Original Research Article

Comparative Study of Unfractionated Heparins and Low Molecular Weight Heparin on Skin Wound Repair

ABSTRACT

Aims: In this study, a comparison was made among the effects of unfractionated heparin from bovine (bovHP) and swine (swi.HP) sources and low molecular weight heparin (LMWH) on skin lesions treatment.
Study design: Wounds were induced on the back of Swiss mice using a punch. Then, the wounds were treated with different heparins for 3 and 7 days.
Place and Duration of Study: Institute of Biomedical Sciences and Animal Breeding Networkand Rodents of the Federal University of Uberlândia, between Abril 2018 and February 2020.
Methodology: Wound closure was performed with a digital caliper. The inflammatory infiltrate as well as the processes of angiogenesis and fibrogenesis were also evaluated.
Results: None of the heparins had a healing effect. Swi.HP showed delay in wound closure and intensification of the inflammatory process compared to bov.HP. Heparins showed a similar response to mast cell infiltrate and angiogenesis. Swine and bovine heparins showed pro-angiogenic activity, although this did not differ between them. Difference between the groups of heparins was observed in relation to the deposition and organization of collagen fibers, with a reduction in fibrogenesis in wounds of the LMWH group compared to the other heparins, especially bov.HP. A better fibrogenic activity was observed for bov.HP.
Conclusion: Thus, we conclude that the source of the heparin used should be considered, as its pharmacological response for wound treatment is quite diverse. Although none of the heparins was able to accelerate wound closure, bov.HP had an anti-inflammatory and pro-fibrogenic effect compared to the other heparins and therefore showed the best response in this experimental model.

Keywords: Bovine heparin, Collagen, Glycosaminoglycan, Healing, Porcine heparin

1. INTRODUCTION

Heparin (HP) is a glycosaminoglycan-sulfated polysaccharide [1] and its chemical and pharmacological structure has aroused interest in the treatment of skin wounds. This fact has been encouraged by studies that describe its affinity for elements of the extracellular matrix (ECM), such as collagen, adhesive molecules and growth factors that promote the chemotaxis of fibroblasts, endothelial cells and keratinocytes [2]. HPs are known to have anti-inflammatory and pro-angiogenic properties that can improve the reconstruction of damaged tissues [2,3].

Commercially available HPs in unfractionated form, when they are isolated and extracted from the intestinal mucosa of swine or bovine lung tissue and as low molecular weight heparin (LMWH) [4]. The diversity of HP extraction sources and the chemical diversity of its structural chain result in different clinical responses [5,6]. This contributes to the controversial results found in the literature. For example, while there are indications that LMWH has no positive effect on the healing of diabetic feet [7], another study concludes that LMWH improves healing when compared with unfractionated heparin [8]. This last work does not describe the source of unfractioned heparin. The same happens with a clinical study that indicates the use of commercial heparins for the treatment of burns, without mentioning their source [9].

Unspecified treatments compromise future HP applications and reduce the fidelity and reproducibility of comparative studies. Thus, this study aims to compare the effects of topical use of bovine, swine and LMWH heparins on wound healing with the aim of understanding the diverse action of these treatments.

2. MATERIAL AND METHODS

2.1 Animal experimentation

This project has been approved by the Ethics Committee on the Use of Animals (CEUA) of the Uberlândia Federal University/Minas Gerais, under the protocol: CEUA nº 045/17. The effectiveness of these drugs were assessed by creating four wounds on the back of 64 Swiss mice, 9 weeks old.

The animals were anesthetized intraperitoneally with Ketamine (80 mg/kg) and Xylazine (5 mg/kg), followed by trichotomy and asepsis of the dorsal region with 70% v/v alcohol. Then, 4 circulares dorsal lesions were induced with the aid of a 5 mm diameter dermatological metallic punch. The skin was removed so that the dorsal muscular fascia was exposed.

2.2 Obtaining the heparins

Unfractionated heparins of bovine origin (ExtrasulExtractosAnimais e VegetaisLtda - Lot: 000392) and swine (Blau Farmacêutica S.A - Lot: 15090326) and LMWH (Henoxaparin - Lot: 16070822) were supplied by Professor Mauro Pavão of the Federal University of Rio de Janeiro.

