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Copaiba oil-resin (*Copaifera langsdorfii* Desf.: Caesalpiniaceae) associated with laser therapy for skin wound treatment in Wistar rats

*Óleo resina de copaíba (*Copaifera langsdorfii *Desf.: Caesalpiniaceae) associada a laserterapia para o tratamento de feridas cutâneas em ratos Wistar*

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ABSTRACT: Alternative protocols for the treatment of skin lesions have been developed with the use of techniques such as photobiomodulation and phytotherapy, aiming to optimize this process. To evaluate the effectiveness of copaiba (*Copaifera langsdorffii*) oil-resin and low-level laser therapy for treating cutaneous wounds, 15 Wistar rats (*Rattus norvergicus*) were used, in whom five 8-mm lesions were produced. The following protocols were applied: negative control group (T1); positive control group (T2); laser therapy with AsGa (904 nm), continuous, focal mode for 10 s, dosage of 4 J/cm² (T3); copaiba oil-resin (T4); and association group (copaiba and low-level laser) (T5). The efficacy of each technique was evaluated based on macroscopic aspects of the lesion, wound healing rate, and histopathological analysis (inflammatory infiltrate and collagen expression). The Kruskal-Wallis test was used for statistical analyses (P> 0.05). Copaiba treatment showed an advantage in type III collagen expression, whereas laser therapy demonstrated an enhanced capacity for tissue regeneration. The significant advantage obtained from the association treatment is the improvement of the macroscopic aspect of the wound, with a reduction in crust formation.

KEYWORDS: Photobiomodulation; Phytotherapy; Skin; Wound Healing.

RESUMO: Protocolos alternativos para o tratamento de feridas cutâneas têm sido desenvolvidos com a utilização de técnicas como a fotobiomodulação e fitoterapia, objetivando acelerar esse processo. Com o objetivo de avaliar a efetividade do óleo-resina de copaíba (*Copaifera langsdorffii*) e do *laser* terapêutico no tratamento de feridas cutâneas foram selecionados 15 ratos Wistar (*Rattus norvergicus*), nos quais foram produzidas cinco lesões cutâneas de 8mm. Os tratamentos empregados foram: controle negativo (T1); controle positivo (T2); laserterapia com AsGa (9,04nm) no modo contínuo, de maneira focal, com duração de 10 segundos e dosimetria de 4 J/cm² (T3); óleo resina de copaiba (T4) e a associação dos tratamentos (copaiba e laserterapia) (T5). A eficácia de cada técnica foi avaliada com base nos aspectos macroscópicos das lesões, taxa de cicatrização e análise histopatológica (infiltrado inflamatório e expressão do colágeno). Os dados obtidos foram analisados pelo teste de Kruskal-Wallis (P>0,05). O tratamento com a copaíba mostrou vantagem na expressão do colágeno tipo III, enquanto a laserterapia demonstrou aumento da capacidade de regeneração tecidual. A vantagem significativa obtida pela associação dos tratamentos foi a melhora do aspecto macroscópico da ferida, com redução na formação de crostas.

PALAVRAS-CHAVE: Cicatrização Cutânea; Fitoterapia; Fotobiomodulação; Pele.

INTRODUCTION

Wound healing is a dynamic process that is divided into three distinct phases: inflammatory, proliferative, and remodeling (MAVER et al., 2015). The events initiated after the injury endure for extended periods; thus, therapeutic intervention aims to reduce the time involved in the process and prevents undesirable consequences, such as scars (ADIELE; ADIELE; ENYE, 2014).

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In addition to the traditional methods, new alternatives intend to improve the results obtained in these protocols. For example, low-power laser therapy (PERCIVAL; FRANCOLINI; PERCIVAL, 2015) photoactivates cellular mechanisms through action on the mitochondrial cytochromes (FARIVAR; MALEKSHAHABI; SHIARI, 2014), in addition to analgesia, reduction of edema, and decreased repair time in damaged tissues (SILVA et al., 2010). The diversity of laser models creates flexible protocols that vary in power, wavelength, frequency, intensity, and dose (J/cm^2) (FARIVAR; MALEKSHAHABI; SHIARI, 2014), which adapt according to the intention of the treatment.

