Effect of Chitosan Gel on Wound Healing: Experimental Study in Donkeys

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ABSTRACT

Chitosan is one of the natural bioactive materials known for its wound healing effect. Healing of equine’s wounds are often delayed and complicated. This study aimed to evaluate efficacy of chitosan gel on healing of equine skin wounds in comparison to povidone-iodine (as control). Six adult male donkeys were used and divided equally into two groups; group A (2x2 cm skin-wound) and group B (4x4 cm skin-wound). Full thickness skin-wounds were created on forearms and shoulders. Wounds at the right side were irrigated using povidone-iodine, while wounds at the left side were dressed using chitosan gel. Clinically, chitosan treated wounds contracted faster than control wounds, and shoulder wounds contracted faster than that of the forearm. By the end of the experiment all chitosan treated wounds were completely healed with intact epidermis. In contrast, 10.4 % and 13.3 % of wound’s area treated with povidone-iodine in group B at the shoulder and the forearm regions, respectively, still unclosed. Grossly, povidone-iodine treated wounds in both regions, were covered with scab in group A, and had an area of granulation tissue in the wound centers in group B. Chitosan treated wounds, in both groups in both regions, were completely covered with cornified epithelium. Microscopically, the re-epithelialization was uncompleted in povidone-iodine treated wounds in both groups in both regions. In contrary, complete epidermal layer was observed in wounds treated with chitosan in both groups in both regions. Finally, chitosan gel may be better than povidone-iodine in healing of equine skin wounds.

1. INTRODUCTION

Skin is the biggest organ of the animal’s body, which separates the organism from its surroundings. As such, it fulfills several physiological functions and contributes most significantly to the individual’s survival. Individual variation influences the thickness and firmness of the skin and so do species, breed, age and regional differences (Schummer et al., 1981). The incidence of traumatic wounds in equines is more prevalent than other species as well as, the healing of these wounds is often delayed and complicated (Caron, 1999). When the skin is destroyed, it tends to gap, as well as, the moisture, electrolytes and proteins in the wound would be lost (Deng et al., 2007).

For treatment of equine lacerated wounds, different topical preparations have been used to improve healing, decrease the incidence of exuberant granulation tissue formation and improve the cosmetic appearance (Wilson et al., 1996; Zhao et al., 2002). The role of bioactive materials in wound healing was investigated by many researchers (Labrude and Becq, 2003; Dai et al., 2011) especially with the increased drug resistance and toxicity as well as, the cytotoxic effect of antiseptics used for wound dressing (Lineaweaver et al., 1985; Cooper et al., 1991).

Chitosan is one of the most known bioactive materials for its wound healing effect (Labrude and Becq, 2003). It is a natural product derived from the polysaccharide chitin (Mukherjee et al., 2003). Chitosan based biomaterials has been utilized in different biomedical applications, such as wound healing preparations, due to its haemostatic, stimulation of healing, antimicrobial and anti-inflammatory effects (Wang et al., 2012; Amin and Abdel-Raheem, 2014). Chitosan promotes
granulation, organization, and normal tissue regeneration, and help in recovering the original tensile strength of the wound (Okamoto et al., 1995; Ueno et al., 2001). Chitosan forms a semipermeable sheet upon the wound that maintains a sterile condition beneath a dry scab, therefore, preventing wound dehydration and contamination (Charernsriwilaiwat et al., 2014; Laura et al., 2014). Bioactive materials contain chitosan, was utilized recently as bone grafting substitutes due to its known osteoconductive and osteogenesis properties (Nandi et al., 2010; Emara et al., 2013). Additionally, chitosan has considerable potential as a wound-dressing material for accelerating wound healing (Park et al., 2016).

The current study was designed to investigate wound healing efficacy of chitosan gel on two locations (shoulder and forearm) and two surface area of donkey's wound model using gross observation and histopathological parameters.

1. MATERIALS AND METHODS
2-1. Preparation of the experimental biomaterials
A- Preparation of 0.1% Chitosan solution
Chitosan solution 0.1 % (w/v) (Yaizu, Suisankagku Co., Japan) was prepared by dissolving in 1.0 % acetic acid solution. It was stirred using magnetic stirrer till complete dissolving.

B- 0.1% Chitosan gel
One gram of carbopol 940 (Beijing Haidian Huiyou Fine Chemical Factory- Beijing, China) was mix with chitosan solution until gel formation, then stored at -20°C until used.

2-2. Experimental design
This study followed the animal welfare guidelines of the faculty of veterinary medicine, university of Sadat city, Egypt.

Animal grouping:
Six adult male Egyptian donkeys (aged between 6-8 years and body weight ranged from 200 to 250 kg) with normal physical examination were used in this study. Animals were housed in covered stalls, allowed free choice grass hay through the day and a one kilogram of concentrates at night, and allowed free-choice water throughout the study. Animals were equally divided into two groups; group A (2x2 cm skin wound) and group B (4x4 cm skin wound).