2.3 Treatment groups

The wounds of 32 mices were topically treated for 3 days, and 32 mice's wound were topically treated for 7 days, once a day. The 32 animals were randomly distributed into 4 groups with 8 animals each. Thecontrol group (CO) received treatment with only 30% vaseline / 70% lanolin.The HPbov group received topical application of bovine heparin, the HPswigroup's wounds were treated with swine heparin and one group was treatment with low molecular weight heparin (LMWH). To prepare the HP gel we incorporated 0,175mg HP/g vaseline 30% and lanolin 70% gel. The dosage used in each group was 2mg/kg.

2.4 Contraction of wounds

Images of the wounds on days 0, 3 and 7 days were obtained with a camera (Sony Cybershot 14,1 DSC W320). The major and minor diameter of the area of the 4 wounds of each animal was measured daily. These measurements were then used to calculate the percentage of wound closure using the following equations:

Wound area = (smaller diameter /2) * (larger diameter/2) * π and area measurement (%) =% wound closure = [1-(Af)/(Af0) X 100], where: Af is the wound area on the evaluated day; Af0 is the initial wound area [10].

2.5 Obtaining samples

The animals were euthanized with deepening anesthetic with 100mg/kg Thiopental (Thiopental®) at the end of the third and seventh day of treatment. The dorsal skin containing the 4 lesions was completely removed with the aid of scissors and then each lesion was removed with an 8 mm surgical punch to ensure total capture of the injured area. After obtaining, samples with the wound area were placed in an ultra-freezer at -80°C for further biochemical analysis. For histological evaluation, samples were fixed in metacarn (methanol, acetic acid and chloroform in the ratio of 6: 3: 1 respectively) and subjected to histological processing.

2.6 Myeloperoxidase (MPO) activity

Myeloperoxidase activity was determined by the technique of Bradley et al., (1982) [11]. Samples were homogenized and, 300µL of it was transferred to microtubes. On these tubes was added 600µL HTAB (Hexadecyltrimethylammonium Bromide - Sigma) 0.75% w/v diluted with pH 6 phosphate buffer. Samples were sonicated for 10 seconds and centrifuged at 5000 rpm for 10 minutes at 4 ° C. The supernatant (200µL) was used for enzyme assay. The reaction followed the orden: 100µl of 0.003% hydrogen peroxide; 100 µl of TMB (3,3', 5,5' tetrametilbenzidine - Sigma) diluted to 6.4 mM in DMSO (dimethyl sulfoxide - Merck) for 1 minute. It was added 100 µl of H2SO4 (sulfuric acid - Merck) 4M to stop the reaction. Then, 200µL were added to the 96 well plate and held in spectrophotometric reading at 450nm. The results were expressed in MPO content (Absorbance at OD/g wet weight of the skin).

2.7 N-acetyl-β-D-glicosaminidase (NAG) activity

N-acetyl-β-D-glicosaminidase lysosomal enzyme is produced by activated macrophages. This technique is based on hydrolysis of nitofenil-p-N-acetyl-β-D-glicosamine (substrate) by N-acetyl-β-D-glicosaminidase, releasing p-nitrophenol [12]. To the samples were added 2.0 mL of saline and 0.9% Triton X-100 (Promega) 0.1% (cream). The samples were homogenized and centrifuged at 3,000 rpm for 10 minutes at 4°C. They were diluted in buffer citrate/phosphate. 100µL from this solution, was replaced, in duplicate, per well (ELISA 96-well plate). Then, added 100 µL of substrate (p-nitrophenyl-N-acetyl-β-D-glicosaminidase Sigma) diluted in citrate/phosphate buffer, pH 4.5 and incubated at 37 for 30 minutes. Finally, 100 µL of 0.2M glycine buffer pH 10.6 were added into the samples and into the curve. The absorbance was measured by microplates reader at 400nm. NAG activity was calculated from a standard curve of p-nitrophenol. Results are expressed in nmol.mL-1/mg wet weight of the skin.

