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STUDY OF THE EFFICIENCY OF ZINC OXIDE NANOPARTICLES ON WOUND HEALING IN GOATS SUFFERING FROM EXPERIMENTALLY INDUCED ZINC DEFICIENCY

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Abstract

Wound healing is a complex procedure, involving a combination of activities of different tissues and cell lineages. The current study was conducted to find the efficiency of zinc oxide nanoparticles (ZnO NPs) on experimentally induced cutaneous wound healing in goats suffering from experimental zinc deficiency. Fifteen local Iraqi breed goats, 5-6 months age and 15.52 ±1.05 kg. Body weight was used. When goats exhibit the signs of Zn deficiency, which induced through increasing the levels of calcium and phosphorus (supplied with ground limestone and calcium di-phosphate for weeks), they were divided randomly into three groups, each of 5 animals. Those of group I (G I) control group were treated by combination of water and glycerin only, while those of group II (G II) were treated by 25mg/kg ZnO NPs, meanwhile animals of group III (G III) were treated by 75mg/kg ZnO NPs once/weekly for to times. Cutaneous wounds were induced surgically under the aseptic condition with local anaesthetization by catting two-circular full-thickness areas from the skin of (3.5-4.0 cm) on the dorsal surface of the back (one area on each side). The diameter of the wounds was measured weekly. Biopsy samples were obtained from edges of the center of wounds 3rd, 7th, 14th and 28th days from the starting of operation. The diameters were at the narrowest levels and were statically significant at the 5th week in comparison with 0-time (time of downing the operation). The diameters of wounds in these treated groups (II, III) were narrower than those in the control group (I). The healing of wounds was better in those of G III on dependence clinical and histological examination follows by these in group II. In conclusion, the ZnO NPs exhibit a significant role in wound healing induced in goats suffering from zinc deficiency.

Key words: Zinc oxide Nanoparticles, healing of wounds, zinc deficiency, goats.

Introduction

In recent years, increasingly engineered Nanoparticles have increasingly been the focus of attention and intense research in various fields of science (Feng *et al.,* 2009). Zinc oxide nanoparticles (ZnO-NPs) that are using in various applications of Veterinary Sciences due to their antibacterial, antineoplastic, wound healing and angiogenic, tissue repair properties and as a food preservative and feed additive. Compared with ordinary ZnO powder, ZnO-NPs have a large specific surface area and small size effect and show wide application potential in microbial inhibition and mold removal (Li. *et al.,* 2012, Cha *et al.,* 2015).

A wound is defined as damage or disruption to the anatomical structure and function of tissues (Robson *et al.,* 2001). Wound healing is a physiological response to injury that is essential across all tissue systems (Nussbaum *et al.,* 2018). It is a dynamic and highly regulated process consisting of cellular, humoral and molecular mechanisms (Reinke and Sorg., 2012). It is an complex process divided into a series of phases including: first phase, is coagulating fibrin clot formation, second phase, is inflammatory response, third phase, including cell proliferation, re-epithelialization, granulation and angiogenesis and in the four phases, matrix remodeling and scar formation (Pei-Hui. *et al.,* 2017) For this efficient and highly controlled repair process to take place, many cells signaling events are required. While the cytokines are key in initiating, sustaining and regulating the post-injury response, these same molecules have been implicated in impaired wound healing, abnormal scar formation and uncontrolled inflammatory response (Pei-Hui, 1997, Branton and Kopp., 1999).

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Drug delivery with ZnO-NPs technology has received great attention for the treatment of wounds due to their effective cell penetration, immunomodulation and antimicrobial capacity (Lansdown *et al.,* 2007, Xiong., 2013, Oyarzun-Ampuero *et al.,* 2015).

The role zinc plays in wound healing can be observed from two sides: first, the impact of zinc deficiency and second, the effect of zinc supplementation (topical/local or systemic) on wound repair (Lansdown *et al.,* 2007, Kogan *et al.,* 2017).

So the current study was conducted to investigate the role of ZnO-NPs on healing skin wounds in goats suffering from experimental zinc deficiency.

Material and Methods

Preparation of animals: Fifteen local Iraqi breed goats, of 5-6 months old and 15.52±1.05 kg. bodyweight was used to conduct the current study on the farm of the College of Veterinary Medicine, University of Diyala. All animals were free from internal parasites, in addition to normal liver and urinary system function. The dependent parameters were a clinical examination of mucous membranes, appetite, behaviors and body condition according to (Constable *et al.,* 2017). The gross examination of skin wounds was done for, to assessment process of wounds healing and detect any abnormal changes or secondary pyogenic infection of wounds.

Zn deficiency was induced by increasing the calcium and phosphorus level in the ration as ground limestone and calcium di-phosphate for 10 weeks (Ibrahim *et al.,* 2016). Blood samples were collected from the jugular vein according to (Pugh., 2002), 5ml in test tubes without anticoagulant to obtain serum. The level of serum Zinc was determined as described by (Fuwa *et al.,* 1964), using the commercial kit for colorimetric determination of zinc in serum (LTA s.r.i, 20060, Bussero (Milan) Italy.

