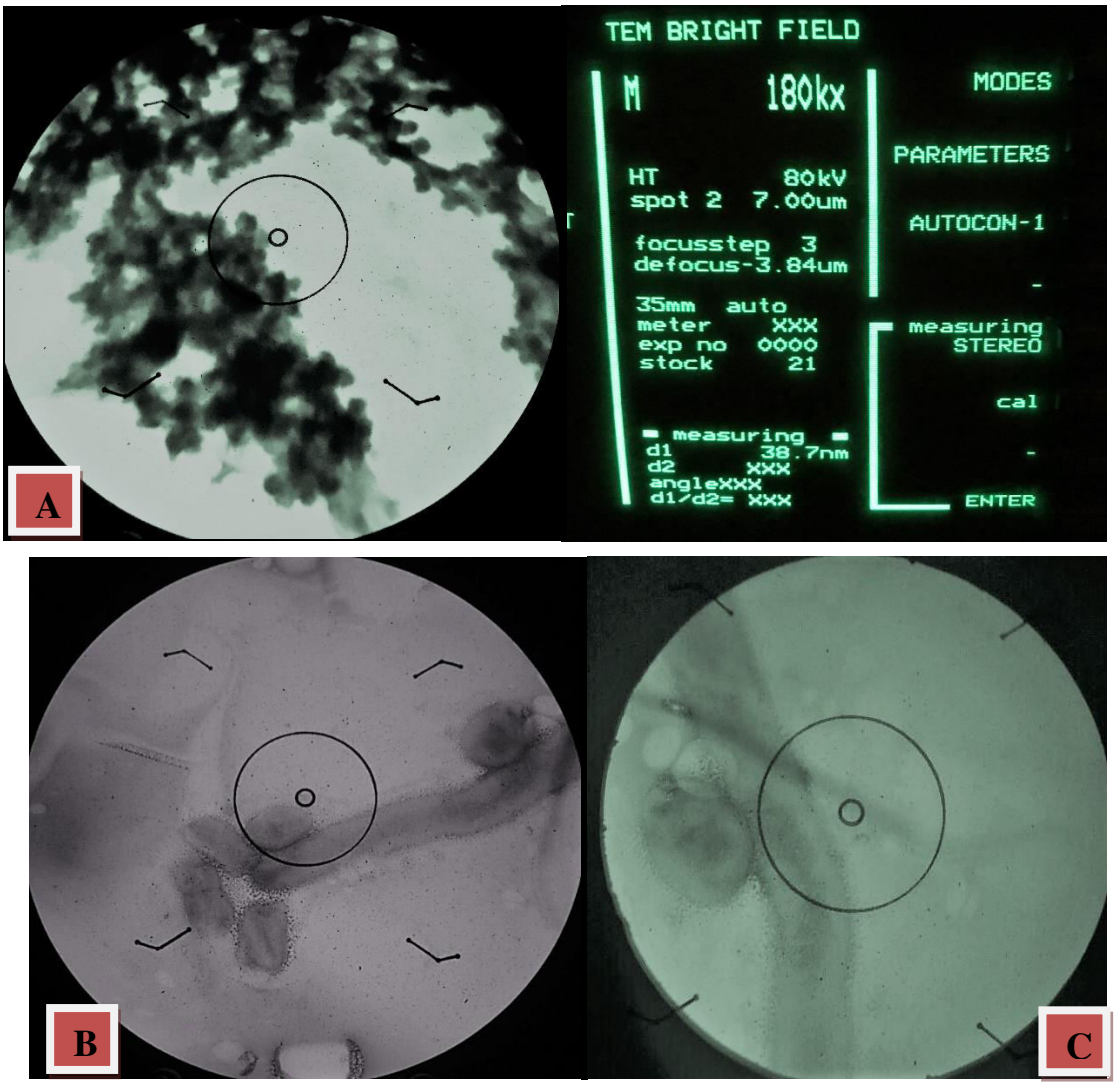

Figure 3 : TEM of A) ZnO NPs under electron microscopes B) *Streptococcus* Spps before treatment Zinc oxide nanoparticles C) *Streptococcus* Spps after treatment Zinc oxide nanoparticles

Result for ZnO NPs (without calcined) almost agree with Barve, A. K. et al. which found the ZnO NPs are spherical shape and the average size approximately 20-25 nm [17].

Atomic Force Electron Microscopy (AFM)

Figure 4 indicates to the atomic force microscopy of ZnO NPs. It shows the sizes equal to 42 nm, and the average diameter is 42 nm for calcined. This result doesn't match with Al-Taie, A. S. et al which found the average particle size 125.77 nm (calcined at 500 °C for 3 hours) [18].

