

**Effects of the topical administration of copaiba oil ointment
(*Copaifera langsdorffii*) in skin flaps viability of rats¹**

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ABSTRACT

PURPOSE: To evaluate the effects of copaiba oil ointment (*Copaifera langsdorffii*) on dorsal skin flaps in rats.

METHODS: Adult male rats (n=30) were distributed into three groups of ten animals each, as follows: GC - control; GCA - absolute control and GT - treated with copaiba ointment. The rats were subjected to dorsal cutaneous skin flap surgery and the animals from the GC and GT received post-operative treatment for eight consecutive days. The animals from the GCA group did not receive treatment while the animals from the GC group received daily topical treatment of ointment without the active ingredient and the animals from the GT group were daily treated with 10% copaiba oil ointment. At the end of each experimental period the lesions were evaluated according to the percentage of necrotic area. Then, fragments from cranial, median and caudal parts were fixed in Bouin's solution and processed for paraffin embedding. The morphology of histological sections (5µm) was evaluated and the number of leucocytes, fibroblasts and blood vessels was also analyzed. The data obtained were submitted to ANOVA test complemented by Tukey-Kramer test (p<0.05).

RESULTS: The necrotic area was lower in the group treated with copaiba ointment when compared to the control groups (GCA>GC and GT), while the morphology showed larger granulation tissue with bulky fibroblasts and collagen fibers more arranged in the GT group. The morphometry showed a significant higher number of blood vessels in the median and caudal parts (GT>GCA and GC), leucocytes in the cranial part (GT>GC>GCA), and also fibroblasts in the median (GT and GC> GCA) and caudal parts (GT>GC and GCA) (p<0.05).

CONCLUSION: The copaiba oil ointment favors angiogenesis and accelerates the viability of random skin flaps in rats.

Key words: Surgical Flaps. Fabaceae. Skin. Ischemia. Rats.

Introduction

Skin flaps are segments of skin taken from a donor area and transferred to a receptor area, temporarily or permanently connected by a pedicle from which comes blood supply. Able to survive due to the maintenance of the circulation system integrity, they provide immediate coverage of a wound, thus avoiding prolonged healing of scab and flaps by second intention¹.

The onset of ischaemia and the consequent necrosis of the skin flaps will depend upon the level of blood supply provided by the vessels of the pedicle. Signs of neovascularization in the skin flap can only be detected from the third post-operative day, since the secondary pedicle is only complete around the ninth day². According to Pavletic³ revascularization is important to keep the skin flap alive for six to seven days in rats and revascularization from the bed of the wound itself is more important than that originating from the edges of it.

Skin flaps are categorized as those with vascular pedicles, those based on a subdermal plexus and standard axial-pattern skin flaps. Subdermal plexus skin flaps, also called random pattern skin flaps, random skin flaps and local skin flaps, are harvested without taking into consideration their inherent vascularization. They are fed by the direct cutaneous artery terminal branches, which is part of the chamber of the panniculus muscle. The standard axial-pattern skin flap incorporates an artery and direct cutaneous veins⁴.

The use of the skin flap combined with pharmacological resources, mainly substances that stimulate angiogenesis, influences the success of reconstructive techniques and thus are commonly employed. Among the pharmacological resources available, it can be mentioned studies that employ vasodilatory and vasoactive drugs, calcium channel blockers and antioxidants^{2,5-7}. Substances such as buflomedil, human growth factor, nicotine, hyaluronidase, pentoxifylline and dimethyl sulphoxide have been studied⁸⁻¹¹. Also, other studies evaluated the effects of certain non-pharmacological resources such as transcutaneous electrical nerve stimulation¹², electroacupuncture¹³ and low-potency lasers¹⁴.

Copaiba oil, extracted from trees of the genus *Copaifera*, from the family Leguminosae-Caesalpinioideae is a substance which has become very important in Brazilian Natural Medicine^{15,16}. Its effects as a healing substance and as an anti-inflammatory agent have been studied in many experimental models. However, its effects in healing models, where angiogenesis is a fundamental factor, have been little studied.

In 2000, in a study that investigated the morphological and morphometric aspects of the healing process of skin wounds treated with copaiba oil (*Copaifera reticulada*) in rats, Brito *et*

*al.*¹⁷ observed an increase in lesion crusting, granulation tissue and the number of blood vessels. Moreover, it has been reported that the copaiba oil (*Copaifera langsdorffii*) had the capacity to reduce the time needed for tissue repair to take place in skin wounds in rats^{18,19}, the formation of granulation tissue²⁰, while it also showed anti-inflammatory and analgesic properties¹⁹⁻²². Lima Silva *et al.*²⁰ and Paiva²¹ demonstrated that *Copaifera langsdorffii* is capable of accelerating the closure of open wounds in rats.

