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Topical treatment with *Copaifera langsdorffii* oleoresin improves wound healing in rats

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Abstract

Copaifera langsdorffii oleoresin (copaiba) has been used in the Amazon as traditional wound healing remedy for centuries. Since its mechanisms of action remain unclear, we investigated its effects on excisional wounds in rats. Wounds were made on the dorsum of animals assigned to three groups: saline, control cream and 10% copaiba cream, and assessed on days 2, 7 and 14 post-wounding morphometrically, histologically and biochemically. Wound healing rate was faster in copaiba than in saline or cream groups. This was corroborated by matrix metalloproteinase (MMP)-2 activity which rose progressively throughout in copaiba group. MMP-9, a marker of inflammation, was not detectable at day 14 in copaiba group, but persisted in the other groups. Moreover, histology showed early population of copaiba-treated wounds by inflammatory cells, and by day 14 this group had less fibroblasts and more organized collagen. Further, copaiba group synthesized collagen faster than saline and cream groups, as evidenced by progressive increases in the amounts of hydroxyproline at days 7 and 14 ($p < 0.012$). These findings suggest that 10% copaiba oleoresin cream promotes wound healing in rats by regulating MMP-2 and MMP-9 activities, stimulating collagen synthesis and promoting tissue remodeling and reepithelialization.

Keywords: wound healing, *Copaifera langsdorffii*, phytotherapy, hydroxyproline, matrix metalloproteinase.

Introduction

Wound healing is a biochemically dynamic process involving precise coordination of innumerable physiological, immunological and cellular pathways [1]. It entails complex interactions between cells, their microenvironment and the extracellular matrix, and it is largely through these interactions that cells are directed to differentiate, proliferate or remain quiescent, as the architecture and function of damaged tissue is reconstituted and the barrier function of the skin is restored [2]. Under normal circumstances, wound healing progresses through overlapping stages that include hemostasis, inflammation, angiogenesis, cell differentiation and proliferation, synthesis and cross-linking of collagen and ultimately tissue re-epithelialization. Successful healing occurs only when these events and associated biochemical processes are precise and perfectly sequential [3, 4].

Impaired wound healing results in lost labor and accounts for a substantial proportion of health care expenditure. By one estimate [5] more than three billion dollars per year is spent on wound care. Although there have been significant advances in wound care within the last three decades, successful healing of chronic wounds remains a conundrum. Available treatment options include cleaning, debriding and topical treatment with antiseptics, antibiotics, and wound healing promoters, such as aloe vera, honey, comfrey, chamomile extract, dexpanthenol, tetrachlorodecaoxide solution, clostebol acetate and cytokines, and other growth factors [6]. The prohibitive cost of growth factors and contemporary pharmaceuticals relative to the resulting levels of success continues to prompt further search for other cost-effective treatment options.

Since time immemorial, medicinal plants have been used to treat various ailment and skin disorders, including cuts, burns and chronic wounds [7]. In recent times, interest has been focused on the Amazon flora because of its biodiversity and its rich resource of phytochemicals and potential pharmaceuticals [8]. Although a large number of herbal products are currently under investigation, our attention has been drawn to plants belonging to *Copaifera spp* genus, which has been used in folk medicine for centuries by Brazil's native Amazonians [9, 10]. Commonly known as copaiba, *Copaifera spp* are trees in the *Leguminosae-Caesalpinoideae* family, reaching 25-40 feet tall and capable of living as long as 400 years. The oleoresin produced by this plant—a detoxifying excretory material naturally used by the plant to defend itself against animals, fungi and bacteria—is traditionally believed to be anti-inflammatory, anti-tumor, anti-tetanus and anti-blenorrhagea. Moreover, it is used as a urinary anti-septic and for wound healing purposes [11]. And there is emerging evidence that copaiba,

indeed, has anti-inflammatory and antimicrobial properties [8, 10]. These findings suggest the need to study the potential value of copaiba in contemporary attempts to develop cost effective wound treatment protocols.

In a previous study [9], we used cultures of fibroblasts to assess the cytotoxicity of *C. langsdorffii* oleoresin and a rabbit ear excisional wound model of injury and repair to assess its healing effects, using different concentrations of oleoresin. We found no cytotoxicity of copaiba oleoresin up to 100 µg/mL. Moreover, a standard pharmaceutical cream with 10% copaiba was not toxic but promoted healing. And more recently, we showed that 10% copaiba cream has antimicrobial properties against *Staphylococcus aureus* and *Streptococcus pyogenes* in a full thickness rat skin wound model [10]. Encouraged by these findings, we examined the wound healing effects of 10% copaiba cream in the same full thickness rat skin wound, a model of contractile wound different from the non-contractile rabbit ear model. Specifically, our aim was to determine the wound healing effect of 10% copaiba cream morphometrically and histologically, and to uncover some of the mechanisms underlying the biochemical effects of copaiba by quantifying collagen synthesis and matrix metalloproteinases activity.

