UNIVERSIDADE ESTADUAL PAULISTA "Júlio de Mesquita Filho" INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

LUCAS FERNANDO SÉRGIO GUSHIKEN

Avaliação do potencial cicatricial de formulações tópicas contendo β-cariofileno em lesões cutâneas de ratos

Botucatu – SP

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biotecnologia, Área de concentração: Biotecnologia aplicada à saúde humana e animal, Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Doutor.

Orientador: Profa. Dra. Cláudia Helena Pellizzon

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"Em algum lugar, algo incrível está esperando para ser descoberto. Quando você faz uma descoberta – mesmo se você for a última pessoa na Terra a ver a luz – você jamais vai se esquecer."

Carl Sagan

Dedicatória

Aos meus pais, Paulo e Isabel, e minha irmã Paula, que sempre me apoiaram em minhas escolhas pessoais e profissionais, sendo meu alicerce, meu refúgio, minha inspiração e fortaleza. Que um dia eu consiga fazer jus a todo o amor, carinho e força que vocês sempre me deram.

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como um adolescente imaturo, deixando pai, mãe e irmã – minha família de sangue – para construir o que jamais pensei ser capaz: uma família de alma. Família sim, pois mesmo eu sendo antissocial e pouco emotivo, posso dizer sem qualquer dúvida, que vocês são parte da minha vida e que hoje sou um pouco melhor como biomédico, pesquisador e ser humano graças a vocês.

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PRÓLOGO

O projeto de doutorado possibilitou o aprimoramento técnico e científico do aluno na área de "Biotecnologia", subárea "biotecnologia aplicada à saúde humana e animal", mediante estudos *in vivo* de atividade cicatricial do β-cariofileno e do óleo-resina de copaíba. Durante o período do doutorado, o aluno realizou as seguintes produções acadêmico-científicas:

Artigos completos publicados em periódicos científicos

1. **Gushiken, Lucas Fernando Sérgio**; Beserra, Fernando Pereira; Bastos, Jairo Kenupp; Jackson, Christopher John; Pellizzon, Cláudia Helena. Cutaneous Wound Healing: An Update from Physiopathology to Current Therapies. Life, v. 11, p. 665, 2021.

2. De Oliveira, Marta H; **Gushiken, Lucas F S**; Pellizzon, Cláudia H; Mancera, Paulo F A. Mathematical modelling, parameter estimation and computational simulation for skin wound healing under *Copaifera langsdorffi* treatments. Computer Methods and Programs in Biomedicine, v. 199, p. 105915, 2021.

3. Pereira Beserra, Fernando; **Gushiken, Lucas Fernando Sérgio**; Vieira, Ana Júlia; Augusto Bérgamo, Danilo; Luísa Bérgamo, Patrícia; Oliveira de Souza, Mariana; Alberto Hussni, Carlos; Kiomi Takahira, Regina; Henrique Nóbrega, Rafael; Monteiro Martinez, Emanuel Ricardo; John Jackson, Christopher; Lemos de Azevedo Maia, Gabriela; Leite Rozza, Ariane; Helena Pellizzon, Cláudia. From inflammation to cutaneous repair: topical application of lupeol improves skin wound healing in rats by modulating the cytokine levels, NF-κB, Ki-67, growth factor expression, and distribution of collagen fibers. International Journal of Molecular Sciences, v. 21, p. 4952, 2020.

4. Pessin, Adriana Bueno Benito; Martins, Regina Helena Garcia; **Gushiken, Lucas Fernando Sérgio**; Pellizzon, Cláudia Helena. Sectorial analysis of the fibrous matrix of vocal folds in the elderly. Journal of Voice, v. -, p. S0892-1997(20)3, 2020.

5. Kauer, Débora Perrone; Alonso, Juliana De Moura; **Gushiken, Lucas Fernando Sérgio**; Lemos, Marivane; Padovani, Carlos Roberto; Rodrigues, Celso Antonio; Alves, Ana Liz Garcia; Watanabe, Marcos Jun; Bastos, Jairo Kenupp; Pellizzon, Cláudia Helena; Hussni,

Carlos Alberto. Experimental skin wound treatment with *Copaifera langsdorffii* Desf Kuntze (Leguminosae) extract and oil-resin in horses. Brazilian Journal Veterinary Res. and Animal Science, v. 57, p. E166095, 2020.

6. Beserra, Fernando Pereira; **Gushiken, Lucas Fernando Sérgio**; Hussni, Maria Fernanda; Ribeiro, Victor Pena; Bonamin, Flávia; Jackson, Christopher John; Pellizzon, Cláudia Helena; Bastos, Jairo Kenupp. Artepillin C as an outstanding phenolic compound of Brazilian green propolis for disease treatment: a review on pharmacological aspects. Phytoterpy Research, v. 2020, p. 1-13, 2020.

7. Beserra, Fernando Pereira; Vieira, Ana Júlia; **Gushiken, Lucas Fernando Sérgio**; De Souza, Eduardo Oliveira; Hussni, Maria Fernanda; Hussni, Carlos Alberto; Nóbrega, Rafael Henrique; Martinez, Emanuel Ricardo Monteiro; Jackson, Christopher John; De Azevedo Maia, Gabriela Lemos; Rozza, Ariane Leite; Pellizzon, Cláudia Helena. Lupeol, a dietary triterpene, enhances wound healing in streptozotocin-induced hyperglycemic rats with modulatory effects on inflammation, oxidative stress, and angiogenesis. Oxidative Medicine and Cellular Longevity, v. 2019, p. 1-20, 2019.

8. Costa, Philipe; Somensi, Lincon Bordignon; Da Silva, Rita De Cássia Melo Vilhena De Andr; Mariano, Luísa Nathalia Bolda; Boeing, Thaise; Longo, Bruna; Perfoll, Ellen; De Souza, Priscila; **Gushiken, Lucas Fernando Sérgio**; Pellizzon, Cláudia Helena; Rodrigues, Débora Munhoz; Bastos, Jairo Kenupp; De Andrade, Sérgio Faloni; Da Silva, Luísa Mota. Role of the antioxidant properties in the gastroprotective and gastric healing activity promoted by Brazilian green propolis and the healing efficacy of Artepillin C. Inflammopharmacology, v. 28, p. 1009-1025, 2019.

9. Kemper, Bernardo; Brandão, Cláudia V S; Rossetto, Victor J V; **Gushiken, Lucas F S**; Padovani, Carlos R; Pellizzon, Claudia H. Autologous and homologous skin grafts treated with platelet-rich plasma (PRP): experimental study in rabbits. Pesquisa Veterinária Brasileira, v. 38, p. 1818-1823, 2018.

10. Souza, M O; **Gushiken, L F S**; Beserra, F P; Pellizzon, C H. Evaluation of the gastroprotective and antioxidant effects of caffeine and caffeic acid on ethanol-induced gastric ulcer. JSM Hepatitis, v. 2, p. 1-5, 2017.

11. **Gushiken, Lucas Fernando Sérgio**; Hussni, Carlos Alberto; Bastos, Jairo Kenupp; Rozza, Ariane Leite; Padovani, Carlos R; Takahira, Regina K; Pellizzon, Cláudia Helena. Hydroalcoholic extract from *Copaifera langsdorffii* has skin wound healing activity in rats. International Journal of Complementary and Alternative Medicine, v. 6, p. 00178, 2017.

12. **Gushiken, Lucas Fernando Sérgio**; Hussni, Carlos Alberto; Bastos, Jairo Kenupp; Rozza, Ariane Leite; Beserra, Fernando Pereira; Vieira, Ana Júlia; Padovani, Carlos Roberto; Lemos, Marivane; Polizello Junior, Maurilio; Silva, Jonas Joaquim Mangabeira Da; Nóbrega, Rafael Henrique; Martinez, Emanuel Ricardo Monteiro; Pellizzon, Cláudia Helena. Skin wound healing potential and mechanisms of the hydroalcoholic extract of leaves and oleoresin of *Copaifera langsdorffii* Desf. Kuntze in rats. Evidence-based Complementary and Alternative Medicine, v. 2017, p. 1-16, 2017.

Capítulos de livros publicados

1. Beserra, F P; **Gushiken, L F S**; Hussni, M F; Pellizzon, C H. Regulatory mechanisms and chemical signaling of mediators involved in the inflammatory phase of cutaneous wound healing. In: Kamil Hakan Dogan. (Org.). Wound Healing - Current Perspectives. 4ed. London: IntechOpen, 2018, p. 1-19.

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3. XVII Workshop de Genética. 2017.

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6. Carcinogênese Experimental – 3 créditos
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8. Microscopia Eletrônica e Ultraestrutura Celular – 4 créditos
9. Propriedade Intelectual e Empreendedorismo – 2 créditos
10. Seminários II - Pesquisa em Andamento – 2 créditos
11. Sinalização Celular – 4 créditos
12. Tópicos Especiais em Biotecnologia: Conhecimento para aprimorar a descoberta e o desenvolvimento de fármacos e biomarcadores – 1 crédito
13. Tópicos Especiais em Biotecnologia: Ferramentas de Informática aplicadas à pesquisa - Introdução ao uso do software Mendeley Reference Manager – 1 crédito

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LISTA DE ABREVIATURAS E SÍMBOLOS

°C: graus Celsius

$AgNO_3$: nitrato de prata

ALT: alanina aminotransferase

ATP: adenosina trifosfato

ANOVA: análise de variância

AST: aspartato aminotransferase

Car: grupo com lesão tratado com emulgel contendo β-cariofileno a 1%

CAT: catalase

CEUA: Comissão de Ética no Uso de Animais

Col: grupo com lesão tratado com colagenase 1.2 UI

Control: grupo sem lesão e sem tratamento

Dex: grupo com lesão tratado com dexpantenol 5%

dL: decilitro

DNA: ácido desoxirribonucleico

Dsg3: desmogleína-3

EGF: fator de crescimento epidermal

ELISA: *Enzyme Linked Immuno Sorbent Assay*

EMBRAPA: Empresa Brasileira de Pesquisa Agropecuária

FAPESP: Fundação de Amparo à Pesquisa do Estado de São Paulo

FCFRP: Faculdade de Ciências Farmacêuticas de Ribeirão Preto

FGF: fator de crescimento de fibroblasto

FGF-2: fator de crescimento de fibroblasto-2

FMVZ: Faculdade de Medicina Veterinária e Zootecnia

FST: grupo com lesão não tratado

g: gramas

Gel: grupo com lesão tratado com emulgel

GPx: glutationa peroxidase

GR: glutationa redutase

GSH: glutationa reduzida

HE: hematoxilina-eosina

HRP/DAB: peroxidase de raiz forte/diaminobenzidina

IBB: Instituto de Biociências de Botucatu

IFN-γ: interferon-γ

IGF-1: fator de crescimento de insulina – 1

IL-10: interleucina-10

IL-1β: interleucina-1β

IL-4: interleucina-4

IL-6: interleucina-6

IL-8: interleucina-8

IU: unidade internacional

kg: quilograma

KGF: fator de crescimento de queratinócitos

L: litro

Lamγ2: laminina-γ2

mg: miligramas

min: minuto

mL: mililitro

MMPs: metaloproteinases de matriz extracelular

MPO: mieloperoxidase

NADPH: nicotinamida adenina dinucleotídeo fosfato reduzida

NeBa: grupo com lesão tratado com neomicina 5 mg/g + bacitracina 250 UI/g

NLC: grupo com lesão tratado com emulsão de carreadores nanoestruturados contendo óleo-resina de *Copaifera langsdorffii* 1%

nmol: nano mol

NSAIDs: anti-inflamatório não estereoidal

PDGF: fator de crescimento derivado de plaqueta

pg: pictogramas

RNA: ácido ribonucleico

ROS: espécies reativas de oxigênio

rpm: rotações por minuto

SNP: polimorfismos de nucleotídeo único

SOD: superóxido dismutase

TIMPs: inibidores de metaloproteinases de matriz extracelular

TGF-β1: fator de crescimento transformador-β1

TNF-α: fator de necrose tumoral-α

UNESP: Universidade Estadual Paulista

USA: Estados Unidos da América

USP: Universidade de São Paulo

VEGF: fator de crescimento endotelial vascular

α-SMA: α-actina de músculo liso

γ-GT: γ-glutamil transferase

µL: microlitro

µm: micrômetro

µm²: micrômetro quadrado

SUMÁRIO

RESUMO

A pele é fundamental para manter a integridade do organismo. A descontinuidade desse sistema promove o mecanismo cicatricial, que pode ser dividido em fases inflamatória, proliferativa e remodeladora. As lesões cutâneas têm grande importância na saúde pública devido à possibilidade de deficiência física ou até mesmo levar à morte. Como consequência de estudos anteriores referentes à atividade cicatricial da *Copaifera langsdorffii*, o sesquiterpeno β-cariofileno e a óleo-resina de *Copaifera langsdorffii* nanoencapsulada foram selecionados para analisar o potencial cicatricial das formulações e seus mecanismos de ação. Para isso, foram utilizados ratos *Wistar* machos divididos em grupos experimentais (n = 5): Controle, FST, Gel, Col, Dex, NeBa, Car e NLC. Os animais foram submetidos à lesão dorsal de 3 cm de diâmetro, tratadas duas vezes por dia durante 3, 7 e 14 dias. As áreas das lesões foram medidas diariamente para verificar a redução macroscópica das lesões e amostras foram retiradas para análises de atividade anti-inflamatória, histopatológicas e imunohistoquímicas. Macroscopicamente foi possível observar a retração das lesões nos tratamentos Car e NLC comparados com FST e Gel. Os resultados de ELISA mostraram o potencial anti-inflamatório dos tratamentos Car e NLC pela redução de citocinas pró-inflamatórias TNF-α, IFN-γ, IL-1β e IL-6 e aumento da citocina anti-inflamatória IL-10. Os resultados das análises bioquímicas para estresse oxidativo mostraram o aumento de GPx nos grupos NeBa, Car e NLC. As análises histopatológicas e imunohistoquímicas mostraram o aumento da imunomarcação de laminina-γ2 e α-SMA, bem como a redução de desmogleína-3 em Car e NLC. Foi observado o aumento da deposição de colágeno nos tratamentos Car e NLC comparados a FST. Sendo assim, a partir dos resultados analisados, confirmou-se a atividade cicatricial do β-cariofileno e da óleo-resina de copaíba em lesões cutâneas, estimulando os mecanismos de retração da lesão mediado por miofibroblastos, o remodelamento da matriz extracelular e a reepitelização mediada por laminina-γ2; além de inibir a atividade inflamatória e o estresse oxidativo local.

Palavras-chave: cicatrização; pele; β-cariofileno; *Copaifera langsdorffii*; carreadores lipídicos nanoestruturados

ABSTRACT

Skin is essential to keep the organism integrity. The discontinuity of this system promotes the healing mechanism, which can be divided into inflammatory, proliferative and remodeling phases. Skin lesions are very important in public health due to the possibility of physical disability or even death. As consequence of previous studies regarding *Copaifera langsdorffii* healing activity, the sesquiterpene β-caryophyllene and the nanoencapsulated *Copaifera langsdorffii* oleo-resin were selected to analyze the healing potential of the formulations and their mechanisms of action. For this, male *Wistar* rats were divided into experimental groups (n = 5): Control, FST, Gel, Col, Dex, NeBa, Car and NLC. The animals were submitted to 3 cm diameter dorsal lesion, treated twice a day for 3, 7 and 14 days. Wound areas were measured daily to verify the macroscopic reduction of the lesions and samples were taken for analysis of anti-inflammatory, histopathological and immunohistochemical activities. Macroscopically, it was possible to observe wound retraction in treatments Car and NLC compared to FST and Gel. ELISA results showed the anti-inflammatory potential of Car and NLC treatments by reducing pro-inflammatory cytokines TNF-α, IFN-γ, IL-1β and IL-6 and increasing the anti-inflammatory cytokine IL-10. Results of biochemical analyzes for oxidative stress showed the increase of GPx in NeBa, Car and NLC groups. Histopathological and immunohistochemical analyzes showed an increase in laminin-γ2 and α-SMA immunolabeling, as well as a reduction in desmoglein-3 in Car and NLC. An increase in collagen deposition was observed in Car and NLC treatments compared to FST. Thus, from the analyzed results, the healing activity of β-caryophyllene and copaiba oleo-resin was confirmed in skin lesions, stimulating the mechanisms of wound retraction mediated by myofibroblasts, extracellular matrix remodeling and reepithelialization mediated by laminin-γ2; besides inhibiting inflammatory activity and local oxidative stress.

Keywords: healing; skin; β-caryophyllene; *Copaifera langsdorffii*; nanostructured lipid carriers

PREFÁCIO

O **CAPÍTULO 1** da tese contém o artigo de revisão no formato da edição especial da revista *Life - Mechanisms Underlying Skin Pathologies*, intitulado: "*Cutaneous wound healing: an update from physiopathology to current therapies*". O presente capítulo traz uma abordagem geral sobre os mecanismos fisiológicos da cicatrização de lesões cutâneas e as principais moléculas envolvidas nas três fases da cicatrização: inflamatória, proliferativa e de remodelamento. Além disso, o capítulo também aborda os mecanismos patológicos da cicatrização de feridas, os fatores de risco para erros no reparo tecidual e os principais tratamentos utilizados atualmente para o tratamento de feridas cutâneas, envolvendo procedimentos cirúrgicos e não cirúrgicos.

O **CAPÍTULO 2** traz o artigo do projeto de doutorado do aluno no formato padrão da edição especial da revista *Oxidative Medicine and Cellular Longevity – Mechanisms, Biomarkers, and Therapeutics involved in Inflammatory Disorders and Tissue Repair 2021*, intitulado: "*Beta-caryophyllene has antioxidant, anti-inflammatory and re-epithelialization activities in a rat skin wound excision model*". Os resultados desse artigo foram obtidos a partir do auxílio aprovado pela FAPESP (Processo n°: 2017/17600-1). Este capítulo mostra o potencial cicatricial *in vivo* da formulação tópica emulgel contendo o sesquiterpeno β-cariofileno a 1% em lesões cutâneas de ratos, comparando o novo fármaco com três medicamentos do mercado com diferentes mecanismos de ação. O estudo mostrou a atividade anti-inflamatória e antioxidante local do fármaco, bem como sua influência acelerando a reepitelização, contração da ferida e remodelamento do tecido.

O **CAPÍTULO 3** representa o segundo artigo do projeto de doutorado do aluno no formato da revista *Journal of Herbal Medicine*, intitulado "*Copaifera langsdorffii oleoresin-loaded nanostructured lipid carrier emulgel improves cutaneous healing by anti-inflammatory and re-epithelialization mechanisms*". Os resultados desse artigo foram obtidos a partir do auxílio aprovado pela FAPESP (Processo n°: 2017/17600-1). O estudo mostrou o efeito do emulgel à base de carreadores lipídicos nanoestruturados contendo óleo-resina de *Copaifera langsdorffii* a 1% em feridas cutâneas de ratos, comparando o novo fármaco com três medicamentos do mercado com diferentes mecanismos de ação. Os resultados do estudo comprovaram o efeito anti-inflamatório do fármaco testado, bem como o estímulo à reepitelização, contração da ferida e remodelamento da matriz extracelular.

O **CAPÍTULO 4** consiste nas considerações finais da tese, apresentando as conclusões dos resultados obtidos durante o doutorado do aluno.