Another form of alternative therapy is herbal medicine, which, according to Ghosh and Gaba (2013), presents antimicrobial and antioxidant effects and promotes mitosis and angiogenesis. Furthermore, it increases collagen production. These properties validate the use of plants such as Copaifera (*Copaifera* spp.) in skin treatment protocols. Sesquiterpenes and diterpenes, components of Copaiba, provide astringent, antimicrobial, and anti-inflammatory properties that promote tissue repair (LEANDRO et al., 2012). Therefore, the effects of copaiba, laser, and their association on induced cutaneous wounds in Wistar rats were studied.

MATERIAL AND METHODS

Following approval by the Ethics Committee for the Use of Animals, number 23107.016568/2015-44, protocol 82/2015, at Federal University of Acre, 15 male Wistar rats (*Rattus norvergicus*), weighing ± 350 g, approximately 100 days old were utilized. They were acclimated for 7 days and maintained at 22 °C, with 50% humidity and a 12-h light-dark cycle. They received rodent pelleted diet and water *ad libitum* throughout the study.

The laser used was gallium arsenide (AsGa), with a wavelength of 904 nm (L42, Ibramed, Amparo, São Paulo) in the focal continuous mode for 10 s and an energy density of 4 J/cm^2 per treatment.

In natura copaiba, oil-resin was obtained from the *Copaifera langsdorffii* tree trunk, which was donated to the study by the extractivist cooperative of the Iaco River Valley's rural producers (COOPERIACO) in Sena Madureira, Acre, Brazil.

The rats were anesthetized using intramuscular ketamine (100 mg/kg) and xylazine (20 mg/kg), followed by mask inhalation of isoflurane vaporized in oxygen to maintain anesthesia. The dorsal surface of the thoracic-lumbar region of each rat was shaved and disinfected using 70% isopropyl alcohol before five excisional wounds were created using a sterile 8-mm skin biopsy punch. Immediately after the procedure, the animals were assigned into five treatment groups, maintained for 14 days, applied at 24-h intervals: negative control, with 0.9% physiological solution (T1); positive control, with solid Vaseline (T2) (Masson-Meyers et al., 2013); low-level laser therapy (T3); copaiba oil-resin (T4); and copaiba oil-resin in association with low-level laser therapy (T5). The model of the study was an experimental group, with each wound, individually, and its 15 animals as repetitions (Figure. 1).

Wounds were macroscopically evaluated by their visual aspects (humidity, crusts, granulation tissue, and inflammatory features), and daily measurement of their area was performed using calipers by the same researcher (SANTOS et al., 2006). Each axis of the ulcer (vertical and horizontal) was evaluated, and the diameters of the ellipse were considered, providing posterior area calculation of the wound healing rate.

Animals were euthanized with an overdose of isoflurane, on days 1, 7, and 14 of the study, in groups of five animals for histopathological evaluation. Skin fragments of wounds were collected individually and preserved in 10% formaldehyde until processing. Following routine histological processing, 4–5 µm paraffin sections were stained using hematoxylin and eosin (HE) for evaluation of inflammatory infiltrate and picrosirius red stain for qualitative and quantitative collagen analysis.

HE results were as follows: acute inflammation (IA), tissue necrosis (NC), granulation tissue (TG), and fibroplasia (FP), described in degrees, from 0 (absent) to 3 (maximum expression). Picrosirius red analysis examined the expression of collagen types from their percentage in tissues. Collagen type I (red) and collagen type III (green) were observed in samples under polarized light. All histopathological analyses were carried out under a microscope (DM1000, Leica,) with a coupled capture camera (DFC295, Leica, Germany) and the ProPlus 4.1 image capture program, with a 4× objective.

The nonparametric Kruskal-Wallis test was employed to compare the treatment groups. The level of statistical significance was set at $p<0.05$.