Material and Methods

Plant Material

The Copaiba oleoresin used in this study was obtained from the trunk of the *Copaifera langsdorffii* tree in Tarauacá, Acre, Brazil, located at latitude 9°41'0" South and longitude 72°5'0" West, as detailed in our previous paper [9].

Animals

Following approval by the Institutional Animal Care and Use Committee (Protocol 09-10#20), University of Wisconsin-Milwaukee, 27 male Wistar rats (*Rattus norvegicus*) weighing 300 to 340g, were obtained from Charles River Laboratories (Wilmington, MA, USA). Rats were fed standard rodent pellet diet and water *ad libitum* and housed in standard sterilized individual polypropylene cages, in a room maintained at 22 C, 50% humidity and 12 hours light-dark cycle throughout the study.

Surgical procedure, experimental design and treatment

Following one week of acclimatization to the animal housing facility, rats were anesthetized with inhaled isoflurane. The dorsal surface of the upper cervico-thoracic region of each rat was shaved and disinfected with 70% isopropyl alcohol before four excisional wounds were created with a sterile 8.0 mm skin biopsy punch (Acu-Punch®, Acuderm, Fort Lauderdale, FL, USA). Then the animals were assigned to three treatment groups: Saline (S), Control cream (Cr) or 10% Copaiba cream (C10). Each group consisted of 9 rats with a total of 36 wounds (4 wounds/animal). On each of days 2, 7 and 14, three rats per group (12 wounds total) were used. First, digital photographs were taken for the Wound Healing Rate analysis (n=12 wounds), before wounds were biopsied. Three biopsies were taken from each rat: one tissue sample was used for histological analysis, one for hydroxyproline and another for matrix metalloproteinases assays. This resulted in three biopsies per analysis per treatment per day of follow-up. Wounds were dressed with sterile 2"x 2" gauze (Dynarex Corp., Orangeburg, NY, USA), held in place with self-adhesive stretch bandage and wrapped with standard athletic tape (Muller Sports Medicine, Inc., Prairie du Sac, WI, USA).

Treatment and dressing changes were done daily, over 14 days. The control cream treatment was a standard pharmaceutical base cream without the active substance (copaiba), while the 10% copaiba cream treatment was the same base cream with 10% copaiba, as previously detailed [10]. We used 10% copaiba cream because our previous studies showed that this concentration of copaiba was suitable for *in vivo* studies. Moreover, we found adverse effects with higher concentrations of copaiba cream or pure copaiba oil [9].

Wound healing rate

Wounds were evaluated on days 2, 7 and 14 post-wounding, using digital photography. To ensure consistency of wound magnification, the camera was placed at a steady distance of 30-cm perpendicularly above each wound. A metric scale placed beside each wound was photographed with each wound as a reference. The visible wound margins were traced on each digital image and the areas in mm^2 were calculated using an image processing analysis software (ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA) [7, 12-14]. The following formula was then used to compute wound healing rates (WHR):

$WHR = A_o - A_n / A_o$, where: A_o: wound area on day 0; n= day 2, 7 or 14.

Histological analysis

Histological studies were performed on days 2, 7 and 14 post-wounding. Rats were euthanized with an overdose of isoflurane, and then an 8.0 mm skin punch biopsy was obtained from the wound area. Wound samples were placed in buffered formaldehyde 10% (w/w), pH 7.4 for 24 hours. Following routine histological processing, 5µm-thick paraffin sections were stained with hematoxylin and eosin (H&E) for qualitative assessment of inflammatory infiltrate, angiogenesis and fibroblasts response to treatment. Then, sections were photographed at 400x with an Olympus® BX41 microscope equipped with an Olympus® DP70 camera (Olympus America Inc., Melville, NY. USA) [15].

Hydroxyproline assay

Wound biopsies taken on days 2, 7 and 14 post-wounding were immediately frozen and stored at -80 C until they were used. The quantitative analysis of hydroxyproline was performed as detailed by Reddy and Enwemeka [16]. Briefly, samples were oven dried overnight at 60 C until a constant weight was attained. The dried samples were transferred to 15 mL plastic tubes and 100 µL of 6N HCl per 1.0 mg dry tissue added, and then homogenized for 10

seconds before being hydrolyzed in HCl for 4 hours at 130 C [14, 16, 17].

