Effect of *Brassica oleracea* in rats skin wound healing[1]

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ABSTRACT

PURPOSE: To investigate the effect of *Brassica oleracea* herbal balsam on the healing of skin wounds in rats.

METHODS: Twenty four rats (Wistar, 60 days, 250 g) were divided into four groups: untreated animals (C) and treated with the ointment (T), subdivided into two experimental times (seven and 16 days). A 3cm^2 skin wound was made in the back of all animals. 100 ml of the *Brassica oleracea* was applied twice a day in T group. Biometric analysis was made with images captured at one, four, seven, ten, 13, and 16 days. At seven and 16 days, animals of each group were euthanized. The wound area removed was processed for histological and histomorphometric analysis to quantify birefringent collagen fibers. Statistical analysis was made considering $p < 0.05$ as significant.

RESULTS: Biometric analysis revealed no significant differences between groups in both experimental times studied. However, histomorphometric analysis showed that the number of type I collagen fibers was significantly higher in the specimens of the group T16 compared to the other groups.

CONCLUSION: *Brassica oleracea* accelerated the wound healing process increasing the number of type I collagen fibers and the maturity of the newly formed tissue.

Key words: *Brassica*. Wound Healing. Skin. Rats.

Introduction

Chronic wounds are a serious public health problem, affecting about 1% of the world population, irrespective of gender, age or race. Despite a multidisciplinary approach, the management of patients with chronic wounds is a major challenge[1]. In addition to their profound effect on the quality of life of affected individuals, about 2% of the health budget is destined to wound care[2-4]. Successful wound management requires an understanding of the wound healing process and the identification and prevention of factors that may delay or interrupt wound healing[2-5]. Early wound treatment permits to reduce public health expenditure and prevents impairment of the quality of life of affected patients[2-5].

Impaired skin integrity leads to the development of a wound that could involves different tissues, from the epidermis to deeper layers such as muscles[3,4]. Immediately after injury, the blood clots forms a scab that protects the injured area[2]. The consequent release of chemical mediators induces an acute inflammatory response characterized by the presence of neutrophils, followed later by macrophages and lymphocytes[2,6-8]. The activation, proliferation and differentiation of undifferentiated mesenchymal cells give origin to activated fibroblasts present in the granulation tissue[6-8]. These fibroblasts become bipolar and secrete extracellular matrix components, including fibronectin, proteoglycans and types I and III collagen fibers[6-10]. Collagen synthesis is induced hours after injury, but only becomes significant after one week and can continue for 12 to 18 months[9-12].

Studies have shown an economically significant effect of plaster made from the leaves of *Brassica* sp on the healing of skin wounds[2-4,13]. *Brassica oleracea* is an edible vegetable whose leaves are used by the population as a skin wound healing agent. Despite its use in folk medicine, scientific studies of this plant are scarce, a fact that has encouraged the present investigation. The herbal balsam Debridan® was fabricated from the active ingredient of *Brassica oleracea* var. *capitata* – glycolic extract 10% and is indicated for the treatment of skin wounds[14,15].

Biometric analysis permits macroscopic assessment of the efficacy of the repair process based on wound contraction and consequent reduction in the wound area[16,17]. On the other hand, histomorphometry is an excellent method for microscopic analysis of the wound area, and permits to quantify collagen fibrils during the different stages of wound healing[9,10,17-19].

The aim of the present study was to evaluate the effect of *Brassica oleracea* var. *capitata* – glycolic extract 10% on the healing of surgical skin wounds in rats using biometric and histomorphometric analyses.

Methods

The present study was approved by the Research Ethics Committee of University Valley of Paraiba (UNIVAP) under the protocol A25/CEUN2010 and in accordance with Federal Law No. 6.638 and the guidelines of the Brazilian College of Animal Experimentation.

All experimental procedures were conducted at the Laboratory of Photodynamic Therapy, Institute of Research and Development (IP&D), UNIVAP.

Twenty-four *Wistar* rats (*Rattus norvegicus, var. albinus*), 60 days old, with a mean weight of approximately 280 g, obtained from IP&D, UNIVAP. The animals were housed in individual cages at a controlled temperature under a 12-h light/dark cycle and received a standard diet comprising pelleted food (Labina; Purina Nutrients Ltda, Sao Paulo-SP, Brazil) and mineral water *ad libitum*. The animals were transferred to the Laboratory of Photoacoustic, UNIVAP, where the experiment was carried out.