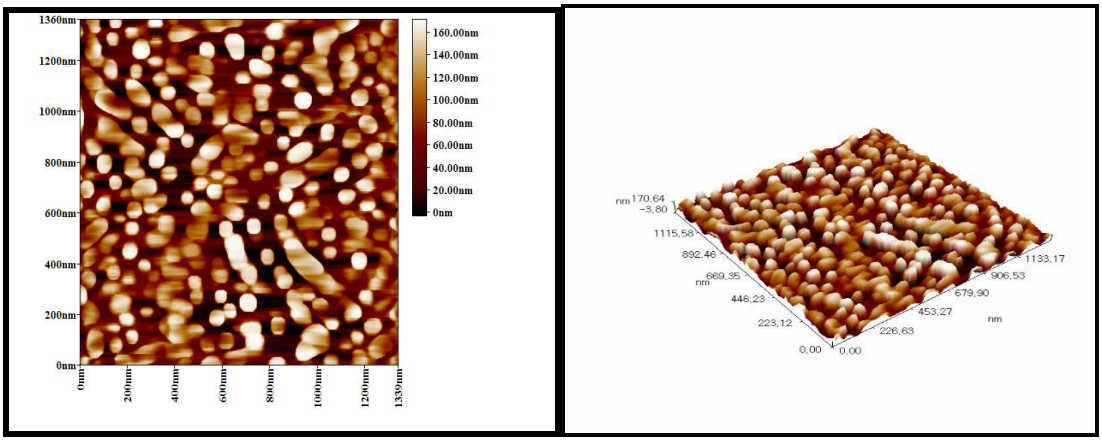

Figure (4) AFM for ZnO NPs

The highly diameter of inhibition zone reached 11mm at concentration (2 mg/ml) compare with other con. Figure (5) can clearly be seen in the TEM picture with external spherical shape and are to a large extent well-separated from one another and the average diameter of the particles is indicated to be 42 nm should be pointed out that each method examines somewhat different aspects of the particle size.

Figure (5):: MIC for ZnO NPs at different concentration on (MHA) at 37°C for 24 hr. Inhibition zone of *S. spp*= 11 mm at concentration 0.5, 1 and 2 mg/ml at 37 ° C for 242 hr.

Histological study

The histopathological changes in group 1 (positave control) showed incomplete regeneration of the epidermal layer in the incision site under cellular debries and neutophils aggrigation (Fig.6A), also the mild neutrophils infilteratin in the dermis and subcutanous tissue (Fig.5B).

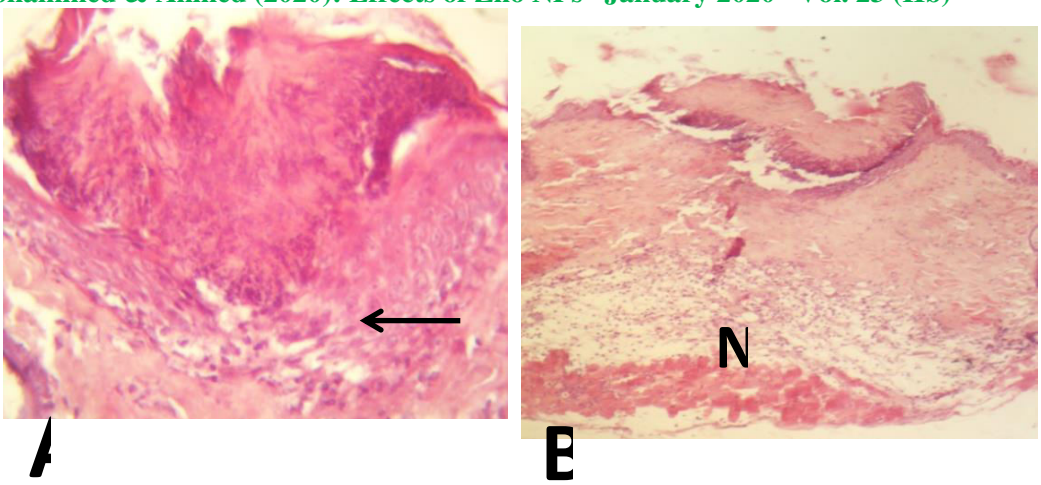

Figure 6: Histopathological section in the skin of group 1 showed (H & E stain): (a) incomplete regeneration of the epidermal (arrow) layer in the incision site under cellular debries and neutophils aggrigation (×400); (b) infilteration of nefutrophils (N) in subcutanous tissue (×100).

The histopathological vary in group 2 (infected grouop) showed severe necrosis and cellular debries in the incision site (Fig.7A) the necrosis extend form epiderms to the dermis with severe neutrophils infilteration in the skin (Fig.7B) and subcutanous tissue. Other section showed abcess in the subcutanous tissue.

