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Effect of Serum Hyaluronic Acid on Wound Healing in Wister Rats

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ABSTRACT

Wound healing is of paramount importance in Veterinary Surgery whenever skin integrity is breached. The faster the healing rate, the better chances to mitigate contamination and infections. There is paucity of information on the use of hyaluronic acid in wound healing in Veterinary medicine. Twenty (20) clinically healthy Wister rats of both sexes were randomly grouped in to four groups (A, B, C, D) of five rats each and allowed to acclimatize for two weeks. Anaesthesia was carried out using a combination of xylazine and ketamine at a dosage rate of 5mg/kg and 50mg/kg respectively, intraperitoneal. Circular skin excision was made on each rat after shaving and scrubbing using 70% ethyl alcohol. Group A rats served as negative control while group B and C served as positive controls povidone iodine and oxytetracycline spray were applied topically respectively, group D served as test group where hyaluronic acid serum was applied topically, healing was monitored for 18 days. Results (macroscopy and histology) shows group D having significant healing rate ($p<0.05$) compared to A, B and C. Hyaluronic acid serum used in this study was seen to have a significant wound healing contraction potential compared to povidone iodine, oxytetracycline spray and the negative control.

Keywords: Wound healing, Hyaluronic acid, Anaesthesia, Wister rats, Intraperitoneal

INTRODUCTION

Hyaluronic acid is a naturally-occurring compound that is composed of repeating units of beta-glucuronic acid and beta-acetylglucosamine. It is classified as a glycosaminoglycan and is not a protein (Fallacara *et al*., 2018). Hyaluronic acid is a member of the glycosaminoglycan family, which makes up a significant portion of the extracellular matrix. It is unique among these compounds due to its straightforward structure and large size. The HA molecule is made up of repeating units of D-glucuronic acid and N-acetyl-D-glucosamine, connected by beta-glycosidic bonds. These repeating units create a long polymer with a molecular weight of up to 5 million kDa (Vigettim *et al*., 2014). The earliest known reference to Hyaluronic Acid was made in 1880 by French scientist Portes, who observed that a type of mucin found in the vitreous body was distinct from other types found in the cornea and cartilage. He named it "hyalomucine" (Boeriu *et al*., 2013). Hyaluronic Acid is known for its exceptional viscoelasticity, ability to retain moisture, biocompatibility and hygroscopic properties (Necas *et al*., 2008/Hyaluronic acid plays a significant role in the extracellular matrix, and influences cell growth, movement and shaping (Fallacara *et al*., 2018). Hyaluronic acid is also present within cells and has been found to have functions within the cell (Litwiniuk *et al*., 2016).

Wound healing is a multi-step, intricate biological process that aims to repair or replace damaged or lost tissue and restore its integrity (Olczyk *et al*., 2014). The process of healing is a complex and overlapping interaction of cells, cytokines, growth factors, and the extracellular matrix (Nyman *et al*., 2019).

A wound is a disruption of the structure and function of cells and tissues caused by various factors such as physical, chemical, thermal, microbial, or immunological damage. This disruption of epithelial integrity can also affect the normal structure and function of the underlying tissue (Mulkalwar *et al*., 2015). To repair injured tissue, a complex series of processes must occur, including multi-cellular migration, proliferation and differentiation, biomolecular interactions, molecular synthesis of matrix components, and a complex signaling network (Stephens *et al.,* 2013; Koschwanez and Broadbent, 2011). One commonly used model for wound healing research is the excisional wound,