The protocol of treatment: Equal volumes of water and Glycerol were mixed, then zinc oxide Nanoparticles were added to the water and Glycerol mixture at suitable concentrations according to (Wang *et al.,* 2018). After the appearance of clinical signs of zinc deficiency on goats, the animals wear randomly divided into 3 groups, 5 in each were. Those of the control group (group I) treated by a combination of water and Glycerol only, while those in the group (II) treated orally by 25mg/kg Nanoparticles Zinc Oxide. Meanwhile, group (III) treated orally by 75mg/kg with Nanoparticles Zinc oxide, once/ weekly for 10 times dose used in the treatment of each group.

Induced wound Surgically: Food was withdrawn for 24 hrs. and water restricted 12 hrs. before surgery. Under light sedation, by using an intramuscular injection of xylazine hydrochloride in a dose of 0.2 mg/kg B.W. and local anesthesia by using an inverted L-shape technique at wound borders with lidocaine hydrochloride 2%, in a dose 1ml for 1cm of tissues. Skin and subcutaneous tissues were removed in two circular full-thickness skin wounds (3.5-4.0 cm) on the dorsal sides of the back of each animal (one wound on each side), 10 cm apart after preparation of the area in a routine surgical manner. The site of operation was covered with gauze dressing.

Wound dimensions measure

Under local anesthetic effect by using lidocaine hydrochloride spray 10% the wound contraction and healing was assessed weekly by measurements of wound size, that were measured by using a ruler graded in millimeters positioned at the borders of wound in clock method according to (Van Rijswijk., 2013) and the means of all wounds were taken in each week of the experiment, for wound contraction evaluation (João De Masia *et al.,* 2016).

Histopathological examination

Biopsies taken from edges and center of wounds which were obtained at 3rd, 7th, 14th and 28th postoperative days (POD), tissues specimens kept and fixed in 10% buffer formalin solution directly submitted to Hematoxylin and Eosin (H α E) stain according to (Anderson and Gordon., 1999).

Statistical analysis

The data were analyzed using analysis of variance (ANOVA) with 2 × 2 factorial in RCBD according to (Gomez and Gomez., 1984), quantitative data are presented as the mean with a standard error of the mean (SEM) and $p<0.05$ was considered to be statistically significant.

Result

The clinical signs of zinc deficiency were loss of the hairs especially on legs, head and flank with rough hair coats. Rough skin, thickened, with dandruff, alopecia, scaling, crusting and hyperkeratosis. Pale mucosa membrane, loss of appetite, pruritus and emaciation. At 6th-week post-treatment, the of an abnormal lesion on the skin disappeared and the skin and heir become nearly in normal appearances. Serum zinc was significantly decreased, starting from the 2nd week till the end of the experimental study, the lowest level 7.61±0.28 µmol/L at 8th week in compared within 0-time (11.34±0.70 µmol/L) (Table 1).

After treatment zinc serum level increased

Table 1: Serum level of Zn in experimentally induced of zinc deficiency in goats.

Parameter	Time				
	0- time	2- weeks	4- weeks	6- weeks	8- weeks
Serum Zn µmol/L	11.34±0.70	9.17±0.43 a	8.94±0.49 a*	7.62±0.17 abc	7.61±0.28 abc

Values are Mean ± SE. a, b, c Means significantly difference level at P< 0.05.

Table 2: Serum levels of Zinc in animal groups use in the study.

Parameter	Gro-ups	Time/week			
		0	3rd	6th	9th
Zinc µmol/L	G I	6.61±0.51aA	6.72±0.99aA	7.39±1.49aA	10.01±3.84aA
	G II	7.18±0.71aA	7.28±1.14aA	8.04±0.65aA	15.82±1.41bB
	G III	7.81±0.29aA	8.33±1.94aA	12.64±1.18bB	16.86±2.38bcB

Values are M ± SEM: a, b, c, d significant difference at a level of P < 0.05 in comparison with in the same group. A,B,C,D significant in the comparison between groups, significance at P < 0.05.

significantly with the highest level was in the 9th week in comparison with the 0-day in groups II and III, but in group, I non significantly increased (Table 2).

The results of circular wounds showed a gradual significant narrowing in its diameters with the narrowest diameters was in the 5th week in comparison with 0-day within each group. While in comparison (between groups), treated groups showed narrowest diameters in comparison with the control values (group I) (Table 3).

Data of macroscopic appearance follow-up in the period of induced wounds showed inflammatory signs in wounded area with systemic reaction, characterized by anorexia, depression and lethargy in the first three days post-operation, then disappeared gradually within first 3 days to become within normal values. At 3rd to 7th days the wound healing showed normal response to injury (inflammatory phase), local inflammatory reaction persisted and were graded from slight to moderate inflammatory swelling with bloody clots formation, inflammatory exudate without signs of infection and the wound itself did not ooze in all groups of animals during this period (Fig. 1).

Fig. 1: Gross appearance in the 3rd -day post-operation.

At 15th day post-operation, diameters of wound were decreased, wounds were remodeled, made up new blood vessels and had eventually scar formation and in 30th day complete closed to the circle of wounds and infused of its adages and significantly decreased in diminution of scar tissue and the treated with Zinc oxide Nanoparticles group III revealed the best wound healing (Fig. 2, 3, 4).