Conceitos gerais sobre a pele, cicatrização e tratamento de lesões cutâneas

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Capítulo I

Cutaneous wound healing: an update from physiopathology to current therapies

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Abstract

The skin is the biggest organ of human body, which acts as a protective barrier against deleterious agents. When this barrier is damaged, the organism promotes the healing process with several molecular and cellular mechanisms, in order to restore the physiological structure of the skin. The physiological control of wound healing depends on the correct balance among its different mechanisms. Any disruption in the balance of these mechanisms can lead to problems and delay in wound healing. The impairment of wound healing is linked to underlying factors as well as aging, nutrition, hypoxia, stress, infections, drugs, genetics, and chronic diseases. Over the years, numerous studies have been conducted to discover the correct approach and best therapies for wound healing, including surgical procedures and non-surgical treatments such as topical formulations, dressings, or skin substitutes. Thus, this general approach is necessary to facilitate the direction of further studies. This work provides updated concepts of physiological mechanisms, the factors that can interfere, and updated treatments used in skin wound healing.

Keywords: skin; wound healing; wound therapies; topical formulation; dressings; skin substitutes

1. Introduction

The skin is the largest organ of humans that acts as the first mechanical barrier between the organism and the external environmental, to protect organism against deleterious agents, control thermal regulation, and regulate water and electrolytes homeostasis [1]. The morphologic structure of skin comprises two layers, the epidermis and dermis [2]. The epidermis is the most external layer of the skin and is divided into four or five sub-layers, depending on the region of the body [2]. Among the cells that constitute the epidermis are by far the major cell type, the keratinocytes, as well as melanocytes, Langerhans cells, and Merkel cells. The dermis is the layer of connective tissue that supports the epidermis, constituted by extracellular matrix proteins (collagens, elastin, proteoglycans, and glycosaminoglycans) synthesized by fibroblasts [3]. If there is a disruption of one or both layers of the skin, the organism starts the wound healing process to regenerate the injured area, involving cellular, molecular and biochemical mechanisms divided in three healing phases: inflammatory, proliferative, and remodeling [4,5]. In this review, we discuss the physiologic mechanism of wound healing, the risk factors that affect healing and provide an update on the main therapies to treat skin wounds.

2. Physiology of skin wound healing

Skin wound healing is a complex process involving interrelated and overlapping mechanisms of cell migration and proliferation, synthesis of extracellular matrix, growth factors and cytokines that coordinate the healing process. Due to this complexity, the wound healing process can be divided in three phases: inflammatory, proliferative, and remodeling phases (Figure 1) [6].

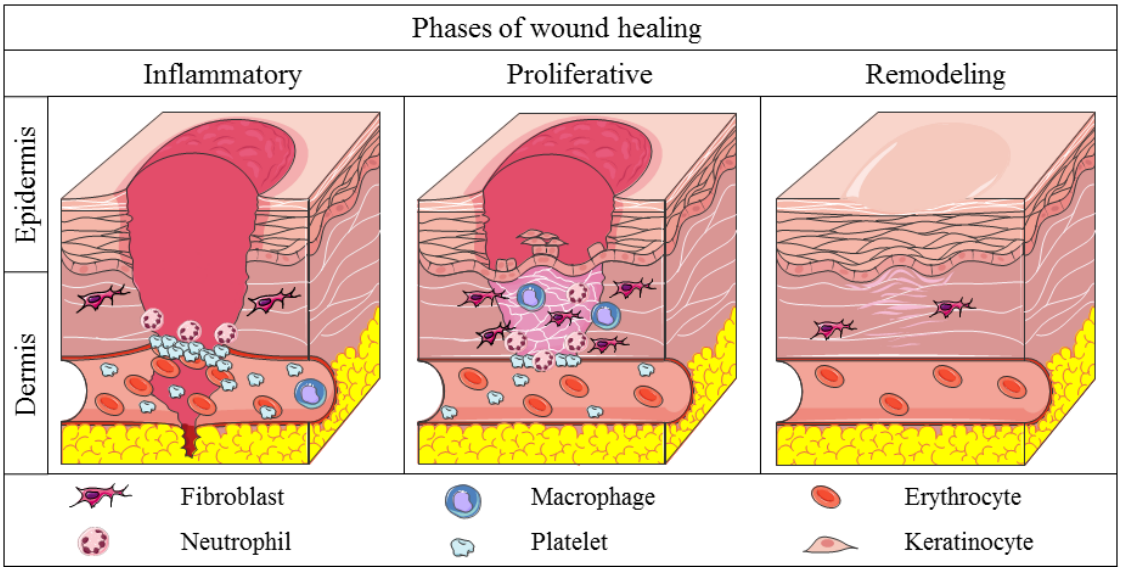

Figure 1. Phases of physiological wound healing. Inflammatory phase: there is the hemostasis of wounded area and acute inflammation through the release of cytokines, growth factors and the migration of leukocytes in the area. Proliferative phase: increase in the migration and proliferation of the keratinocytes, fibroblasts, endothelial cells and leukocytes in the wound. Increase in the synthesis of extracellular matrix components and improve of angiogenesis and re-epithelialization mechanisms. Remodeling phase: extracellular matrix remodeling, with substitution of collagen III for collagen I. Increase in the activity of MMPs. Apoptosis of provisional endothelial cells, fibroblasts and myofibroblasts of the injury.

The inflammatory phase of wound healing begins with the activation of platelets, which synthesize the compounds responsible for fibrin clot formation, restoring the local hemostasis and acting as a provisional extracellular matrix for the migration of blood cells [5]. Simultaneously, the platelets and injured cells release cytokines and growth factors such as IL-1β (interleukin-1β), TNF-α (tumor necrosis factor-α), FGF (fibroblast growth factor), and PDGF (platelet derived growth factor) which attract leukocytes to the region [4,6]. Initially, neutrophils migrate to the injured area, starting the debridement of necrotic tissue and the phagocytosis of pathogenic antigens. Furthermore, the neutrophils release pro-inflammatory cytokines as IL-1β, TNF-α, IL-6 (interleukin-6) and IL-8 (interleukin-8), which attract other inflammatory cells to the wounded area (Table 1) [7]. The neutrophils also release VEGF (vascular endothelial growth factor) and IGF-1 (insulin growth factor-1) which activate the local proliferation of fibroblasts, keratinocytes, and endothelial cells [7,8]. After a few days, macrophages start to migrate to the wound region to continue the debridement of necrotic tissue and phagocytosis of deleterious antigens, and secrete growth factors and cytokines which coordinate the subsequent mechanisms of the wound healing [5].

Table 1. Main cytokines involved in the inflammatory phase of skin wound healing. . Adapted from Holloway *et al.* 2011 [9].

Cytokine	Secretory cells	Biologic effect
Pro-inflammatory cytokines		
IFN-γ	Macrophage, neutrophil, lymphocyte T	Macrophage activation/Decrease of collagen synthesis/Synthesis of MMPs
IL-1β	Macrophage, neutrophil, keratinocyte	Chemotaxis of fibroblast and keratinocyte/Synthesis of MMPs
IL-6	Macrophage, keratinocyte neutrophil	Chemotaxis of macrophages and neutrophils/Proliferation of fibroblasts
IL-8	Macrophage, neutrophil, fibroblast	Chemotaxis of macrophages and neutrophils/Synthesis of collagen
TNF-α	Macrophage, neutrophil	Cytotoxicity of macrophages and neutrophils/Synthesis of MMPs
Anti-inflammatory cytokines		
IL-4	Lymphocyte T, basophils, mast cells	Decrease of TNF-α, IL-1β and IL-6/Proliferation of fibroblasts/Synthesis of collagen
IL-10	Lymphocyte T, macrophage, keratinocyte	Decrease of TNF-α, IL-1β and IL-6/Inhibition of macrophages and neutrophils

The proliferative phase of wound healing is characterized by the intense migration and proliferation of cells and synthesis of granulation tissue, comprised of provisional extracellular matrix, macrophages, endothelial cells, and fibroblasts [10]. The cells in the injury secrete FGF, VEGF, EGF (epidermal growth factor), and TGF-β1 (transforming growth factor-β1) to promote the proliferation of fibroblasts, keratinocytes, and endothelial cells (Table 2). Fibroblasts also synthesize compounds of provisional extracellular matrix, including type III collagen, proteoglycans and fibronectin, in order to support cell migration into the area [5,6]. The restructuring of vascularization at the wound begins immediately after the injury, but has higher activity in the proliferative phase, providing oxygen and nutrients needed for the migration and proliferation of cells and synthesis of extracellular matrix compounds. Secreted

mediators as VEGF and angiopoietins stimulate the proliferation of endothelial cells and restructuring of the vascular system at the wound site [11]. During the proliferative phase, re-epithelialization occurs to close the epithelial gap and restores the barrier function of the skin [5,12]. Firstly, the keratinocytes at the border of wounds are stimulated by growth factors, resulting in the proliferation and differentiation of the keratinocytes. This stimulus triggers the loss of keratinocyte adhesion molecules, inhibiting the physical contact with desmosomes and hemidesmosomes, and increasing the migration of these cells through the extracellular matrix [8,12].

Table 2. Growth factors involved in skin wound healing. Adapted from Bennett *et al.* 2003 [13].

Growth factor	Secretory cells	Biological effect
EGF	Macrophages/Keratinocytes	Proliferation of fibroblasts and keratinocytes
FGF-2	Fibroblasts/Endothelial cells	Proliferation of fibroblasts and keratinocytes
IGF-1	Fibroblasts/Endothelial cells/Neutrophils	Proliferation and differentiation of keratinocytes, fibroblasts and endothelial cells
KGF	Fibroblasts	Proliferation and migration of keratinocytes
PDGF	Macrophages/Platelets	Activation of neutrophils and fibroblasts/Proliferation of fibroblasts and endothelial cells
TGF-β1	Platelets/Macrophages/ Fibroblasts/Keratinocytes/ Neutrophils/Endothelial cells	Angiogenesis/Extracellular matrix remodeling/Fibroblast differentiation
VEGF	Neutrophils/Endothelial cells/Platelets	Angiogenesis

The last stage of skin wound healing is the remodeling phase, which depends on the mechanisms started in early phases. There is a decrease of granulation tissue, substitution of

provisional extracellular matrix and apoptosis of provisional cells that migrated to the area [5]. The fibroblasts are stimulated by TGF-β1 to differentiate into myofibroblasts, acquiring a contractible phenotype and decreasing the wounded area due to the multiple points of connection of myofibroblast proteins to collagen fibers [14]. Additionally, the proteins of provisional extracellular matrix are degraded by MMPs, metal-dependent proteases synthesized by local cells to remodel the wounded extracellular matrix proteins [15]. Thus, the fibroblasts in remodeling tissue synthesize type I collagen, elastin, and other compounds of permanent extracellular matrix, resulting in higher resistance and flexibility in the regenerated skin [8].

3. Pathological healing

Wound healing is a physiological process of vertebrates to maintain organism homeostasis and involves perfect interactions of numerous cell types and molecules [5]. The imbalance of these interactions can result in the occurrence of errors in the healing mechanisms and lead to the impairment of wound healing [16].

One of the errors that delays the wound healing is the chronic inflammatory state [17]. Due to the imbalance of pro- and anti-inflammatory mediators, there is an exacerbated recruitment of neutrophils and macrophages to the region, with the overexpression of inflammatory cytokines [18,19]. As result, there is the increase in leukocyte recruitment to the region and the release of reactive oxygen species (ROS) by inflammatory cells [17,20]. The overproduction of ROS inflicts cellular damage, interfering with proliferation/differentiation of keratinocytes and fibroblasts in the wounded area and leading to cell apoptosis [21]. Furthermore, the ROS cause the degradation of growth factors involved in healing mechanism, decreasing the quantity and bioavailability of these molecules [20–22]. Moreover, the increased in pro-inflammatory cytokines affects the subsequent mechanisms of wound healing, increasing MMPs and other proteases that impairs cell proliferation/migration and decreases the accumulation of extracellular matrix components [18,23,24].

In physiologic cicatrization, there is the perfect balance between the proliferation/activation and maturation/apoptosis of blood vessel during the proliferative phase of healing [25]. However, the imbalance between pro- and anti-angiogenic factors promotes the decrease of neovascularization and blood flow in the area, delaying the subsequent mechanisms from proliferative and remodeling phases [26].

Delay in the re-epithelialization is another error that can occur in wound healing. As a consequence of chronic inflammation and reduced vascularization, keratinocytes from

pathological wound edges acquire a hyperproliferative state because of the overexpression of β-catenin/c-myc pathway [27,28]. Moreover, these keratinocytes differ from normal wound keratinocytes because they are hyperkeratotic and parakeratotic and express low levels of keratins 1, 2 and 10 [29–31]. In addition, the migratory potential of the keratinocytes in abnormal healing is impaired [29,31]. The molecular mechanism of this poor migratory potential of keratinocytes is related to the proteolytic degradation of growth factors and extracellular matrix proteins mandatory for the migration, as well as the reduced expression of laminin 3A32, a precursor of laminin 5 associated with keratinocyte migration [12,31].

Impairment in remodeling is other important failure of wound healing. The cells of the injury synthesize excessive amounts of MMPs and other proteases, degrading not only extracellular matrix components, but also cell surface receptors, growth factors, and cytokines [32,33]. In addition, inhibitors of metalloproteinases (TIMPs) are reduced, contributing to protease deregulation in these injuries [32,33]. As a consequence, there is a degradation of important molecules from extracellular matrix such as collagen, elastin, fibronectin and chondroitin sulfate, impairing proliferation and migration of the cells [20,23]. Furthermore, the overproduction of MMPs and the degradation of cytokines and growth factors lead to a cycle of extracellular matrix degradation/synthesis, resulting in the impairment of extracellular matrix remodeling mechanism [20,33].

4. Factors that affect the wound healing

The human body is susceptible to numerous local and systemic conditions, which can negatively affect skin repair through various mechanisms, leading to a delay in the process. The major conditions that interfere with wound healing are discussed below (Figure 2) [34].

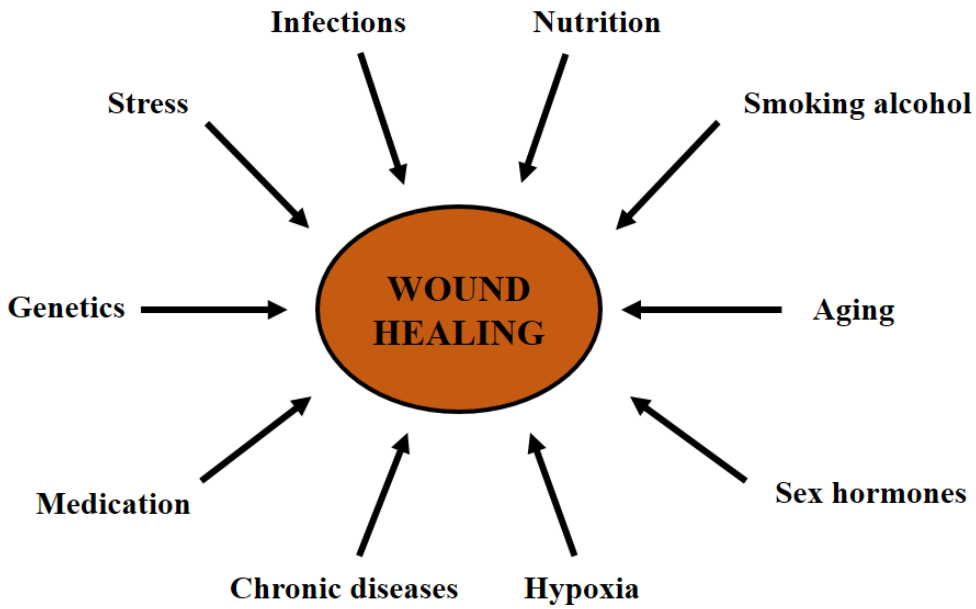

Figure 2. Factors that affect wound healing. Common situations that delay skin wound healing.

4.1. Hypoxia

Oxygen is required for ATP synthesis, which is essential for cell metabolism and survival. When an injury occurs, there is a decrease in local oxygen supply due to the vascular disruption [34]. The hypoxic wound microenvironment is important because it provides the release of mediators that coordinate the mechanisms of angiogenesis, re-epithelialization, and synthesis of growth factors and cytokines [34–36]. However, hypoxia leads to the synthesis of reactive oxygen species (ROS) and pro-inflammatory cytokines that can impair the healing process. Furthermore, the correct oxygen concentration is necessary to prevent wound infection, improve the angiogenic response, and increase fibroblast and keratinocyte differentiation, proliferation and migration [35]. Therefore, the correct balance of oxygenation is necessary to avoid the impairment and consequent delay of wound healing.

4.2. Nutrition

Wound healing requires numerous vitamins, minerals, fatty acids, carbohydrates, and proteins to perform the correct regenerative process [37]. Malnutrition impairs healing by prolonging inflammation, decreasing angiogenesis, phagocytosis and fibroblast metabolism, and prolonging the extracellular matrix remodeling [38]. Some of the essential nutrients that are important for wound healing are the omega-3 fatty acids (modulating the arachidonic acid pathway and cell membrane synthesis), vitamin A (improving the proliferation of keratinocytes), vitamin C, and carbohydrates (responsible for collagen synthesis) [37,38].

Proteins and aminoacids such as arginine, cysteine, methionine, and glutamine modulate the immune cell activity and control the collagen synthesis. Zinc is a cofactor for RNA and DNA biosynthesis, with pivotal role of proliferating cells of wound [39]. Iron act as cofactor in collagen synthesis and its deficiency impairs extracellular matrix remodeling. In addition, iron has important role in oxygen transport and hypoxia, as part of the hemoglobin molecule [37,39].

4.3. Infection

When the skin is injured, there is the possibility of local bacterial infection, resulting in the delay of healing process [38]. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and other *Streptococci* species are largely responsible for wound infection. In response to infection, the human organism starts an inflammatory mechanism, with the migration of leukocytes and cytokine release [34]. However, the phagocytic activity of leukocytes leads to the release of endotoxins by the bacteria, resulting in local necrosis and inflammation due to the increase of pro-inflammatory cytokines, higher metalloproteinase activity and decrease of growth factors release [10,40]. Although an immediate inflammatory response is an initial physiologic mechanism of healing, chronic inflammation impairs the healing process, affecting re-epithelialization and delaying wound retraction and tissue remodeling [10,34].

4.4. Stress

Stress can interfere negatively on wound healing, as well as in many systemic diseases, through the deregulation of endocrine hormones. Stress acts in the nervous system and hypothalamus increasing the release of epinephrine, norepinephrine, cortisol and glucocorticoid hormones [34,41]. These molecules promote a decrease of cytokine release and leukocyte immune response, resulting in the impairment of the inflammatory mechanism and the delay of the healing process [41].

4.5. Age

Aging is one of the major risk factors related to the impairment of skin wound healing. Due to metabolic and systemic changes during aging, the epidermal layer becomes thinner as people age [37]. There are several alterations in the inflammatory response of elderly people, such as a delay of leukocyte migration to the area, decrease of macrophage phagocytic activity, and reduction of growth factor/cytokine release [37,38]. Furthermore, aged people also present with delayed re-epithelialization, delayed angiogenesis, and a decrease of fibroblast activity and collagen remodeling [34].

4.6. Sex hormones

Estrogen shows anti-inflammatory activity through a decrease of leukocyte infiltration and pro-inflammatory cytokine release [42]. Moreover, estrogen molecules demonstrate influence over keratinocyte and endothelial cell proliferation and migration, increasing the rate of re-epithelialization and angiogenesis of wound healing [43]. In contrast, androgen hormones (testosterone and 5α-dihydrotestosterone) have a chronic inflammatory effect in skin wounds, delaying the healing process by increasing inflammatory cytokines level and leukocyte migration [42,43].

