

Wound-Healing Activity of Green and Chemical Zinc Oxide Nanoparticles (ZnO-NPs) Gels in Equine Wounds: A clinical Study

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Abstract

This study was aimed to evaluate the wound healing activity of both green and chemical Zinc oxide Nanoparticles in equine. Ten animals (nine donkeys and one horse) suffered from traumatized infected or non-infected wounds were used in this study. The visible signs of wound infection were evaluated in six cases, while the other four cases were admitted with fresh recent wounds. Animals were topically treated once a day in and day out and monitored for three weeks. It was observed that, the macroscopic wound contraction percents in animals treated with green Zinc oxide Nanoparticles gel in 1st, 2nd and 3rd weeks were 45%, 76%, and 93.6% respectively. While, those which treated with chemical Zinc oxide Nanoparticles gel were 40.4%, 67.2%, and 90.6% respectively. The study revealed that, green Zinc oxide Nanoparticles gel accelerated the wound healing and cleared the wound infection faster when compared to chemical Zinc oxide Nanoparticles gel. It was concluded that, topical green Zinc oxide Nanoparticles gel can significantly accelerate the process of wound healing and clear the wound infections in wounded equine from clinical field cases.

Keywords: Green ZnO-NPs; Gel; Wound healing; *Lawsonia inermis* extract; Clinical efficacy; Equine.

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Introduction

Wound healing is a dynamic, complex and well-organized process consists of four stages; inflammation, re-epithelization, wound contraction and remodelling. They are continuing without line of demarcation between them (Stadelmann et al., 1998; Sehn et al., 2009). The first aim of agents for wound healing is to accelerate the healing rate with minimal scar formation (Oryan et al., 2018). The wound healing affected by several variables, including blood supply, size of the wound, infection (Sehn et al., 2009), age, sex, and diseases as diabetes (Mathews and Binnington, 2001). Topical zinc accelerates the healing of small and acute skin wounds (Soderberg et al., 2001), as well as stimulates re-epithelialization, reduces inflammation and bacterial growth (Agren, 1990).

Zinc oxide (ZnO) is characterized by photocatalytic and photo-oxidizing capacity against chemical and biological species (Sharma et al., 2010). ZnO nanoparticles have both antifungal and antibacterial effect; they are effective against Gram-positive and Gram-negative bacteria (Azam et al., 2012). ZnO nanoparticles are synthesized by physical, chemical and green methods (Ekosse et al., 1986; Afifi et al., 2015). The use of toxic chemicals involved in the chemical method which are harmful to the environment (Dhandapani et al., 2014). Green ZnO-NPs have a very strong antibacterial effect at a very low concentration against Gram-negative and Gram-positive bacteria when compared to chemical ZnO-NPs (Hazra et al., 2013; Vimala et al., 2013). Plant parts like leaf, stem and root, fruit, and seed are used for green synthesis of ZnO-NPs. Using plant extracts is a cheap process and takes very little time (Heinlaan et al., 2008). This study aimed to evaluate the clinical efficacy of topical green and chemical ZnO-NPs gels for wound healing in equine.

Materials and Methods

1- Ethical statement

All experimental procedures in this study were approved by the Ethical Committee of South Valley University, Qena, Egypt. All procedures were done after oral consent from animals' owners for drug applications and pictures.

2- Chemicals

Zinc nitrate hexahydrate (Zn (NO₃)₂*6H₂O) 99% (Oxford), Sodium tripolyphosphate, potassium dichromate, and acetic acid of AR grade were used without any further purification. Methanol was purchased from lab scan analytical sciences, Poland. Activated charcoal powder was procured from ADWIC Co, Egypt. Carbopol 940® was purchased from Loba Chemie Company, India. All other chemicals and solvents were locally purchased and were used of an analytical grade.

3- Plant Materials

Leaves of *Lawsonia inermis* (Henna) were purchased from a local market from Aswan city (latitude 24°5'15'' N; longitude 32°53'56'' E), the southern part of Egypt and identified by Flora Department, Faculty of Science, South Valley University where a voucher specimen was deposited. Leaves were ground into fine powder by using an electric mixer.