2-3. Surgical procedures
On the day of surgery, tetanus prophylaxis and flunixin meglumine (50 mg/ 45 kg, IV, Schering-Plough, Germany), were administered to the animals, and an IV jugular catheter placed for induction of general anesthesia. Skin of the shoulders and the forearms were clipped and aseptically prepared with povidone-iodine. Animals were anesthetized using xylazine hydrochloride (2% xylazine hydrochloride "xylaject"; ADWIA, 10th of Ramadan City, Egypt) at dose of 1.1 mg/kg, IV, and ketamine (Ketalar®, Gracure pharmaceutical Ltd, Bhiwadi, Rai, India) at dose of 2.2 mg/kg, IV. Anesthesia was maintained with the intravenous combination of xylazine, ketamine and guaifenesin (650mg, 1300mg, and 50g, respectively) in 1 liter of sterile saline. The operated donkeys were secured in lateral recumbency while the skin was relaxed and undistorted. Full-skin thickness wounds, 2x2 cm, were created in {both region areas} of group A, and 4x4cm, were created in {both region areas} of group B. Wounds at the right side were irrigated using 2.5% Povidone-iodine (as a control), while wounds at the left side were dressed using chitosan gel. Wounds were covered using a non-adherent dressing. Wound dressing was proceeded day after day for the first 10 days then every 4 days till the end of the experiment.

2-4. Assessment of wound healing
Macroscopic evaluation
Digital photographs were taken of all wounds after the area had been carefully cleaned using saline to visualize wound margins. The width and the length of wounds were measured using digital caliber. The surface area was estimated using the following formula:

\[ \text{Wound surface area} = \text{Length (mm)} \times \text{Width (mm)} = \ldots \text{mm}^2 \]

The rate of wound contraction was evaluated by determination of the unclosed area of the wound as a function of time. It was calculated using the following formula:

\[ \text{Wound surface area percent relative to day zero (WSAP)} = (\text{wound surface area x 100})/ (\text{surface area of the excision at day zero}) \]

\[ \text{Wound contraction percent (WCP)} = 100\% – \text{WSAP} = \ldots\% \]

Histopathological examination:
After complete wound healing, animals were euthanized under the effect of thiopental sodium anesthesia (Thiopental®: EPICO Co., A.R.E). Skin samples were obtained from the whole healed wound tissues and the surrounded intact skin. Samples were fixed in 10% neutral buffered formalin for 72 hours and then trimmed and processed for haematoxylin and eosin staining. Histological photos were taken by using Leica EC3 digital camera.

2. RESULTS
3-1. Clinical observation:
No signs of adverse reaction were recorded throughout the experiment. Each wound retained the original square periphery to slightly stellate with an around central granulation bed until complete epithelialization occurred. During the first 2 weeks’ post wounding (pw), all wounds underwent expansion with rapid increase of the wounds surface area. Wound contraction started at the 14th day PW and proceeded rapidly during the rapid healing phase approximately from 14th day till the 34th day PW. Compared with control wounds, chitosan treated wounds contracted at higher rate, as well as, wounds at the shoulder region contracted faster than that of the forearm region.

In group A (2x2 cm); on day 14th PW, wound contraction of the chitosan treated wounds at the shoulder region reached 42%, which was 34 % higher than the counterpart control wounds. At the forearm region wounds started to contract at day 18th PW. At this day, the contraction percent were 23% and 34% in control and chitosan treated wounds respectively. On day 22 PW, wound contraction increased up to 65% in both shoulder and forearm regions in chitosan treated wounds, which was about 26.5% and 16.5% higher than the counterpart control wounds in both regions, respectively. Chitosan treated wounds at the shoulder and forearm regions were completely healed at day 38 and 42 PW, respectively. Chitosan treated wounds at the shoulder and the forearm regions, respectively still unclosed (Charts, 3 & 4 and Photos, 5a, 6a. 7a & 8a).

3-2. Pathological findings:
Gross examination of skin wounds of group A (2x2 cm) in both shoulder and forearm regions, which treated with 2.5% povidone-iodine, revealed that wounds in both regions are covered with scab (Photos 1a and 2a). Microscopically, the re-epithelialization was uncompleted over a granulation tissue which fill the wound cavity in both regions (Photos 1b & 2b). Additionally, gross examination of skin wounds of group B (4x4 cm) in both shoulder and forearm regions, which treated with 2.5% povidone-iodine, revealed that large area of granulation tissue still appears in the center of wounds (Photos 5a & 6a).