Based on positive results with copaiba oil in various models of healing, and taking into account the need for therapeutic resources to improve the viability of skin flaps, this study sought to evaluate the macroscopic and morphometric aspects of random skin flaps treated with 10% copaiba oil (*Copaifera langsdorffii*) ointment in rats.

Methods

The study protocol was approved by the Animal Ethics Committee of the Federal Rural University of Pernambuco (UFRPE) (process n° 23082.014123/2011). At the end of the experiment the animals were euthanized, prepared and discarded according to the requirements of ethical principles for experimental work of the Brazilian College of Animal Experimentation (COBEA), Sao Paulo, Brazil, 1991.

Thirty adult male rats (*Rattus norvegicus albinus*), weighing 250g, at three months of age were obtained from the Morphology and Physiology Department of Federal Rural University of Pernambuco (UFRPE). The animals were housed in individual boxes with commercial chow (Presence®, Purina) and water *ad libitum*, maintained at 23-25°C, under 12 hour light/dark cycle, in the animal colony at the Pharmacy Department of Rural Federal University of Pernambuco (UFRPE).

The rats were then randomly divided into three groups of ten animals each according to the application of the ointment. GCA - absolute control with no treatment; GC - (control) that received topical treatment with ointment containing vaseline and glycerine as a vehicle and GT - the animals received topical treatment with 10% copaiba oil ointment. Euthanasia was performed by overdose of anesthetics.

Surgical procedure

All animals were anesthetized with a combination of xylazine (20 mg/Kg) and ketamine (100 mg/Kg), administered (right gastrocnemius muscle) intramuscularly. They were subsequently placed in the prone position, their limbs were

immobilized with adhesive tape and the dorsal thoracic region was shaved and antisepsis was performed with topical alcoholic chlorhexidine 0.5%. A surgical pen with a rectangle cranial base of 3 cm by 8 cm length, limited by the lower cranial the paddles angles was also used¹⁰. The skin flap was elevated using an incision with a number 15 scalpel blade in the delimited area. After being dissected and removed from the adjacent muscle-aponeurotic plan by using blunt scissors, the skin flap was released from the surrounding tissues, brought up to the relevant area and sutured to it using a simple interrupted suture at 4.0 monofilament nylon line. The skin flap comprised superficial fascia, a fleshy panicle, subcutaneous tissue and skin.

Immediately after the dorsal skin flap surgical procedure, the rats from GT group were subjected to the topical treatment with 10% copaiba oil ointment. This procedure was repeated every 24 hours, making a total of eight treatments the GC group rats were treated topically with ointment containing only the “vehicle” (vaseline and glycerine) for the same period of time. The GCA animals were not treated.

Preparation of the copaiba oil and ointment extract

The copaiba oil was extracted from native trees of *Copaifera langsdorffii* (Leguminosae/Caesalpinioideae) registered by the Mandeville Herbarium (Herbário Mandevilla) of the Faculty of Agrarian Sciences of the University Centre of Patos in the state of Minas Gerais in Brazil.

Two preparations were made, the test ointment and the control. The preparation of the control ointment contained only glycerine and vaseline, while the test ointment was prepared by adding 10% copaiba oil to the control ointment. After these manipulations, samples were collected for microbiological examination.

Morphology and morphometry

At the eighth day of treatment, the animals were anaesthetized with isoflurane and photographed with a digital camera (Sony w70) at a focal distance of 20 cm. These photographs were taken at the resolution of 640 X 480 pixels, 24 bits of colour, with demarcation of the necrotic area and total area and later calculation of the percentage of the necrotic area (Percentage (%) off lap necrosis = area equivalent of necrotic tissue/total area to the flaps x100%).

Fragments were collected for histological analysis from distinct areas: cranial (containing healthy tissue), median

(between the cranial and caudal areas) and the caudal (including the area of necrosis). The fragments were fixed in Bouin’s solution for 24 hours, dehydrated in increasing concentrations of ethyl alcohol and diaphanized in xylene. Samples were then included in paraffin. Samples of longitudinal sections (5µm thick) were obtained parallel to the greater axis of fragments and stained with hematoxylin-eosin (H.E) and Gomori trichrome for morphological and histometric analysis.