For surgical proceedings the animals were weighed and anesthetized by intramuscular administration of 10% ketamine hydrochloride (Dopalen®, 0.1 ml/100 g body weight) and 2% xylazine hydrochloride (Calmium®, 0.1 ml/100 g body weight). An area of 6 x 4 cm (length x width) was shaved in the dorsal region by drawing an imaginary line caudally from the lower margin of the ear. Antisepsis of the area was performed with 4% alcohol-based iodine. In the center of the shaved area, a surgical skin lesion (Ø=3 cm), 1cm below the bone prominence, was created using a metallic circular marker to delimit the area, and with the aid of a surgical scalpel, the dorsal muscle fascia was exposed. For pain control, animals received aspirin (100mg/kg weight) diluted in water, until euthanasis. The animals were randomly divided into two groups of 12 animals each, subdivided in two experimental times: control and treated animals (C and T, respectively), and the experimental times of seven and 16 days. The wound was cleaned daily with 0.9% saline, followed by topical application of 100ml *Brassica oleracea* var. *capitata* – glycolic extract 10% (Debridan®) twice a day for the treated group (T).

Biometric and histomorphometric analysis

For biometric analysis, images of the skin wounds at one, four, seven, ten, 13 and 16 days after surgery were captured with an Optical SteadyShot DSC-W350 digital camera (14.1 megapixels), and positioned at a distance of 15 cm. The Image J program was applied to digital images, 25 x magnification, to calculate the diameter of the wound area (cm^2).

Six animals of each group (C and T) were sacrificed at days seven and 16, using the anesthetic procedure described above, followed by intracardiac administration of 0.5 ml of potassium chloride. Specimens of the wound area were removed, fixed in 10% formalin, and sent to the Department of Pathological Anatomy, Sao Paulo University Hospital, for slide preparation according to routine procedures. Slices of 5µm were stained with hematoxylin-eosin and picrosirius, and analyzed under a polarized light microscope using the ACT-@U program.

Birefringent collagen fibers were quantified in the dermis (µm^2) using digital images of the histological sections (x400) captured with a Nikon Digital Sight DS-5M digital camera coupled to a microscope (Nikon-Optizon x0.8-2.0). The Image Pro Plus Program has been used to measure the wound area and histomorphometric analysis at x25 magnification.

Statistical analysis

The normality of the data was tested by the Kolmogorov-Smirnov test using the OriginPro® 8.0 SRO program. Differences between groups were evaluated by one-way ANOVA for parametric data (Minitab®), followed by the Tukey post-test. $p<0.05$ was considered to be significant.

Results

Macroscopic biometric analysis

Figure 1 presents the macroscopic aspects of the wounds over the time course of the experiment. No sign of abscess formation in the early phase (seven days) or hypertrophic scars in the final phase (16 days) was observed. The digital images of the wounds permitted to evaluate wound area progression in the groups of the experimental times of seven days and 16 days.

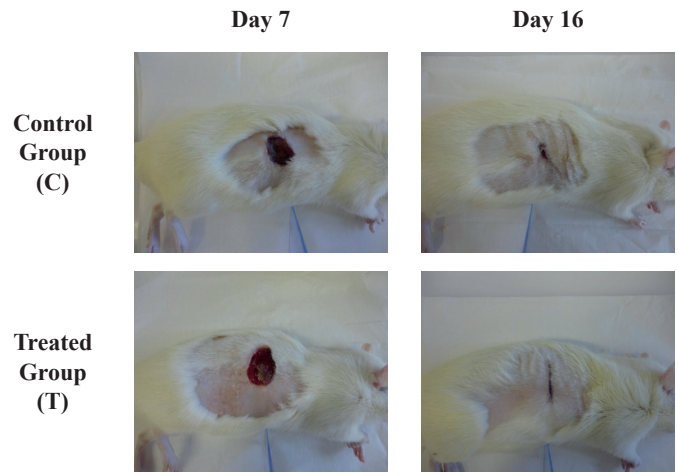

FIGURE 1 - Size of the wound area: control (C7; C16) and treated (T7; T16) groups at seven and 16 days experimental times.

Figure 2 shows the graphic comparative of wound areas between the different groups and experimental times studied. At seven days after surgery, the reduction in wound diameter was greater in group C7 than in T7 group (64.34% versus 55.59%, respectively). The wound was almost completely closed in both groups after 16 days (reduction of 96.7% and 95.85% in C16 and T16, respectively). However, comparison of the biometry results showed no significant differences. It should be noted that the wounds of animals that received herbal treatment were cleaned and treated daily, which may have determined the slight difference observed in the absolute values.