### Table 1. Effect of chitosan gel in comparison to povidone-iodine as dressing for donkey's wound on restoration of skin histological architecture of shoulder region

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group A (2x2 cm)</th>
<th>Group B (4x4 cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Povidone-iodine</td>
<td>Chitosan</td>
</tr>
<tr>
<td>Amount of granulation tissues(^a)</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>Inflammatory infiltrates(^b)</td>
<td>Few</td>
<td>Few</td>
</tr>
<tr>
<td>Epidermal closure(^c)</td>
<td>Non</td>
<td>Complete</td>
</tr>
<tr>
<td>Epidermal differentiation(^d)</td>
<td>Early</td>
<td>Late</td>
</tr>
<tr>
<td>Keratin layer formation(^e)</td>
<td>Non</td>
<td>Formed</td>
</tr>
</tbody>
</table>

\(^a\)Profound, moderate, scanty, or absent.  
\(^b\)Plenty, moderate, or a few.  
\(^c\)Pasal layer of the epidermis to assess the newly formed epidermis.  
\(^d\)Early, spinous epidermal differentiation; late, granular epidermal differentiation.  
\(^e\)Non, not formed, formed with deformity, or formed.
Table 2. Effect of chitosan gel in comparison to povidone-iodine as dressing for donkey’s wound on restoration of skin histological architecture of forearm region

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group A (2x2 cm)</th>
<th>Group B (4x4 cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Povidone-iodine</td>
<td>Chitosan</td>
</tr>
<tr>
<td>Amount of granulation tissues*</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>Inflammatory infiltratesb</td>
<td>Few</td>
<td>Few</td>
</tr>
<tr>
<td>Epidermal closure c</td>
<td>Incomplete</td>
<td>Complete</td>
</tr>
<tr>
<td>Epidermal differentiationd</td>
<td>Early</td>
<td>Late</td>
</tr>
<tr>
<td>Keratin layer formation*</td>
<td>Thin layer</td>
<td>Formed</td>
</tr>
</tbody>
</table>

*aProfound, moderate, scanty, or absent.  
*bPlenty, moderate, or a few.  
*cPasal layer of the epidermis to assess the newly formed epidermis.  
*dEarly, spinous epidermal differentiation; late, granular epidermal differentiation.  
*eNon, not formed, formed with deformity, or formed.

Chart 1: The average contraction % of the control and chitosan gel treated wounds of the shoulder region (group A: 2x2 cm)

Chart 2: The average contraction % of the control and chitosan gel treated wounds of the forearm region (group A: 2x2 cm)

Chart 3: The average contraction % of the control and chitosan gel treated wounds of the shoulder region (group B: 4x4 cm)

Chart 4: The average contraction % of the control and chitosan gel treated wounds of the forearm region (group B: 4x4 cm)
Microscopically, large area of granulation tissue in the center of the wounds can be detected and the epithelialization were weak (Photos 5b & 6b).

On the other hand, gross examination of skin wounds of group A (2x2 cm) in both shoulder and forearm regions which treated with chitosan gel revealed that, wounds in both regions are covered with cornified epithelium (Photos 3a & 4a). Microscopically, complete epidermal layer over a granulation tissue which fill the wound cavity were observed in wounds of both regions (Photos 3b & 4b). Additionally, gross examination of skin wounds of group B (4x4 cm) in both shoulder and forearm regions which treated with chitosan gel revealed that wounds in both regions are completely covered with cornified epithelium (Photos 7a & 8a). Microscopically, complete epidermal layer over a granulation tissue which fill the wound cavity were detected in the wound of shoulder region but this epidermal layer branched deeply in the granulation tissue (Photos 7b). In the wound of the forearm region, completed epidermal layer over a granulation tissue that fill the wound cavity was observed (Photos 8b).

3. DISCUSSION
The incidence of traumatic wounds is higher in equine than other species and healing usually delayed and complicated (Caron, 1999). With skin loss and inability to primary wound closure, treatment of traumatic skin wounds to heal by second intention is the only available option (Lindsay, 1990; Auer and Stick, 1999). With increased drug resistance and cytotoxic effect of antiseptics used for wound dressing (Cooper et al., 1991; Greenwood, 1995), the need for a safe and effective dressing also increased. Chitosan hydrogel might be one of the most useful and protective forms which allow contraction of the wound in a suitably moist healing environment, as well as, possess most of the properties of an ideal wound dressing materials (Labrude and Becq, 2003; Obara et al., 2003; Stashak et al., 2004).

Many researchers use chitosan as bioactive materials for its wound healing effect but in different concentration, 0.5% in dogs (Ueno et al., 1999), 1% in rats (Hima Bindu et al., 2010), 1:3% in mice (Laura et al., 2014) and 4% in rats (Tírcia et al., 2013). In the present study, chitosan was applied at 0.1% concentration according to Abou El Ella et al. (2007), with promising effectiveness, so could be avoid the possibility of chitosan complication.