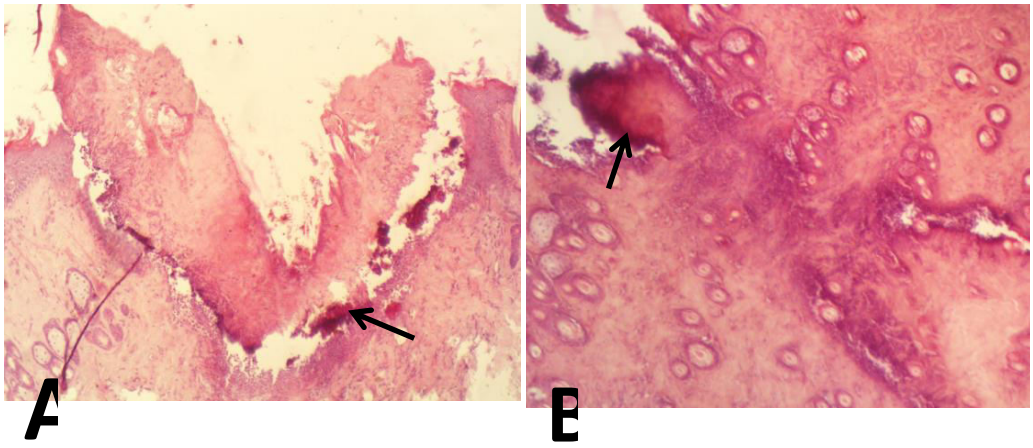

Figure 7: Histopathological section in the skin of group 2 showed (H & E stain; ×200): (a) severe necrosis (arrow) and cellular debries in the incision site; (b) necrosis (arrow) and infilteration of neutrophils in the dermis.

The 3rd group (treated group) showed complete regeneration of the epithelial cell of the epidermis with mild degeneration and increase cellulartiy of the connective tissue of the derims (Fig.5A), other section showed mild hyperplasia of epithelial cells of the epidermis (Fig.5B).

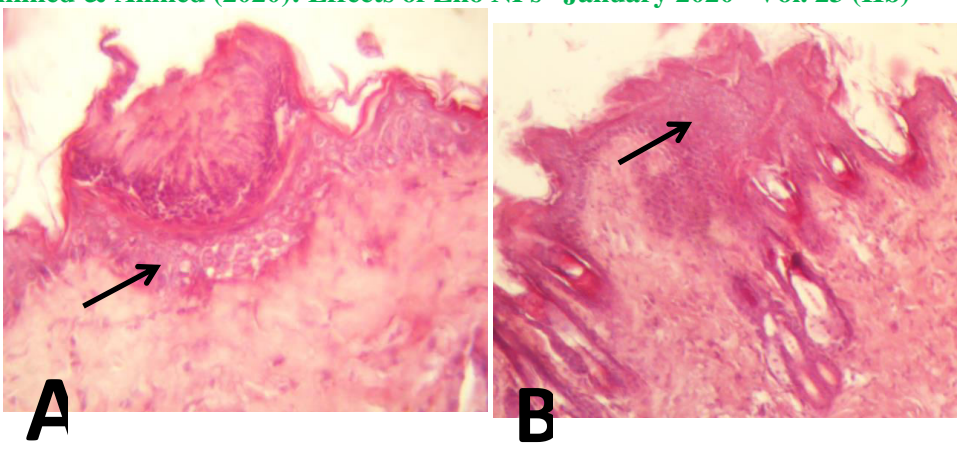

Figure 5: Histopathological section in the skin of group 3 showed (H & E stain; ×200): (a) complete regeneration of the epidermal layer in the incision site (arrow) under cellular debries and neutophils aggrigation and increase cellulartiy of the dermis layer; (b) mild hyperplasia of epithelial cells of the epidermis

In vivo study showed that the histological changes in the skin of mice of the first group distinguished by infiltration of inflammatory cells (neutrophils) during 48 hr of injury and this revealed the first phase of normally wound healing, in addition to fibrin plug and fibrous connective tissue infiltration in the dermis and these responses was a part of the normal skin healing in immune component animal (19). *S. pyogenes* cause infection of skin and soft tissues with diverse clinical entities (20), so histopathological changes in the skin of mice in the second group showed severe damage in epidermis, dermis and subcutaneous tissue and this may be contributed to the exotoxine streptolysin O (SLO) which is produced by all strains of *S. pyogenes* and it is toxic to many cell types such as leukocytes, endothelial cells and fibroblasts (21), in addition to hyaluronidase which degrades hyaluronic acid found in the ground substance of connective tissue for nutritional purposes (22) and facilitate the spread of the bacteria. In the 3rd group, treated the infected skin using ZnO NPs lead to good healing process compared with the infected group (G2) and this may be due that silver nanoparticles does not seem to negatively affect the proliferation of fibroblasts and keratinocytes, leading to the restoration of normal skin since ZnO NPs have interesting effects in wound healing (23). As ZnO nanoparticles are used increasingly in various commercial products as well as in biological and medical applications, it is more important than ever to study their possible toxicological effects on humans. The result agrees with Lin, W. et al [(24, also agrees with Wang, B. et al [25)

Conclusion:

In conclusion, treatment of *Streptococcus spp* infected skin with silver nanoparticles concentration (2 mg/ml) limit the skin damage and localized the lesion to the incision site with good healing process. The present study has shown the antibacterial effect of ZnO nanoparticles on *Streptococcus spp* so reduced the bacterial load of wounds. Based on present findings, local application of ZnO nanoparticles may help wound healing processing due to the reduction of bacterial load

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