On day 40th, the wounds disappeared and become as normal skin and hair in group III, but the other group's little markets appear in wound location. The current study also reported that wounds in those of group III were somehow similar to normal skin, with less hypertrophic scarring and nearly normal hair on the skin surface. Although, the clinical sings showed that similar in wounds healing of treated and control groups but, treated with Zinc oxide Nanoparticles group III revealed the best wound healing (Fig. 5, 6, 7).

In the current study, the histological examination was performed *via* a comparison between non treated (control group) and treated wound healing at different times and different concentrations of substances. At day 3 indicated presence of reactive cells, accumulation of exudates, the proliferation of fibrous connective tissue were observed to understand the normal healing process, the infiltration of reactive cells including neutrophils, macrophages and lymphocytes were present in control and treated groups. The progress of wound healing in the control section showed a large area of dermal tissue uncovered by

Table 3: Wounds dimension parameters in animal groups uses in the study.

Parameter	Groups	Time/ week				
		1st	2nd	3rd	4th	5th
Wounds dimension mm	G I	32.75±1.05bcdA	29.75±1.49bcdB	20.87±1.80bcB	14.25±0.25bB	9.37±1.43aBC
	G II	32.87±1.68bcdA	29.62±1.24bcdB	20.62±1.46bcB	9.75±0.52bA	6.5±0.45aB
	G III	32.62±1.37bcdA	28.37±2.61bcdB	18±1.77bcA	9.62±2.89bA	3±1.41aA

Values are M ± SEM: a, b, c, d significant difference at a level of P < 0.05 in comparison with in the same group. A, B, C, D significant in the comparison between groups, significance at P < 0.05.

epidermal tissue. This area was observed covering with the necrotic debris and accumulation of thin fibrin and it was also invaded with immature blood capillaries and occupied by granulation tissues (Fig. 8). At the same time wound healing in those treated by 25mg/kg, Zinc Oxide Nanoparticles revealed some detectable progression. This progression was observed *via* the development of new matrix deposition along with aggregation of many proliferating fibroblasts and less amount of necrotic debris. The same results were indicated in wound healing in those

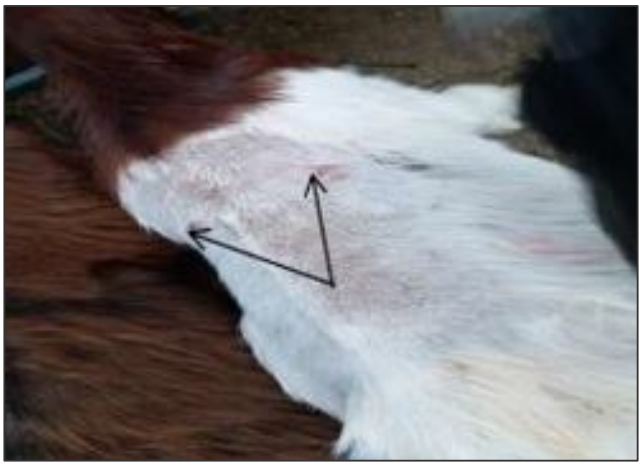

Fig. 2: Gross appearance of wounds in 30th-day post-operation (G I)

Fig. 3: Gross appearance of wounds in 30th-day post-operation (G II)

Fig. 4: Gross appearance of wounds in 30th-day post-operation (G III)

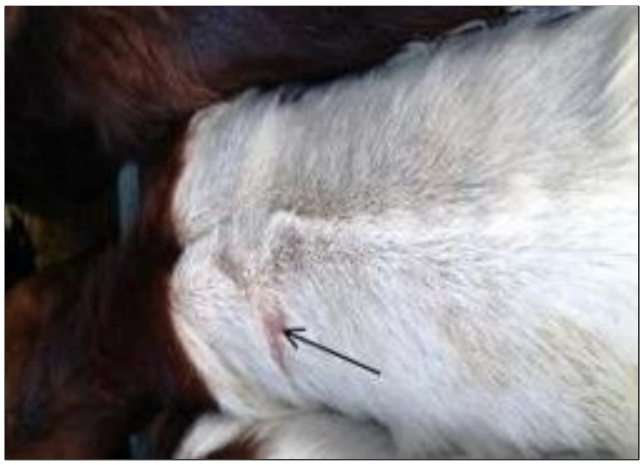

Fig. 5: Gross appearance of wounds in 40th-day post-operation (G I)

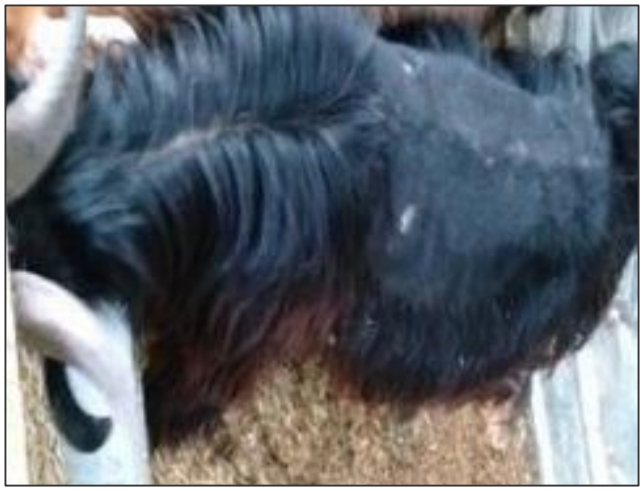

Fig. 6: Gross appearance of wounds in 40th-day post-operation (G II)