2.8 Hemoglobin dosage (Hb)

Hemoglobin content allows indirect evaluation of neovascularization. The skins were homogenized by Ultra Stirrer homogenizer (80 ultramodel – 8.000-30.000 rpm) in 2.0mL of a specific chromogenic reagent for hemoglobin (Drabkin reagent kit Hemoglobin dosage - LABTEST) [13,14]. The samples were centrifuged for 40 minutes at 10,000g at 4°C and, the homogenates were filtered through 0.22µm millipore filter (Hydrophilic Membrane GV-DURAPORE) attached on filter support (SWUINNY - stainless steel for syringe). A 200µL aliquot of samples and standards were added to a 96 well plate, in duplicate, read on spectrophotometric at a wavelength of 540nm. The hemoglobin concentration of each

sample was calculated from a known standard curve (Labtest) and the results expressed as concentration of hemoglobin (µg) per milligram wet weight of skin.

2.9 Histological analysis

After fixation in metacarn and histological processing, 5 µm sections were obtained from each sample. Histological analyzes were performed for general tissue evaluation on slides stained with hematoxylin and eosin (H.E). For the quantification of mast cells, blood vessel diameter and collagen, the slides were stained respectively with toluidine blue, gomori trichrome and picrosirius red staining [15]. Sections stained with picrosirius red were examined under polarizing (collagen fiber differentiation) and non-polarized (total collagen) microscopy under a Nikon eclipseTi microscope with an optical camera attached. For this analysis, a 20X objective was used. For the analysis of mast cells and blood vessels, photomicrographs were obtained with a 40X objective in a Leica Microsystems Inc microscope. (Wetzlar). For all analyses, 10 different areas were captured and the measurement was performed in the Image J program [16].

2.10 Statistical analysis

Statistical analysis and graphs were performed in Prism program 5. Control group and treatments were evaluated statistically through mean, standard deviation, one-way ANOVA and Bonferroni post-test. Statistical difference was considered for$P \leq 0.05$.

3. RESULTS

3.1 Swine heparin promotes delayed wound closure and intensifies the inflammatory process

During treatment, only swine heparin changed the time required for wound closure. Macroscopic images of the wound closure follow-up are shown in the figure 1A,while the graph representing the percentage of closing can be found in **figure 1B**. After 3 days, the animals in the swi.HP group (22.7±4.3) showed delayed wound closure compared to the CO (46.0±3.5; $P \leq 0.05$) and bov.HP (40.9±2.0; $P \leq 0.01$).This delay in healing continued after 4 days of treatment, with values of 39.8±3.6 for swi.HP, 58.4±2.5 for CO ($P \leq 0.05$) and 59.4±3.1 for bov.HP groups ($P \leq 0.01$).The delay in wound closure was also observedin swi.HP after 5 days, with 55.3±3.7, value lower compared to the closure observed in wounds in the CO group (68.6±1.4) ($P \leq 0.01$).

The inflammatory process was measured by MPO and NAG markers, indicating, respectively, the activity of neutrophils and macrophages. There was no significant difference on MPO activity in the bov.HP and LMWH groups compared to the control after the third and seventh day of treatment. On the other hand, wounds treated with swi.HP (119.6±31.7) showed higher MPO activity than the values found for the bov.HP(12.6±9.7) and CO (11.69±9.7) groups after 3 days of treatment. After 7 days, the increase in this activity was maintained in swi.HP (48.0±15.0) only when compared to CO (5.7±3.7), therefore, there was no difference between swi.HP and bov.HP**(Figure 1C).** In all evaluations for MPO the *P*value was 0.05.

NAG activity was reduced in bov.HP (14.1±1.8) after the third day of treatment, when the control showed a value of 27.7±2.1. Macrophage activity in wounds treated with swi.HP (24.5±3.4) was higher than the value observed for the CO group (12.4±1.4), after 7 days of treatment. In this period, swi.HP also showed greater macrophage activity in relation to

bov.HP group (12.4±2.0) **(Figure 1D).** For all assessments of NAG activity, statistical difference was observed with *P*≤0.01.