4.7. Chronic diseases

There are many diseases affecting cardiovascular, respiratory, or immunologic systems that can impair skin wound healing by interfering with inflammation, angiogenesis, re-epithelialization, and matrix remodeling mechanisms [38]. Diabetes mellitus, for example, is a multifactorial systemic disease, which shows notably impairment of wound healing. The disease affects leukocyte migration and activation and increases pro-inflammatory cytokines release, resulting in chronic inflammation [37]. Diabetes also adversely influences skin microvasculature, leading to a hypoxic environment and decreased angiogenesis. Moreover, the disease modifies keratinocyte and fibroblast proliferation and differentiation, delaying re-epithelialization and extracellular matrix remodeling [37]. Obesity is another chronic disease that causes several complications in skin wound healing. Pressure and venous ulcers associated with hematoma, oedema, seroma formation and local infection are common hallmarks in obese wounds [44,45]. The cellular and molecular mechanisms associated with the impairment of obese wound healing are related to reduced microperfusion at the skin, an excessive release of pro-inflammatory cytokines, and decreased immune response [34,44].

4.8. Medication

Currently, there are approved drugs that can have unfavorable influence on wound healing, by interfering with coagulation cascade, inflammatory mechanism, or cell proliferation [34]. Corticosteroids are used routinely as anti-inflammatory agents and also to modulate immune response, but the systemic anti-inflammatory effect of steroids induces a decrease in growth factors and cytokines which modulates other mechanisms of healing, as well as the decreasing of fibroblast proliferation [46]. Non-steroidal anti-inflammatory drugs (NSAIDs) are used systemically to treat inflammation and pain but demonstrate a negative impact on

wound healing by decreasing fibroblast proliferation, reducing wound retraction and delaying angiogenesis. When administered topically, NSAIDs formulations improve wound healing and reduce local pain [4,47]. Chemotherapeutic drugs can also interfere with skin wound healing because the mechanism of action of these molecules decreases cellular metabolism and proliferation. As consequence, there is a decrease in re-epithelialization, angiogenesis, collagen synthesis, and delay of wound retraction [46].

4.9. Smoking

It is well-known that smoking is related to the increase of risk of several diseases, including the impairment of skin wound healing, with studies proving that cigarette compounds like tobacco, nicotine, carbon monoxide, and hydrogen cyanide affect the mechanisms of healing [48]. Hypoxia is one of the major mechanisms for smoking-related impairment of wound healing, decreasing the proliferation of erythrocytes, oxygenation, blood flow, and angiogenesis in wounded tissue [34]. Smoking also increases platelet aggregation and adhesiveness, increasing blood viscosity, which results in a higher risk of thrombosis and embolism. Furthermore, cigarette compounds are involved in the decrease of fibroblast migration, proliferation and collagen remodeling [34,45]. Smoking has influence in immune system, decreasing neutrophil, macrophage, and lymphocyte activity, resulting in a higher risk of infections [34,48].

4.10. Alcohol

Chronic or acute intake of alcohol contribute to the impairment in skin wound healing [34]. One of the mechanisms responsible is the suppression of host immunity and increase in susceptibility to infections. Studies revealed the influence of alcohol on inflammation, initially by decreasing neutrophil recruitment/activity and pro-inflammatory cytokines, then subsequently by promoting the chronic elevation of cytokines and leukocyte in the later stages of healing [45]. Moreover, alcohol consumption has an influence on the proliferative phase, reducing angiogenesis in the wounded area through the low expression of VEGF receptors [34,45]. As consequence, a hypoxic environment occurs in the region with the formation of oxidative stress molecules and free radicals. Alcohol intake also impairs the remodeling mechanism, decreasing collagen synthesis and altering the concentration of extracellular matrix metalloproteinases [34].

4.11. Genetic predisposition

Cutaneous wound healing can be affected by genetic factors that impair the tissue repair. Keloid, for example, is a wound repair condition that has a strong genetic influence, with an increased occurrence in African, Asian, and Hispanic ancestry and minor occurrence in Caucasian population [49]. Moreover, studies suggest that there is a major risk of keloid in mutated genes that overexpress collagen deposition and in susceptible loci, like rs8032158 SNP in *NEDD4* gene on chromosome 15 [34]. Ehlers-Danlos syndrome comprises several disorders with defective connective tissue and collagen synthesis, characterized by skin fragility and hyperflexibility, joint hypermobility, and impaired wound healing [50]. Mutations in *COL3A1* and *COL5A1* genes lead to nonfunctional collagen III and V, with alterations in extracellular matrix remodeling [37,50]. Epidermolysis bullosa is a group of genetic skin diseases characterized by fragility in dermo-epidermal junction and separation of cutaneous layers, resulting in blisters with impaired healing [51]. The existing genetic alterations in all types of epidermolysis bullosa can be found in *COL16A1* (type XVI collagen) and *FN1* (fibronectin) genes, extracellular matrix molecules related to the remodeling mechanism of skin wound healing [37].

5. Wound treatments

According to the World Health Organization, millions of people who suffer with pathologic wounds annually require medical care [52]. Currently, there are several treatments for skin injuries, comprising surgical procedures, non-surgical therapies, and pharmacologic agents, with costs of $12 billion annually and that will reach $35 billion in 2023 [52]. However, depending on the size and type of wound, the existing wound therapies are not effective. Below, we discuss the current and new alternatives to the enhancement of skin wound healing [53].

5.1. Surgical procedures

There are some surgical options to treat wounds, depending on the etiology and type of injury. Skin grafts are the most common surgical alternative to the partial treatment of burns or chronic wounds and can be divided in three different categories: autografts, allografts, and xenografts [54]. The surgical gold standard to the restoration of a wound is the autograft, in which a healthy suitable area of skin from the same person is transplanted to the injured area [55]. The autologous tissue allows the reorganization of local vasculature and restoration of epidermal function and has the advantage of no immune rejection. However, the use of autografts has some limitations, such as insufficient normal skin sites for autologous transplant

in wounds that cover a large area, consequent scarring, and painful healing [54,55]. Allografts are an alternative to autograft and can be defined as the transplantation of a suitable skin from another person. On the other hand, xenografts are skin samples from a different animal species that are transplanted to humans [55]. These therapies have many deficiencies involving not only the possibility of scarring and painful healing, but also the possibility of cross-infection and immune rejection of the transplanted tissue [54].

The surgical debridement of devitalized tissue is an alternative to extensive necrotic life-threatening wounds. Debridement of nonviable tissue is important to prepare the wound bed, avoid the impairment of healing and accelerate the extracellular matrix remodeling [56]. This is a fast and effective procedure undertaken with the patient under anesthesia. Although the effectiveness and agility of surgical debridement, this method has increased risks because of the general anesthesia [56].

The use of flaps is other surgical therapy to skin wounds. A skin flap is composed by skin and subcutaneous tissue partly detached and moved to cover a nearby wound, preserving the blood supply [57]. Blood flow maintenance is the main advantage of skin flaps compared with grafts, providing nutrients to cell proliferation/differentiation, cytokines and growth factors that control the healing. However, any microvasculature disturbance may results in flap failure and impairs the healing [58]. The anesthetic-dependence as an invasive therapy is other disadvantage of flaps compared with non-surgical treatments. Then, a high knowledge of skin flaps physiology and biomechanics is essential to the success of this method [58,59].

5.2. Non-surgical therapies

Current non-surgical therapies for skin wounds include topical formulations, dressings, scaffolds, and skin substitutes. These treatments generally fit into the following categories: manage infection and inflammation; correct balance of the wound moisture and exudate; debride the wound site or control the progress of re-epithelialization and wound contraction [60].

5.2.1. Topical formulations

Topical dermatological treatments can be defined as the application of a formulated drug to the skin [61]. The topical route of administration is the most commonly used one to treat skin wounds, with several advantages compared to systemic drugs, such as avoidance of first pass metabolism and systemic side effects, easy drug application, and suitability for self-medication. However, depending on the formulation, there is the possibility of discomfort, skin irritation,

and allergenic reactions [61,62]. Topical formulations include creams, gels, emulsions, ointments, pastes, suspensions, lotions, foams, and sprays. There are also emerging approaches to improve the stability and efficacy of topical treatments, like particulate carriers, liposomes, nanoparticles, and biopolymers [63,64].

Topical antibiotics have been widely used in skin wounds. Creams and ointments containing neomycin, bacitracin zinc, polymyxin B sulfate, povidone-iodine, metronidazole, or silver sufadiazine act in the prevention and treatment of infections in wounds, with effectiveness against several bacteria such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae* [2,65,66]. The topical antimicrobials were developed to prevent wound infection and its complications by stopping the functions of or destroying the microorganisms [10]. The local route was preferred instead of systemic antimicrobials because of the higher risk of bacterial resistance. Moreover, the use of formulations containing these antibiotics in skin wounds can accelerate the healing process, avoiding water loss through the injury and maintaining the moisture in the microenvironment [65]. These drugs are most effective in the inflammatory phase of wound healing and must be suspended when the wound is free of infection, due to the occurrence of skin hypersensitivity reactions and allergic contact dermatitis. [10,67].

Dexpanthenol, used as emulsions or ointment, is an analogue of pantothenic acid that improves the healing process by controlling the proliferation of fibroblasts and the synthesis of granulation tissue. The molecule also accelerates re-epithelialization, by promoting the proliferation and migration of the keratinocytes from the border of the wounds. Re-epithelialization is dependent on a perfect balance among cytokines, growth factors, and other mediators stimulated by dexpanthenol [68,69]. For this reason, dexpanthenol improves epithelialization, by controlling the proliferative and migratory profile of the keratinocytes from the border of the wounds, with major effectiveness during the proliferative phase of wound healing [69].

Collagenase ointment is the most used enzymatic debriding agent for skin wounds and have been used effectively to treat skin wounds for decades [70]. Collagenase is an enzyme derived from bacteria *Clostridium histolyticum* that breaks down denatured collagen, promoting enzymatic debridement of necrotic tissue and extracellular matrix proteins in full-thickness wounds [60]. Therefore, the collagenase formulation has its best activity with the tissue debridement and extracellular matrix remodeling, during the remodeling phase of wound healing [10]. Besides the debridement mechanism, studies indicate that collagenase ointments

increase the proliferation and migration of endothelial cells and keratinocytes, with possible involvement in angiogenesis and re-epithelialization mechanisms [60].

In the last decades, growth factors have been tested topically in wounds because of their benefits in healing. However, conventional formulations have low bioavailability caused by rapid clearance of growth factors [71]. As a result, emerging approaches such as nanoparticles and carriers are combined with topical formulations to improve the stability and bioavailability of growth factors as new treatments [71–73]. Becaplermin (Smith & Nephew Inc, Fort Worth, TX, USA) is a recombinant human PDGF dispersed in sodium carboxymethylcellulose-based gel which shows clinical benefits for patients with diabetic foot ulcers, stimulating neutrophil, monocyte, and fibroblast chemotaxis and proliferation [73]. In vitro and in vivo studies involving the topical application of KGF conjugated with gold nanoparticles show the promising healing potential of this formulation, enhancing the re-epithelialization through keratinocytes migration and proliferation [71,72]. Topical FGF also has being used experimentally as skin wound therapy. According to a study carried out by Xu et al. (2020), the topical application of FGF10-loaded microspheres emulsion improves collagen synthesis and angiogenesis [74].

5.2.2. Dressings

Dressings protect the injured area from exogenous agents and mechanical trauma, reducing the risk of infections and accelerating the healing process [10]. In the past, dressings functioned to keep the wound dry, removing the wound exudate. However, nowadays, it has been shown that a warm and moist microenvironment can accelerate healing [75]. Therefore, a good dressing must provide protection to the wounded area, remove the excess of exudate in the wound, allow the gaseous exchange, keep a moist microenvironment in the wound bed, and be easy to use [76]. For this reason, recent dressings were developed in order to overcome the disadvantages of previous. Recent dressings can be classified according to the polymeric materials which they are synthesized, including alginate, films, foams, hydrocolloids, hydrofibers, and hydrogels [62,75,77]. Studies suggest that the polymeric structure of dressing components have positive effects in healing, such as fibroblast proliferation and collagen deposition mediated by calcium alginate dressing, and the antibacterial film resulting from hydrogels and hydrocolloids wound interactions [62,75,78,79].

There is a different type of dressing synthesized with biomolecules that combine tissue engineering and nanotechnologies using biological polymers (collagen, elastin and hyaluronic acid), growth factors and drugs [10,75,80]. These biological dressings mimic extracellular

matrix and key molecules in cellular proliferation/migration/differentiation [75]. Natural compounds such as propolis, honey, quercetin, and silk fibroin can be associated with films or nanofibers dressings to improve their biocompatibility, antibacterial potential, remove the excess of exudate, and maintain wound moisture. In addition, in vitro and in vivo experiments confirm the influence of these biological dressings in re-epithelialization and collagen deposition [81–84]. Antibiotic drugs and composites can be associated with biological dressings to improve their healing activities. Studies involving doxycycline and graphene oxide-associated dressings accelerated wound healing, improving re-epithelialization and collagen remodeling, and enhancing the molecules antibacterial activity [85,86]. Nanotechnologies have been used in dressings for growth factor incorporation, ameliorating growth factors bioavailability and stability. According experimental studies, EGF and FGF-incorporated dressings accelerate angiogenesis, extracellular matrix remodeling and re-epithelialization, with great potential in skin wound treatment [87–92].

5.2.3. Skin substitutes

Skin substitutes mimic the normal skin, providing a protective semi-permeable barrier to the wounded area, enhancing the healing and facilitating skin regeneration [55]. Besides improve the wound healing and decrease the risk of local infections, skin substitutes also reduce the mortality and morbidity associated with chronic wounds [55,93]. Skin substitutes can be synthesized by materials distributed in three categories: scaffolds, growth factors and cells. Three-dimensional scaffolds consist act as an extracellular matrix analogue, allowing the adhesion, proliferation, migration and differentiation of the cells present in the wound [93]. Acellular skin substitutes are scaffolds with no cells, providing a template for keratinocytes, fibroblasts, and endothelial cells from the wound. Some examples of polymers used to synthesize the scaffolds are alginate, chitosan, collagen, elastin, fibronectin, glycosaminoglycans, and hyaluronic acid [93–95]. Growth factors (EGF, FGF, PDGF, TGF-β, and VEGF) can be found in skin substitutes, enhancing the proliferation and migration of endothelial cells, fibroblasts and keratinocytes of the wound [55,93]. Alloderm™ and Matriderm® are commercially available cell-free dermal substitutes composed of a three-dimensional lyophilized collagen scaffold, recommended in burn or chronic wounds [96]. Integra™ and Biobrane® are also acellular dermal substitutes with a three-dimensional type I collagen and chondroitin sulfate scaffold as extracellular matrix analog and a silicone film as epidermis analog. These commercial skin substitutes are recommended in the treatment of partial and full thickness wound, burns, or chronic ulcers [96,97].

Cellular skin substitutes have cells in the constitution and although the cells are not necessary to synthesize the skin substitutes, the addition of fibroblasts, keratinocytes, macrophage, endothelial cells, and stem cells improve the control of the regenerative mechanisms [98–100]. Bioseed-S®, Epicel®, EpiDex®, and MySkin™ are cellular epidermal skin substitutes composed by an autologous cultured keratinocyte layer seeded on scaffolds (silicone, petrolatum gauze, allogeneic fibrin), mimicking the epidermal barrier [97,101]. As epidermis analogues, these therapies are suitable as temporary wound coverage in patients who lose a big part of their total body surface area, accelerating the wound re-epithelialization. However, there are some disadvantages such as long-time preparation, hyperkeratosis and scar possibility [55,97]. When it is necessary a greater mechanical stability, dermal substitutes are more suitable than epidermal [101]. Dermagraft™ and Transcyte™ are cellular commercial dermal substitutes composed by cultured allogeneic neonatal skin fibroblasts seeded in type I collagen/silicone film [55,96,97]. These extracellular matrix analogues and their fibroblasts secrete growth factors and proteins that enhance reepithelialization by the patient's keratinocytes in partial- and full-thickness wounds [55]. In some injuries, the treatment with epidermal (keratinocyte) or dermal (fibroblast) substitutes alone can result in improper healing. Thus, more sophisticated cellular skin substitutes have been developed, mimicking epidermal and dermal layers and providing growth factors, cytokines and extracellular matrix components for initiation/regulation of wound healing [101]. Apligraf™ and OrCel™ are two commercial products containing a type I collagen scaffold seeded with allogeneic neonatal skin keratinocytes and fibroblasts [102,103]. Both therapies are used in partial and full thickness burns, chronic wounds and diabetic foot ulcers, stimulating wound repair through FGF- and KGF-related mechanisms [55,97]. Alternatively, Tiscover™ (A-Skin) consists of an autologous full-thickness cultured skin, used in the treatment of chronic therapy-resistant wounds [55,97]. The major disadvantages of dermo-epidermal cellular substitutes are their high cost and the possibility of tissue rejection, with necessity of new studies and technologies to overlap these problems [101].

6. Conclusions

The use of different therapies to treat skin wounds and the development of new drugs for wound healing requires the comprehension of the physiology of normal healing and the alterations in pathological healing. As discussed in this review, skin wound healing is composed of overlapping and interdependent mechanisms—re-epithelialization, inflammation,

angiogenesis, wound contraction, and extracellular matrix remodeling—and the imbalance among the factors that control these mechanisms can lead to a delay in the healing process, or even to unhealed wounds. Consequently, the understanding of the mechanisms that comprise the healing process, the factors that can affect the healing and the different options of therapies to wounds can provide a strong basis for the development of new drugs and technologies to skin wound treatment. Nowadays, the most used therapies for skin wounds are topical formulations containing antimicrobial, anti-inflammatory, or debriding drugs and topical growth factors improved by nanoparticles and nanostructured carriers. Furthermore, biological molecules and nanotechnology have been used to synthesize recent dressings and skin substitutes to obtain better results in tissue regeneration. Future perspectives in skin wound healing should include therapies that comprise mechanisms of all healing phases. Numerous advances have been made in the last years in order to discover new drugs, biocompatible dressings and non-immunogenic skin substitutes. With tissue engineering advances, stem cells technologies, bioinformatics and miRNAs regulation, more studies are required for the development of safe, efficient, and low-cost wound therapies.

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Atividade cicatrizante de formulação tópica contendo β-cariofileno em lesões cutâneas de ratos

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Capítulo II

Beta-caryophyllene has antioxidant, anti-inflammatory and re-epithelialization activities in a rat skin wound excision model

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Abstract

The skin is a critical organ for the maintenance of the integrity and protection of the organism. When a wound occurs, a sequence of healing mechanisms is triggered to reconstruct the wounded area. β-caryophyllene is a sesquiterpene in *Copaifera langsdorffii* oleoresin with antioxidant and anti-inflammatory potential. On the basis of previous studies with *C. langsdorffii*, β-caryophyllene was selected to evaluate its wound healing potential and pharmacological mechanisms. The excision wound model was used with male *Wistar* rats and macroscopic, histological, immunohistochemical and biochemical analyses were performed with skin samples, comparing the β-caryophyllene-treated group with reference drugs. The results showed macroscopic retraction of the wounds treated with β-caryophyllene. Biochemical assays revealed the antioxidant and anti-inflammatory mechanisms of the β-caryophyllene-treated group with increasing levels of IL-10 and GPx and decreasing levels of pro-inflammatory molecules, including TNF-α, IFN-γ, IL-1β and IL-6. After β-caryophyllene treatment, histopathological and immunohistochemical assays showed enhanced re-epithelialization mediated by laminin-γ2 and desmoglein-3 as well as a role for this sesquiterpene in a remodeling mechanism with increased collagen content. These findings indicated the wound-healing potential of β-caryophyllene in rat skin wounds, which may be useful in the future as a treatment for skin wound healing.