Following adjustment to neutral pH, 10 µL hydrolizate was added to a 96-well microplate. Standard solutions with known concentrations of hydroxyproline were prepared: 1.0, 2.0, 4.0, 6.0, 8.0, 10, 20, 40, 60, 80 and 100 µg/mL, and 10 µL of each standard solution was added to the microplate. Then, 90 µL of 0.056M Chloramine T was added to each sample before incubation at room temperature for 25 minutes. Ehrlich's reagent (100 µL 1M) was added to the oxidized samples followed by incubation at 60°C for 20 minutes and chromophore development [14, 16].

The samples were homogenized at 20 rpm for 10 minutes and the absorbance of reddish purple complex measured at 550 nm at room temperature using a multi-detection microplate reader (Synergy™ HT, Biotek. USA). Absorbance values were plotted against the concentrations of standard hydroxyproline, and the presence of hydroxyproline in unknown tissue homogenates was determined from the standard curve [16]. Tests were done in triplicate to foster accuracy of results.

Detection of matrix metalloproteinases activity

Sample preparation

wound biopsies stored at -80°C were thawed, kept in an ice bath and weighed. Then, they were sectioned, placed in buffer and homogenized at 25,000 rpm twice for 20 seconds each using a Polytron PT 1200E tissue homogenizer on ice. The homogenate was centrifuged at 4°C for 20 minutes and the supernatant stored at -80°C in aliquots of 500 µL until they were used.

Protein determination

A protein assay kit based on the Bradford method (Cayman Chemical. Ann Harbor, MI, USA) was used to determine protein content colorimetrically. The standard curve was determined using the following concentrations of bovine serum albumin (BSA): 0.0, 5.6, 7.5, 10.1, 13.3, 18, 24, 32 and 40 g/mL. Each sample was diluted by a factor of 100 in deionized water and added to the microplate wells. Protein determination assay reagent was added and the microplates were incubated at room temperature for 5 minutes. Tests were performed in duplicates, and absorbance measured at 595 nm in a microplate reader (Synergy™ HT, Biotek. USA).

Gelatin zymography

from the protein determination, the equivalent volume was calculated in order for each sample to contain 20 µg of total proteins, to be used for zymography. The equivalent volume (µL) of total protein of each sample was mixed with an equal volume of the sample buffer (62.5 mM Tris-HCl, 4.0% SDS, 25% glycerol, 0.01% bromophenol blue; pH 6.8) prior to loading to the gels [18, 19]. Samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% SDS-PAGE gels copolymerized with gelatin (1.0 mg/mL) (BioRad, Cat. 161-1185, Richmond, CA, USA) under non-reducing conditions without prior boiling. Gel loading was design to contain samples from the three treatment groups of each of the three periods of follow-up. Thus, each gel had samples loaded as: S, Cr and C10-treated wounds from days 2, 7 and 14 post-wounding. Twenty microliters of a molecular weight marker (Novex® Sharp Unstained Protein Standard, Invitrogen, Carlsbad, CA, USA) were also loaded to each gel. Zymography was performed in triplicate. The apparatus was set to run at 100 V, for 90 minutes at 4 C. After completion of electrophoresis, gels were washed in 2.5% Triton X-100, rinsed in developing buffer (50mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35 (30%); pH 7.5) and incubated in developing buffer at 37°C for 16 hours to allow the MMPs to digest the gelatin substrate [19-21].

Following incubation, gels were stained (0.5% Coomassie brilliant blue R-250, 40% methanol, 10% glacial acetic acid, water) at room temperature for 1 hour. Gels were then destained (40% methanol, 10% glacial acetic acid and 50% water) until the zones of proteolysis had cleared. Protease activity was detected as clear (unstained) bands against a blue background [22]. Gels were washed in deionized water, and incubated in a drying solution (30% methanol, 5% glycerol, water) at room temperature for 4 minutes before they were placed in acrylic molds in between cellophane sheets and allowed to dry for 24 hours.

The dried gels were then scanned and the relative pixel density of each band was measured (ImageJ software). Matrix metalloproteinases were identified according to the molecular weight marker and the enzymatic activity was quantified by the intensity of proteolysis bands determined by the area of the peaks corresponding to each band [21, 23].

Statistical analysis

Wound healing rates, hydroxyproline and matrix metalloproteinases data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used to compare groups using SPSS version 18. Bonferroni *post hoc* tests were then used to pin-point groups that differed statistically. The level of statistical difference was set at $p<0.05$.