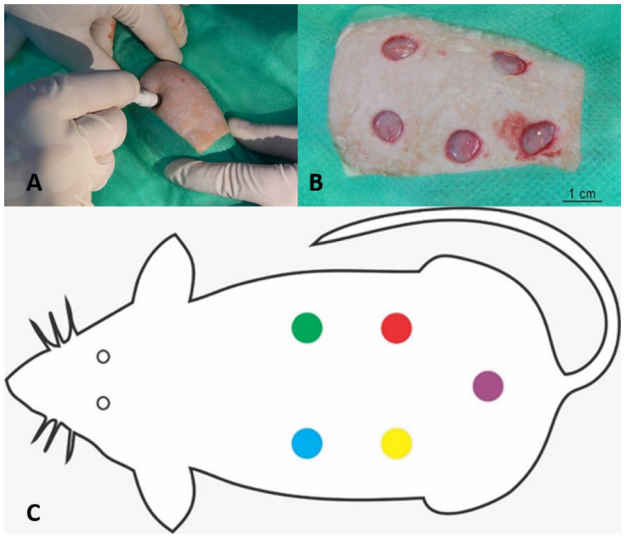

Figure 1. The surgical procedure to produce five excisional wounds with a sterile 8-mm skin biopsy punch in the thoracic-lumbar region of each Wistar rat (A e B). Experimental design of wounds and treatments: negative control (T1); positive control (T2); low-level laser therapy (T3); copaiba oil-resin (T4); low-level laser therapy and copaiba oil-resin association (T5) (C).

RESULTS

Wounds treated with the negative control and laser therapy groups demonstrated an earlier reduction in their area in macroscopic observations. Excessive crust formation in ulcers treated with copaiba oil-resin resulted in inferior wound contraction, delaying the healing process. Compared with the copaiba oil-resin group, the association group presented better tissue contraction and a smaller number of crusts. All wounds were completely healed and did not show inflammation or crusts by day 14 (Figure. 2).

On day 1 post-injury, the wound healing rate did not differ among treatment groups; although in absolute numbers, T1 had minor areas, and T4 had a greater wound size (Table 1). These results may be a consequence of the brief time spent between the procedure and the measurement. However, this tendency remained during the study, showing T1 as most effective for area reduction, as seen by day 7, when the negative control exposed the minor wounds, and those treated with copaiba oil-resin, and the association group, larger ulcers, equivalent to T2, indicating the inferior ability of injury contraction (Kruskal-Wallis test; $p<0.05$). Similar findings remained until day 14 when 0.9% physiological solution produced a greater wound healing rate, and lesions treated with T4 or T5 had the poorest results. Most wounds were re-epithelialized by day 14, showing no differences and revealing all protocols capable of healing wounds.

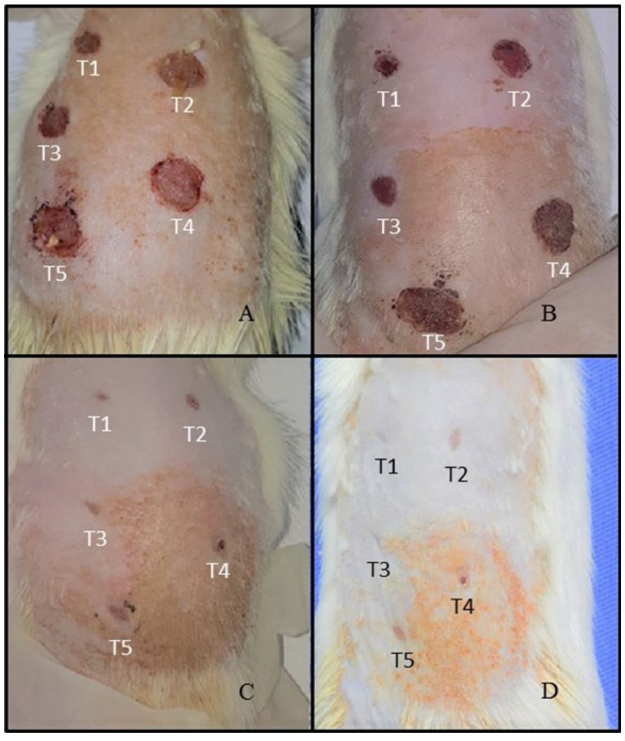

Figure 2. Macroscopic aspects of wounds produced with a sterile 8.0-mm skin biopsy punch, in Wistar rats treated with 0.9% physiological solution (T1); solid vaseline (T2); low-level laser therapy (T3); copaiba oil-resin (T4); copaiba oil-resin in association with low-level laser therapy (T5), on day 1 (A), 5 (B), 10 (C) and 14 (D) post-procedure.