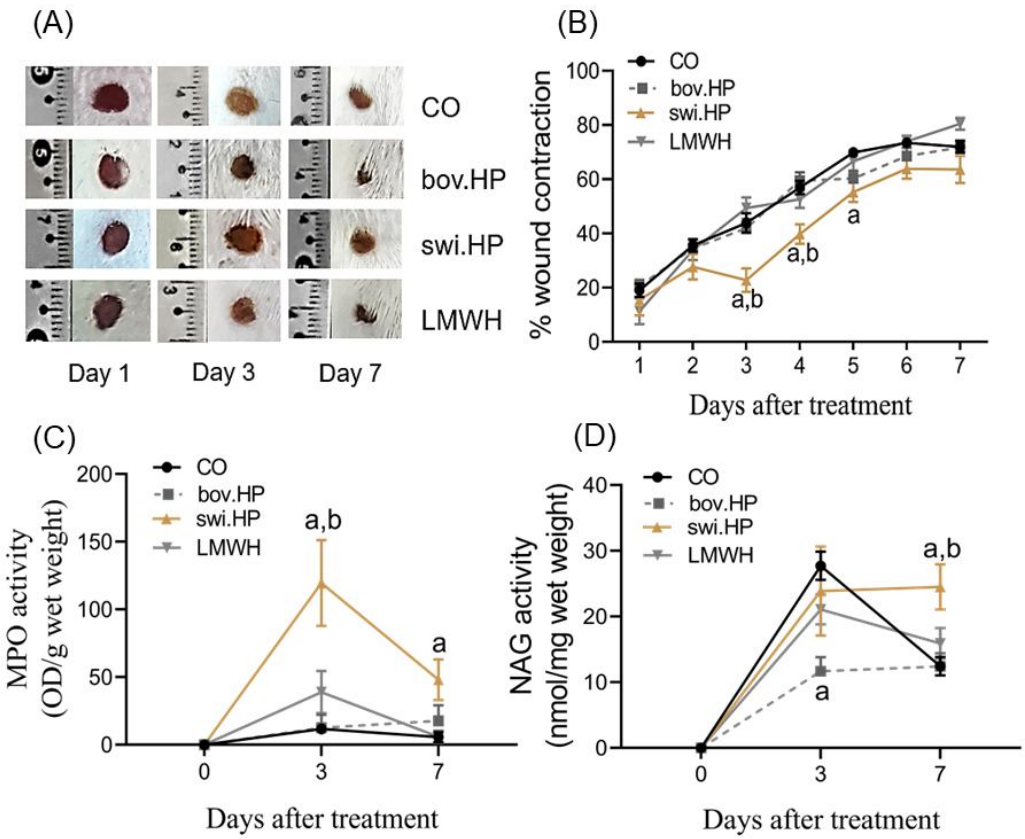

Figure 1. Wound contraction and inflammatory process in wounds treated. (A) Macroscopic images of wound closure and (B); graph of wound contraction percentage (C); MPO activity (neutrophils) and (D) NAG activity (macrophages). The group are bovine heparin (bov.HP), swine heparin (swi.HP) and low molecular weight heparin (LMWH). (a) represents statistical difference in relation to the CO group and (b) represents a statistical difference in relation to the bov.HP group.

3.2 Wounds treated with different heparin show similar results for mast cells and angiogenesis

After the third day of treatment, the number of mast cells in wounds treated with bov.HP was higher (2.8±0.1) than the value found in the CO group (1.2±0.4) (*P*≤0.01). There was no statistical difference between the groups after the seventh day of treatment **(Figure 2A and 3A).**

Angiogenesis was evaluated bydiameter of blood vessels and hemoglobin quantification. In both evaluations, there was a difference in heparins with the control group. However, there was no difference between the heparin groups (bov.HP, swi.HP and LMWH). After 3 days of treatment, the hemoglobin value was higher in the swi.HP group (75.8±20.7) compared to the CO group (6.75±0.6) (*P*≤0.01)**(Figure 2B).** After 7 days, wounds treated with

bov.HPshowed higher hemoglobin values (48.7±9.2) than the values obtained for the control group (22.5±5.1) (*P*≤0.05) **(Figure 2B).**

There was no statistical difference on the diameter of blood vessels in the initial period, 3 days. Larger blood vessels were observed only in the bov.HP group (111.3±1.6) compared to the CO group (100±3.4) (*P*≤.01) after 7 days of treatment **(Figure 2C and 3B).**