Results

Gross macroscopic observations

Two days post-wounding, wounds treated with control cream or 10% copaiba cream appeared moist compared to wounds treated with saline (Figure 1A). By day 7, saline-treated wounds had firmly attached scabs while wounds treated with control cream were inflamed and those treated with 10% copaiba cream showed important decrease in wound area. By day 14, saline-treated and 10% copaiba cream-treated wounds had re-epithelialized; those treated with 10% copaiba cream had healed fully with firm scar tissue.

Wound healing rate (WHR)

Two days post-wounding, the WHRs for wounds treated with control cream and 10% copaiba cream were lower compared with

saline-treated wounds (p=0.006, p=0.002, respectively) (Figure 1B). By day 7, WHRs were higher for saline and 10% copaiba cream groups compared with control cream-treated wounds, with the 10% copaiba cream treated wounds healing significantly faster (p=0.005; 10% copaiba cream vs. control cream). Most wounds were re-epithelialized by day 14

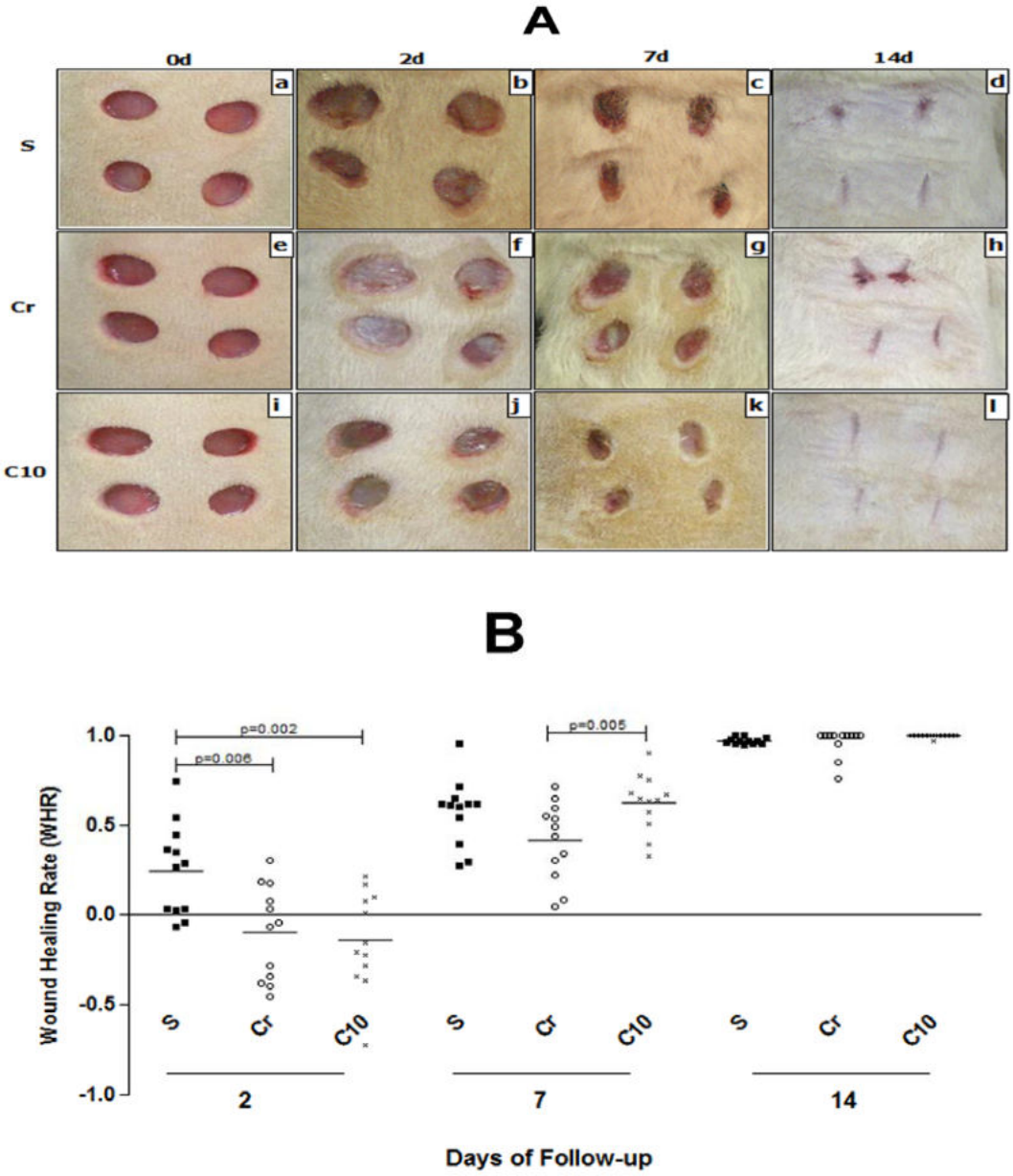

Figure 1. Representative images of the wounds and wound healing rates during follow-up.