Measured areas of each wound provided the median of each treatment, and the statistical analysis (Kruskal-Wallis) was performed, in which equal letters indicated no significant difference.

Inflammatory infiltrates analysis showed no difference between treatments on day 1. However, although no statistical differences were observed in the treatments, T4 showed minor tissue necrosis in absolute numbers (Table 2). By day 7, for acute inflammation and tissue necrosis parameters, T1 and T3 had the best results, whereas fibroplasia indicated the best outcomes for treatments T3 and T5 (Table 3). The final evaluation for inflammatory infiltrates on day 14 displayed wounds of the positive control group, with minor results for TG, indicating late tissue proliferation (Table 4), whereas no differences were found in the remaining factors.

Table 1. Area (mm) of surgically induced cutaneous wounds, by 8.0-mm skin biopsy punch in Wistar rats, treated with group with 0.9% physiological solution (T1); solid vaseline (T2); low-level laser therapy (T3); copaiba oil-resin (T4); copaiba oil-resin in association with low-level laser therapy (T5), on days 1, 7 and 14 postoperatively.

	Day 1	Day 7	Day 14
T1	0,25 (0,21-0,37) c	0,01 (0,0035-0,02) c	0 (0-0) a
T2	0,28 (0,23 - 0,37) abc	0,04 (0,03-0,07) ab	0,01 (0-0,005) a
T3	0,28 (0,25 - 0,32) bc	0,03 (0,02-0,08) b	0 (0-0) a
T4	0,37 (0,28 - 0,5) a	0,07 (0,03-0,13) ab	0,001 (0-0,0005) a
T5	0,32 (0,31 - 0,38) ab	0,07 (0,04-0,12) a	0,02 (0-0,01) a

Table 2. Histopathological analysis of the inflammatory infiltrate of surgically induced skin wounds, by 8.0-mm skin biopsy punch in Wistar rats, treated with 0.9% physiological solution (T1); solid vaseline (T2); low-level laser therapy (T3); copaiba oil-resin (T4); copaiba oil-resin in association with low-level laser therapy (T5), of samples collected on day 1 of treatment. Parameters analyzed: acute inflammation (AI); tissue necrosis (TN); granulation tissue (GT); and fibroplasia (FP).

Treatment	Parameter			
	AI	TN	GT	FP
T1	2 (2-3) a	2 (1-2) ab	1 (0-1) a	0 (0-1) a
T2	2 (2-3) ab	2 (1,5-3) ab	1 (0,5-1) a	0 (0-0,5) a
T3	3 (3-3) ab	2 (1,5-3) a	1 (0,5-1) a	0 (0-0,5) a
T4	2 (1-3) ab	1 (0,5-2) b	1 (0-1) a	0 (0-2) a
T5	3 (2-3) b	2 (2-2) ab	1 (0-1,5) a	1 (0-1) a

0= absence; 1=mild; 2=moderate; 3=severe. Levels provided the median of each treatment, and the statistical analysis (Kruskal-Wallis) was performed, in which equal letters indicated no significant difference.

Table 3. Histopathological analysis of the inflammatory infiltrates of surgically induced skin wounds by an 8.0-mm skin biopsy punch in Wistar rats, treated with 0.9% physiological solution (T1); solid vaseline (T2); low-level laser therapy (T3); copaiba oil-resin (T4); copaiba oil-resin in association with low-level laser therapy (T5), of samples collected on day 7 of treatment. Parameters analyzed: acute inflammation (AI); tissue necrosis (TN); granulation tissue (GT); and fibroplasia (FP).

Treatment	Parameter			
	AI	TN	GT	FP
T1	0 (0-0) c	0 (0-0) c	2 (1,5-3) a	2 (2-2,5) abc
T2	1 (0-1) abc	1 (0-1) ab	3 (3-3) a	2 (1-2) c
T3	0 (0-0,5) bc	0 (0-0,5) bc	2 (1,5-3) a	3 (2-3) a
T4	1 (0-2) a	1 (1-2) a	3 (2-3) a	2 (1-2) bc
T5	1 (0-1) ab	1 (0-2) a	2,5 (0-3) a	2,5 (2-3) ab

0= absence; 1=mild; 2=moderate; 3=severe. Levels provided the median of each treatment, and the statistical analysis (Kruskal-Wallis) was performed, in which equal letters indicated no significant difference.