Keywords: wound healing; skin; β-caryophyllene; sesquiterpene; anti-inflammatory; antioxidant

1. Introduction

Skin wounds are major health problems that affect millions of people every year, causing physical and psychological deficiencies when not treated correctly (Javanmardi et al. 2020). The comorbidities associated with unhealed wounds increase every year, and the costs of healing treatments have reached billions of dollars worldwide (Sen 2019). The wound-healing process involves overlapping and interdependent mechanisms (inflammation, epithelialization, angiogenesis, wound retraction and matrix remodeling) to reconstruct the skin (Morin et al. 2012). When there is an imbalance among these mechanisms, the healing process enters a pathologic state, resulting in errors of healing, such as hypertrophic scars and unhealed wounds (Karppinen et al. 2019). To prevent pathologic mechanisms of wound healing, there are several treatments on the market used to promote cutaneous healing by acting as antimicrobial or anti-inflammatory agents, improving tissue debridement, epithelialization and/or remodeling mechanisms. However, the existing treatments may not be efficient in treating cutaneous wounds depending on the type, extension and location of the injury (Ward and Saffle 1995). Therefore, studies have focused on discovering alternative drugs that accelerate skin wound healing without scarring (Rennert et al. 2013).

β-caryophyllene (trans-(1,9)-8-methylene-4,11,11-trimethylbicycloundec-4-ene) is a natural bicyclical sesquiterpene found in several plants and essential oils, including *C. langsdorffii* (Leguminosae) oil (Cho et al. 2015). β-caryophyllene is a volatile compound that is poorly soluble in water and has high pharmaceutical potential due to its analgesic, antioxidant, antimicrobial and anti-inflammatory activities (Dahham et al. 2015; Jung et al. 2015; Gertsch et al. 2008). Previous studies on *C. langsdorffii* have suggested its healing potential in a 10% formulation (Gushiken et al. 2017). Therefore, our group tested a 1% β-caryophyllene emulgel formulation to analyze the wound-healing potential and the mechanisms of action of this new formulation in a rat excision wound model.

2. Materials and Methods

2.1. Chemicals and reagents

The reference drugs neomycin sulfate (5 mg/g) + bacitracin zinc (250 IU/g), dexpanthenol 5% and collagenase 1.2 IU were purchased from pharmaceutical industries. SOD, CAT, GSH, GPx and silica gel 60H chromatoplates were obtained from Sigma-Aldrich Chemicals (Saint Louis, USA). ELISA kits of TNF-α, IFN-γ, IL-1β, IL-6 and IL-10 were bought from R&D

Systems (Minneapolis, USA). Biochemical colorimetric kits for quantification of AST, ALT, γ-GT, urea and creatinine were purchased from Interteck-Katal (Belo Horizonte, Brazil). The primary antibodies Lamγ2 was purchased from Santa Cruz Biotechnology (Dallas, USA). Dsg3, Ki-67, α-SMA antibodies and immunohistochemistry reveal kits were purchased from Abcam (Cambridge, USA). Sepineo P600, propylene glycol and methyldibromo glutaronitrile/phenoxyethanol were obtained from Spectrum Chemical Manufacturing Corporation (New Brunswick, USA). Labrafac lipophile WL 1349 was purchased from Gattefossé (Lyon, France).

2.2. Extraction and isolation of β-caryophyllene

The extraction and isolation of β-caryophyllene have been previously reported by Ribeiro *et al.* (2019) (Ribeiro et al. 2019). Briefly, *C. langsdorffii* oleoresin was collected in the northern and southeastern regions of Brazil, and the plant voucher was identified by Silvana Tavares Rodrigues at the Herbarium from EMBRAPA Amazônia Oriental (SPFR 10120). One hundred milliliters of a volatile fraction of oleoresin was added to 500 mL of water and subjected to hydrodistillation for 12 hours using a Clevenger-type apparatus. Then, the volatile fraction was subjected to a spinning band distillation process to obtain fractions rich in β-caryophyllene. β-caryophyllene was purified from these fractions by classical column chromatography packed with Sigma-Aldrich 60H silica gel impregnated with $AgNO_3$ with a gradient of hexane-ethyl acetate used as the mobile phase.

2.3. Formulation of emulgel and 1% β-caryophyllene emulsion

The emulgel was synthesized homogenizing Sepineo P600 (3% w/w), propylene glycol (5% w/w), Labrafac lipophile WL1349 (10% w/w), methyldibromo glutaronitrile/phenoxyethanol (0.1% w/w) and water at room temperature (± 25°C) with a Polytron PT 10-35 GT (Kinematica, Switzerland) at 600 rpm. The 1% β-caryophyllene emulsion was synthesized homogenizing the β-caryophyllene (1% w/w) with the emulgel at room temperature (± 25°C). The rheological behavior of the emulgel was analyzed in a Rheometer R/S plus (Brookfield) equipped with a C50-1 spindle and RHEO Software 2000 version 2.8. The sample behavior was monitored at a constant temperature (25°C) using a water bath/circulator.

2.4. Animals

In total, 105 male 7-week-old *Wistar* rats weighing 250 ± 20 g (Central Animal House, UNESP, Botucatu) were used in the experiments. The animals were housed individually for one week before the experiments and subjected to a temperature of 23 ± 2°C and a 12-hour dark-light cycle, and the animals had free access to food and water until the experimental procedure were initiated. This study was approved by the Ethics Committee on Animal Use at São Paulo State University under protocol 976/2017.

2.5. Experimental protocol of excision wound

The rats were anesthetized with intraperitoneal ketamine (0.08 mg/100 g) and xylazine (0.04 mg/100 g). The hair of their back was shaved and a full-thickness skin wound excision was made in the dorsum (in the subscapular area) using a 3 cm diameter punch. The wound placed in this area could not be reached by the animals, which prevented self-licking (Lim, Levy, and Bray 2006). Each rat was topically treated twice per day for three different experimental periods: 3, 7 or 14 days (n = 35 animals/period). After each period, the animals were euthanized, and samples of the skin wounds and blood were collected for analyses. The animals were allocated into the following seven groups (n = 5/group): FST, NeBa, Dex, Col, Emulgel, Car and Control.

2.6. Wound contraction analysis

To measure the contraction of the wounded area, each wound was photographed using a digital camera with a scale bar on days 0, 3, 7 and 14 after the excision wound was induced (day 0). A morphometric analysis was performed through measurement of wounded areas using specific software, and the percentage of wound contraction of each rat was calculated using the following formula:

$$\text{Wound contraction (\%)} = \left(\frac{\text{initial wounded area} - \text{wounded area in the analyzed day}}{\text{initial wounded area}}\right)*100$$

2.7. Hepatic and renal toxicity

Immediately after the euthanasia of each animal, blood was collected and centrifuged for 15 minutes at 6000 rpm and 4°C. The supernatant was collected and used to determine liver and kidney toxicity using AST, ALT and γ-GT (IU/L) parameters as well as the concentrations of urea and creatinine (mg/dL).

2.8. Inflammatory and oxidative stress mediators

Samples of wounded skin were collected to quantify the IFN-γ, IL-1β, IL-6, IL-10 and TNF-α inflammatory cytokines and the molecules involved in oxidative stress, including SOD, CAT, GPx and GSH. The skin samples were homogenized (1:5 m/v) in phosphate buffer (pH 7.4) and centrifuged for 15 minutes at 10000 rpm and 4°C. The supernatant of each sample was collected, and the cytokines were quantified by ELISA as described by the supplier. The results were expressed in pg/mg of protein. The following molecules involved in oxidative stress pathways were quantified through biochemical assays according to protocols: SOD (IU/mg of protein) (Winterbourn et al. 1975), CAT (IU/mg of protein) (Aebi 1984), GPx (nmol NADPH/min/mg of protein) (Yoshikawa et al. 1993) and GSH (nmol/mg of protein) (Faure and Lafond 1995).

2.9. Histological parameters

Skin samples from each rat were fixed with 10% buffered formaldehyde, embedded in paraffin, sectioned (5 µm) and stained with HE and Masson's trichrome stain. Each section was submitted to morphometric analysis via light microscopy. With HE staining, the epidermis thickness (µm), number of total cells in the epidermis and dermis (µm²) and the quantity of blood vessels in the dermis (number of blood vessels) were analyzed. With Masson's trichrome staining, the collagen content of the dermis (µm²) was analyzed. The border and center regions of the wounds were analyzed with five different photomicrographs of each region in the sections. AvSoft BioView Spectra software was used to perform the analysis.

2.10. Immunohistochemistry

Skin samples from each rat were processed routinely (fixation with 10% buffered formaldehyde, embedded in paraffin and sectioned; 5 µm thickness). Immunohistochemistry was then performed with primary antibodies against laminin-γ2 (1:200 µL), desmoglein-3 (1:200 µL), Ki-67 (1:100 µL) and α-SMA (1:400 µL) according to the protocols of a specific HRP/DAB detection kit. The areas (µm²) and positive cells (count of positive cells) for each antibody were quantified in the border and center of wounds with five different photomicrographs for each region of the sections. AvSoft BioView Spectra software was used to perform the analysis.

2.11. Statistical analysis

The data are expressed as the means ± standard deviation. Two-way ANOVA with Bonferroni post-test was used in the analysis of wound contraction. The inflammatory mediator, oxidative stress and local toxicity data were submitted to one-way ANOVA with Tukey's post-hoc test. Histological parameters and immunohistochemical data were analyzed according to the Kruskal-Wallis test with Dunn's post-test. GraphPad Prism 5.01 software (GraphPad Software Inc., San Diego, USA) was used to perform the analyses with a significance of 5%.

3. Results

3.1. Emulgel and 1% β-caryophyllene emulsion stability test

The rheological behavior of emulgel and 1% β-caryophyllene emulsion showed a non-Newtonian characteristic, with pseudoplastic behavior ($n < 1$). This result demonstrates that, with the increase of the shear rate, the viscosity reduces (data not shown).

3.2. Wound contraction analysis

The contraction of the wounds was analyzed on days 3, 7 and 14 (Figure 1). The data showed macroscopic contraction of wounds and a decrease in local edema in the groups treated with NeBa and Car for three days compared to the FST and Emulgel groups. After seven and fourteen days of treatment, the Col and Car formulations showed the best results in terms of macroscopic contraction compared to the FST and emulgel formulations. Furthermore, in both periods of treatment, the rats treated with Car showed decreased fibrinous exudate compared to the other groups.

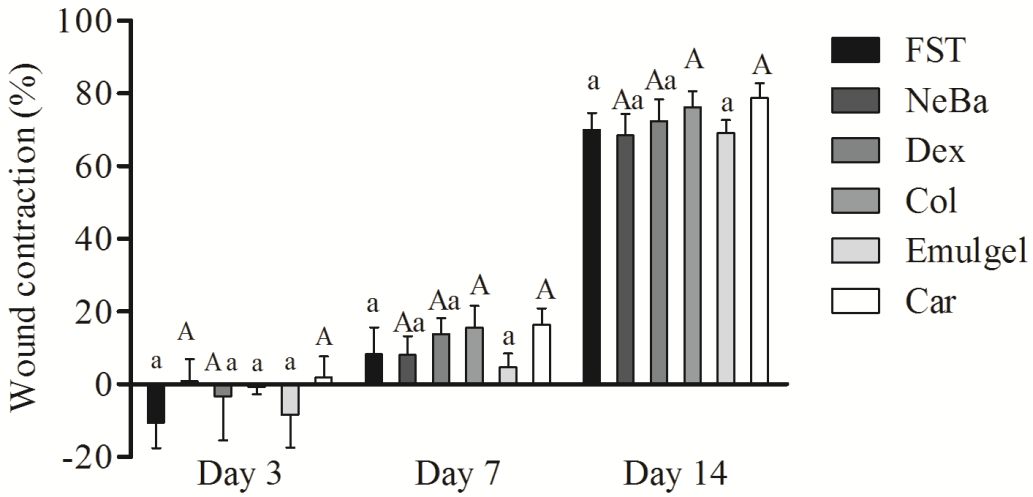

Figure 1. Wound contraction (%) in FST, NeBa, Dex, Col, Emulgel and Car treatments during 3, 7 and 14 days. Equal letters represents no statistical difference. Capital letters indicate statistical difference compared to groups with small letters, according to two-way ANOVA followed by Bonferroni test (n = 5).

3.3. Hepatic and renal toxicity

To analyze the hepatic and renal toxicity of the formulation containing β-caryophyllene, the liver enzymes (AST, ALT and γ-GT) and kidney proteins in blood (creatinine and urea) were evaluated. No significant differences among the values of all the treatment measurements and the normal parameters were found during the three experimental periods of treatment (Table 1).

Table 1. Systemic toxicity analysis data for liver (AST, ALT, γ-GT) and renal (creatinine, urea) parameters in the serum of rats treated for 14 days.

Groups	AST (IU/L)	ALT (IU/L)	γ-GT (IU/L)	Creatinine (mg/dL)	Urea (mg/dL)
FST	143 ± 28	62 ± 12	1.2 ± 0.4	0.30 ± 0.03	44 ± 2.1
NeBa	138 ± 17	65 ± 7.7	1.1 ± 0.3	0.31 ± 0.04	44 ± 6.2
Dex	150 ± 28	80 ± 17	0.9 ± 0.3	0.27 ± 0.03	43 ± 5.6
Col	153 ± 29	63 ± 12	1.0 ± 0.2	0.30 ± 0.05	45 ± 4.6
Emulgel	146 ± 6.1	65 ± 8.5	1.1 ± 0.2	0.26 ± 0.04	44 ± 4.7
Car	124 ± 14	60 ± 9.5	1.1 ± 0.2	0.31 ± 0.03	42 ± 7.6
Control	147 ± 25	65 ± 9.1	1.0 ± 0.2	0.30 ± 0.02	44 ± 4.9

3.4. Quantification of inflammatory mediators

The concentrations of the IFN-γ, IL-1β, IL-6, IL-10 and TNF-α cytokines as evaluated by ELISA are shown in Figure 2. The level of IFN-γ was reduced in the Car group compared to the FST group after three days. In the two other treatment periods, the NeBa, Dex and Col commercial formulations, as well as the tested Car drug, decreased the levels of IFN-γ compared to the FST and Emulgel. After three days, a reduction in the IL-1β level in NeBa and Dex treated groups compared to FST group was observed. Within seven days, decreased levels of IL-1β were observed in the NeBa, Dex, Col and Car groups compared to the FST and Emulgel groups. In the last period of treatment, the Car group showed decreased IL-1β concentrations compared to all wounded groups. The IL-6 levels were decreased in the NeBa, Dex and Car groups after three and seven days of treatment compared to the FST and Emulgel groups. The levels of IL-10 were not different among the treatments on days three and seven. In the last period of treatment, an increase in IL-10 was observed in the NeBa, Dex, Col and Car treatment groups compared to the FST and Emulgel treatment groups. The TNF-α levels were reduced in the NeBa, Dex and Car groups compared to the FST and Emulgel groups on day fourteen, and there were no significant differences in the two other periods (Figure 2).

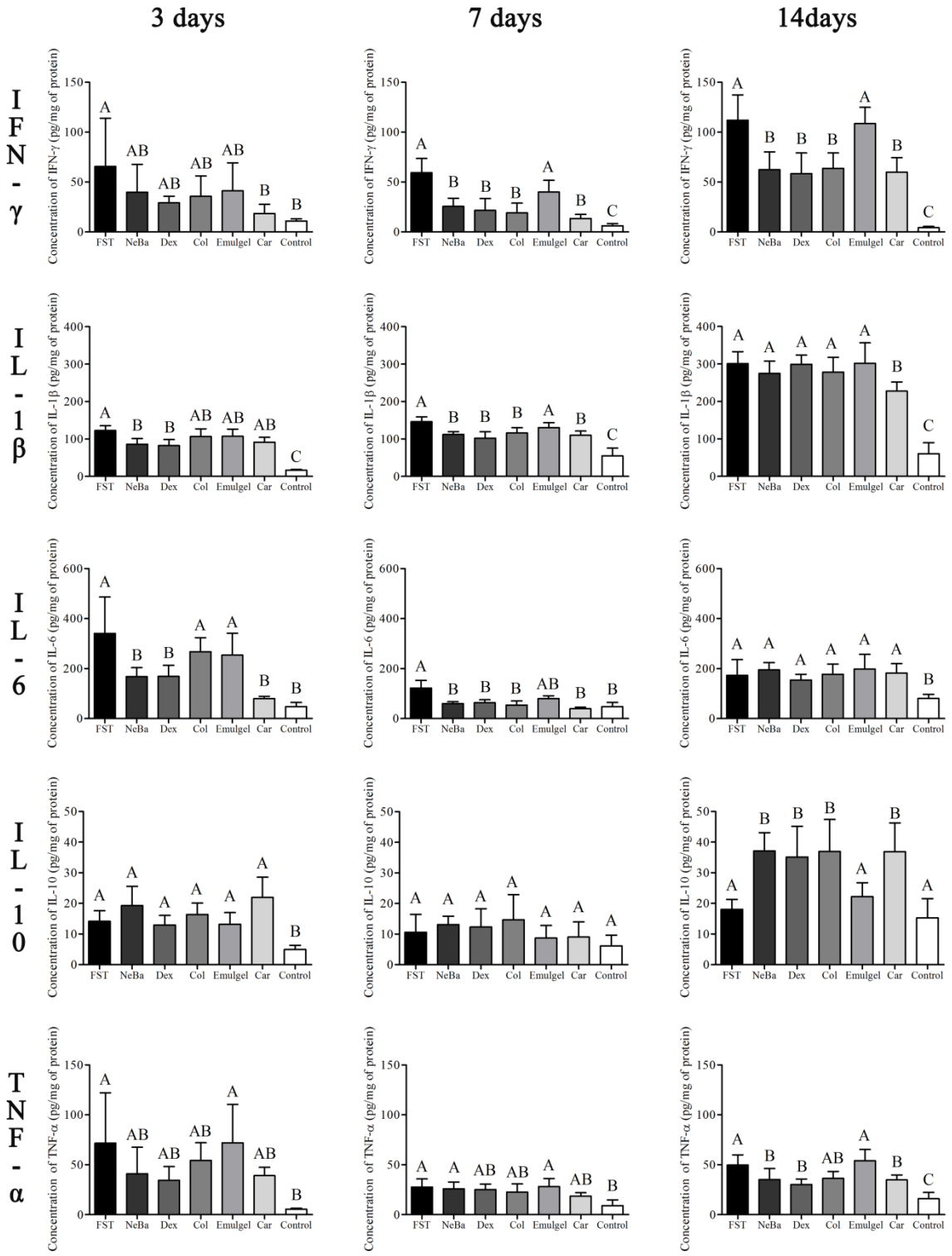

Figure 2. Concentrations of IFN-γ, IL-1β, IL-6, IL-10 and TNF-α (pg/mg protein) in cutaneous wounds at 3, 7 and 14 days. Equal letters show no statistical difference and different letters indicate statistical difference compared to the other groups, according to one-way ANOVA followed by Tukey test (n = 5).