The material was analyzed and photographed using an optical microscope (BX-51 Olympus). Quantification of the blood vessels, leucocytes and fibroblasts were performed in the center of the lesion in an area of 0.66 mm² (imaging), with the aid of an image analyzer (Imagelab 2000® system) in a Windows operational system. The preparation of histologic slides as well as the image analysis was carried out in the Histology area of the Department of Animal Morphology and Physiology (DMAF) of the Federal Rural University of Pernambuco.

Statistical analysis

The data was evaluated by ANOVA complemented by Tukey-Kramer test (p<0.05). Statistical analysis was performed using Assistat software, version 7.6 beta2.0.

Results

The chemical analysis of the oil was carried out via the technique of gas-chromatography coupled mass spectroscopy (CG/EM). The CG/EM analysis for copaiba oil provided the chromatogram below (Figure 1 and Table 1). The identification revealed the presence of 16 compounds, making up 99.99% of the oil. Of these, 62.82% correspond to sesquiterpenic hydrocarbons, 10.63% was diterpene and 26.54% methyl ester.

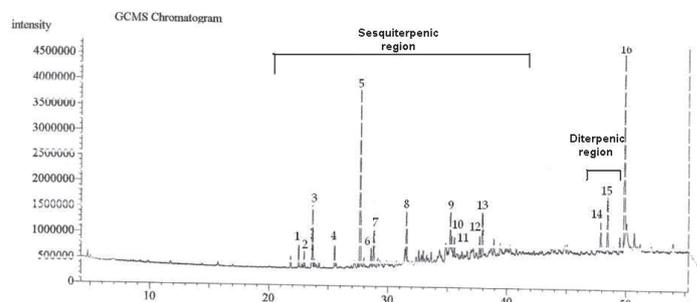


FIGURE 1 - Copaiba oil (*Copaifera langsdorffii*) chromatogram.

TABLE 1 - Chemical composition of copaíba oil tested in the experiment.

	Compound	Retention index (IR)	Relative %
1	α -cubebene	1359	2.37
2	Ciclosavitene	1370	1.51
3	α -Copene	1386	5.80
4	β -cariophyllene	1429	2.45
5	γ -muurolene	1480	22.73
6	γ -Patchoulene	1501	1.59
7	Trans- β -guaiene	1506	4.32
8	α -cedrene epoxide	1575	4.95
9	Zizanone	1670	4.41
10	(Z)- α -santalol	1673	1.39
11	Mustakone	1678	2.08
12	curcumenol	1734	2.45
13	eremofilone	1741	6.77
14	Abieta-8,12-diene	2028	3.79
15	Caurene	2046	6.84
16	Methyl 9-octadecenoate	2091	26.54
	Total		99.99

Macroscopically, there was a lower percentage of necrotic area in the GT group compared with the CGA (Figures 2 and 3). The GT had a less hardened necrotized area with a yellower colour in the delimited area (Figure 2). During collection of samples for histological examination, the tissues with surface necrosis from the GT group appeared more irrigated on the dermic side. The GT animals had areas of necrosis that varied from 7% to 30%. The GC animals had areas of necrosis which varied from 17% to 48% and the GCA group, variations from 33% to 50% (Figure 3).