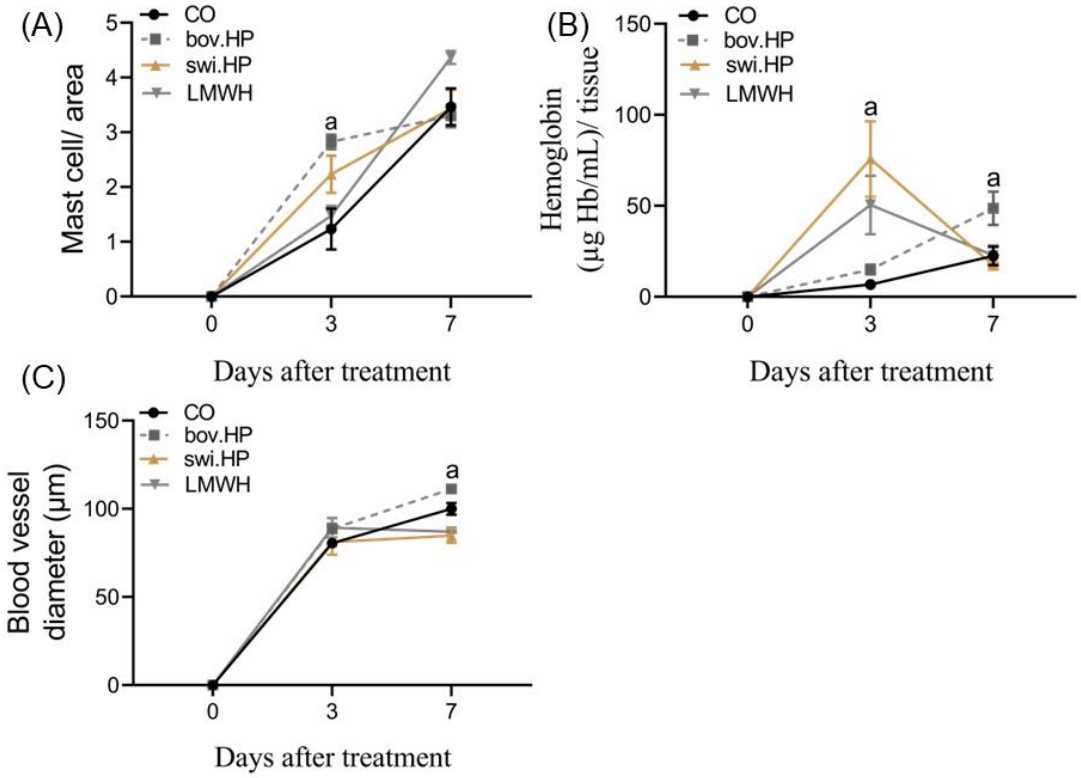

Figure 2. Mast cells infiltrated and angiogenesis. (A) quantification of mast cells; (B) hemoglobin values and (D); blood vessel diameter values in wounds treated with bovine heparin (bov.HP), swine heparin (swi.HP) and low molecular weight heparin (LMWH). The control group is represented by CO. (a) means statistical difference in relation to the CO.