which is created by surgically removing the epidermis, dermis, and subcutaneous fat. This model allows for the study of various aspects of the healing process such as bleeding, inflammation, formation of granulation tissue, re-epithelialization, angiogenesis, and remodeling (Nauta *et al.*, 2013; Wong *et al.,* 2011). The wound area can be recorded and monitored over time, and the rate of healing can be calculated based on the size of the wound. For histological analysis, animals can be euthanized or locally anesthetized, and biopsies can be collected, processed, and examined for the distance between the wound margins, characteristics of the granulation bed, and collagen organization (Koschwanez and Broadbent, 2011; Wong *et al*., 2011). The materials and techniques used in this model are relatively simple and practical, making it easy to apply topical agents and investigate their effects on the healing process (Wong *et al*., 2011). Various animals such as mice, rats, rabbits, and pigs can be used in excisional wound healing research, and multiple wounds can be created on each animal. For example, up to two wounds can be inflicted in mice (Caetano *et al.,* 2015), four wounds in rats (Leite *et al.,* 2014), four or more in pigs (Fries, 2005), and six wounds in rabbits (Masson-Meyers *et al.,* 2013). In veterinary practice in Nigeria, a number of topical agents have been used to enhance healing, with povidone iodine and oxytetracycline spray among the most commonly used. However, there is limited information on the use of hyaluronic acid serum to promote healing at both gross and histological levels in Nigeria.

MATERIALS AND METHOD

Experimental animals

Twenty Wister rats of equal weight (0.13±0.00kg) and of both sexes, were obtained and acclimatized for two weeks. The rats were randomly grouped into four (A, B, C, D) of five rats each.

Ethical statement

This research was carried out according to institutional use of laboratory animals approved by the faculty of Pharmacy, University of Maiduguri.

Experimental procedure

Anaesthesia

The rats were physically restrained and anaesthetized using a combination of Xylazine (2% Xyl-M2, VMD n.s/s.a, Arendonk, Belgium) and Ketamine hydrochloride (5% Ketamin, Vital healthcare pvt. Ltd, Mumbai, India) at a dosage of 5mg/kg and 50mg/kg respectively. They were then placed on dorsal recumbency to expose the ventral abdominal area. The anaesthetic agents were administered intra-peritoneally on the mid caudo-ventral abdominal region to achieve anaesthesia.

Wound Creation

The middle of each rat's back was shaved and cleaned with 0.3% chlorhexidine gluconate (0.3% w/v Purit® antiseptic, Saro lifecare Ltd, Ibadan, Nigeria). solution. The surgical site was then disinfected with 70% isopropyl alcohol (70% Gauze®, Gauze Pharma and Lab Ltd, Awka, Anambra, Nigeria). A sterilized plastic washer of 15mm diameter was placed on the surgical site of each rat, and a full-thickness circular incision was made with a surgical blade around the washer to remove the skin. Digital pressure with gauze was used to stop bleeding. Initial measurement of excision site and after excision shown in plate 1 below.

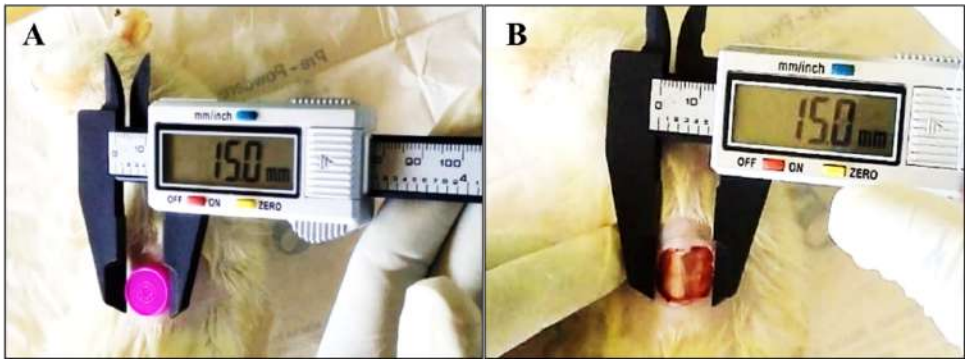

Figure 1: Initial measurement (A), Wound creation (B)