4- Extract Preparation

Dried leaves of *Lawsonia inermis* were soaked in 80% Methanol at a ratio of 1:10 in Erlenmeyer flask with periodic shaking for 24 h. The methanolic extract was decanted by sieve and cotton followed by filtration using Whatman No. 1. Before filtration, activated charcoal was added and left for 15 min for chemical depigmentation. The filtrate was vacuum

dried using a rotary evaporator (Heidolph, Germany) at 45 °C.

5- Preparation of ZnO-NPs by Chemical method

Solution A (0.1 M of zinc nitrate hexahydrate [Zn (NO₃)₂.6H₂O] was dissolved in deionized water) and solution B (0.4 M of sodium hydroxide in deionized water), were prepared. Solution A put in a water bath at 60°C under gently stirring, and solution B was dropped into solution A at a slow rate. The mixture was sealed under the heating and stirring for 2 hours. Then the precipitation was separated and washed several times. Finally, the products were dried at 60°C for about 8 hours.

6- Preparation of ZnO-NPs by Henna extract

0.1 M of zinc nitrate hexahydrate [Zn (NO₃)₂.6H₂O] was dissolved in 40 mL distilled water, and then 20 mL of Henna extract was added. The mixture was mixed well for 2 hours and the precipitate was separated and washed several times. Finally, the products were dried at 60°C for about 8 hours.

7- Preparation of Green/Chemical ZnO-NPs Gels

0.2g of ZnO-NPs dissolved in 20ml acetic acid 1% by mixing on a hot plate with metallic stirrer for 90 min at 60°C, followed by cooling. 100ml of D.W was poured in a mixer and then 1g of Carbopol 940® was added slowly followed by the addition of ZnO-NPs solution till the formation of the gel at a concentration of 0.2%.

8- Study Animals and Treatment protocol

The study was carried out on ten animals (nine donkeys and one horse), their age ranged from 1 – 3.5 years old, were admitted to the Veterinary Teaching

Hospital, South Valley University, Egypt during 2019.

All animals were clinically examined; three donkeys were admitted with old and septic wounds at tail base, three donkeys were suffered from old and septic wounds at the lateral aspect of the abdomen, one donkey with a recent wound at the lateral aspect of the abdomen, two donkeys exhibited traumatized wounds on the lateral aspect of the thoracic region and one horse exhibited a traumatized wound on the leg.

Animals were randomly divided into two groups; the first group includes one horse and four donkeys were treated by green ZnO-NPs gel topically on the wounds. The second group includes five donkeys that were treated by chemical ZnO-NPs gel topically on the wounds. The wound surface was covered with a thin piece of sterile gauze followed by a protective bandage. The gel was applied once a day in and day out and all treated animals were monitored for three weeks.

9- Treatment procedures

Before application of the gel, the wound area was prepared according to the type of wound (old or recent);

In recent wound: Hemorrhages were controlled by packing and the wound area was aseptically prepared.

In old wound: Unhealthy tissues were surgically debrided and freshened, Hemorrhages were controlled by packing.

9.1 Wound Length

Wound length was measured by a graduated ruler.

9.2 Evaluation of Wound Healing

Evaluation of wound healing was based on:

- 1- Wound morphology during healing.
- 2- Wound contraction % was determined on day 7, day 14 and day 21 according to the equation described by (Ramsey et al., 1995).

Wound Contraction (WC %) = $\frac{W_0 - W_1}{W_0} \times 100$ Where; W_0 = the initial wound measurement, W_1 = the final wound measurement after healing.

9.3 Visible Signs of Wound Infections

Visible signs of Wound Infection were evaluated visually to check edema, exudate, and absence of pus, odor, healthy and morbid granulation tissue.

10- Statistical analysis

Data were expressed as mean \pm standard deviation by SPSS version 16. Statistical significance was assessed by one-way ANOVA. Significance was set at $P \leq 0.05$ and the graphs were done by Excel.

Results

All wounds were clinically evaluated for three weeks after the start of the application of the gel. Wound management strategies applied such as the surgical debridement in the case of the old wounds which were considered very important for the removal of dead and necrotized tissues which provide a suitable environment for the action of the gel for good and clean healing. Any hemorrhage could have resulted from surgical debridement, was arrested immediately and controlling by packing after clearance of the wound. However, the hemorrhage continued for a while after the application of first dressing changing and stopped completely in the second dressing changing. After treatment by three to five weeks, the wound surface appeared bright red, moist and the wound area was decreased in size rapidly in treated cases with green ZnO-NPs in compared with those animals treated with chemically synthesized ZnO-NPs gel.