3.5. Oxidative stress analysis

The CAT activity, GPx activity, SOD activity and GSH concentration are shown in Figure 3. The activity of CAT was decreased in the Col and Car groups compared to the FST and

Emulgel groups on day fourteen, with the same level of Control, demonstrating the normality of the enzyme in the groups. During the first two periods of treatment, there was no significant difference in the CAT activity. The GPx activity was increased in the NeBa, Col and Car treatment groups compared to the other groups during the seven-day treatment period with no differences on days three and fourteen. The GSH concentration and SOD activity was not different among the wounded groups in any period (Figure 3).

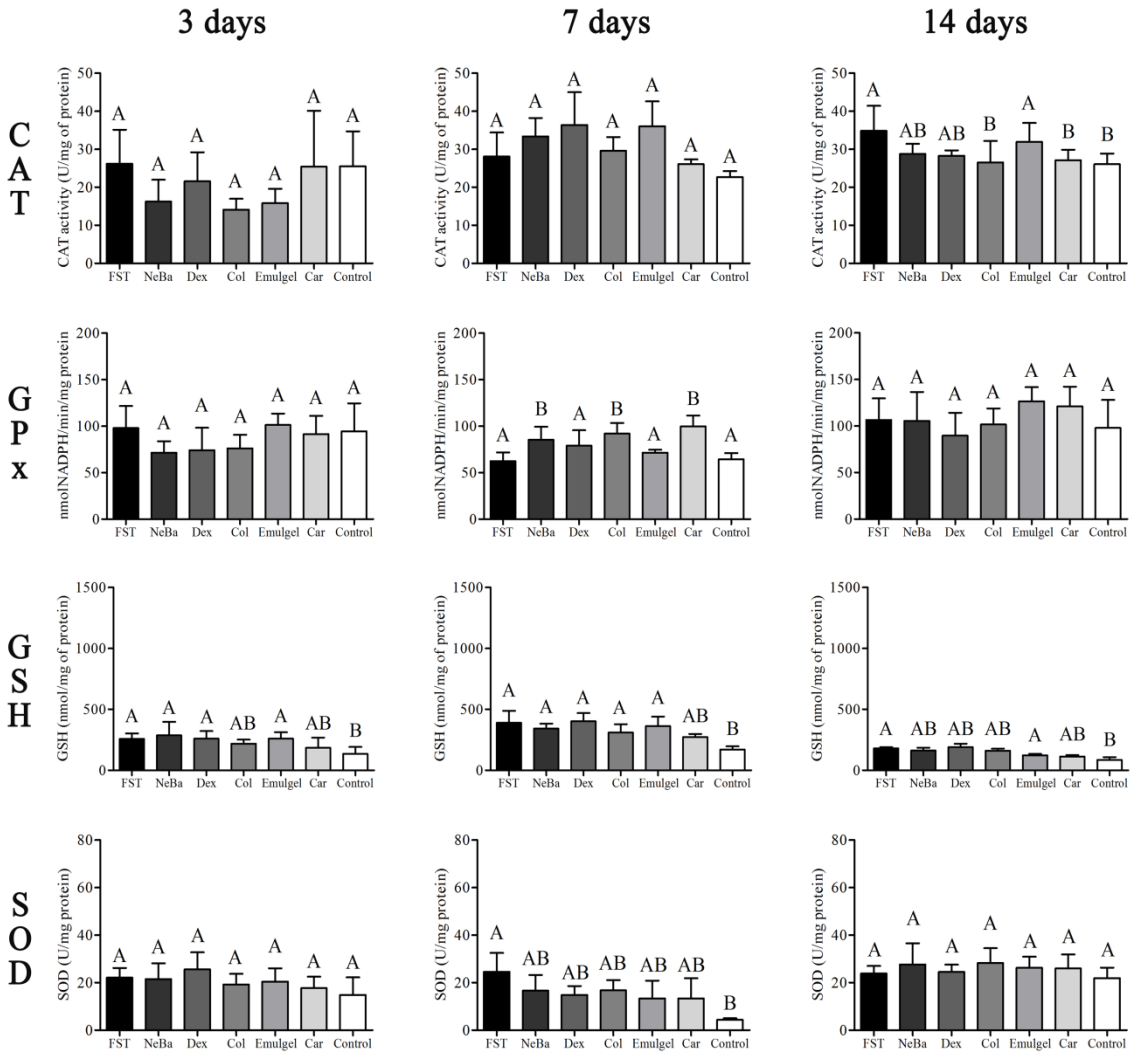

Figure 3. Concentrations of CAT, GPx, GSH and SOD in skin wounds at 3, 7 and 14 days. Equal letters show no statistical difference and different letters indicate statistical difference compared to the other groups, according to one-way ANOVA followed by Tukey test (n = 5).

3.6. Histological parameters

There was no difference in the quantification of cells from the epidermis, border of center of the dermis among the wounded groups in any period of treatment (Supplementary materials 1 – 4). The morphometric analysis showed a reduction in the epidermis thickness of the animals

treated with the NeBa, Dex, Col and Car formulations compared to that of the animals in the FST and Emulgel groups after seven and fourteen days. Moreover, the results of the Car group on day fourteen were similar to those of the Control group, resulting in normal epidermis thickness in the Car treatment (Figure 4, Supplementary materials 2). The quantification of blood vessels in the border and center of the wounds did not reveal a significant difference during any period of treatment (Supplementary materials 3 – 5). The quantification of total collagen in the border of the wounds showed no difference on days three, seven and fourteen. In the central area of wounds, there was an increase in collagen amount in the Car group compared to the FST and Emulgel on day three, with a similar concentration compared to Control. After seven and fourteen days, no differences were observed (Figure 5, Supplementary materials 6 and 7).

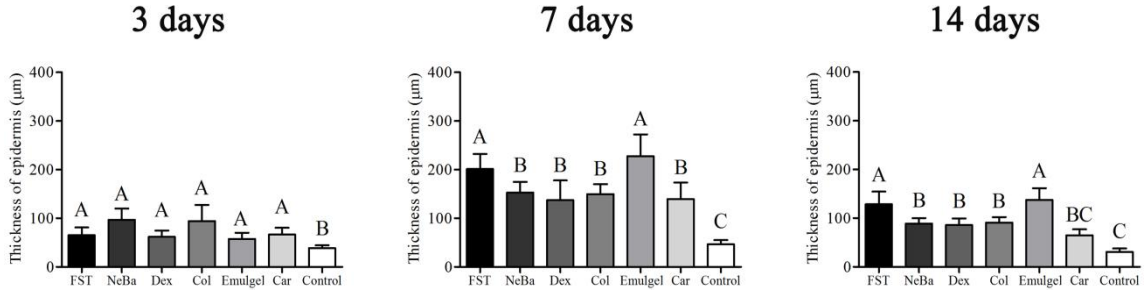

Figure 4. Thickness of epidermis (µm) of FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days. Equal letters show no statistical difference and different letters indicate statistical difference compared to the other groups, according to the Kruskal-Wallis test, followed by the Dunn post-test (n = 5).

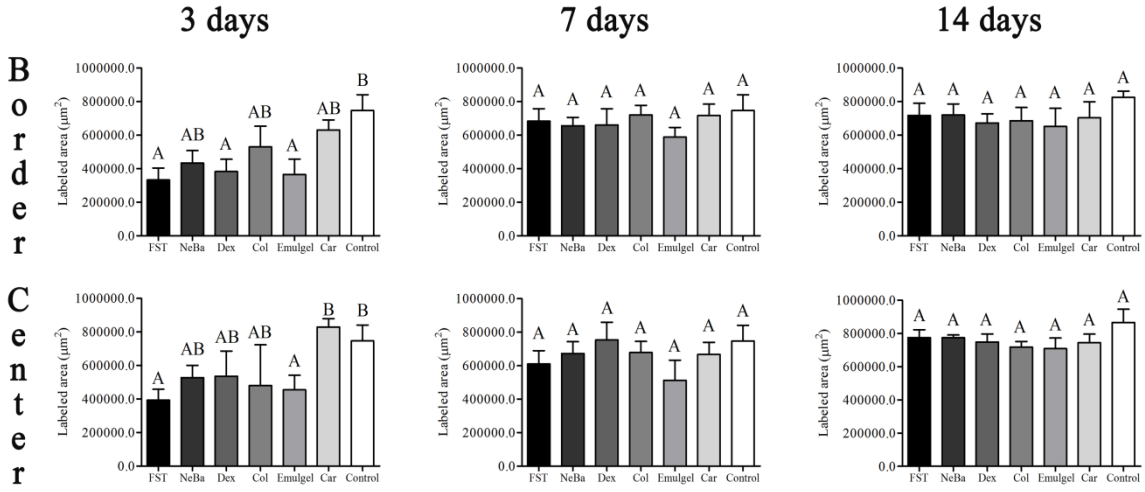

Figure 5. Quantification of collagen (µm²) at the border and center of wounds of FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days. Equal letters show no statistical difference and different letters indicate statistical difference compared to the other groups, according to the Kruskal-Wallis test, followed by the Dunn post-test (n = 5).

3.7. Immunohistochemistry

There was an increase in α-SMA-immunolabeled fibroblasts in the NeBa, Dex, Col and Car samples compared to FST and emulgel samples after three and seven days of treatment with no differences on day fourteen. However, the commercial treatments and Car formulation showed results similar to the control, indicating normalization of α-SMA in these groups (Figure 6, Supplementary materials 8). Immunolabeling for Dsg3 showed a reduction in the labeled area of the NeBa and Car groups compared to the FST group after three days. There was a decrease in Dsg3 immunolabeling in the Col and Car groups compared to the FST and Emulgel groups after seven days. There was no significant difference in the last period of treatment (Figure 6, Supplementary materials 9). There was an increase in Lamγ2 immunolabeling in the Col and Car groups compared to the FST and Emulgel groups on day three and an increased area in the Car group compared to the FST and Emulgel groups on day fourteen (Figure 6, Supplementary materials 10). There were no significant differences among the wounded groups in the number of Ki-67-immunolabeled cells in the epidermis, border region of central region of the dermis after three, seven and fourteen days (Supplementary materials 11 – 14).

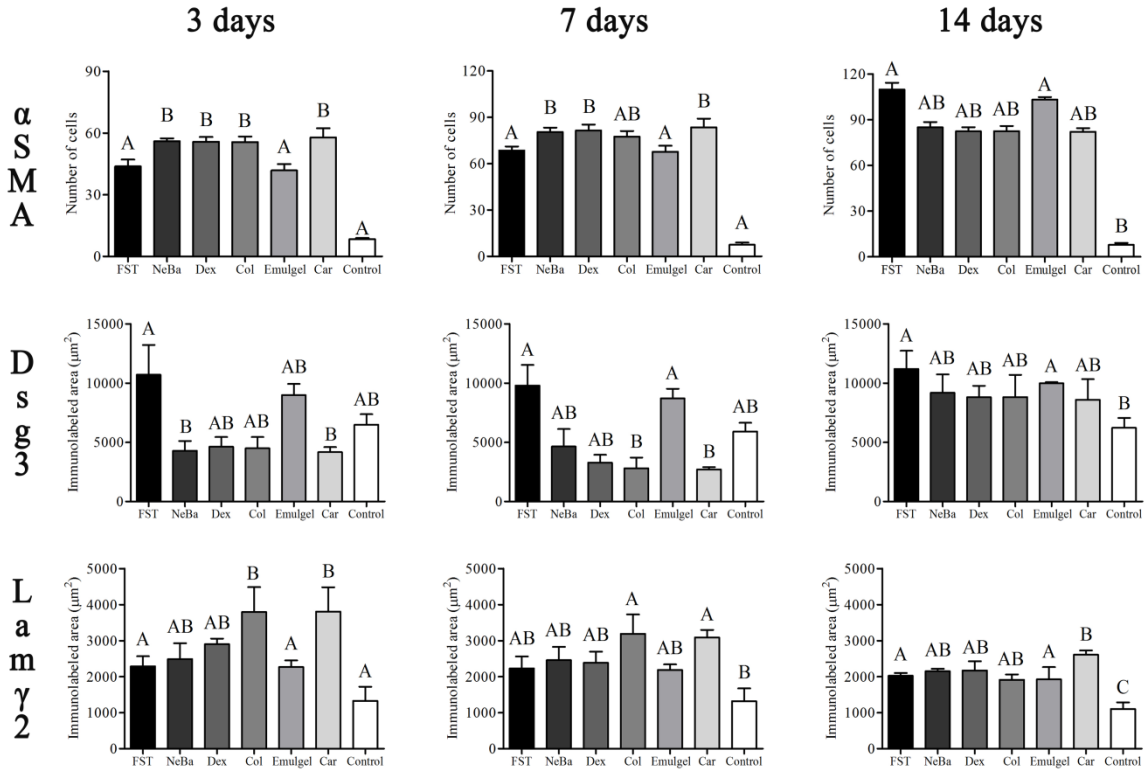

Figure 6. Immunolabeling of α-SMA, Dsg3 and Lamγ2 in wounds of FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days. Equal letters show no statistical difference and different letters indicate statistical difference compared to the other groups, according to the Kruskal-Wallis test, followed by the Dunn post-test (n = 5).

4. Discussion

β-Caryophyllene is a sesquiterpene in many consumable plants and essential oils with related antioxidant, anti-inflammatory and antimicrobial potentials (Dahham et al. 2015; Jung et al. 2015; Gertsch et al. 2008). Previous studies using *C. langsdorffii* oil resin as a treatment for skin excision wounds have shown that the best effective concentration of the oil resin was 10%, and the phytochemical profile of the oil resin has been determined (Gushiken et al. 2017; Sousa et al. 2011). Therefore, we calculated the concentration of the sesquiterpene based on these studies to synthesize the emulgel formulation for this study, resulting in a concentration of 1%. The present study confirmed the wound-healing activity of an emulgel formulation containing 1% β-caryophyllene in a rat excision model, demonstrating the antioxidant and anti-inflammatory activities as well as the improvement in remodeling and re-epithelialization mechanisms mediated by the sesquiterpene.

There are some topical drugs on the market with different mechanisms of action to treat wounds aiming to reduce the time of cutaneous healing and treat errors in tissue repair. Topical formulations containing neomycin and bacitracin zinc sulfate are used in the initial phase of skin wounds to prevent injury from infection, one of the major factors of delayed wound healing (Ward and Saffle 1995). Dexpanthenol (5%) is also used in skin injuries with previous studies reporting the proliferative potential of keratinocytes and fibroblasts with this treatment (Gorski et al. 2020). Another common drug used to treat cutaneous wounds is collagenase, a protease of *Clostridium histolyticum,* which has been show to play a role in the debridement of provisional extracellular matrix and to have a remodeling mechanism that makes it useful to treat wounds in the clinic (Tallis et al. 2013; Mekkes, Zeegelaar, and Westerhof 1998). Therefore, these three drugs were used in our study as positive controls to compare the new treatment containing β-caryophyllene.

AST, ALT and γ-GT are enzymes that catalyze reactions in some peptides and are highly expressed in hepatocytes. When drugs cause a toxic effect in the liver, there is an increase in these enzymes in blood flow, and they are used in the evaluation of hepatic toxicity (Giannini, Testa, and Savarino 2005). Creatinine and urea are proteins physiologically synthesized by the body and are filtered by the kidneys for elimination from the organisms. The increase in plasma levels of these proteins represents the instability of renal filtration due to a toxic effect (Traynor et al. 2006). The plasma levels of hepatic and renal proteins of β-caryophyllene and positive controls were similar to those measured for physiological quantification (Control), demonstrating that the local treatment containing the sesquiterpene did not have systemic toxicity.

Cutaneous injury results in the synthesis and release of mediators of the hemostatic cascade and the death of cells in the wounded area with the local release of reactive oxygen species (ROS) (Murthy et al. 2013). Although the released free radicals and ROS have important roles in the antimicrobial pathway, exacerbated release of ROS can cause chronic inflammation and impairment in the healing process, resulting in hypertrophic scars or unhealed wounds (Kwon et al. 2012). To control the concentration of ROS, the cells of the area synthesize antioxidant mediators that are involved in the superoxide and hydrogen peroxide pathways. Superoxide radicals are highly reactive molecules that are processed into hydrogen peroxide, a less reactive molecule, by SOD (Winterbourn et al. 1975). However, at high concentrations, hydrogen peroxide is a toxic compound to cells. Thus, hydrogen peroxide is metabolized by CAT and GPx (with consumption of GSH), resulting in water and oxygen molecules (Yoshikawa et al. 1993; Soman et al. 2010). Previous studies have reported the *in vitro*

antioxidant potential of β-caryophyllene (Dahham et al. 2015; Calleja et al. 2013), and our findings showing that GPx increased activity confirmed the antioxidant potential of β-caryophyllene in a rat excision wound model as well as in the NeBa and Col positive controls.

The inflammatory mechanism is essential for the correct healing of skin wounds. The IL-1β, IL-6 and TNF-α pro-inflammatory cytokines are involved in cell differentiation and proliferation, coordinating the synthesis of granulation tissue, angiogenesis, re-epithelialization and collagen remodeling mechanisms (Serra et al. 2017). Furthermore, these cytokines are associated with IFN-γ to enhance the migration to and proliferation of leukocytes at the wound, improving the debridement of necrotic tissue and the phagocytosis of antigens (Wagner and Wehrmann 2007). IL-10 is another interleukin involved in the inflammatory mechanism of skin wounds, acting as an anti-inflammatory mediator inhibiting the synthesis of pro-inflammatory cytokines and playing a role in angiogenesis (Sato, Ohshima, and Kondo 1999). However, the imbalance among inflammatory cytokines can lead to a chronic inflammation process, resulting in errors in the subsequent healing mechanisms and the impairment of wound healing (Zhao et al. 2016). In the present study, the cytokine quantification data showed that the anti-inflammatory activity of β-caryophyllene in cutaneous wounds was due to the reduction in IFN-γ, IL-1β, IL-6 and TNF-α levels as well as the increase in IL-10 level with anti-inflammatory potential similar to NeBa, Dex and Col.

Another mechanism of wound healing involves the contraction of wounds with the interaction of fibroblasts and extracellular matrix proteins resulting in wound closure (Agha et al. 2011). TGF-β1 is a growth factor involved in mechanisms in all phases of wound healing, including the differentiation of fibroblasts. Through TGF-β1 stimulation, fibroblasts located at the border of injuries synthesize α-SMA, an intracellular stress fiber protein, acquiring a contractile phenotype and differentiating into myofibroblasts (Darby et al. 2014). The myofibroblasts bind to the collagen I extracellular fibers and initiate the contraction of the stress fibers, reducing the wounded area. (Darby et al. 2014). Thus, the role of the β-caryophyllene formulation in wound contraction was validated through macroscopic evaluation, in which a decrease in wounded area was observed in groups treated with β-caryophyllene, neomycin/bacitracin and collagenase. Moreover, the immunohistochemical data of α-SMA suggested that the increase of wound contraction of these groups was mediated by α-SMA as indicated by the increase of immunolabeling of this protein in these treatments.