Experiment

Group A served as the negative control group, where the wound was left to heal without any topical agent application. Groups B and C served as the positive control groups, where the wounds were treated with oxytetracycline spray (Terramycin®, Zoetis Ltd, Sandton, South Africa) and povidone iodine (10% Povidone Iodine®, Sirmaxo chemicals pvt. Ltd, Maharashtra, India), respectively. Group D served as the test group, where the wounds were treated with hyaluronic acid serum (Laikou®, Shantauwesheda cosmetics co. Ltd, Guangdong, China). The wound healing process was observed and scored daily; the topical agents were applied for 10 days. Wound contraction rate was measured every 48 hours using a digital composite caliper (Electronic digital caliper®, mitech metrology co Ltd, Gwangzhou, China) for 18 days after the surgery for % mean change in diameter, dryness, borders and crust formation.

Wound Scoring

Diameters of wounds created were recorded on days 0,2,4,6,8,10,12,14,16 and 18 using a digital composite caliper. Mean percentage change in diameter for each group was calculated by the formula below.

A unique wound scoring system described by Jamadagni *et al*. (2016) was adopted and used in this study (Table 1).

$$\frac{\text{(Initial Wound Size – Current Wound Size)}}{\text{Initial Wound Size}} \times 100 = \%\ \text{Mean change in diameter}$$

Table 1: Wound Healing Scoring System

Observations	Score
Dryness	
▪ Fresh/heavy blood or pus on wound surface: Initially, fresh blood oozes out of wound which later stops due to hemostasis.	1
▪ Blood or pus on wound surface (less bleeding than score1).	2
▪ Apparently dry but blood or pus oozes on moping: Pus is formed under a thin layer of crust.	3
▪ Little dry or watery discharge.	4
▪ No blood or completely dry surface	5
Borders	
▪ Initially, borders are swollen due to inflammation and hemorrhages.	1
▪ In case of infection of wound, the borders are moist and rose with blood or pus. A scanty crust formation may take place at the borders.	2
▪ Proliferation of granulation tissue is seen at the borders.	3
▪ Crust formation has taken over the borders. Hence, borders are uneven and dry.	4
▪ Dry and sharp borders. No major abnormality visible	5
Crust formation	
▪ Initiated at the borders (primary or secondary crust)	1
▪ Apparently dry surface but occupied by scanty crust formation.	2
▪ Crust with folds/primary crust/crust not so hard is visible (improved as compared to score 2)	3
▪ Hard crust about to fall. This crust may be removed during observations (improved as compared to score 3)	4
▪ Secondary crust formation	5

Histological Assessment of Wound Healing

An elliptical section of skin was removed during surgery at the point where the incision sites were joined to assess the healing process. The sample was collected in a sterile manner, preserving the epidermis and dermis in formalin. The wound edges were embedded in paraffin and cut into thin sections. The sections were then stained with hematoxylin and eosin to examine the morphology. Computer software, ImageJ, was used to measure the amount of collagen present 18 days after the procedure.

15mm diameter circular wounds were created on the experimental animals with a margin of error of +1 to +2 in 2 rats in group A (negative control) and group D (test group) due to the nature of the skin. The wounds were allowed to heal on their own in all groups. Hyaluronic acid serum was applied to the Test group and Povidone iodine and Oxytetracycline spray were applied to the positive control groups for a period of ten days.

Data Analysis

Data obtained were presented as mean ± standard deviation/percentage mean and analyzed using one way analysis of variance (ANOVA) with GraphPad prism version 6.0. Values of $p < 0.05$ show statistical evidence that there is a difference between the data in question under a 95% confidence interval.

RESULTS

Wounds experimentally created on days 0, 4, 10, 14 16 and 18 of each group are presented in Figure 2 below. At day 0, all wounds created were moist with fibrin seals. After application of Oxytetracycline spray, povidone iodine and hyaluronic acid to groups B, C and D respectively, group B and C wounds appear drier after 2 hours compared to D retaining its moist appearance. At day 2, wound borders in groups A, B and C were discrete while indistinct in group D. Wound contraction rates in all groups were distinct and relatively comparable in groups A, B and C while group D wounds contracted more at days 4, 6 and 8. At day 10, group A wound has a diameter of 9.7mm, B and C having 7.1mm and 6.5mm respectively while D has a better contraction diameter of 4.7mm in diameter. By day 12 to 14, contraction rates in all the groups were half the values compared to the last 4 days. At day 16, group D wounds contracted fully leaving a scab while wounds in groups A, B and C were having values of 3.2mm, 2.9mm and 2.0mm in diameter respectively. Wound healing was complete in group D rats while Scabs were seen on wounds in groups A, B and C respectively.