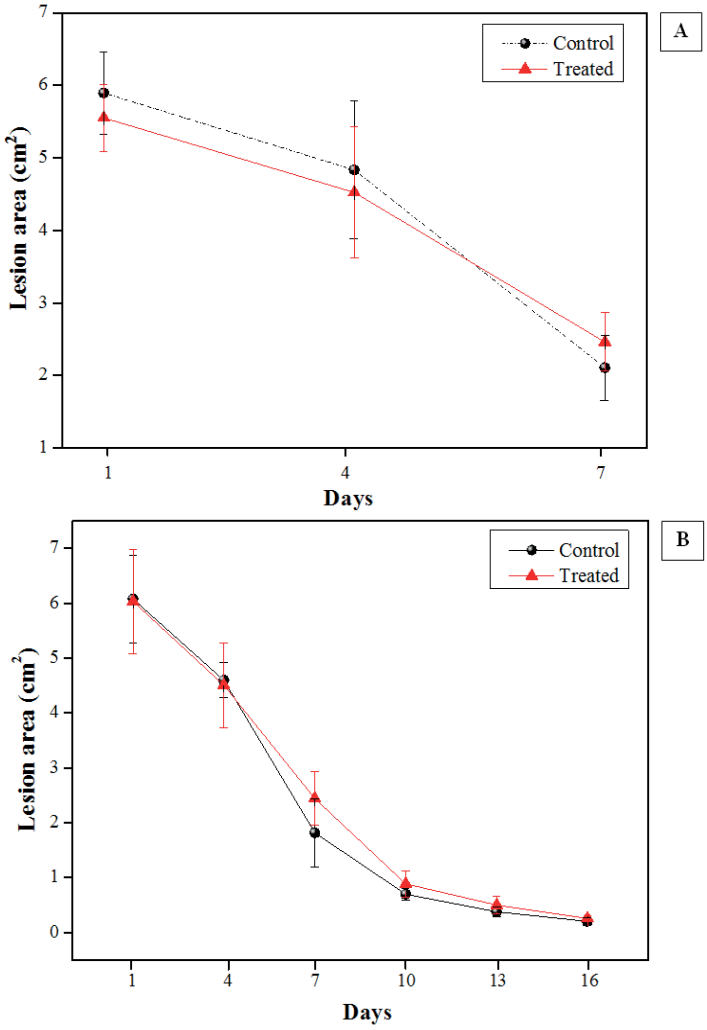

FIGURE 2 - Biometry analysis (cm^2) of the experimental groups: **A**) C7 x T7; **B**) C16 x T16.

Histomorphometry

The wound area (µm), number (µm^2) and distribution (types I and III) of collagen fibers were evaluated in the histomorphometric analysis. Figure 3 and Table 1 show the

absolute values of the wound area. It is possible to observe the lower values in the control groups at seven days (7.83 µm) and 16 days (4.12 µm) compared to the treated groups (T7 = 8.64 µm and T16 = 4.91 µm, respectively). In addition, the best results, i.e., the smallest wound area, were observed for specimens of group C16 (Table 1). However, these results were not statistically significant.

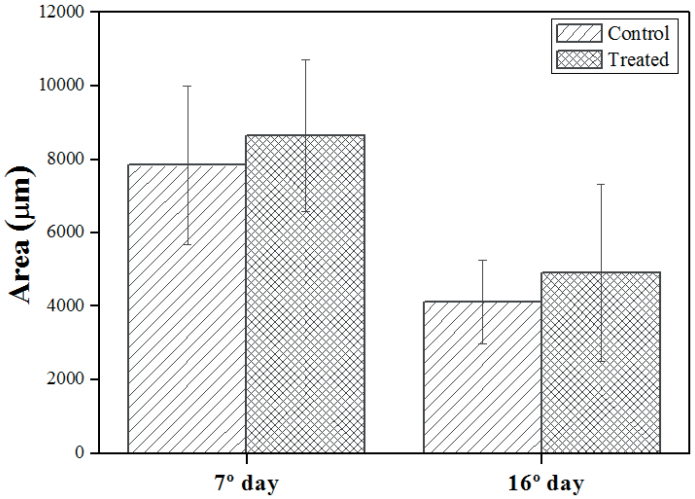

FIGURE 3 - Histomorphometric results of the wound area in the different groups and experimental times studied (x25).