Re-epithelialization is important in cutaneous wound healing with the proliferation and migration of keratinocytes from the border of injuries. First, proteinases dissolve adhesion molecules among keratinocytes, such as desmoglein-3. These cells proliferate and synthesize

the anchoring protein, laminin-γ2, to assist in keratinocyte migration through the extracellular matrix, coordinating the re-epithelialization mechanism (Fisher and Rittié 2017; Rötzer et al. 2016). However, the overexpression of proliferating mediators causes the hyperproliferation of keratinocytes at the wounds with an increase of epidermis thickness as a marker of keloid formation (Limandjaja et al. 2020). Therefore, the histological results of epidermis thickness and the immunohistochemical data of keratinocyte proliferation (Ki-67), desmoglein-3 and laminin-γ2 demonstrated that β-caryophyllene and collagenase treatments enhanced the re-epithelialization mechanism in which desmoglein-3 and laminin-γ2 involved during the migration of cells. Our findings corroborated the results of Koyama et al. (2019), who reported enhanced re-epithelialization of skin wounds after treatment with β-caryophyllene oil in mice (Koyama et al. 2019).

Finally, the provisional extracellular matrix is replaced by permanent tissue to initiate the remodeling mechanism. In this process, type III collagen and other components of the provisional matrix are metabolized by extracellular matrix metalloproteinases. Simultaneously, compounds, such as type I collagen, elastin and proteoglycans, are synthesized and organized as more resistant tissues, and excess myofibroblasts, fibroblasts and endothelial cells proliferate during healing and undergo apoptosis (Gurtner and Chapman 2016). Our histological evaluation results of Masson's trichrome staining suggested the role of β-caryophyllene in skin repair, increasing collagen synthesis in the central area of wounds during the first period of cutaneous healing with better results in the remodeling mechanism compared to the reference drugs.

5. Conclusions

Considering our results, we conclude that the emulgel formulation containing 1% β-caryophyllene enhances *in vivo* skin wound healing through antioxidant, anti-inflammatory, wound contraction, re-epithelialization and remodeling mechanisms. Our results demonstrated the promising potential of β-caryophyllene in skin wound therapy compared with three reference drugs, with greater results in remarkable mechanisms during the whole healing instead of one mechanism from reference drugs. Furthermore, the analysis of hepatic and renal parameters confirmed the safety of the tested formulation by showing that there was no systemic toxicity. Therefore, this study provides good evidence that 1% β-caryophyllene has great potential for use in treating full-thickness skin wounds, even when compared to other drugs.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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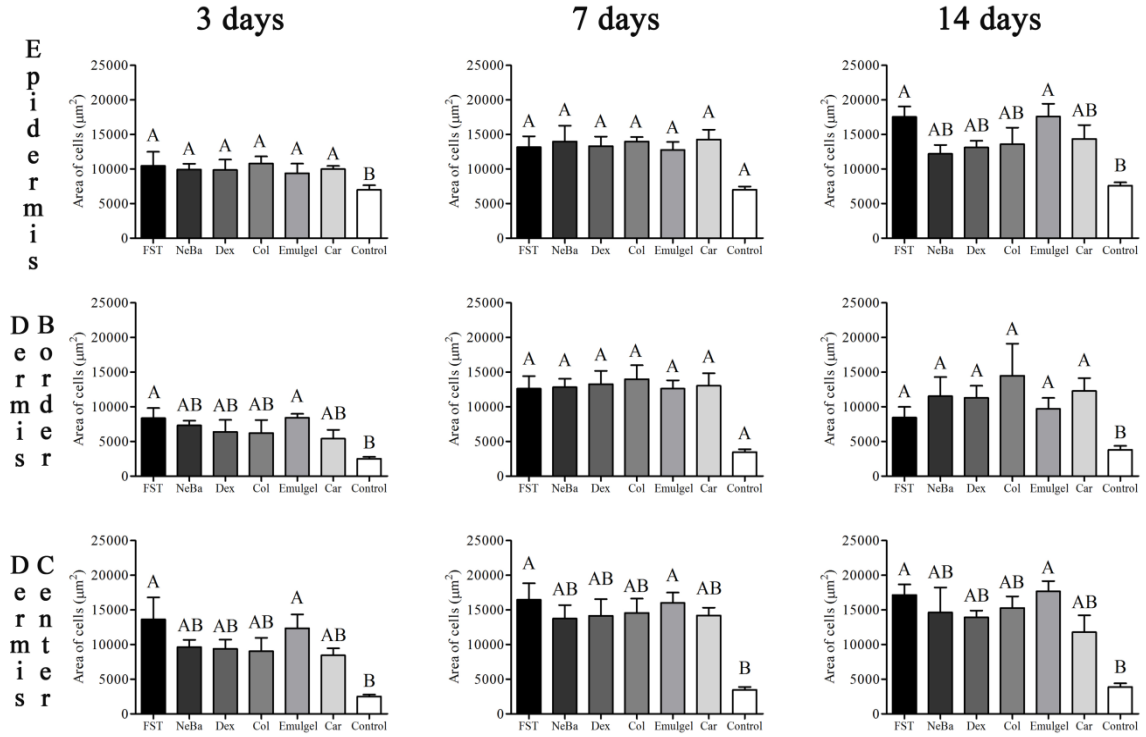
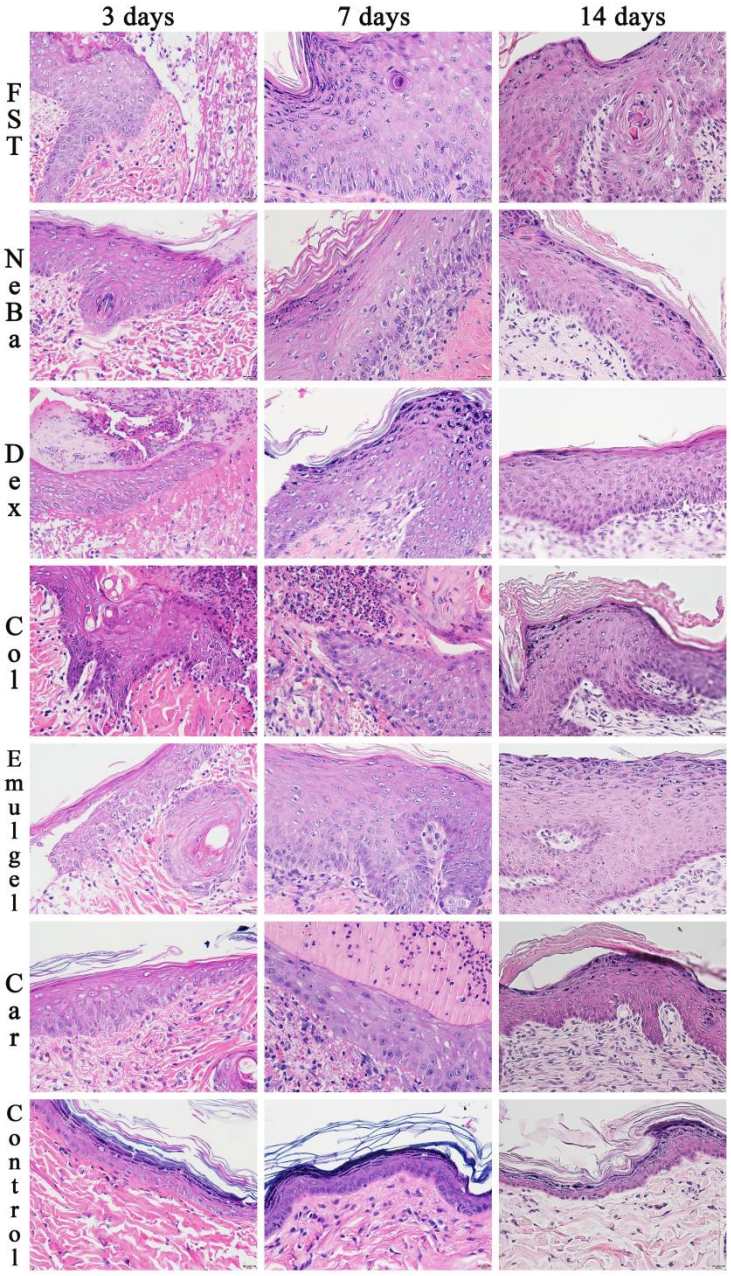
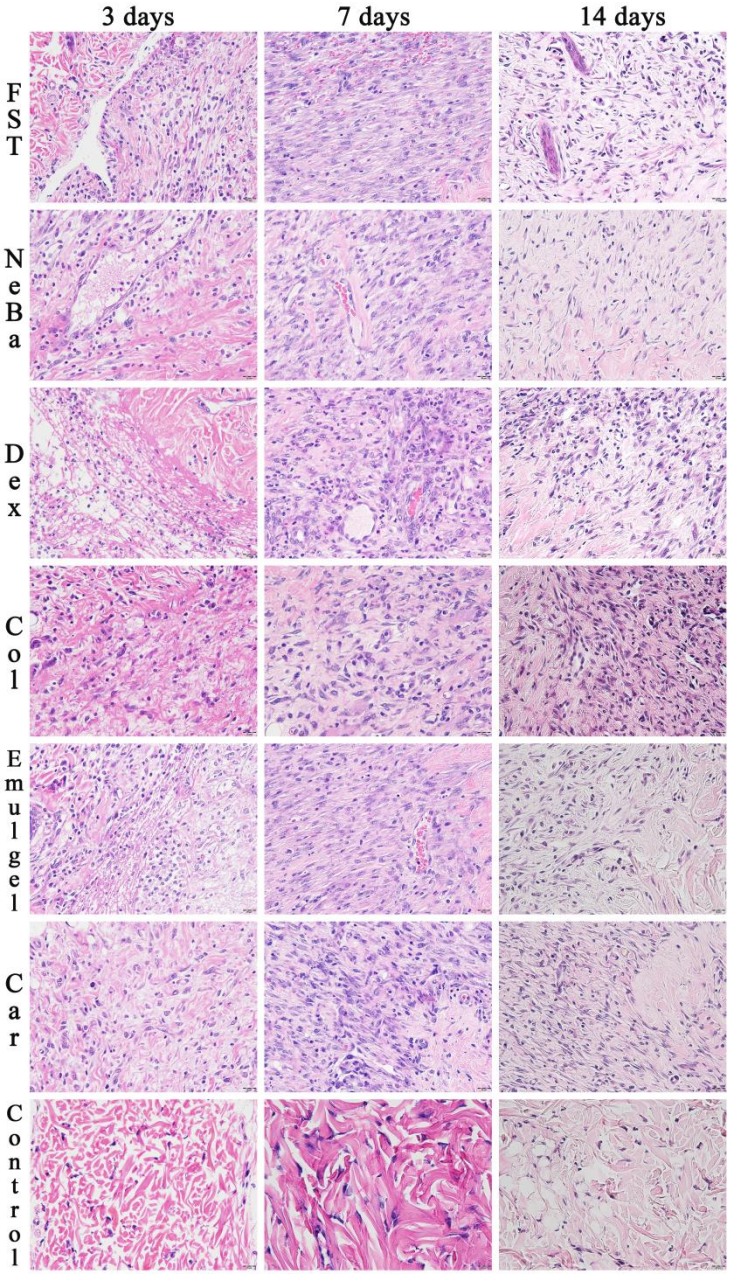
Supplementary Materials



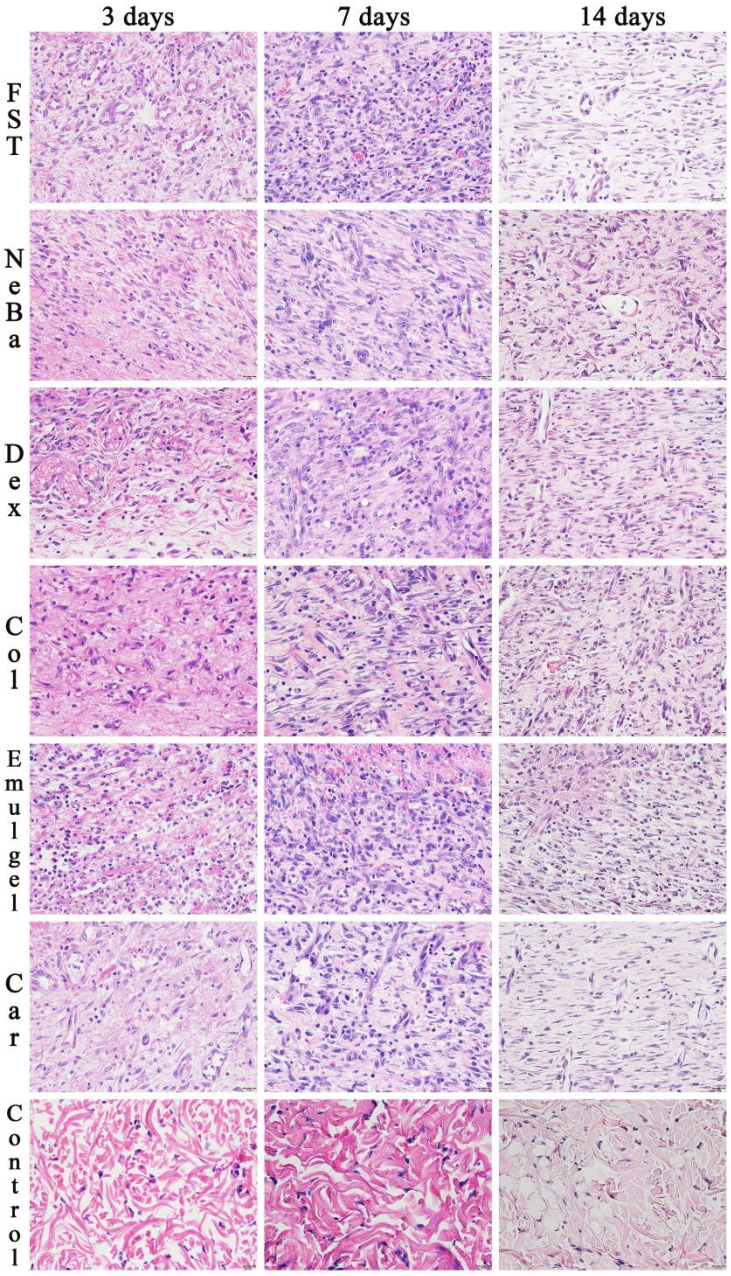
Supplementary materials 1. Quantification of cells (µm^2) in the epidermis, border and center of the dermis in wounds of FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days. Equal letters show no statistical difference and different letters indicate statistical difference compared to the other groups, according to the Kruskal-Wallis test, followed by Dunn post-test (n = 5).



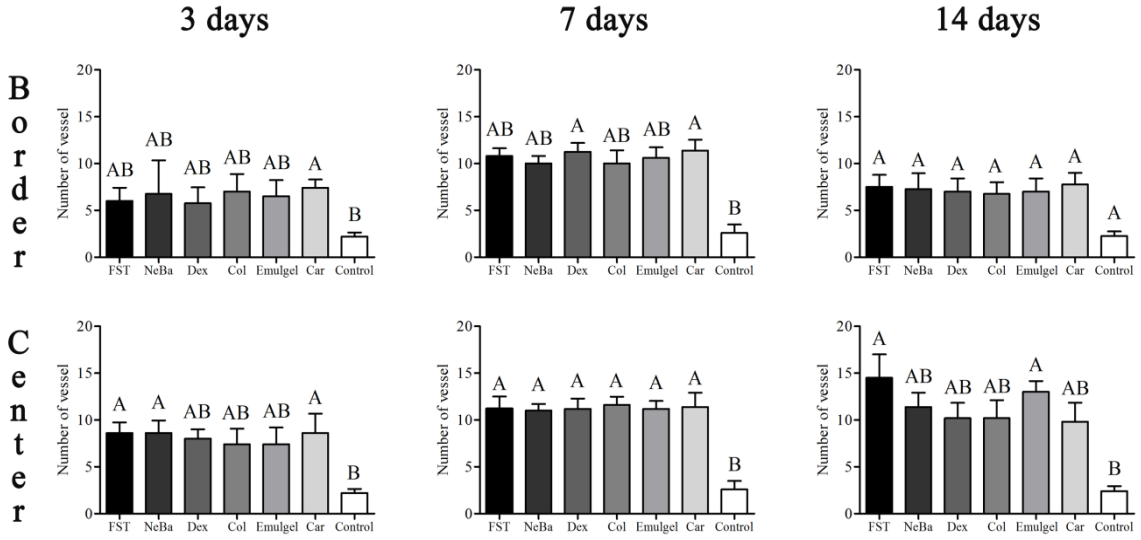
Supplementary materials 2. HE photomicrographs of the epidermis in FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days.



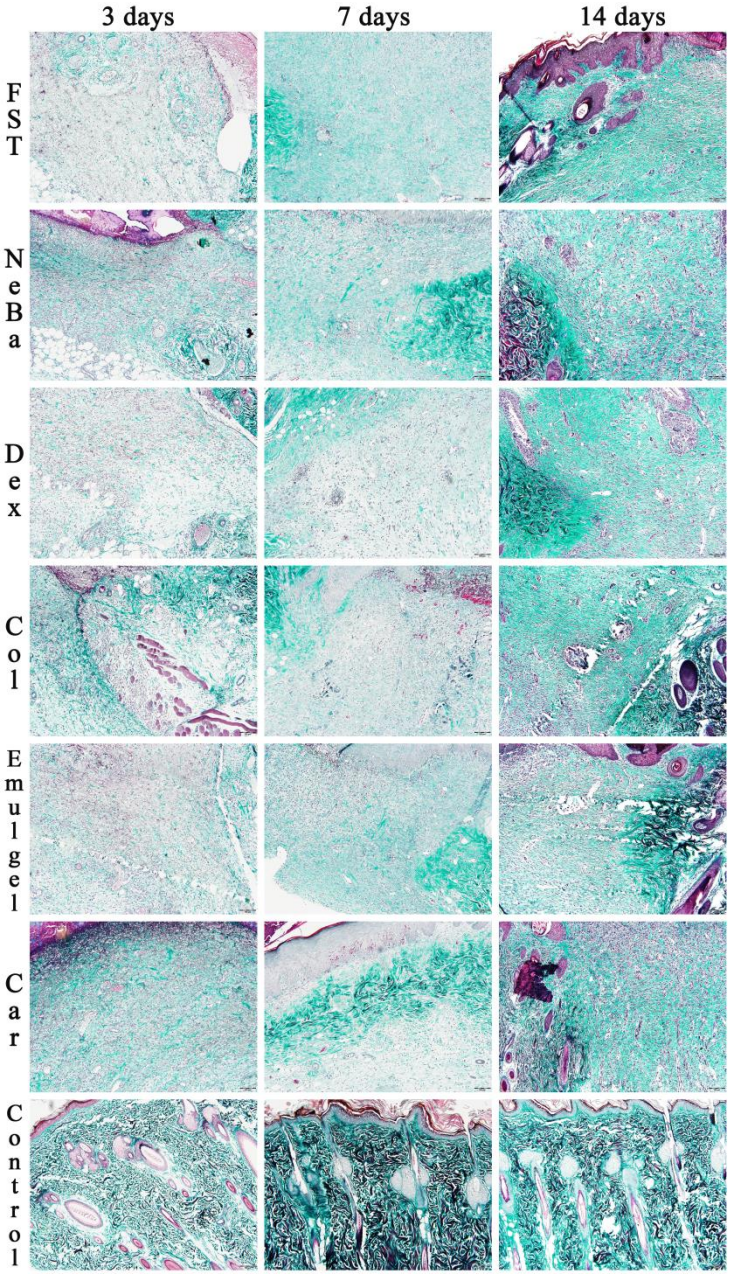
Supplementary materials 3. HE photomicrographs of the border of the wounds in the dermis of FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days.