Wounds mean contraction diameters recorded on days 0,2,4,6,8,10,12,14,16 and 18 were expressed as total mean percentage for each rat. The total mean percent change in contraction is given on Table 1. Test group (D) has a mean total contraction rate of 35.02% which differs significantly ($p<0.05$) compared to the negative control group (A) with a mean total of 21.82% and the positive control group 2 (C) with a mean total of 23.52%. Positive control group (B) has a mean total contraction rate of 27.98% which differs significantly ($p<0.05$) compared to negative control group (A).

Figure 2: Macroscopic presentation of wound contraction from day 0 to day 18 in rats.

Table 1: Total mean percent change in wound contraction rates from day 0 to 18 in Wister rats post intervention. All data were pulled from day 0 to 18 per group.

	Contraction rate (%) Mean±SD			
	A	B	C	D
Total %	21.82±3.7[a]	27.98±4.7[bc]	23.52±2.9[ac]	35.02±3.4[b]

P<0.05 (p=0.0058, p=0.0004) [abc]= Level of significance
(A=Negative control, B=Positive control 1, C=Positive control 2, D=Test, n=5)

Based on Jamadagni *et al*. (2016) wound scoring system, the results are presented in Figures 3, 4, and 5. The Test group's wound dryness had a mean score of 4 at 0-6 days and increased to 5 at 13-18 days, while the negative control group had the lowest mean score of 1.5 at 0-6 days and peaked to 2 at 13-18 days. The mean wound dryness of the negative control group differed significantly from the test group at 7-12 days (p=0.0082). The positive control groups 1 and 2 had initial dryness mean scores of 1.8 and 2, respectively, and reached their highest levels of 3.1 and 3.3 at 0-6 and 13-18 days. In all groups, the mean wound dryness varied significantly within each group at designated day intervals (p<0.05) (Figure 3). The Test group's wound border appearance had a mean score of 2.4 at 0-6 days, which decreased to 5 at 13-18 days. The positive control groups 1 and 2 had initial mean scores of 2.2 at 0-6 days, which later increased to 4.6 and 4.8 at 13-18 days, respectively. The negative control group's wound borders had an initial mean score of 2 at 0-6 days and peaked at 4.5 at 13-18 days. In all groups, the mean wound border

appearance varied significantly within each group at designated day intervals ($p<0.05$) (Figure 4).

The crust formation on the wounds during healing presented an initial mean score of 2 on days 0-6 and peaked to 5 at 13-18 days for the test subjects. The positive control groups 1 and 2 had initial mean scores of 1.5 and 1.7 at 0-6 days and peaked to 4.6 and 4.8 at 13-18 days, respectively. The negative control group had an initial mean score of 1.4 at days 0-6 and peaked to 4.5 at 13-18 days. In all groups, the mean wound crust formation varied significantly within each group at designated day intervals ($p<0.05$) (Figure 5).