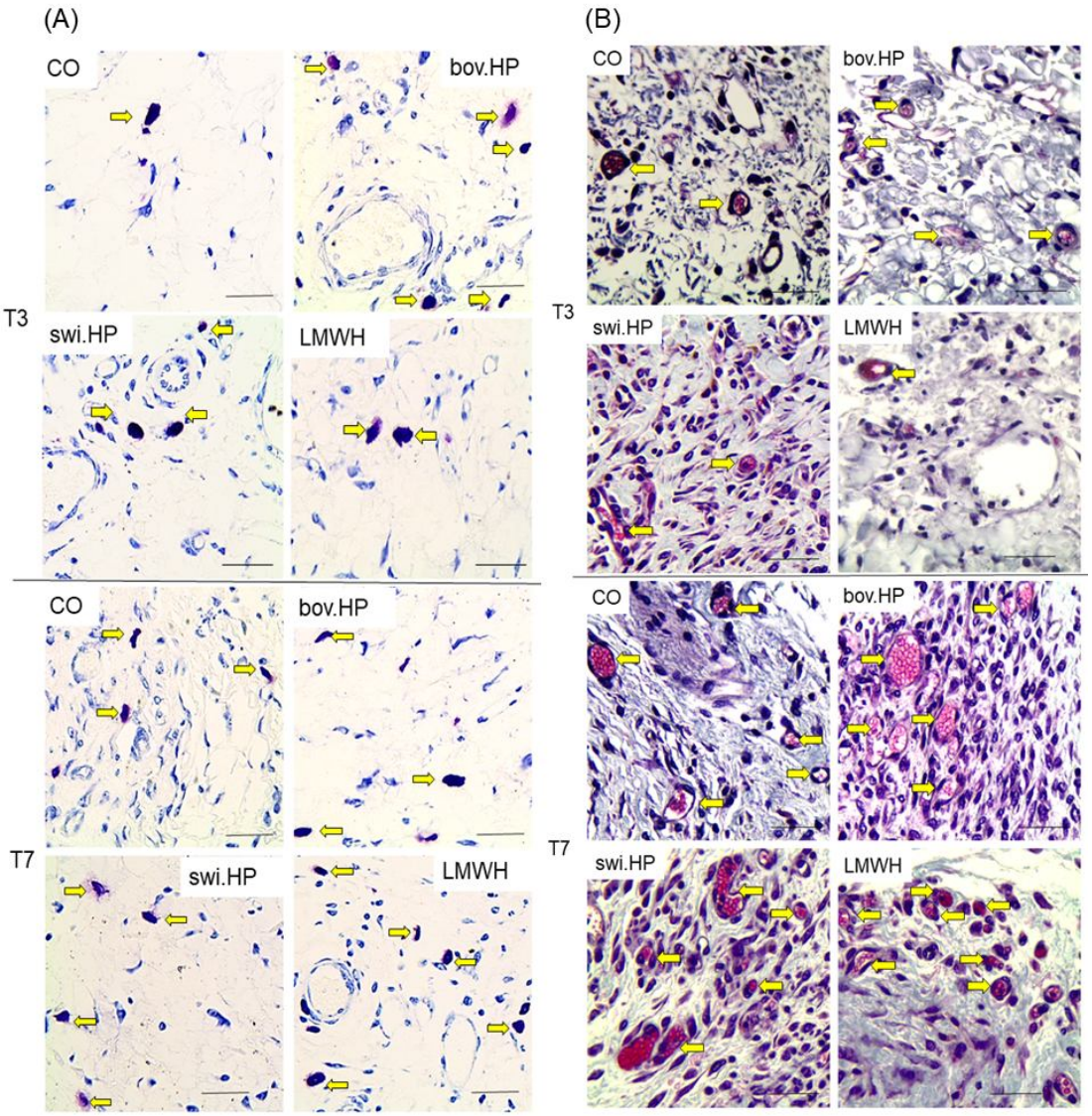

Figure 3. Mast cells infiltrated and angiogenesis. (A) Mast cells and (B); blood vessels indicated by the yellow arrow in photomicrographs of slides stained with toluidine blue and gomori's trichrome, respectively. The images represent areas of wounds treated after 3 (T3) and 7 days (T7). The groups are CO (control), bov.HP (bovine heparin), swi.HP (swine heparin) and LMWH (low molecular weight heparin) (400x magnification).

3.3 Wounds treated with LMWH show reduced collagen deposition in wounds compared to bovine heparin

The organization of collagen fibers was evaluated under polarized light and the immature collagen, recently produced, was called type III collagen and the collagen deposited later, with thicker fibers, was type I collagen. In wounds of 3 days, the treatment with bov.HP

(*P*≤0.05) was able to increase both types of fibers, collagen I (556217±56304) and collagen III (176517±18166) compared to the CO group, which presented respectively 243737±48468 and 70022 ±7845. Both types of fibers were reduced in the LMWH group in the same period, in relation to bov.HP. LMWH showed 122855±25766 (*P*≤0.001) for collagen I and 79988±28907 (*P*≤0.5) for collagen III. Also in this period, type III collagen fibers were also different between the swi.HP and bov.HP groups, with a reduction in the swi.HP group (41786±3431) (*P*≤0.01) **(Figure 4A and 4B).**

With 7 days of treatment, the main difference in relation to the organization of collagen fibers was observed in the LMWH group in which they were reduced. For type I collagen, LMWH (534651±79060) was lower than the CO group (943141±121113) and bov.HP (1014436±55504), both considering *P*≤0.05. Considering type III fibers, LMWH (272378±51664) was reduced when compared to all other groups treatment, CO (673511±41253) (*P*≤0.001), bov.HP (537819±40826) (*P*≤0.5) and swi.HP (554456±55226) (*P*≤0.01) **(Figure 4A and 4B).Figure 4C** shows photomicrographs of wounds used to quantify collagen fiber.