Table 4. Histopathological analysis of the inflammatory infiltrate of surgically induced skin wounds by an 8.0-mm skin biopsy punch in Wistar rats, treated with 0.9% physiological solution (T1); solid vaseline (T2); low-level laser therapy (T3); copaiba oil-resin (T4); copaiba oil-resin in association with low-level laser therapy (T5) of samples collected on day 14 of treatment. Parameters analyzed: acute inflammation (AI); tissue necrosis (TN); granulation tissue (GT); and fibroplasia (FP).

Treatment	Parameter			
	AI	TN	GT	FP
T1	0 (0-0) a	0 (0-0) a	0 (0-1) b	2,5 (1-3) a
T2	0 (0-0) a	0 (0-0) a	1 (1-2,5) a	3 (1,5-3) a
T3	0 (0-0) a	0 (0-0) a	0 (0-1) b	2 (2-3) a
T4	0 (0-0) a	0 (0-0) a	0 (0-1) b	3 (3-3) a
T5	0 (0-0) a	0 (0-0) a	0 (0-1) b	2 (2-2) a

0= absence; 1=mild; 2=moderate; 3=severe. Levels provided the median of each treatment, and the statistical analysis (Kruskal-Wallis) was performed, in which equal letters indicated no significant difference.

Collagen type I analysis revealed a minor advantage in wounds treated with negative control on day 1, although statistical differences were absent between the treatments. By days 7 and 14, treatments were identical according to the statistical tests, although the greater expression of this molecule was found in wounds treated with T5 on day 7 and T1 on day 14.

Quantification of type III collagen on day 1 revealed wounds treated with negative control as inferiors, while other protocols were equal (Table 5). By day 7, no differences were found between the treatment groups. However, on day 14, higher expression of type III collagen was detected in T4 and T5 samples, with the presence of oil-resin, indicating that Copaiba stimulates this molecule (Figure. 3). However, this advantage was considered equivalent to the T2 and T3 groups. The negative control, in this evaluation, showed poorer results, corroborating the initial findings, even though equal to T2 and T3, according to statistical analysis.

DISCUSSION

The study showed that the association between low-level laser therapy and copaiba oil-resin provides advantages in comparison with the T4 protocol, enhancing the macroscopic aspect of the wounds, with a minor amount of coating. This may indicate that laser therapy can minimize the deleterious effects caused by copaiba phytochemical properties, allowing the anti-inflammatory potential of both therapeutic alternatives to be exacerbated.

The copaiba composition, formed mainly by sesquiterpenes (approximately 90% of the plant constitution), has an anti-inflammatory function, and diterpenes, which have antimicrobial activities, contribute to this plant's healing characteristics. These components also play an important role in promoting wound contraction and increasing the rate of epithelialization (LEANDRO et al., 2012; MASSON-MEYERS et al., 2013). Despite the vegetal therapeutic qualities, wound healing rate, differing from that found by Masson-Meyers et al. (2013), revealed that phytotherapy exhibited inferior results, presenting a delay in wound contraction and epithelialization, whereas the aforementioned study showed superior results using copaiba. This can be derived from the oil-resin physical qualities, in which viscosity tends to form extensive crusts on the skin, hindering wound contraction. In addition, the characteristics of the experimental model may have influenced the outcomes since the T4 and T5 wounds were placed where more tissue dead space was found due to loss of skin.

Regardless of using the same plant, pharmaceutical forms were distinct in both studies: Masson-Meyers et al. (2013) employed 10% copaiba cream, which may have improved the benefits of phytotherapy on the skin; in contrast, the present study used the *in natura* configuration to develop unfavorable crust formation. Vieira et al. (2008) observed extensive crust and delayed epithelialization when applying copaiba oil-resin *in natura*, confirming the deleterious factor of its unprocessed form. Additional to formulation differences, herbal chemical composition variation should be considered, caused by factors such as geography, harvest, climate, cultivation, age of vegetables, and storage conditions (MOSSI et al., 2009).

Low-level laser therapy was less efficient than the negative control in reducing the size of the wounds, diverging from what was found by Silveira et al. (2007), whose study demonstrated the advantages of using an AsGa laser (904 nm), despite the absence of other treatments, even in the control group, for comparison. This observation may be due to the experimental model since T1 and T3 are quite close, and laser effects could influence adjacent tissue.