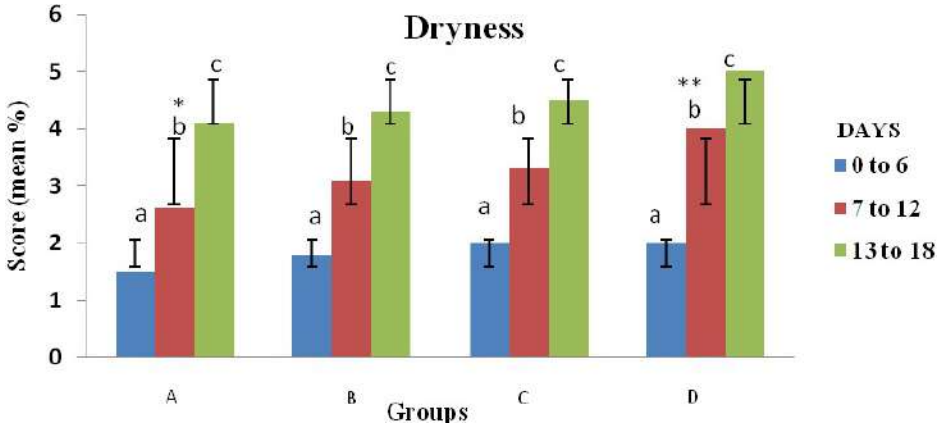

Figure 3: Wound mean dryness variations score (A=Negative control, B=Positive control 1, C=Positive control 2, D=Test). *p=0.0028, a,b,c=significant difference ($p<0.05$) within groups %.

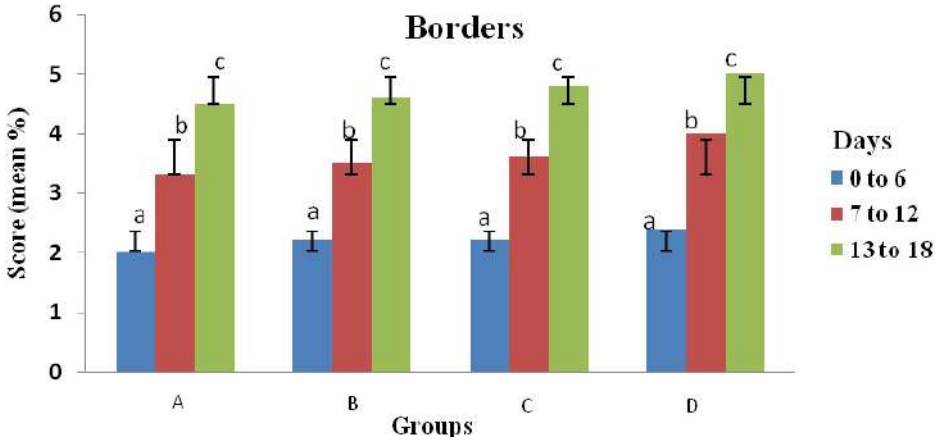

Figure 4: Wound mean borders variations score (A=Negative control, B=Positive control 1, C=Positive control 2, D=Test). a,b,c=significant difference ($p<0.05$) within groups %.

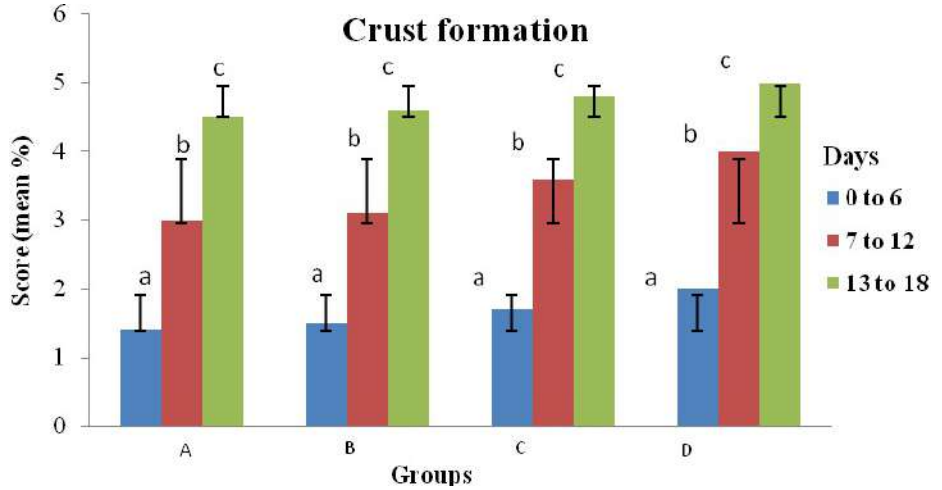

Figure 5: Mean crust formation variations score (A=Negative control, B=Positive control 1, C=Positive control 2, D=Test). a, b, c=significant difference ($p<0.05$) within groups %.