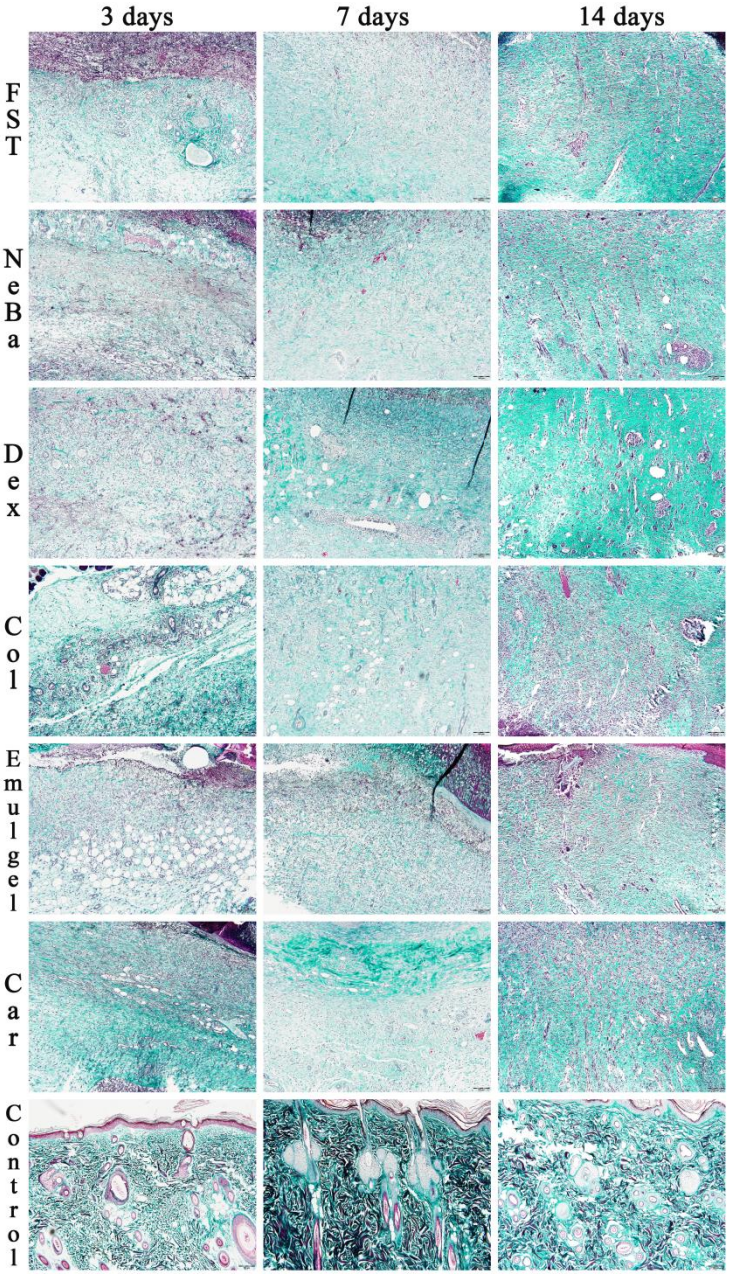
Supplementary materials 4. HE photomicrographs of the center of wounds in the dermis of FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days.



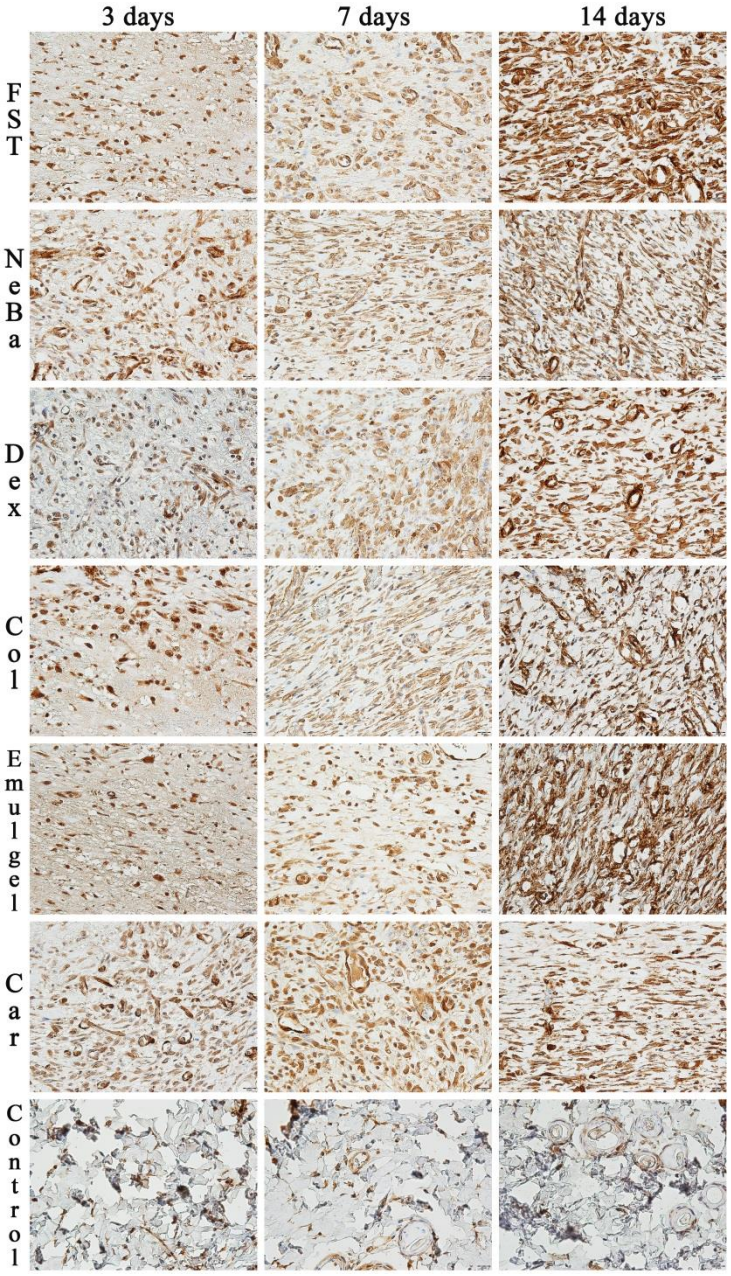
Supplementary materials 5. Number of blood vessels in the border and center of the dermis in wounds of FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days. Equal letters show no statistical difference and different letters indicate statistical difference compared to the other groups, according to the Kruskal-Wallis test, followed by the Dunn post-test (n = 5).



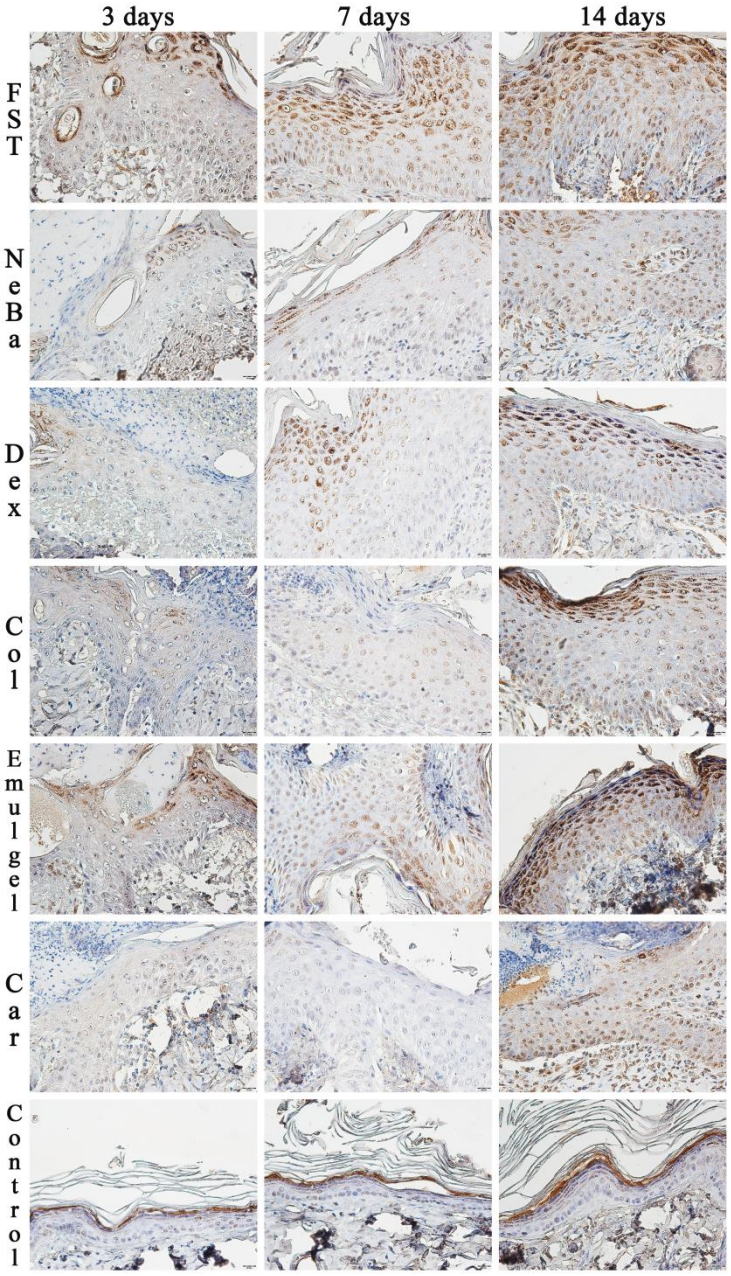
Supplementary materials 6. Masson's trichrome photomicrographs of the border of the wounds in the dermis of FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days.



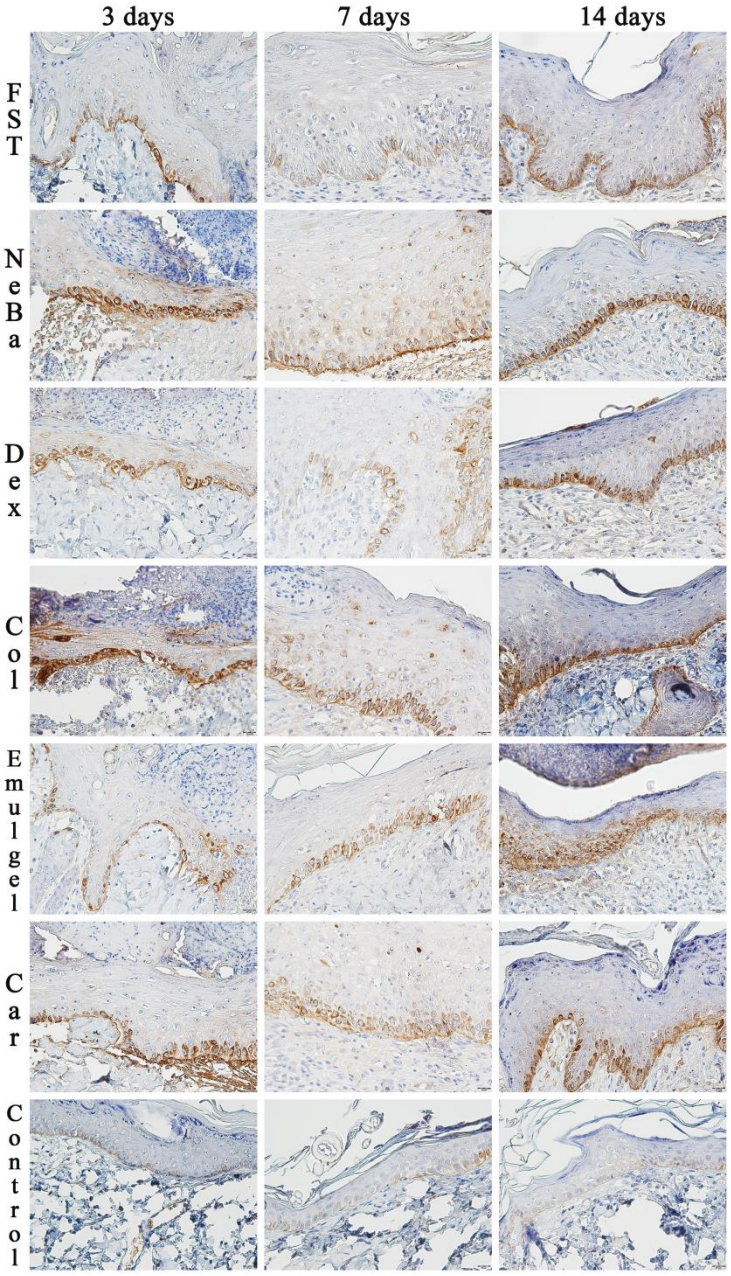
Supplementary materials 7. Masson's trichrome photomicrographs of the center of the wounds in the dermis of FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days.



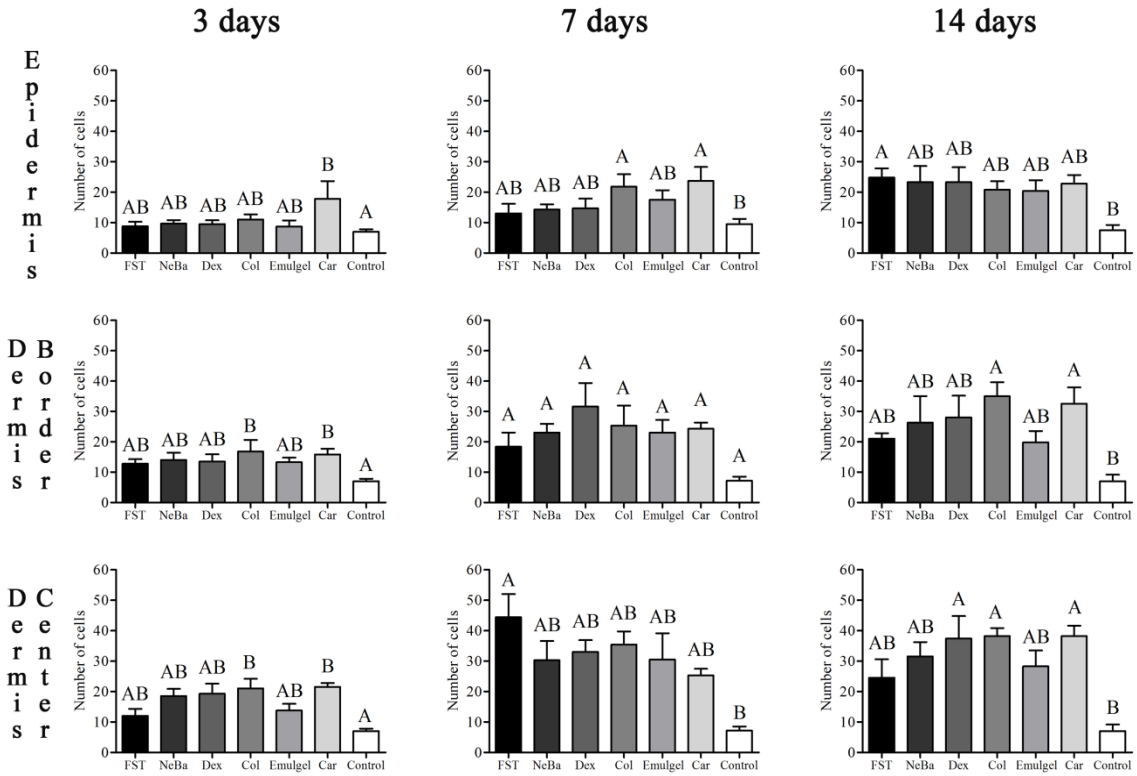
Supplementary materials 8. Photomicrographs of the immunolabeling of α-SMA in wounds of FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days.



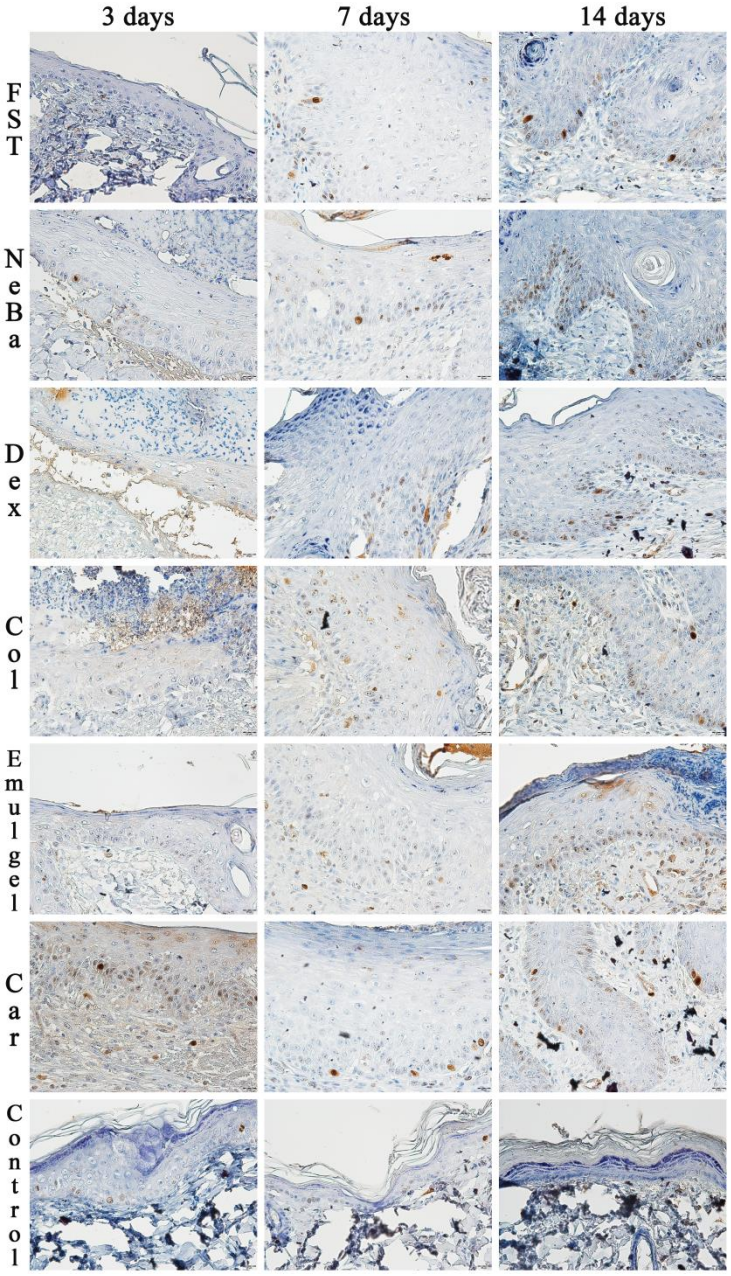
Supplementary materials 9. Photomicrographs of the immunolabeling of Dsg3 in wounds of FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days.