TABLE 1 - Wound area and number of thin and thick collagen fibers in the control and treated groups seven days (C7 and T7) and 16 days (C16 and T16) after surgery.

	Wound area (µm)[a]	Thick fibers (μm^2)[b]	Thin fibers (μm^2)[c]
C7	7.83±2.15	0.042±0.017	0.014±0.013
T7	8.64±2.07	0.035±0.013	0.002±0.003
C16	4.12±1.13	0.058±0.015	0.019±0.018
T16	4.91±2.40	0.096±0.027(*)	0.015±0.015

Results are reported as the mean ± standard deviation
[a]Wound area: x25
[b]Thick collagen fibers: x400
[c] Thin collagen fibers: x400
(*)p<0.05, significant

The number and type of birefringent collagen fibers per μm^2 were determined by polarized light microscopy at the different experimental times studied (Table 1, Figures 4 and 5). Thin (type III) and thick (type I) collagen fibers could be distinguished. Types I and III collagen fibers appeared by day 4 or 5 of wound healing. Differences in the predominant fiber type and in the number of collagen fibers were observed depending on the stage of the tissue repair process.

The number of thin fibers (Table 1, Figure 4) was higher in group C7 (mean: 0.014) than in group T7 (mean: 0.002), indicating a predominance of type III collagen fibers at the beginning of the inflammatory process.

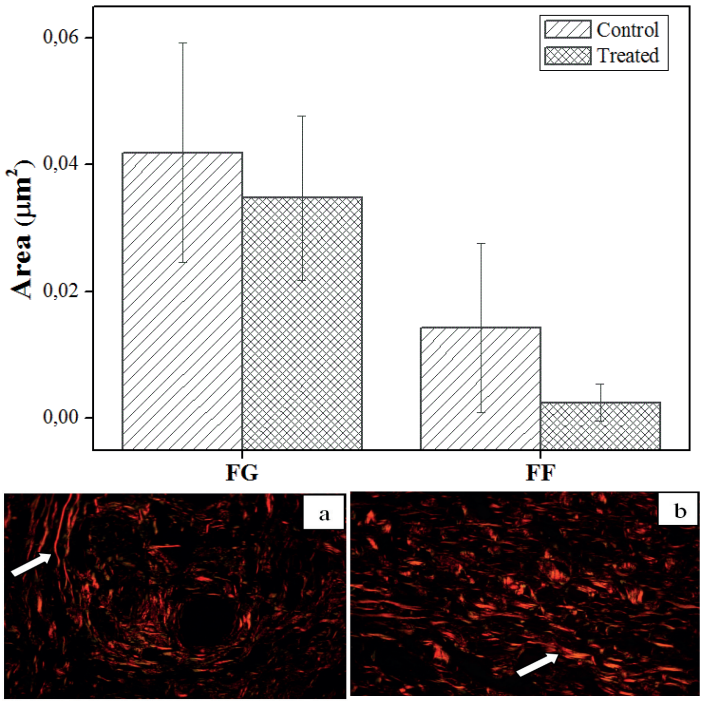

FIGURE 4 - Photomicrographs of thick and thin collagen fibers: seven days experimental time. The arrow indicates: (**a**) thin fibers in the control group (C7); (**b**) thick fibers in the treated group (T7). FG: thick collagen fibers; FF: thin collagen fibers. Picro-sirius, x400.

The predominance of type I collagen fibers (Table 1, Figure 5) was observed on day 16, especially in group T16 (mean: 0.096) ($p<0.05$), when compared to group C16 (mean: 0.058).

The present results indicate a greater tensile strength of the wounds of animals treated with the herbal ointment when compared to the untreated groups (Figures 5a and 5b).