Histological Assessment of Wound Healing

An elliptical section of skin was removed during surgery at the point where the incision sites were joined to assess the healing process. The sample was collected in a sterile manner, preserving the epidermis and dermis in 10% formalin. The wound edges were embedded in paraffin and cut into thin (5µ) sections. The sections were then stained with hematoxylin and eosin to examine the morphology at X100, X400. Computer software, ImageJ, was used to measure the amount of collagen present 18 days after the procedure. Figure 6 (A) shows a high level of fibroblasts and fibrin with a limited amount of collagen, (B) shows a moderate amount of collagen with less fibrin and improved collagen production, (C) shows a high amount of collagen, consistent with type III collagen, and (D) shows a large amount of collagen, similar to type I collagen."

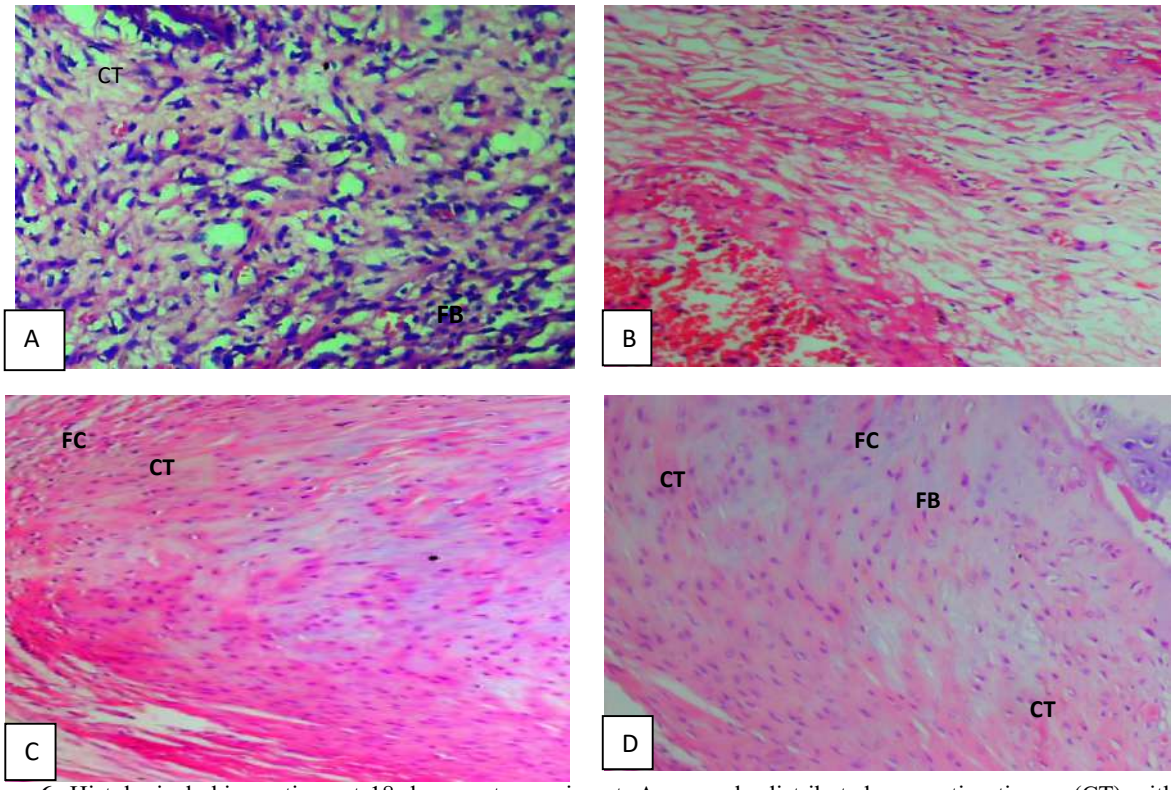

Figure 6: Histological skin sections at 18 days post experiment, A; sparsely distributed connective tissues (CT) with low collagen deposition and presence of numerous fibroblast (FB), B; moderate connective tissue (CT) distribution with presence of few fibroblast (FB), C; dense collagen and connective tissue (CT) with mature fibrocyte (FC) and D; fully formed fibrocytes (FC) and densely distributed connective tissue (CT).

DISCUSSION

Healing occurred without complications in all groups except for excessive crust formation in one rat in the negative control group and another in the positive control group 2. The rate of healing was evaluated using various methods for 18 days. The test group, where hyaluronic acid serum was applied, had a faster healing rate compared to the positive control groups and the negative control group. This agrees with findings of Leite and Frade (2021) where they compared hyaluronic acid with saline and chlorhexidine on skin abrasion in rats. The negative control group had the slowest healing rate, while the positive control group 1 had a better healing rate than the positive control group 2. The test group maintained a moist appearance on the wound surface compared to the positive controls and negative control, which appeared dry and glassy. This is because wounds tend to heal faster in a wet or moist environment, as it promotes faster re-epithelialization and reduces scar formation. This was demonstrated by Junker *et al*. (2013). The wounds in the test group also healed completely on day 18 faster than the other groups without scar formation. This may be due to hyaluronic acid's ability to activate keratinocytes, which are vital in the epithelialization process. Reports by Kawano *et al*. (2021) revealed that wounds treated with hyaluronic acid led to decreased scarring. The mean wound contraction rate for