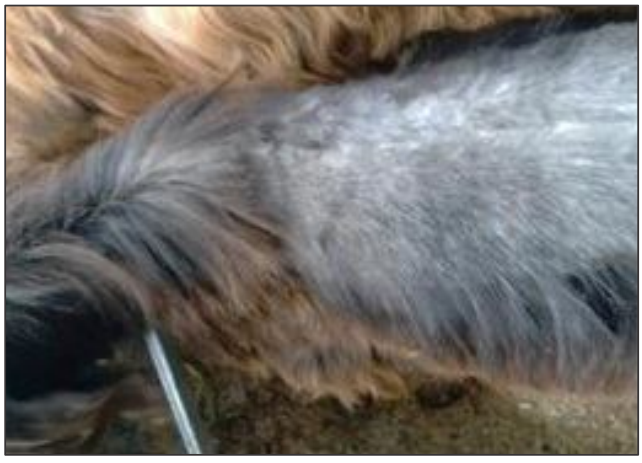

Fig. 7: Gross appearance of wounds in 40th-day post-operation (G III)

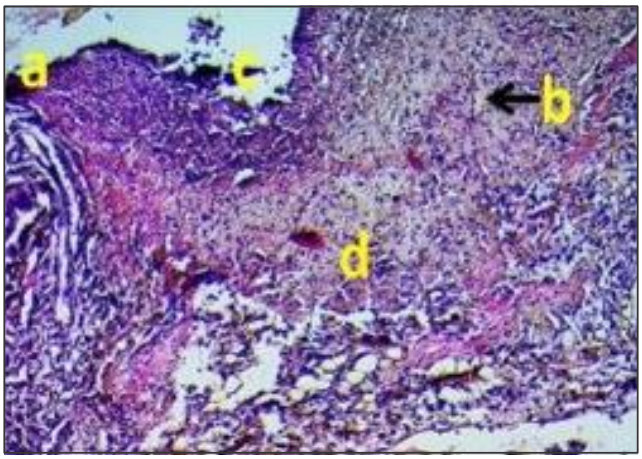

Fig. 8: Photomicrograph shows the progression of normal wound healing (control (G1)) at day 3, (a) represents the thin fibrin, while (b) indicated the infiltration of inflammatory cells (c) indicated necrotic tissues, whereas (d) represents granulation tissue. (H & E: 40 X).

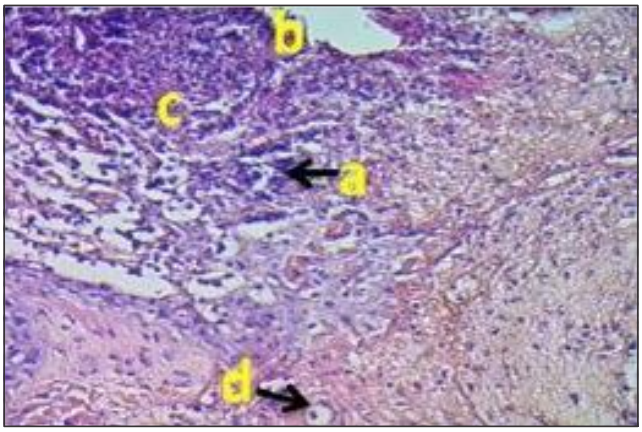

Fig. 9: Photomicrograph illustrates the process of wound healing (G2) at day 3, (a) indicated the infiltration of inflammatory cells, (b) indicated necrotic tissues, whereas (c) represents granulation tissue, (d) blood vessels. (H & E: 40 X).

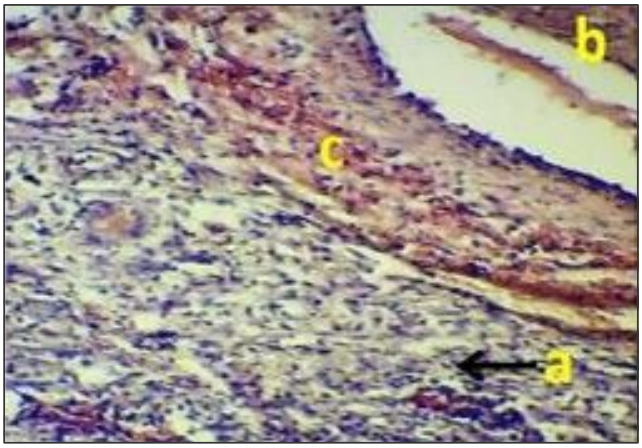

Fig. 10: Photomicrograph illustrates the process of wound healing (G3) at day 3, (a) indicated the infiltration of inflammatory cells, (b) indicated necrotic tissues, whereas (c) represents granulation tissue. (H & E: 40 X).

treated by 75mg/kg Zinc Oxide Nanoparticles, but with much more progression in the formation and organization of collagen fibers and proliferating fibroblast, it was also showed that there were moderate amounts in the numbers of inflammatory cells (Fig. 9, 10).