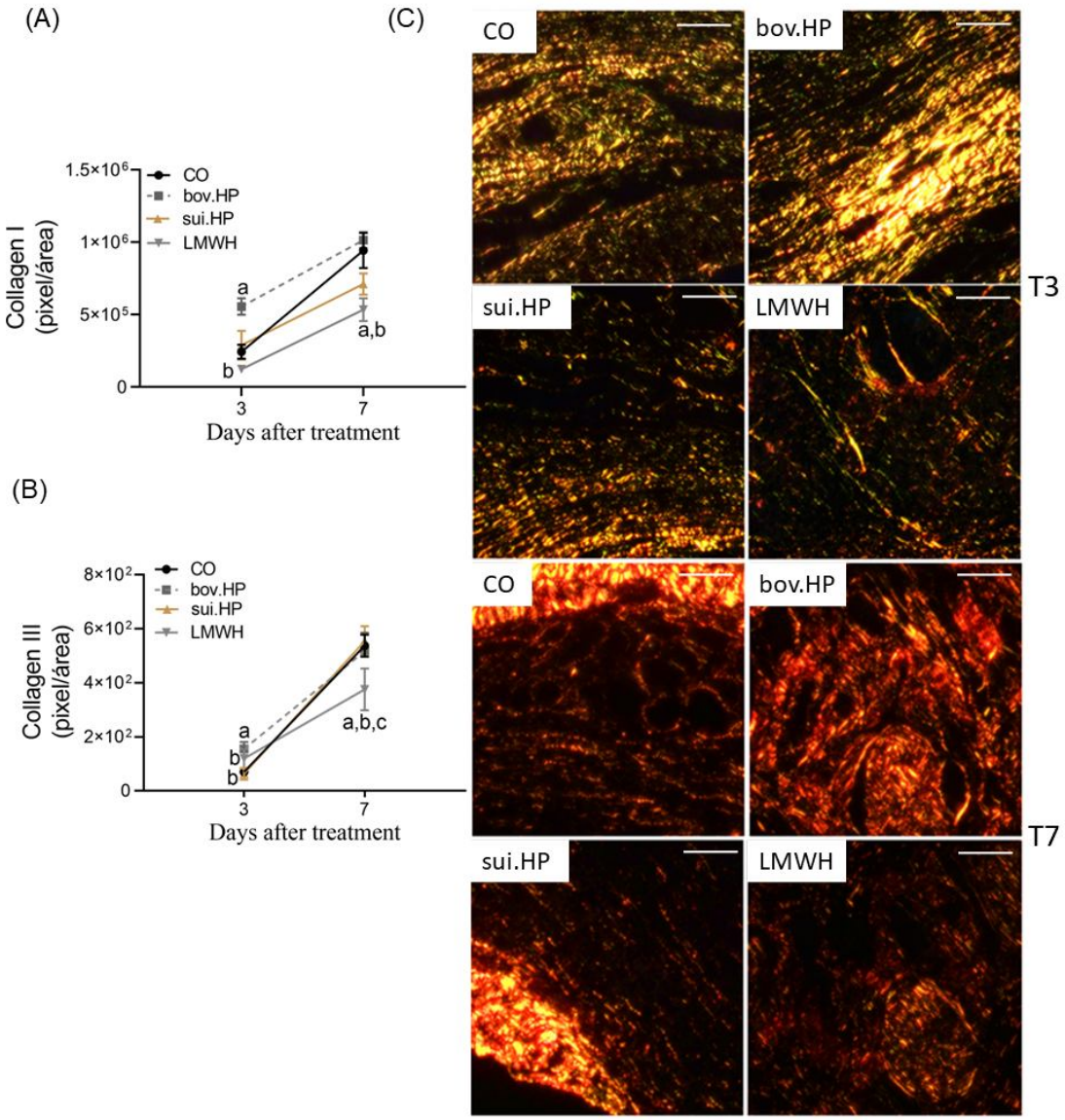

Figure 4. Organization of collagen fibers. Graphs with the quantification in pixels of collagen fibers type I (A); and type III (B) in photomicrographs of slides from groups CO (control), bov.HP (bovine heparin), swi.HP (swine heparin) and LMWH (low molecular weight heparin). Slides were stained with picrosirius red and evaluated under polarized light. Type I fibers are shown in red-orange and type III fibers in green. The overlap of both fibers is shown in orange color (400x magnification).

4. DISCUSSION

Although the use of heparin has shown a positive effect in a clinical trial of burns [9]. In our study, none of the types of heparins were able to accelerate the wound closure process until

the 7th day of treatment. Comparative evaluation demonstrated a delay in the closure of wounds treated with swine heparin compared to bovine heparin.

The effect of heparins on the modulation of the inflammatory process has been reported and its effects usually directly and indirectly mediate several chemical factors involved in inflammation, which may influence the activity of macrophages and neutrophils, activity of growth factors and platelet aggregation [9]. A pharmacological response was also observed differently between bovine and swine heparin, in relation to inflammation. In this sense, wounds treated with swine heparin showed greater inflammatory infiltrate with increased neutrophils after 3 days of treatment and macrophages after 7 days of treatment compared to wounds treated with bovine heparin.

Although the cellular infiltrate plays an important role in the initial repair, the exacerbated and prolonged leukocytes infiltrate can promote the chronic process [17]. From there, these data suggest that swine heparin increases the inflammatory response on skin lesions and promotes a delay in wound closure.

Simultaneously, at the end of the resolution of the inflammatory process, we have the proliferative stage in which one of the events is angiogenesis and fibrogenesis. Neo-angiogenesis has an early onset because inflammatory cells require interaction with blood vessels to enter into the wound [18], as well as their importance in the supply of nutrients and oxygen for the growing tissue [19]. Based on the existing literature, 7 days after injury corresponds to the period of intense angiogenesis [2]. In response to the aforementioned factors, wounds treated with swine heparin resulted in a previous increase in angiogenesis that may have contributed to the greater influx of neutrophils in this period.