(A) Wounds on days 0, 2, 7 and 14 respectively, arranged by treatment: Saline (S) (a-d); Control cream (Cr) (e-h); 10% Copaiba cream (C10) (i-l). (B) Effect of topical treatments on wound healing rates (WHR). Results are presented as mean ± SEM (n=12).

Histological analysis

On day 2, inflammatory infiltrate (leukocytes) were observed in each group (Figure 2-Panels a, d and g), while on day 7, each group had evidence of fibroblast proliferation, but wounds treated with saline (Figure 2-Panel b) and 10% copaiba cream (Figure 2-Panel h) still had evidence of inflammation. Compared to the saline or the control cream group, 10% copaiba cream-treated wounds had less fibroblasts and more organized collagen fibers at day 14 (Figure 2-Panels c, f and i).

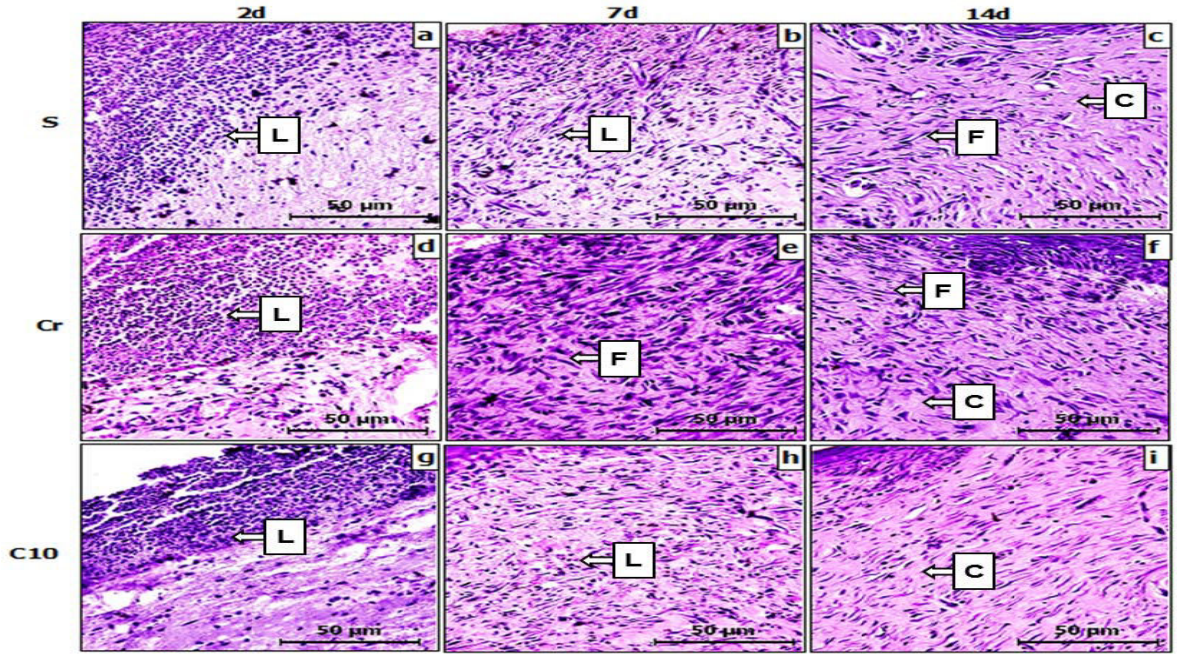

Figure 2. Hematoxylin and eosin stained sections of the wounds during follow-up.

Wound sections on days 2, 7 and 14 respectively: Saline (S) (a-c); Control cream (Cr) (d-f); 10% Copaiba cream (C10) (g-i). Magnification 400x. L: leukocytes; F: fibroblasts; C: collagen fibers.

Hydroxyproline analysis

Hydroxyproline assay showed an overall increase in collagen synthesis from day 2 to day 14 in each group (Figure 3). However, only treatment with 10% copaiba cream induced a statistically significant increase in collagen synthesis between days 2 and 7 (p=0.012), 2 and 14 (p=0.012) and 7 and 14 (p=0.012). Treatment with 10% copaiba cream engendered more collagen synthesis than any of the other two groups, increasing collagen synthesis 75.7% between day 2 and 7, and 83.7% between day 2 and day 14. The corresponding increases in collagen synthesis for saline and control cream treated wounds were 62.1% and 49.1% [day 2 vs. day 7], and 79.1% and 71.1% [day 2 vs. day 14).