On day 7 indicated that the progression of wound healing in the control section was observed still covering with the necrotic debris and accumulation of fibrin and granulation tissues, fibroblast increased and inflammatory cells decreased in comparison with control groups at day 3 (Fig. 11). While in wound healing treated by 25mg/kg Zinc Oxide Nanoparticles revealed some visible development. This development was observed *via* increasing the amounts of matrix deposition along with aggregation of many proliferating mature fibroblasts, blood capillary and less amount of inflammatory cells.

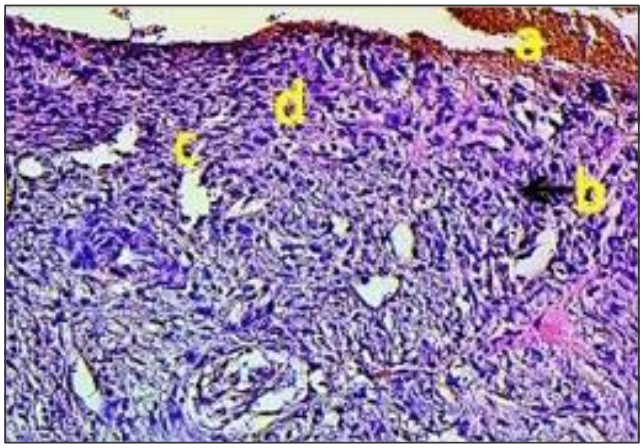

Fig. 11: Photomicrograph shows the progression of normal wound healing (control (G1)) at day 7, (a) represents the thin fibrin, while (b) indicated the infiltration of inflammatory cells, (c) indicated fibroblast, whereas (d) represents granulation tissue. (H & E: 40 X).

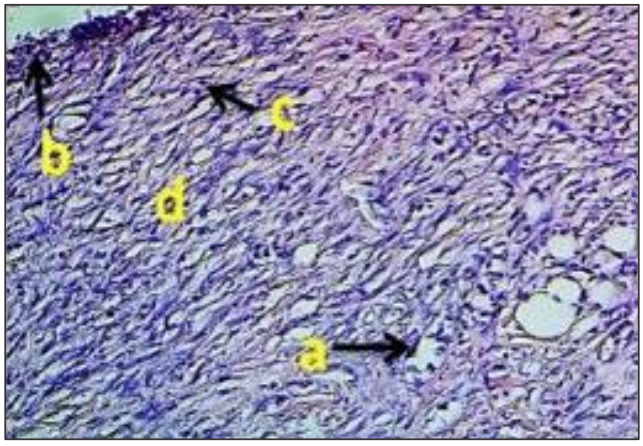

Fig. 12: Photomicrograph shows the progression of wound healing (G2) at day 7, (a) represents blood vessels, while (b) indicated mild infiltration of inflammatory cells, (c) indicated fibroblast, whereas (d) represents granulation tissue. (H & E: 40 X).

The identical results were revealed in wound healing treated by 75mg/kg Zinc Oxide Nanoparticles, but with much more progression in the formation and organization of mature fibroblast and formation of hyperplastic area (scar tissue) it was also showed that there were moderate amounts in the numbers of inflammatory cells (Fig. 12, 13).

On day 14 the progress in wound healing showed the clear generation of thin epidermal tissue. This layer of re-epithelization of epidermal tissue covering the wound with scar tissue formation, addition to that the collagen and fibroblast in dermal tissue appeared more organization and replacement of the initial fibrin matrix with collagen-rich granulation tissue, decreased inflammatory cells (Fig. 14). While, in wounds healing treated by 25mg/kg Zinc Oxide Nanoparticles at the same time revealed further detectable progression, which includes thick layer from re-epithelization of epidermal tissue completely covering the wound, collagen and fibroblast more organization along with aggregation of many mature fibroblasts, blood capillary and less amount of inflammatory cells. The alike consequences were indicated in wound healing treated by 75mg/kg Zinc Oxide Nanoparticles, an addition that it was observed an obvious formation and organization of mature fibroblast, blood capillary and formation of new hair follicles (Fig. 15, 16).

At day 28 the control group section showed a clear and a complete re-epithelization but still, there was a remaining of scab and observed some localization of inflammatory cells. The dermis showed remodeling in the connective tissue, but it was also showed a delay in

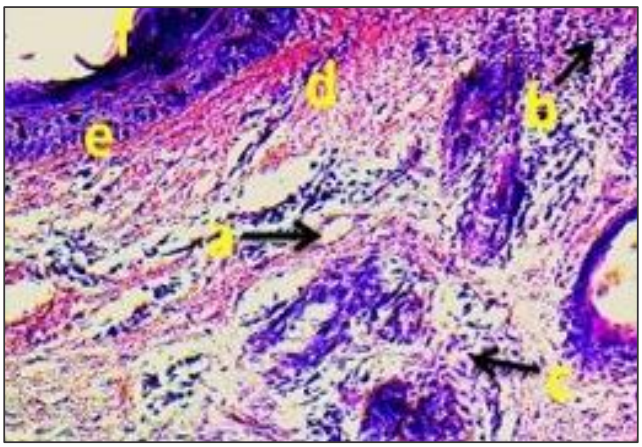

Fig. 13: Photomicrograph shows the progression of wound healing (G3) at day 7, (a) represents blood vessels, while (b) indicated mild infiltration of inflammatory cells, (c) indicated fibroblast, whereas (d) represents granulation tissue, (e) mild re-epithelization, (f) scar tissue. (H & E: 40 X).