Although we observed a pro-angiogenic effect of the evaluated heparins, this response did not differ between them, but only in comparison to untreated wounds. Specifically for swine heparin, this effect has been observed in the study of Long et al. (2017)[20]. They revealed that vascular density was significantly higher in wounds treated with swine heparin compared to healthy tissue, indicating an influx of blood vessels into the wound [20].

At the same time angiogenesis occurs, fibroblasts migrate into the tissue, initiate collagen synthesis and replace the provisional fibrin matrix [19]. Healthy dermis contains 80% of collagen type I and 25% of collagen type III. In the granulation tissue, the lesion expresses 40% of type III collagen [21]. The response of heparin treatments on fibrogenesis was varied. The main difference observed was in the reduction of collagen deposition in in the treatment with low molecular weight heparin in relation to other heparins, especially bovine heparin. In addition to the intense inflammatory response, the reduction in collagen deposition in the swi.HP group after 3 days of treatment may have contributed to the delay in wound closure. The improvement in the organization of type III collagen fibers and also of the late deposition fibers, with greater tension, type I collagen in wounds treated with bovine heparin, reflects a pro- fibrogenic activity of this heparin in relation to swine and low molecular weight heparins.

4. CONCLUSION

Treatment with unfractionated and low molecular weight heparins resulted in a different response in the parameters of wound healing, inflammation and fibrogenesis, with no difference in the angiogenic action. According to our study, none of the heparins evaluated had a positive effect on wound healing. On the other hand, our results do not recommend the use of swine heparin, since this, in addition to not contributing, this resulted in a delay in

the healing process.Factors such as increased inflammatory infiltrate and modulation in collagen production may have contributed to this result. Regarding the positive aspect, we concluded that some bovine heparin had an effect on the organization of collagen fibers. However, this activity was not enough to lead to an improvement in healing.

ETHICAL APPROVAL (WHEREEVER APPLICABLE)

“All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee”

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