It was observed that, the macroscopic wound contraction percents in animals

treated with green ZnO-NPs gel in 1st, 2nd and 3rd weeks were 45%, 76%, and 93.6% respectively. While who's treated with chemical ZnO-NPs were 40.4%, 67.2%, and 90.6% respectively as shown (Table 1, Figure 1)

Table 1.: Mean + SD of wound contraction % in equine wounds treated with Green and Chemical ZnO NPs gels

Time in weeks	Green ZnO NPs treated group	Chemical ZnO NPs treated group
1 st	45 \pm 4.4	40.4 \pm 5.3
2 nd	76 \pm 2.5	67.2 \pm 7.5
3 rd	93.6 \pm 4.8	90.6 \pm 4.6

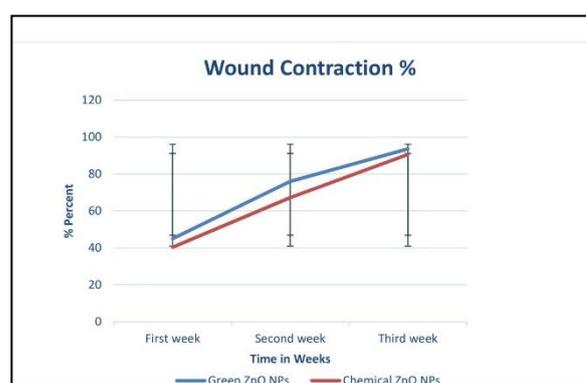


Fig (1): Wound contraction percents in treated animals with Green and Chemical ZnO NPs gels.

It was observed that the macroscopic wound contraction percents in animals treated with Green ZnO NPs gels in 1st, 2nd and 3rd weeks were 45%, 76%, and 93.6% respectively, while that in animals treated with chemical ZnO NPs gels were 40.4%, 67.2%, and 90.6% respectively.

Infected wounds were recorded in six cases of donkeys explaining the work nature of these animals in Egypt, where those hardly work in poor animal management circumstances. These infected wounds were

clinically estimated into three categories; wounds with mild signs of wound infection, wounds with moderate signs of wound infections, and wounds with severe signs of wound infections as shown (Table 2).

By the 1st week, the visible signs of wound infections were disappeared in most cases as shown (Figure 2, 3, 4). The two infected wounds treated with green ZnO-NPs gel characterized by severe and moderate signs of infection which disappeared after only one week of gel application. While, the four infected wounds treated with chemical ZnO-NPs gel characterized by mild and moderate signs of infection in two cases which disappeared after one week of gel application, and severe signs of infection in the other two infected cases which disappeared after two weeks of gel application.



Fig (2): Photograph of a traumatic infected wound. Where, a male donkey suffering from an infected wound at the right lateral side of the abdomen perhaps as a result of owners' hitting. Photograph was taken before any surgical debridement.

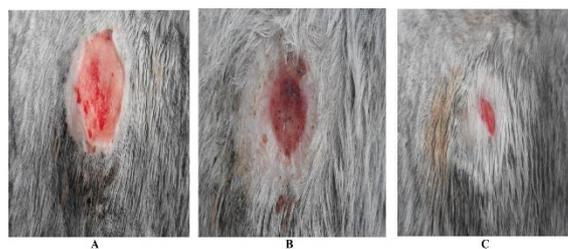


Fig (3): Healing stages of an infected cutaneous wound in a donkey treated with chemical ZnO NPs gel after surgical debridement. (A) 1.5 years old male donkey suffering from an infected wound at the right thoracic region after surgical debridement. (B) The same case after two weeks of treatment by chemical ZnO NPs gel showed a decrease in wound size and the tendency for closure (C) The same case after 21 days of treatment showed non-infected and decreased wound size.



Fig (4): Healing stages of a non-infected cutaneous wound in a horse treated with Green ZnO NPs gel after surgical debridement. (A) 2.5 years old horse suffering from a non-infected wound at the right hind limb. (B) The same case after four weeks of treatment by green ZnO NPs gel showed an amazing result to the owner in a very mobile part in his horse.