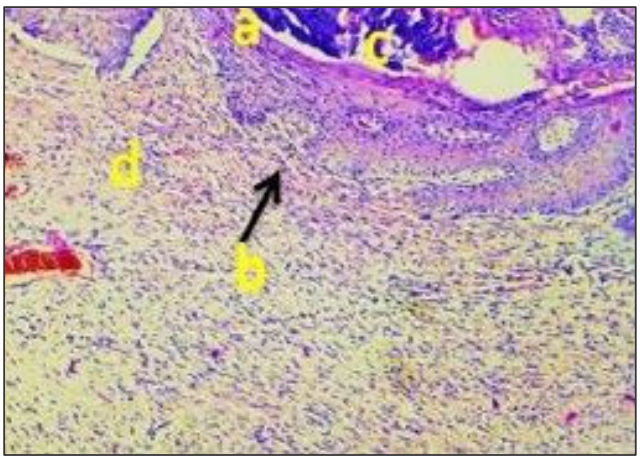

Fig. 14: Photomicrograph shows the progression of normal wound healing (control (G1)) at day 14, (a) thin epidermal tissue, while (b) indicated fibroblast, whereas (c) scar tissue, (d) infiltration of inflammatory cells. (H &E: 40 X).

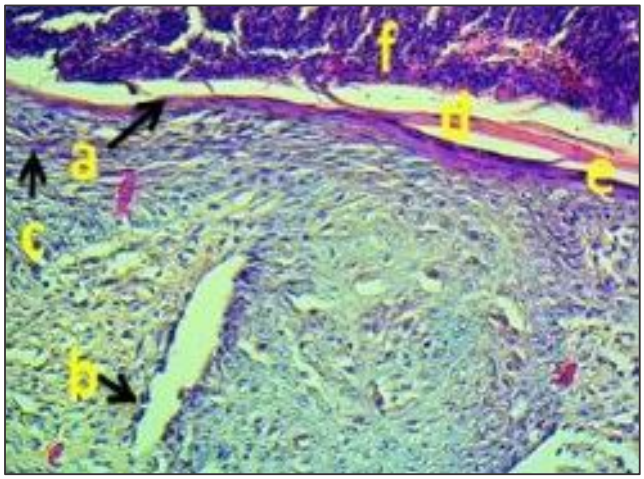

Fig. 15: Photomicrograph shows the progression of wound healing (G2) at day 14, (a) thin epidermal tissue, while (b) represents blood vessels, (c) indicated fibroblast, whereas (d) fibrin, (e) mild re-epithelization, (f) scar tissue. (H & E: 40 X).

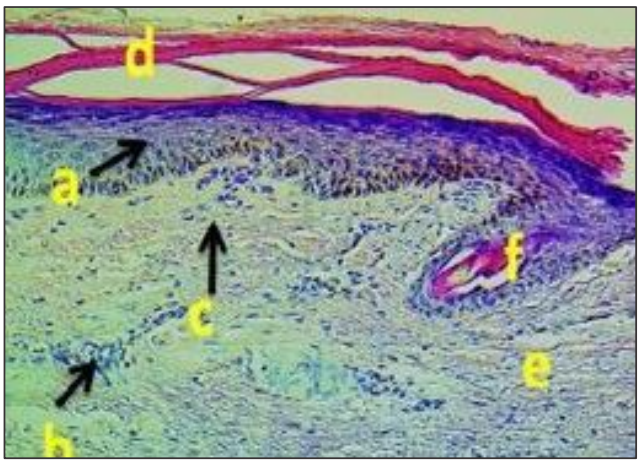

Fig. 16: Photomicrograph shows the progression of wound healing (G3) at day 14, (a) epidermal tissue, while (b) represents blood vessels, (c) indicated fibroblast, whereas (d) fibrin, (e) organization of collagen fibers, (f) generation of new hair follicle. (H & E: 40 X).

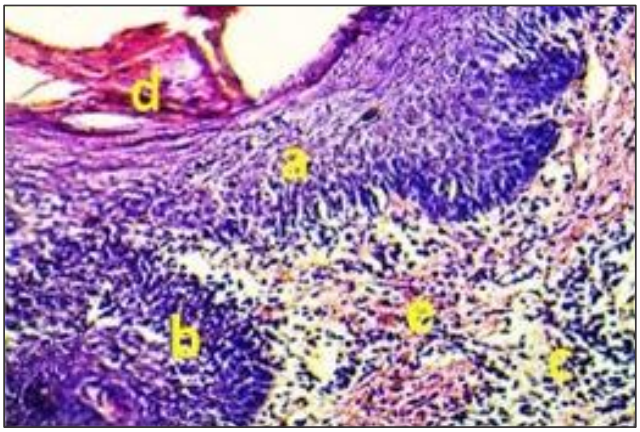

Fig. 17: Photomicrograph shows the progression of normal wound healing (control G1) at day 28, (a) epidermal tissue, while (b) represents massive infiltration with inflammatory cells, (c) indicated inflammation accompanied with localization of inflammatory cells, whereas (d) scar tissue. (H & E: 40 X).