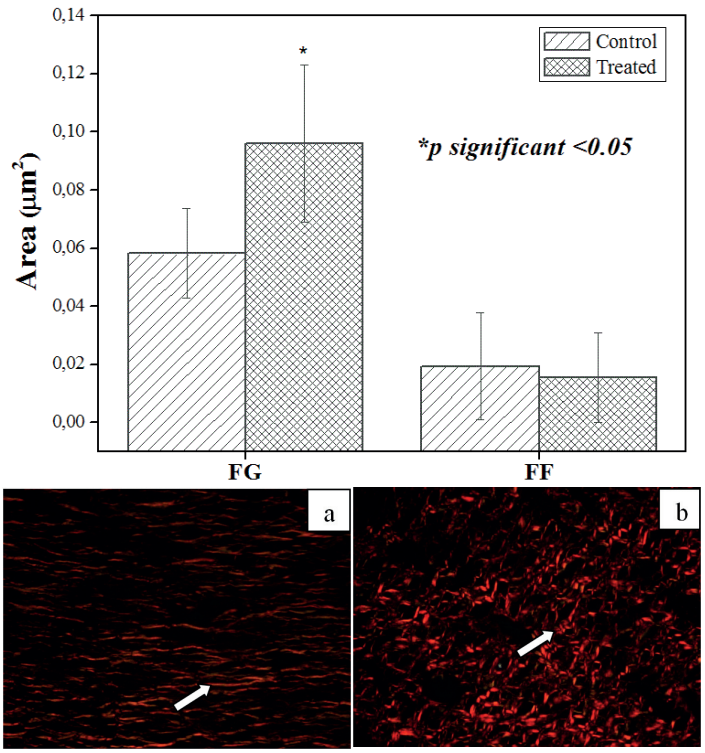

FIGURE 5 - Photomicrographs of thick and thin collagen fibers: 16 days experimental time. The arrow indicates: (**a**) thin fibers in the control group (C16); (**b**) thick fibers in the treated group (T16). FG: thick collagen fibers; FF: thin collagen fibers. Picro-sirius, x400.

Discussion

Tissue injuries, when reaching the different layers of the skin, alter their anatomical and physiological structure and trigger the process of tissue repair. Wound healing can occur by first intention or by second intention, when the injury and tissue destruction are extensive[2,4,6].

Type I collagen accounts for 80 to 90% of the extracellular matrix of intact skin, whereas type III collagen corresponds to only 10 to 20%[11,12]. At the beginning of the repair process, the extracellular matrix consists of 30% type III collagen, resulting in a more fragile matrix. Collagen fibers are birefringent and therefore appear bright against a dark background when analyzed under a polarized light microscope[9,10,18]. This technique permits the use of collagen fibrils to characterize the different stages of wound healing[9,18].

In addition, the collagen fibrils present in this matrix are strongly glycosylated, thin and show a parallel and not intermingled orientation[1,12,19]. After one week, the strength of the extracellular matrix is 3% of that of intact tissue. Collagenases and proteases cleave and degrade collagen fibers. This process is supplied by the deposition of collagen, increasing the thickness and organization of collagen fibrils and contributing to a more resistant matrix. After three weeks, tissue strength has increased by 30% and the typical original strength is reached by three months (80%)[1,11,12].

About 80 to 90% of intact skin is formed by type I collagen[19]. However, in the case of extensive tissue injury, type III collagen predominates in the extracellular matrix during the early stage of wound healing, which is characterized by thin, parallel and not intermingled fibrils[9,10]. The number of thin fibers was higher in group C7 than in group T7, indicating a predominance of type III collagen fibers at the beginning of the inflammatory process[1,11,12,19]. These fibers are gradullay absorbed and replaced with type I collagen fibers, increasing the strength, organization and thickness of the extracellular matrix as well as the number of cross-links among fibrils. As a consequence, a predominance of type I collagen fibers was observed on day 16, especially in group T16 when compared to group C16. Since an increase in the number of type I collagen fibers increases the tensile strength of newly formed tissue[1,12,16,19].

The monitoring of the progress of wound healing is necessary since wound contraction is greater during the maturation phase and consists of centripetal movement of the edges[13,20]. Several instruments are used for macroscopic evaluation of this process such as rulers, pachymeters, photographs and mathematical modeling[14,20].

We observed a greater tensile strength of the wounds of animals treated with the herbal ointment when compared to the untreated groups. According to Gantwerker and Hom and Montes, the presence of type III collagen fibers indicates the early phase of wound healing, as observed for specimens of the control group.

Since the tissue repair process comprises different stages and the thickness of type I collagen fibers indicates a more advanced stage, the present findings demonstrate that treatment with the herbal ointment yielded the best results. These were confirmed by the predominance of type I collagen fibers in the group treated for 16 days, suggesting that in these specimens the wound healing process was in a more advanced stage and the colagen fibers presented major grade of maturation when compared to the observed in the control group.

Conclusion

Treatment of wounds in skin with *Brassica oleracea var. capitata* – glycolic extract 10% (Debridan®) showed a significant increase in the number of mature collagen fibers after 16 days of treatment, accelerating the healing process, in rats.

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