the test group, where hyaluronic acid serum was applied, was faster than the negative control group (A) and positive control group 2 (C), but not different from positive control group 1 (B). This is because hyaluronic acid is a major component of the extracellular matrix of the skin and improves re-epithelialization, granulation tissue formation, and angiogenesis (Leite and Frade *et al*., 2021). Moreover, hyaluronic acid's hydrophilicity, porosity, and swelling support exudate absorption, cell migration, and proliferation. The faster reduction in the mean contraction rate in the test group (D) may be due to these properties (Litwiniuk *et al*., 2016) and thus possess a superior healing power compared to the negative and positive control groups (A, B, and C).

Crust formed on wound surfaces of groups A, B and C on days 6 to 12 appeared more bulkier compared to a thin crust formed on group D. This agrees with findings of Leite and Frade (2021) where crust formed on wound abrasions in rats treated with hyaluronic acid was less compared to crust formed on abrasions treated with saline and chlorhexidine solution. While wound surfaces in groups A, B and C lost their moisture contents appearing dry and glassy with discrete borders, group D wounds retained their moisture contents appearing moist with indefinite borders throughout the healing process. This finding agrees with properties of hyaluronic acid on wounds as described by Alven and Aderibigbe (2021); Papakonstantinou *et al*. (2012).

Histologically, skin sections in group D showed fully formed fibrocytes with dense collagen connective tissues suggestive of type III collagen compared to skin sections in group B and C with less fibrocytes and collagen deposition while skin sections in group A has scanty fibrocytes and sparse collagen tissues suggestive of type I collagen. This finding agrees with findings by Toole (2004) and Li *et al*. (2006) where hyaluronic acid has been shown to provide a framework for angiogenesis and fibroblast migration creating the environment for collagen deposition and wound maturation indicating healing.

Conclusion

In this study, it was observed that the hyaluronic acid serum had a greater ability to promote wound contraction and therefore better healing of wound created compared to other topical treatments in rats. Additionally, the use of oxytetracycline spray resulted in better wound healing outcomes than povidone iodine and the negative control group.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contribution

AMA conceived this work, HPM DL and DLM participated in sampling and data analysis, AII and GB were involved in the laboratory work. All authors read and approved the final draft of the manuscript.

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