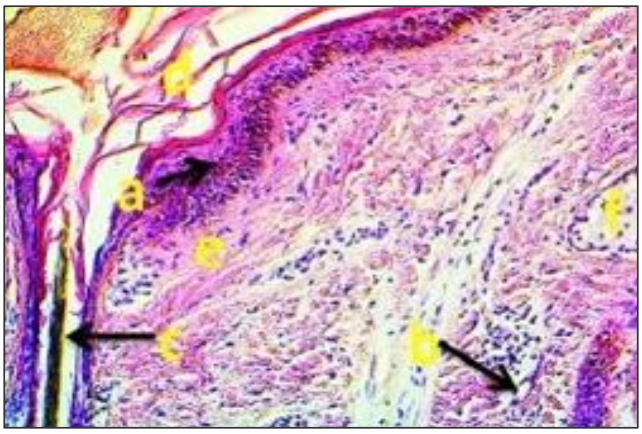

Fig. 18: Photomicrograph shows the progression of wound healing (G2) at day 28, (a) epidermal tissue, while (b) represents blood vessels, (c) hair follicular, whereas (d) fibrin, (e) organization of collagen fibers,(f) sebaceous gland. (H & E: 40 X).

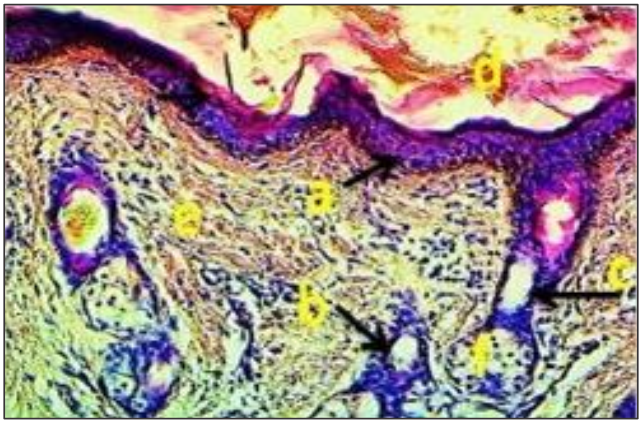

Fig. 19: Photomicrograph shows the progression of wound healing (G3) at day 28, (a) indicaAtes of epidermal tissue, while (b) represents blood vessels, (c) hair follicular, whereas (d) scar tissue, (e) organization of collagen fibers,(f) sebaceous gland. (H & E: 40 X).

the organization of interconnecting collagen fibers to each other and new hair follicular growth and sebaceous glands (Fig. 17). Compared with the treatment of group with 25mg/kg Zinc Oxide Nanoparticles and 75mg/kg Zinc Oxide Nanoparticles respectively, revealed there was a complete re-epithelization and with final skin layers. In this staged the healing showed complete clearance from the inflammatory cells and new generation of skin, this has appeared obviously in wound healing treated with 75mg/kg than 25mg/kg (Fig. 18, 19).

Discussion

Zinc is an essential trace element that is required by all cells in animals as well as its effective roles in numerous enzymatic reactions, on the other hand, deficiency of Zn are associated with reduced growth rate, poor immune function, decrease reproductive performance, as well as affecting skin in severe cases (Chan *et al.,* 1998, Mozaffari and Derakhshanfar., 2007).

In this study macroscopic findings showed that wounds healing in goats have high intended to reaper and the healing process is with limited inflammatory reaction in the site of wounds in all groups. In the other way, the groups treated by ZnO NPs showed faster healing and better response than control group, this results agree with the results of studies of Lansdown *et al.,* (2007) and Kogan *et al.,* (2017) that attribute it to zinc plays important role in wound healing and treated zinc deficiency results in improved wound healing compared to those with zinc deficiency. Also, the results of current study which documented important dose amount of ZnO NPs in wound healing and similar to results was reported in study by Al-Zubaedi., (2017) which was showed that better healing is achieved in a concentration 20% followed by that of 10% by using silver nanoparticles in cutaneous wounds in rabbits. The addition this study consistent with the study that indicated significant results of wound contraction rate, epithelialization and histopathology of the healed tissues of rats confirmed the promising wound healing property of ZnO NPs (Ekta *et al.,* 2018).

Moreover at day 3 histological results in this study indicated presence of reactive cells (neutrophils, macrophages and lymphocytes), accumulation of exudates, regeneration of epidermis, proliferation of fibrous connective tissue were observed to understand the normal healing process, these results revealed to normal response in early stage of wounds healing it may be because released molecules are chemo-attractants for neutrophils which enter the wound site and increased endothelial permeability, the same as also has been reported by (Reinke and Sorg., 2012, Werner and Grose., 2003).