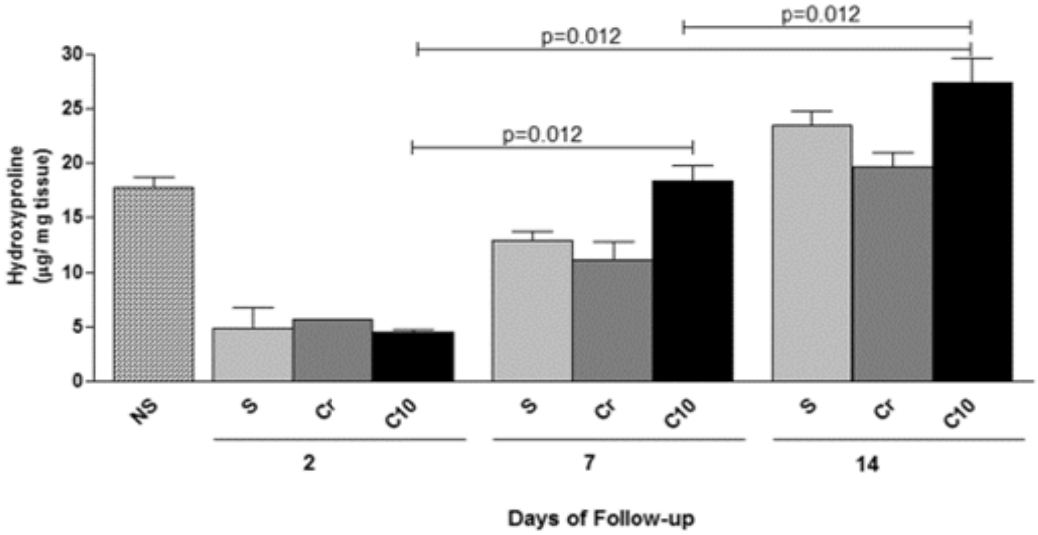

Figure 3. Concentration of hydroxyproline during follow-up on days 2, 7 and 14.

Saline (S), Control cream (Cr) and 10% Copaiba cream (C10). Analysis of hydroxyproline content in non-wounded rat skin (normal skin) (NS) is included as a reference. Results are presented as mean ± SEM (n=3).

Matrix metalloproteinases analysis

As shown in Figure 4 (a representative zymogram gel), gelatin zymography revealed clear proteolytic bands with molecular weights of 66 kDa, 72 kDa and 92 kDa, suggesting the presence of matrix metalloproteinases (MMP) 2 active, pro-MMP-2 and MMP-9, respectively

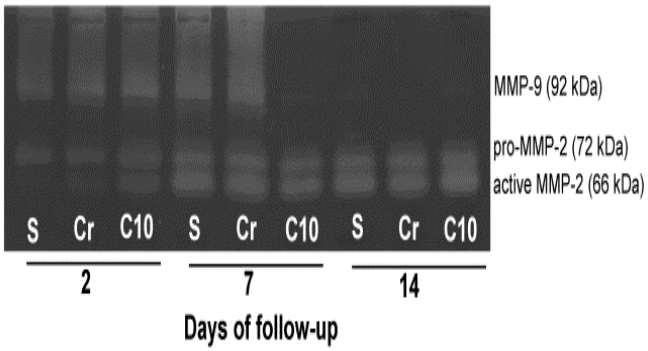

Figure 4. A representative zymogram gel.
Gel shows proteolytic activity of MMPs present in wound biopsies on days 2, 7 and 14: Saline (S), Control cream (Cr) and 10% Copaiba cream (C10).

Digital quantification of the bands with ImageJ software showed increased active MMP-2 activity in wounds treated with saline and 10% copaiba cream overtime, and a decrease for control cream treated wounds between days 7 and 14 (Figure 5A). Active MMP-2 increased progressively in the saline treated group, reaching statistical significance by day 14 ($p<0.05$) but not by day 7. In the cream and 10% copaiba cream treated groups, active MMP-2 was significantly high by day 7 ($p<0.05$; $p<0.01$), remaining significantly high at day 14 in the 10% copaiba treated group but declining slightly in the cream control group. Compared to day 2, active MMP-2 rose 93.2% by day 7 and remained virtually at the same level (93.4%) by 14 in the copaiba treated group. The rise for the saline group was 58.7% by day 7 and 78.1% by day 14; while that of wounds treated with cream were 70.1% and 57.8%, respectively. In contrast, pro-MMP-2 activity remained at a constant level in each group (Figure 5B), except for moderate statistically insignificant increases in the cream treated group between days 2 and 7, and in the 10% copaiba treated group between days 7 and 14.