Swelling of wound bed and centrifugal tension forces occurred from the surrounding skin retracted the wound edges and resulted in increasing of the wounds surface area during the first 14th days PW (Bertone et al., 1985). In the current study, wound contraction started at 14th day PW and proceeded rapidly during the following 4-5 weeks. The extent and the rate of wound contraction during this phase (the rapid healing phase) of wound healing depends upon the degree of myofibroblasts organization and the contraction of its actin contents (Jacobs et al., 1984 & Wilmink et al., 1999b).

Our clinical observation revealed that, chitosan treated wounds contracted at higher rate compared with the povidone-iodine treated wounds. This may be attributed to the biochemical effects of chitosan in wound healing, which includes; increases the expression and activities of growth factors, fibroblast activation and stimulation of wound granulation and fibrous tissue synthesis (Abou El Ella et al., 2007; Muzzarelli, 2009; Jayakumar et al., 2011; Wang et al., 2012). In this study, the rate of wound contraction varied between different body areas; it was higher at the shoulder compared with the forearm. This may be attributed to the differences in cellular inflammatory response and organization which, is a part of the regional anatomic and physiologic difference between these two locations (Wilmink et al., 1999a, b).

The rate of wound healing is determined by the sum of both wound contraction and epithelization (Jacobs et al., 1984). Epithelization of the wound occur by proliferation and migration of keratocytes to cover the surface of the wound. Concerning our histopathological results; epithelization was complete by the end of the observation period (42:50-day PW) and covered the whole surface of chitosan treated wounds compared with povidone-iodine treated wounds, which was incomplete. There is an intimate relation between both wound contraction and epithelization. Contraction of the wound stop when epithelization is complete. On the contrary, epithelization should be activated when contraction ceased because of the increased reactive forces of the surrounding skin on the wound edge to overcome the contractive forces of the wound myofibroblasts.
Figures 1a-4b: Wound healing, shoulder and forearm regions, donkey, group A (wound, 2x2cm). Figures 1a and 2a: Gross photographs of skin wounds treated with povidone-iodine in shoulder and forearm regions, respectively. Wounds in both regions are covered with scab. Figures 1b and 2b: Microscopic photomicrographs of skin wounds treated with povidone-iodine in shoulder and forearm regions respectively. Partial re-epithelialization was recorded over a granulation tissue which fill the wound cavity. Epidermis (black arrows), granulation tissue (stars), angiogenesis (white arrows), hair follicle (H), sebaceous gland (Se), sweat gland (S), scab (arrowheads), HE stain, X10. Figures 3a and 4a: Gross photographs of skin wounds treated with the chitosan gel in shoulder and forearm regions, respectively. Wounds in both shoulder and forearm regions are covered with cornified epithelium. Figures 3b and 4b: Microscopic photomicrographs of skin wounds treated with chitosan gel in shoulder and forearm regions, respectively. Complete epidermal layer was recorded over a granulation tissue which fill the wound cavity. Epidermis (arrow), granulation tissue (star), scab (arrowhead), HE stain, X10.
Figures 5a-8b: Wound healing, shoulder and forearm regions, donkey, group B (wound, 4x4cm). Figures 5a and 6a: Gross photographs of skin wounds treated with povidone-iodine in shoulder and forearm regions, respectively. Epithelial-uncovered granulation tissue still appears in the center of wounds of shoulder and forearm regions. Figures 5b and 6b: Microscopic photomicrographs of skin wounds treated with povidone-iodine in shoulder and forearm regions, respectively. The wounds were observed partial re-epithelialization over a granulation tissue which fill the wound cavity. Epidermis (arrows), granulation tissue (stars), hair follicle (H), sebaceous gland (Se). HE stain, X10. Figures 7a and 8a: Gross photographs of skin wounds treated with chitosan gel in shoulder and forearm regions, respectively. Wounds in both shoulder and forearm regions are covered with cornified epithelium. Figures 7b and 8b: Microscopic photomicrographs of skin wounds treated with chitosan gel in shoulder and forearm regions, respectively. 7b) Wounds were recorded complete but underlying branched epidermal layer over a granulation tissue which fill the wound cavity. Epidermis (arrows), granulation
In such cases epithelization, must be relied upon to cover the remaining granulation bed (Swaim 1980; Jacobs et al., 1984). Finally, using minimum concentration of chitosan (0.1%), with shortest time (42–50 day), in the current study relative to other studies for complete healing in donkey’s wound, give hopeful idea in handling of wound dressing in another species of animal particularly equine one.

4. Acknowledgment
We appreciate the support rendered by the Faculty of Veterinary medicine, University of Sadat City, Egypt.

5. Conflict of Interest Statement
The authors declared that they had no conflicts of interest with respect to their or authorship or the publication of this article.

6. REFERENCES