But the same time the treatment of groups with 25mg/kg ZnO NPs and 75mg/kg ZnO NPs respectively, revealed to progression was observed *via* development of new matrix deposition along with aggregation of many proliferating fibroblasts and less amount of necrotic debris, These results agreed with Padmavathy and Vijayaraghavan., (2008), which revealed ZnO has both antibacterial and anti-inflammatory properties and accelerates the healing of both acute and chronic wounds. Also this experiment indicated that decreased inflammatory reaction in treated groups in comparison with control group, this may be correlated to the ability of zinc in modulating both innate and adaptive immune response, additional to alters immune responses in a multitude of ways ranging from myeloid-derived cells and inflammatory signaling to lymphocyte differentiation and antibody production (Pei-Hui *et al.,* 2017). Similarly, a recent trial reported that zinc could participate in the modulation of monocyte differentiation into pro-inflammatory (M1) or immune-regulatory/wound healing (M2) macrophages (Dierichs *et al.,* 2017). Another explanation could be the connection with the decreases of an inflammatory reaction, addition to ZnO NPs maybe has an effect on bacteria when used in different concentrations and it showed the ability of high concentration as compared to the low amount (Khitam *et al.,* 2018).

The regulated and promotion phases of wound healing depend on several factors (Guo and Dipietro., 2010, Dhivya *et al.,* 2015). One of these factors is the availability of appropriate trace elements serving as enzyme cofactors of structural components in tissue repair (Lansdown *et al.,* 1999). Zinc has a significant function in regulated and promotion and also on many cells over the entire process of wound repair (Pei-Hui *et al.,* 2017) these results agree with results of current study by Bao and his coworkers and also can explain the role of zinc supplementation in reduced plasma levels of oxidative stress markers, decreased *ex vivo* production of inflammatory cytokines, chemokine's and reduced secretory cell adhesion molecules which represent important biomarkers of cell damage-associated inflammation in endothelium and platelets (Bao *et al.,* 2010, Prasad., 2014). The mains observation of histological imaging at day 7th including many proliferating mature fibroblasts, blood capillary and formation of hyperplastic area (scar tissue) with much more progression in treated groups, these changes in cells proliferation similar to findings which were revealed to that the moment when granulation tissue begins to cover the wound surface marks the transition to the proliferative phase and represented by activation of fibroblasts which produce collagen and other extracellular matrices, such as neoangiogenesis (Negut *et al.,* 2018) and the results of current study are parallel with studies which reported that ZnO NPs play significant role in angiogenesis (Manuja *et al.,* 2012, Li and Chang., 2013).

Development and highly maturation of fibroblasts in groups treated by ZnO NPs may be because of increase fibroblasts drawn from healthy dermis, bone marrow progenitor cells, circulating fibroblasts and multipotent cells in the dermis (Shaw and Martin, 2009). Granulation tissue is only temporary and will be replaced during the remodeling phase. It is characterized by dense vascularization, fibroblast and my fibroblast populations and macrophages (Iocono *et al.,* 1998), these results synergy with the current study in the role of zinc information of granulation tissue (Maywald *et al.,* 2017).

In the current study on day 14, histological findings showed a clear generation of the thin skin tissue layer. This layer of re-epithelium of skin tissue covering the wound with the formation of scar tissue. These facts were more advanced in groups treated with ZnO NPs, especially in the 75mg/kg treated group, where serious hair follicle development was observed. Observations of this study are consistent with the studies that indicated the importance of ZnO NPs in promoting keratinocyte migration, thus improving Re-epithelialization (Ekta *et al.,* 2018, Vijayakumar *et al.,* 2019).

Looking at the histological results on day 28 of the present study, it is clear that the ZnO NPs are very close in shape to normal skin and the dermis and sub-dermal layers appear to cover the wound and in this staged the healing showed complete clearance from the inflammatory cells and new generation of skin this results comparable with Aksoy *et al.,* (2010), which they reveled that ZnO NPs accelerate the healing of both chronic and acute wounds, because of its epithelialization and bacteriostatic properties. On other hand, these results are very similar with the results mentioned by Naraginti *et al.,* (2016) which they indicated that appearance of fibroblasts, complete re-epithelialization, neovascularization and fewer inflammatory cells were observed in the tissue obtained from the ZnO NPs treated group, which formed the basis for the increase of collagenation at the wound site.

Among the parameters used for the analysis of healing wounds including cell proliferation, re-epithelialization, collagen deposition, granulation, angiogenesis, matrix remodeling and scar formation (Pei-Hui *et al.,* 2017). Studies of Xiong., (2013) and Oyarzun-

Ampuero *et al.,* (2015) described the advances in drug delivery with ZnO technology has received considerable attention for the treatment of wounds due to their effective cell penetration, immunomodulation and antimicrobial capacity. This fact could be has a main role in the process of regeneration and wound healing in the current trial. To sum up, wound healing is influenced by many factors as in this study the concentration, particle size and time for giving the zinc oxide. Shortly may be more evidence that is viable to confirm this statement.

Conclusion

To sum up, wounds healing is influenced by many factors as in this study the concentration, particle size and time for giving the zinc oxide. Shortly may be more evidence that is viable to confirm this statement.

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