MMP-9 activity decreased progressively in the saline treated group, reaching statistical significance by day 14 ($p<0.001$) (Figure 5C). Similarly, control cream treatment resulted in a progressive decrease of MMP-9, reaching statistical significance by day 7 ($p<0.05$) with further decline by day 14 ($p<0.01$). Treatment with 10% copaiba cream significantly reduced MMP-9 between days 2 and 14 ($p<0.05$) and also between days 7 and 14 ($p<0.05$). MMP-9 activity was undetectable for this group on day 14.

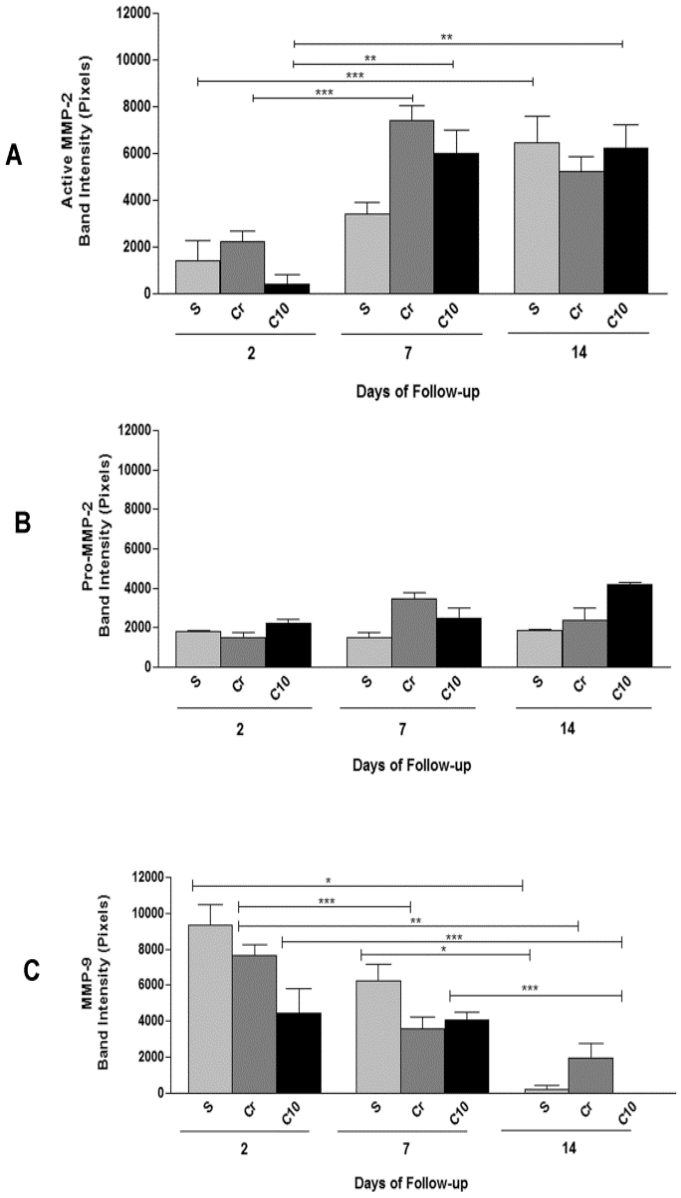

Figure 5. Profiles of matrix metalloproteinases activities.
(A) active MMP-2, (B) pro-MMP-2, (C) MMP-9, on days 2, 7 and 14: Saline (S), Control cream (Cr) and 10% Copaiba cream (C10). Results are presented as mean ± SEM of band intensity from the zymograms (n=3). Statistical differences: (*) $p<0.001$; (**) $p<0.01$ and (***) $p<0.05$.

Discussion

Copaiba oleoresin has been popularly used as an alternative for wound healing for centuries by Brazil's native Amazonians because of the perception that it has tissue repair properties; but few studies have investigated this traditional belief [24]. Our study shows that treatment with 10% copaiba cream enhances wound healing by stimulating collagen synthesis, promoting tissue remodeling, regulating matrix metalloproteinases-2 and -9 activities and improving reepithelialization.