Table 2: Visible signs of wound infection before treatment and after 1st, 2nd and 3rd weeks of treatment

Green ZnO NPs gel					
Animal	Wound site	Clinical score of clearance of Infection			
		Before treatment	After one week	Two weeks	Three weeks
Horse	Leg	-	-	-	-
Donkey	Tail base	+++	-	-	-
Donkey	Lateral aspect of abdomen	-	-	-	-
Donkey	Lateral aspect of abdomen	++	-	-	-
Donkey	Lateral aspect of thoracic region	-	-	-	-
Chemical ZnO NPs gel					
Animal	Wound site	Clinical score of clearance of Infection			
		Before treatment	After one week	Two weeks	Three weeks
Donkey	Tail base	++	-	-	-
Donkey	Lateral aspect of abdomen	+++	++	-	-
Donkey	Lateral aspect of abdomen	+	-	-	-
Donkey	Tail base	+++	+	-	-
Donkey	Lateral aspect of thoracic region	-	-	-	-
(-) indicate the absence of signs of wound infections. (+) indicate the presence of mild signs of wound infections. (++) indicate the presence of moderate signs of wound infections. (+++) indicate the presence of severe signs of wound infections.					

Discussion

Chronic wounds are these wounds that fail to heal (Adam and Southwood, 2006). So, the wound healing aims to restore the normal tissue anatomy and function (Davidson and DiPietro, 2006). The high incidence and prevalence of trauma wounds in horses, besides that the wounds impair the daily function and appearance of equine with obvious financial and welfare implications (Singer et al., 2003)

motivate many researchers for seeking agents to possess the effective wound healing activity.

Recently, the equine wounds of the distal portion of the limbs take the attention of the clinicians because they have undergone prolonged complex healing. Infection is the most common factors which result in a delayed wound healing in horses (Hendrickson, 2012). Staphylococcus aureus, Pseudomonas aeruginosa, and β -hemolytic streptococci are common bacteria found in infected and clinically

non-infected wounds (Harding and Edwards, 2004, Davis et al., 2008). The wound infection delays the healing process and may cause wound breakdown, herniation of the wound in addition to complete wound dehiscence (Alexander, 1994).

Our results showed that, the use of green ZnO-NPs as a topical gel for cutaneous wound healing, was more effective in compared with chemical ZnO-NPs gel in the acceleration of wound healing. Green synthesis of ZnO-NPs using Lawsonia leaves extract stimulates and accelerates the wound healing process.

Previous reports indicated the potential role of *Lawsonia inermis* in the wound-healing activity. *Lawsonia inermis* stimulates wound epithelization at a faster rate and decreases wound size (Taweepraditpol et al., 2017).

Phytochemicals (Budhiraja and Sudhir, 1987; Tsuchiya et al., 1996; Sakarkar et al., 2004; Nayak et al., 2006; Wagini et al., 2015; Ali et al., 2016) in henna leaves have an important role in the stimulation of angiogenesis, the proliferation of endothelial cells (Kirkland and Marzin, 2003; Jeyaseelan et al., 2012) and accelerating wound healing process (Kelmanson et al., 2000) by activation of angiogenesis, re-epithelization, accelerating the wound contraction rate (Yadav et al., 2013; Mohammad, 2016) and increasing the granulation tissue by increasing collagen fibers and fibroblasts and decreasing inflammatory cells infiltration (Yadav et al., 2013). Henna leaves have anti-bacterial effect (Kelmanson et al., 2000) especially against gram-positive bacteria, antioxidant, immunomodulatory (Mikhaeil et al., 2004; Ben Hsouna et al., 2011), anti-inflammatory (Ali et al., 1995), hemostatic effect (Kluytmans et al., 1997). The antimicrobial activity of *Lawsonia inermis* Extract was reported against both gram-positive and

gram-negative bacteria (Muhammad and Muhammad, 2005; Hussain et al., 2011).

Zinc is an important microelement for accelerating the wound healing process (Lin et al., 2018) through increasing epithelialization (Agren et al., 1991; Agren, 1999; Sazegar et al., 2011) by up-regulating endogenous growth factors, notably insulin-like growth factor-I (Tarnow et al., 1994; Li et al., 2006) and decreases necrotic material at the site of the wound (Apelqvist et al., 1990; Keefer et al., 1998) when used topically. In addition, its deficiency has been linked to impaired wound healing process (Lansdown et al., 2007; Kogan et al., 2017). Zinc oxide possesses antimicrobial activity (Ann et al., 2014; Pasquet et al., 2015).