Table 5. Quantification through a percentage of type I (I%) and type III (III%) collagen of surgically induced skin wounds by an 8.0-mm skin biopsy punch in Wistar rats, treated with 0.9% physiological solution (T1); solid vaseline (T2); low-level laser therapy (T3); copaiba oil-resin (T4); copaiba oil-resin in association with low-level laser therapy (T5), on days 1, 7and 14 postoperatively.

Treatment	COLLAGEN TYPE					
	I%			III%		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
T1	37,5 (20-70) a	25 (20-32,5) a	25 (15-57,5) a	10 (5-15) a	25 (15-35) a	10 (7,5-15) a
T2	20 (12,5-42,5) ab	15 (7,5-55) a	20 (10-52,5) a	30 (25-30) b	25 (7,5-35) a	25 (15-32,5) ab
T3	20 (5-45) ab	20 (10-35) a	20 (12,5-47,5) a	30 (25-30) b	25 (17,5-32,5) a	25 (17,5-37,5) ab
T4	15 (10-20) b	10 (5-40) a	20 (12,5-22,5) a	25 (27,5-32,5) b	20 (10-42,5) a	35 (17,5-42,5) b
T5	10 (7,5-20) b	30 (15-37,5) a	10 (5-25) a	20 (17,5-27,5) b	10 (7,5-20) a	35 (27,5-42,5) b

Percentages provided the median of each treatment, and the statistical analysis (Kruskal-Wallis) was performed, in which equal letters indicated no significant difference.

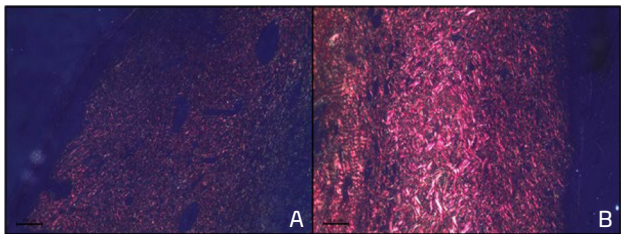

Figure 3. Expression of type I (bright red) and III (green) collagen in wounds produced with an sterile 8.0-mm skin biopsy punch in Wistar rats, treated with 0.9% physiological solution (A); and copaiba oil-resin in association with low-level laser therapy (B), on day 14 post-procedure.

Necrosis reduction in wounds treated with copaiba oil-resin on day 1 revealed the advantages of copaiba in wound healing protocols, corroborating the findings of Estevão et al. (2013), who showed the lowest rates of necrosis in cutaneous flaps treated with copaiba oil-resin.

The advantages observed in the resolution of the inflammatory process using a laser were similar to those noted by the authors who tested the AsGa laser. Demir, Balay, and Kirnap (2004) observed increased collagen synthesis and the enhanced strength of the scar tissue, in addition to reducing the inflammatory phase, in agreement with our findings that exposed minor necrosis degree and superior fibroplasia expression in both treatments using this method (T3 and T5) on day 7, although statistically equivalent to T1.

Our results of collagen quantification, in which a therapeutic protocol using copaiba resulted in denser and more organized collagen fibers, corroborate the findings of Silva (2013), who showed better results in type III collagen expression in wounds treated with copaiba. Masson-Meyers et al. (2013) had more evident effects, with differences in the collagen synthesis, demonstrated in hydroxyproline assays, with superior results in wounds treated with copaiba, which is in agreement with our study that indicates a tendency for increased expression of these molecules in treatments that included phytotherapy.

Samples presented a greater amount of type III collagen than type I collagen on day 14, indicating that collagen fibers had not yet been replaced, as expected in the remodeling phase. This information emphasizes the need for further studies to gather broader information.

All treatments achieved positive results, with complete wound epithelialization, except for the positive control, which demonstrated macroscopic and histopathological disadvantages.

CONCLUSIONS

Macroscopic aspects of wounds imply that the association between laser therapy and phytotherapy is favorable for reducing crust formation. Additionally, it was suggested that copaiba when used alone or in combination with laser therapy promoted the enhancement of type III collagen expression in cutaneous wounds on day 14 post-procedure.

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