Histological examination of the wounds revealed early population of all wounds by inflammatory cells. By day 7, these cells had been replaced by fibroblasts, but saline and copaiba treated wounds continued to show evidence of inflammation. Copaiba treated wounds synthesized collagen rapidly, and quicker than the other two groups, as evidenced by higher amounts of hydroxyproline at days 7 and 14. Our results also show that wound healing rate was similarly faster following copaiba treatment. This is not just consistent with the observation that a higher concentration of hydroxyproline correlates with a faster rate of wound healing [7, 17], but correlates very well with our histological observations and matrix metalloproteinase-2 activity.

The presence of mature and organized collagen fibers, observed in our histological analysis of samples from wounds treated with 10% copaiba cream, along with the increase in hydroxyproline, suggests that copaiba oleoresin promotes fibroblast proliferation and extracellular matrix synthesis during wound healing.

Our zymographic analysis showed a modulation of metalloproteinases 2 and 9 activity in the samples. MMP-2, a marker of cellular activity of conjunctive tissue is normally present in the skin, in basal levels, while MMP-9, a marker for assessing the inflammatory phase of wound healing, is expressed mainly by neutrophils and macrophages, and is related to the inflammation following tissue damage [20, 25].

Pro-MMP-2 activity show moderate increases in the cream treated group between days 2 and 7, and in the 10% copaiba treated group between days 7 and 14. In contrast, active MMP-2 increased progressively in 10% copaiba cream-treated wounds. These findings are in accordance with the hypothesis that MMP-2 has an important role in the remodeling phase and also on the homeostasis of collagen. Moreover, they are consistent with our histological findings, as well as our quantification of collagen synthesis in each group. During the last phases of repair there is an increase of pro-MMP-2 and persistence of MMP-2 [18, 26-28]. The progressive increase in the activity of pro- MMP-2 and persistence of active-MMP-2 observed from days 7 to 14 in wounds treated with 10% copaiba cream correlates very well with the increase in collagen synthesis observed in this group; indicating that copaiba promotes healing.

MMP-9 activity was not detectable by day 14 in copaiba treated wounds, and this suggests that treatment with copaiba accelerated inflammatory response to injury compared to saline or cream treatment. Furthermore, the low levels of MMP-9 activity in copaiba treated wounds at days 2 and 7 provide further evidence that inflammatory response to injury was moderate and seemed modulated given the consistently lower levels of MMP-9 in copaiba treated wounds relative to the other two groups.

Wounds treated with saline and control cream continued to show MMP-9 activity on day 14; more so in the cream treated group. Lingering inflammation is associated with continuing TNF- and subsequently MMP-9 activities [29]. This prevents migrating keratinocytes from forming new attachments to a newly synthesized basement membrane. And as our histological findings and data on WHR and hydroxyproline analysis indicate, saline treated and cream treated wounds healed at a different rate and synthesized less collagen than copaiba treated wounds.

The biologically active mediators of wound healing in copaiba are not clearly understood. However, high resolution gas chromatography-mass spectrometry (HRGC-MS) of the oleoresin used in this study showed a mixture of sesquiterpenes (75%) and diterpenes (25%) [30]. The main compounds among sesquiterpenes were shown to be -caryophyllene (51%), followed by -humulene (8.52%). Among diterpenes, the main compounds were 11-acetoxy-copalic acid (5.23%), 11-hydroxy-copalic acid (4.8%), copalic acid (4.69%) and agatic acid (3.32%) [30]. Studies on medicinal plants have shown that phytochemical constituents like flavonoids, triterpenoids and tannins promote wound healing [14, 31]. Moreover, it has been suggested that the biological activities of copaiba oleoresin may be ascribed to the complex mixture of sesquiterpenes and diterpenes [32]. Terpenoids could promote wound healing because of their astringent and antimicrobial properties, which appear to be responsible for wound contraction and an increased rate of epithelialization [33]. The wound healing activity may be attributed to their individual activities or the synergistic effect of bioactive molecules [26].

Conclusion

Our findings suggest that 10% copaiba cream promotes wound healing by modulating and moderating inflammation, stimulating fibroblast proliferation, advancing collagen synthesis, and modulating MMP-2 and MMP-9 activities. This is evidenced by early appearance of a larger amount of inflammatory mediator cells, their rapid replacement by fibroblasts, synthesis of higher amounts of hydroxyproline, differences in the levels of MMPs at various stages, and the higher wound healing rate of copaiba treated wounds compared to cream or saline treated control wounds.

Author's Contributions

All authors have made substantial contributions to this work. All authors participated in conception and design, discussed the content and implications and commented on the manuscript at all stages. All authors have read and approved the final manuscript.

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