Recently, the involvement of nanoparticles in wound treatment protocols is increased. Some metal nanoparticles are used because of their therapeutic activity or as a drugs vehicle (Gupta et al., 2012) like, ZnO-NPs application in the wound healing field (Bai et al., 2003).

The potential wound-healing activities of *Lawsonia inermis* extract and ZnO NPs motivate us for using of *Lawsonia inermis* leaves extract in green synthesis of ZnO-NPs in our study, due to the role of *Lawsonia inermis* in the stimulation of re-epithelization and its antibacterial effect which improve the efficacy of ZnO-NPs.

Nanoparticles have been synthesized by physical, chemical and green methods (Parthasarathy et al., 2016; Mahmoud and Shalaby, 2019). The chemical method is characterized by being a more expensive method and the using of toxic chemical agents that produce a large amount of waste products affecting the environment and the handling person (Rosi and Mirkin, 2005; Princy, 2012; Dhandapani et al., 2014). While, the physical method involves the use of costly equipment, high temperature and pressure (Chandrasekaran et al., 2016).

Green or Biogenic synthesis of nanoparticles with the use of plant extract shows better advancement than chemical and physical methods as it is a lesser toxic, cost-effective, environmental friendly (Rosi and Mirkin, 2005; Vidya et al., 2013).

The chemical and physical methods for the synthesis of ZnO-NPs have multiple disadvantages (Jain et al., 2009; Hussain et al., 2016). While, green synthesis procedures have several advantages such as simple, inexpensive, good stability of nanoparticles, less time consumption, non-toxic byproducts, and large-scale synthesis (Lakshmi et al., 2017). Using natural extracts of plant parts is a very ecofriendly, cheap process and it does not involve usage of any intermediate base groups. Also, it takes very little time, does not involve the usage of costly equipment and precursor (Heinlaan et al., 2008). Plants are the most preferred source of NPs synthesis because they lead to the production of stable, varied in shape and size NPs (Qu et al., 2011).

We could explain our results in light of the previous reports which proved that, Zinc oxide nanoparticles accelerate collagen synthesis and wound shrinkage and reducing scars after healing wounds (Saremi et al., 2016). Zinc ions (Zn^{2+}) which released from ZnO can enhance keratinocyte migration towards the wound site so promote the healing (Kumar et al., 2012). The released zinc ions (Zn^{2+}) (Aydin Sevinç and Hanley, 2010; Premanathan et al., 2011) showed its antibacterial activity by interacting with the bacterial cell membrane leading to bacterial cell death (Liu et al., 2009), or by active transport inhibition, amino acid metabolism, and enzyme system disruption leading to bacterial growth stoppage (Sirelkhatim et al., 2015). ZnO nanoparticles are effective antibacterial agents on Gram-positive and Gram-negative bacteria (Emami-Karvani and

Chehrazi, 2011) showing high antimicrobial activity against *Pseudomonas aeruginosa* (Aysa and Salman, 2016).

Our study results were proved by (Agren, 1990; Jaiswal et al., 2015) who indicated that, the topical application of ZnO-NPs stimulates angiogenesis and re-epithelization of the wound, scar tissue formation, and reduce inflammation and bacterial growth (Agren, 1990), while the healing was delayed in infected wound (Sehn et al., 2009). Green ZnO-NPs have a very antimicrobial effect against Gram-positive and Gram-negative microorganisms when compared with chemically synthesized ZnO-NPs (Hazra et al., 2013; Vimala et al., 2013).

Here, we documented that, the infected wounds treated by both green and chemical ZnO-NPs showed healing with a clearance of visible signs of infection, accelerating re-epithelization and contraction of old and recent wounds of equine.

Conclusions

It concluded that, green synthesis of zinc oxide nanoparticles using *Lawsonia inermis* leaves extract accelerates the rate of wound healing in addition to its antimicrobial effect when compared with chemically synthesized zinc oxide nanoparticles. The old and recent wounds which treated by green ZnO-NPs gel, showed faster healing than those treated with chemically prepared ZnO-NPs gel.

Author's contributions

AAM, AAA, and ASS put the design of the study and supervised the study procedures. KEW prepared the green and chemical ZnO nanoparticles. SAM carried out henna extraction. AAM carried out all study experiments (as a part of her Ph.D. thesis), analyzed data, prepared and reviewed the original draft of the manuscript. All authors read and approved the final manuscript.

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