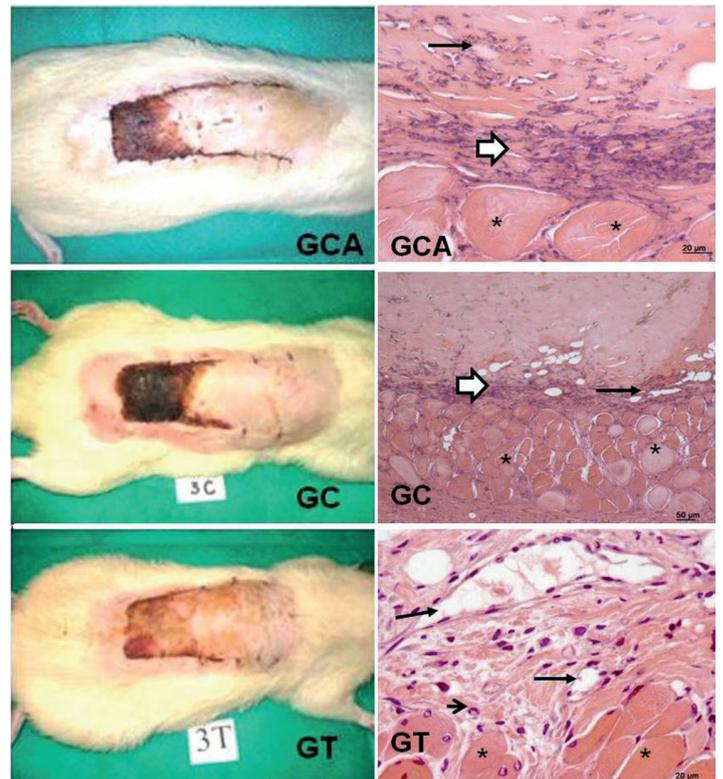


FIGURE 2 – Photographs of rats of each group showing the macroscopic aspects of skin flap (left column). Photomicrographs of part of caudal portion of skin flap of an animal from each group (right column). Note presence of cells undergoing process of necrosis (long arrows), muscle cells (*) and few blood vessels (short arrow). GCA – absolute control group; GC – control group; GT = treated with copaiba oil ointment group. HE x400.

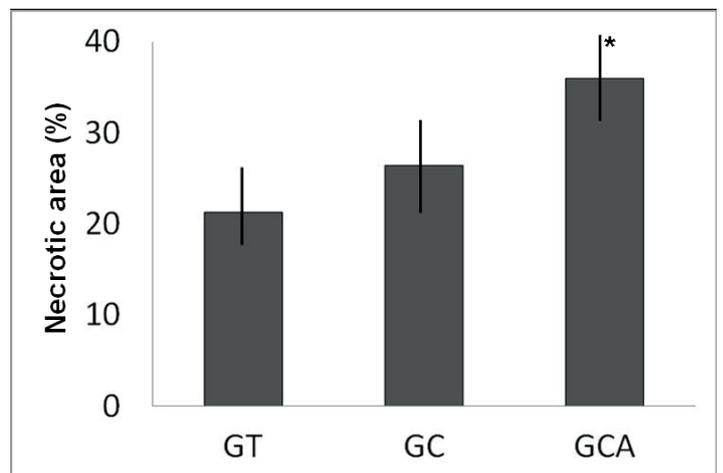


FIGURE 3 - Graph showing the percentage of necrotic area in skin flaps of rats (expressed as mean \pm SD) (GCA>GT; p<0.05). GCA – absolute control; GC – control; GT = treated with copaiba oil ointment.

The analyses of variance showed that there was a significant difference (p<0.05) for the percentage of necrotic area (GCA > GC and GT) (Figure 3), number of blood vessels in the median and caudal parts (GT > GCA and GC), number of

leucocytes in the cranial (GT > GC and GCA), median (GT and GC > GCA) and caudal parts (GT > GC > GCA) and number of fibroblasts in the median (GT and GC > GCA) and caudal parts (GT > GC and GCA) (Table 2). There was no significant difference among the groups for blood vessel, no fibroblast count in the cranial parts ($p > 0.05$) (Table 2). The GT group had higher means for the number of blood vessels in the caudal and median parts of the flap, however, there was no difference between the medians in the cranial region of the flap (Table 2).

TABLE 2 - Number (expressed as mean \pm SD) of vessels, leucocytes and fibroblasts in the median, caudal and cranial parts of flaps.

	N° of vessels			Leucocytes			Fibroblasts		
	GCA	GC	GT	GCA	GC	GT	GCA	GC	GT
Cranial	5.2 \pm 0.3	5.1 \pm 0.2	5.8 \pm 0.2	3.9 \pm 0.2 ^b	3.7 \pm 0.2 ^b	5.68 \pm 0.3 ^{a*}	3.9 \pm 0.1	4.14 \pm 0.1	4.22 \pm 0.1
Media	4.7 \pm 0.5 ^b	4.1 \pm 0.5 ^b	8.3 \pm 2.1 ^{a*}	3.5 \pm 0.2 ^b	4.70 \pm 0.3 ^{b*}	6.2 \pm 1.5 ^{a*}	3.0 \pm 0.1 ^b	3.7 \pm 0.3 ^a	4.6 \pm 0.5 ^{a*}
Caudal	3.0 \pm 0.2 ^b	2.6 \pm 0.1 ^b	7.1 \pm 1.2 ^{a*}	2.5 \pm 0.3 ^c	4.75 \pm 0.2 ^b	6.26 \pm 0.4 ^{a*}	2.2 \pm 0.2 ^b	2.0 \pm 0.2 ^b	4.2 \pm 0.5 ^{a*}

GCA – absolute control; GC – control; GT = treated with copaiba oil ointment; a>b>c; * $p < 0.05$.

Discussion

The period of post-operative observation of the skin flaps was set to eight days. By then, the area of necrosis was already defined, the process of angiogenesis had already set in and there was neovascularisation from the bed and the perimetral area of the skin flap as also reported by Guimaraes *et al.*²³

A dorsal cranial-based pattern skin flap of dimensions at 8 cm (length) by 3 cm width (proportion 2.6:1) was defined to study necrosis, revascularization and histological alterations. Earlier, Campos *et al.*⁷, used skin flaps with a length x width ratio of 1:1. In later studies, however, for greater effectiveness this was changed according to the blood supply upon the area operated. The type of flap studied in this research had a sufficiently increased length-width ratio to evaluate the effects of a venous return under hampered conditions. The setting-in of ischaemia and subsequent necrosis depends upon the blood supply provided by the blood vessels in the pedicle.

Brito *et al.*¹⁷ in an experiment of healing of skin wounds in rats using copaiba oil (*C. multijuga*), reported that the oil was capable of increasing the vascular network by the 7th post-operative day, which is in accordance with our findings. Eurides *et al.*¹⁸ observed an increase in granulation tissue formation and blood vessels during the process of tissue repair in healing wounds in rats, demonstrating that copaiba oil (*Copaifera langsdorffii*) is

capable of increasing vascularization. Our results were similar to those reported and reinforce the evidence for copaiba oil's useful properties.

The increase in concentration of fibroblasts and blood vessels in the animals of the GT group in this experiment is in accordance with the proliferation phase for repair tissue. This second phase of the healing process is characterised by the processes of granulation, including capillary formation and the production of collagen by the fibroblasts. The presence of a large number of leucocytes in the median and caudal portion of the GT animals is explained by the presence of necrotic cells, stimulating leucocyte production as a result of phagocytosis. It was observed that copaiba oil was effective in the healing of the flaps, making the tissues more viable during the time taken for tissue repair to take place²⁴.

The chemical analysis carried out on the copaiba oil used in this study showed a chemical profile similar to that of the copaiba oils researched by Veiga Junior and Pinto²⁵. Santos *et al.*²⁶ worked with various copaiba oils from native trees of the Amazon and identified oils originating in the Amazon basin, with a predominance of diterpenes, as identified in the oil tested. The diterpenes copalic acid, found by Veiga Junior *et al.*²⁷ in all the commercial oils studied, was not identified by Gramosa and

Silveira²⁸ in study on the species *Copaifera langsdorffii* and was not identified in this study either. The chromatogram confirmed the authenticity of the oil and its type because the chemical constituents identified in the oil tested and the oil of *C. langsdorffii* collected in the municipality of Crato, state of Ceará were similar to those found in this experiment²⁸.

The mechanism of action of the active ingredients in copaiba oil is still not yet totally clear²⁹. The majority of its anti-bacterial and anti-fungal properties is related to the products of secondary metabolism, found in essential oils such as terpenoids and phenolic compounds, which also in their pure form exhibit antimicrobial properties. Terpenes are the main chemicals responsible for the fragrance and medicinal and culinary uses of plants²⁷. It is suggested that the results of this research resulted from the chemical properties of one or more of the components of the oil tested, which, due its synergetic action, lead to an increase in neoangiogenesis.

With reference to its use “in natura”, the advantages of the ointment included its ease of application without being excessively oily and a decrease in concentrations of phytotherapeutic compounds.

Conclusion

The copaiba oil (*Copaifera langsdorffii*) increased the viability of the skin flaps by decreasing the area of necrosis and was responsible for a greater proliferation of blood vessels in the median and caudal parts of the skin flap, revealing an improved process of tissue repair.

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Received: Aug 20, 2013

Review: Oct 18, 2013

Accepted: Nov 18, 2013

Conflict of interest: none

Financial sources: Pernambuco Research Foundation (FACEPE), Minas Gerais Research Foundation (FAPEMIG) and National Council of Technological and Scientific Development (CNPq).

¹Research performed at Laboratory of Experimental Surgery, Federal Rural University of Pernambuco (UFRPE), Brazil.