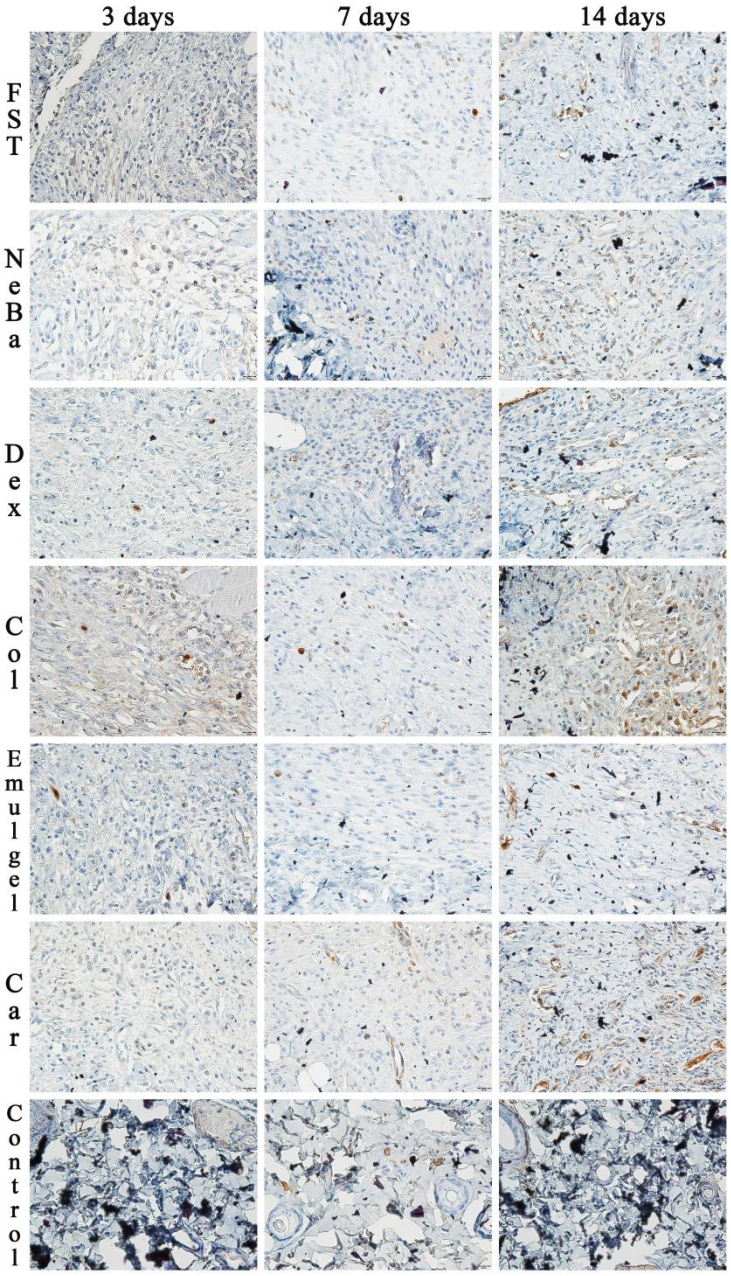
Supplementary materials 10. Photomicrographs of the immunolabeling of Lamγ2 in wounds of FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days.



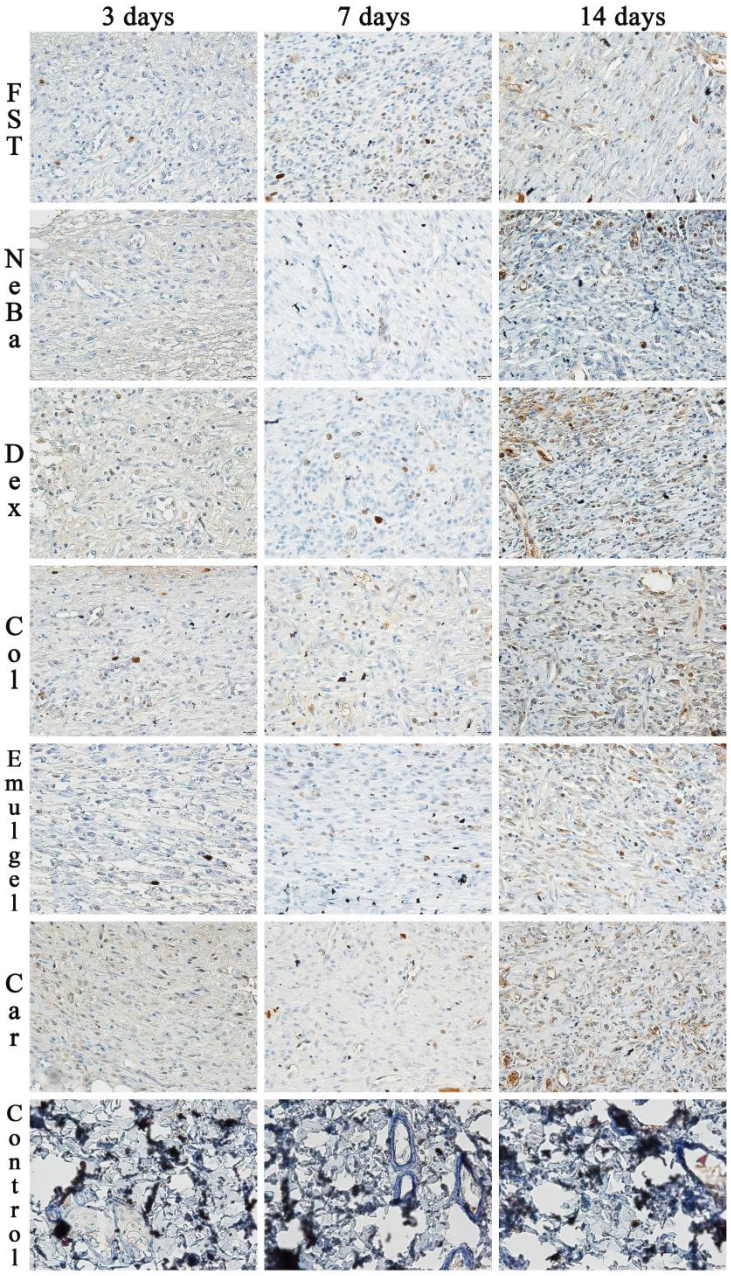
Supplementary materials 11. Ki-67 immunolabeling of proliferating cells in the epidermis, border and center of the dermis in FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days. Equal letters do not show statistical difference and different letters indicate statistical difference compared to the other groups, according to the Kruskal-Wallis test, followed by the Dunn post-test (n = 5).



Supplementary materials 12. Photomicrographs of the immunolabeling of Ki-67 in the epidermis of FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days.



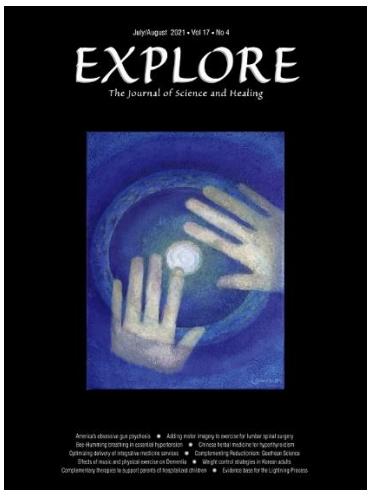
Supplementary materials 13. Photomicrographs of the immunolabeling of Ki-67 of the border of the wounds in the dermis of FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days.



Supplementary materials 14. Photomicrographs of the immunolabeling of Ki-67 of the center of the wounds in the dermis of FST, NeBa, Dex, Col, Emulgel, Car and Control groups during 3, 7 and 14 days.

Atividade cicatrizante de formulação tópica à base de carreadores lipídicos nanoestruturados contendo óleo-resina de *Copaifera langsdorffii* em lesões cutâneas de ratos

(Artigo submetido à revista *EXPLORE: The Journal of Science & Healing* ISSN 1550-8307)



Capítulo III

Copaifera langsdorffii oleoresin-loaded nanostructured lipid carrier emulgel improves cutaneous healing by anti-inflammatory and re-epithelialization mechanisms

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Abstract

Background and Objectives: The skin is essential to keep the integrity of the organism. The disruption of this organ promotes a wound and the organism starts the healing to reconstruct the skin. *C. langsdorffii* is a tree used in folk medicine to treat skin affections and other diseases, with antioxidant and anti-inflammatory properties. The oleoresin of the plant was associated to nanostructured lipid carriers aiming evaluate the healing potential of this formulation, comparing the treatment with reference drugs used in wound healing. *Materials and methods:* Male *Wistar* rats were used to perform the excision wound model, with the macroscopical analysis of wound retraction. Skin samples were used in histological, immunohistochemical, and biochemical analyzes. *Results:* The results showed the macroscopic retraction of the wounds in oleoresin-treated group, mediated by α-smooth muscle actin (α-SMA). Biochemical assays revealed the anti-inflammatory mechanism of oleoresin-treated group increasing IL-10 concentration and decreasing pro-inflammatory molecules TNF-α, IFN-γ, IL-1β and IL-6. Histopathological and immunohistochemical results showed the improving of reepithelialization and tissue remodeling in *C. langsdorffii* group, mediated by increase of laminin-γ2, decrease of desmoglein-3 and increase of collagen remodeling. *Conclusions:* These findings indicate the wound healing potential of nanostructured lipid carriers associated to *C. langsdorffii* oleoresin in skin wounds, which can be useful as an alternative treatment for skin wounds.

Keywords: wound healing; skin; nanostructured lipid carrier; *Copaifera langsdorffii*; anti-inflammatory

Abbreviations

α-SMA: α-smooth muscle actin

γ-GT: γ-glutamyl transferase

ALT: alanine aminotransferase

ANOVA: analysis of variance

AST: aspartate aminotransferase

CAT: catalase

Col: wounded animals treated with collagenase 1.2 IU

Control: animals without lesion and treatment – physiologic pattern

Dex: wounded animals treated with dexpanthenol 5%

ELISA: enzyme-linked immunosorbent assay

FST: wounded animal without treatment

GPx: glutathione peroxidase

GSH: reduced glutathione

HE: hematoxylin and eosin

HRP/DAB: horseradish peroxidase/diaminobenzidine

IBB: Institute of Biosciences of Botucatu

IFN-γ: interferon-γ

IL-10: interleukin-10

IL-1β: interleukin-1β

IL-6: interleukin-6

IU: International Units

MMP: extracellular matrix metaloproteinases

NeBa: wounded animal treated with neomycin 5 mg/g + sulfate bacitracin zinc 250 IU/g

NLC: wounded animals treated with 1% *Copaifera langsdorffii* oleoresin loaded in nanostructured lipid carriers

ROS: reactive oxygen species

SOD: superoxide dismutase

TNF-α: tumor necrosis factor-α

UK: United Kingdom

UNESP: São Paulo State University

USA: United States of America

Introduction

The skin is the first protective barrier of vertebrates against deleterious agents. When there is a disruption in this barrier, the organism starts the wound healing process, activating overlapping and dependent mechanisms to reconstruct the skin[1]. Currently, several drugs on the market help with wound healing, avoiding errors and optimizing the process. One of the drugs used in skin wound healing is a formulation of neomycin and bacitracin used to treat skin lesions specifically, especially at the beginning of wound healing, to avoid infection[2]; dexpanthenol, which improves the proliferation of keratinocytes and fibroblasts in skin wounds[3]; and collagenase, an enzyme that acts in the wound debridement and remodeling phase of wounded tissue[4]. However, depending on the location, extent and type of lesion, the existing treatments may not be efficient[5]. Therefore, several studies have discovered new alternative drugs that accelerate skin wound healing without errors in tissue repair, including medicinal plants and natural products[6].

One of these plants is *C. langsdorffii* Desf. (*Leguminosae*), popularly known as "copaiba", whose oleoresin is used in folk medicine to treat skin wounds[7]. In previous studies, our group proved the wound healing potential of *C. langsdorffii* oleoresin and some mechanisms of action in a cream formulation[8]. However, the cream formulation has low acceptance by patients, and there is poor physicochemical stability with oleoresin[9]. Furthermore, researchers have developed new strategies to improve the absorption and effectiveness of drugs through the encapsulation of drugs in nanoparticles, such as nanostructured lipid carriers, which improve the biocompatibility, drug release and stability of the incorporated drugs[10]. Therefore, our group tested a new formulation with 1% copaiba oleoresin loaded in a nanostructured lipid carrier to analyze the wound healing potential and mechanisms of action of this formulation in a rat excision wound model.

Materials and Methods

Extraction of oleoresin

The extraction of *C. langsdorffii* oleoresin and its purification were reported by Ribeiro *et al.* (2019)[11], and the characterization of the plant was described by Souza *et al.* (2011)[12]. Briefly, oleoresin was collected, and the plant voucher (SPFR 10120) was identified by Silvana Tavares Rodrigues. The volatile fraction of oleoresin was obtained by hydrodistillation using a Clevenger-type apparatus.

Preparation and characterization of nanostructured lipid carriers with *C. langsdorffii* oleoresin

A nanostructured lipid carrier containing 1% *C. langsdorffii* oleoresin was made as described by Pivetta *et al*. (2018) with a few modifications[13]. The oil phase, prepared with 200 mg of Illipe butter (Polytechno Indústrias Químicas Ltda, Guarulhos, Brazil) and *C. langsdorffii* oleoresin (1% w/w), was heated to 60°C. An aqueous solution of Pluronic F68 (0.5% w/v) (Sigma-Aldrich, Missouri, USA) at 60°C was added to the oil phase, followed by sonication (13 mm probe and 40% amplitude). Afterwards, the dispersion was cooled to 25°C, forming a nanostructured lipid carrier with oleoresin. The size, polydispersity index and zeta potential of these nanostructures were determined by dynamic light scattering using a Zetasizer Nano ZS90 (Malvern Panalytical, Malvern, United Kingdom). The measurements were made with samples diluted in 1 mM KCl solution.

Preparation and characterization of the topical formulation containing copaiba oleoresin-loaded nanostructured lipid carriers

The emulgel was made by homogenizing Sepineo P600 (3% w/w) (Spectrum Chemical, New Brunswick, USA), propylene glycol (5% w/w) (Spectrum Chemical, New Brunswick, USA), Labrafac lipophile WL 1349 (10% w/w) (Gattefossé, Lyon, France), methyldibromo glutaronitrile/phenoxyethanol (0.1% w/w) (Spectrum Chemical, New Brunswick, USA) and water. Afterwards, a *C. langsdorffii* oleoresin-loaded nanostructured lipid carrier dispersion (30% w/w) was added to the emulgel and homogenized at 25°C. The rheological behavior of the emulgel containing copaiba oleoresin-loaded nanostructured lipid carriers was analyzed in a Rheometer R/S plus (AMETEK Brookfield, Middleborough, USA) equipped with a C50-1 spindle and RHEO Software 2000 version 2.8. The sample behavior was monitored at 25°C using a water bath/circulator. The time of the upward curve was 120 s, with shear rates ranging from 0 to 1000 s^{-1}, and 120 s to the downward curve with shear rates ranging from 1000 to 0 s^{-1}[114].

Animals

Ninety male *Wistar* rats (*Rattus norvegicus*) weighing 250 ± 20 g were used in the experiments. The animals were supplied by the Central Animal House, UNESP, Botucatu, and acclimated in individual cages under controlled conditions (23 ± 2°C, 12 hours' dark-light cycle, food and water *ad libitum*) until the experimental procedures were performed. All experiments were approved by the Ethics Committee on Animal Use (IBB/UNESP) under protocol 976/2017.

Excision wound model and experimental protocol

After the acclimation period, the animals were anesthetized with intraperitoneal ketamine (80 mg/kg) and xylazine (4 mg/kg), their dorsum was shaved, and a lesion was made in the dorsum (in the subscapular area) using a 3 cm diameter punch. The wound placed in this area could not be reached by the animals, which prevented self-licking[15]. Afterwards, each rat was randomly distributed into six groups (n = 5/group): FST, NeBa, Dex, Col, NLC, and control. After the surgical procedure, the wounds of the FST, NeBa, Dex, Col and NLC groups were topically treated every day twice a day, and the control group was used as the reference pattern of normal skin (without excision and treatment) during three different experimental periods: 3, 7, or 14 days (30 animals/period). After each treatment period (3, 7, and 14 days), the animals were euthanized, and wound and blood samples were collected for biochemical, immunoenzymatic, immunohistochemical and histological analyses.

Macroscopic analysis

To determine the macroscopic reduction of lesions, the wounded area was photographed every day using a scale bar during each experimental period. The wounded areas were measured using specific software, and the percentage of wound retraction was calculated according to the daily retraction of the lesions and compared to their initial size according to the formula: wound retraction (%): {(initial wounded area – wounded area in the analyzed day)/initial wounded area}*100.

Histopathological analysis

The wound samples were fixed with 10% buffered formalin, processed in paraffin, sliced (5 µm thickness) and stained in HE and Masson's trichrome. HE staining was used to analyze the number of cells, epidermis thickness and number of blood vessels. Masson's trichrome was

used to analyze the total amount of collagen and its deposition in the dermis. For each sample, the border and the center of the wounds were analyzed and photographed in five different fields. The measurements were made using AvSoft BioView Spectra software.

Immunohistochemical analysis

The wounded skin samples were fixed with 10% buffered formalin, processed in paraffin and sliced (5 µm thickness). Subsequently, the slices were processed according to the protocol of a mouse- and rabbit-specific HRP/DAB detection kit micropolymer (Abcam, Cambridge, USA) using primary antibodies against laminin-γ2 (1:200 µL) (Santa Cruz Biotechnology, Dallas, USA), desmoglein-3 (1:200 µL) (Abcam, Cambridge, USA), Ki-67 (1:100 µL) (Abcam, Cambridge, USA) and α-SMA (1:400 µL) (Abcam, Cambridge, USA). For the analysis, all areas with immunolabeling corresponding to the primary antibodies were measured, and the positive cells were counted. The measurements were made using AvSoft BioView Spectra software.

ELISA

The samples were also used to quantify pro- and anti-inflammatory cytokines. For this, IFN-γ, IL-1β, IL-6 and TNF-α were used as proinflammatory biomarkers, and IL-10 was used as an anti-inflammatory biomarker. First, the samples were homogenized in a 1:5 proportion (m/v) using phosphate saline buffer at pH 7.4. The homogenate was centrifuged for 15 minutes at 10000 rpm and 4°C. After centrifugation, the supernatant was collected for each sample and used to quantify the concentration of each cytokine by ELISA as described by the protocols of each kit (R&D Systems, Minneapolis, USA). The results were expressed in pg of cytokine/mg of protein.

Oxidative stress assays

To determine the antioxidant potential of the treatments, the activity of SOD, CAT and GPx and the concentration of GSH were analyzed. The samples were homogenized in a 1:5 proportion (m/v) using phosphate buffer at pH 7.0. The homogenate was centrifuged for 45 minutes at 12000 rpm and 4°C. After centrifugation, the supernatant was collected for each sample and used to analyze the activity of SOD[16], CAT[17] and GPx[18] and quantify GSH[19].

Toxicological analysis

The blood of each animal was collected immediately after euthanasia and centrifuged for 15 minutes at 6000 rpm and 4°C. The parameters of liver and kidney toxicity were evaluated through quantification of AST, ALT and γ-GT activities (IU/L) and the concentrations of urea and creatinine (mg/dL) (Interteck-Katal, Belo Horizonte, Brazil).

Statistical analysis

The percentage of retraction data were subjected to two-way ANOVA, followed by the Bonferroni posttest. Antioxidant, anti-inflammatory and toxicity analyses were subjected to one-way ANOVA and Tukey's post hoc test. The results of histological and immunohistochemical analysis were subjected to the Kruskal-Wallis test, followed by the Dunn posttest. The analyses were performed by GraphPad Prism software, with a significance of 5%.

Results

Characterization of the topical formulation containing copaiba oleoresin-loaded nanostructured lipid carriers

Nanostructured lipid carriers with *C. langsdorffii* oleoresin exhibited a size of 204.06 ± 8.3 nm, a low polydispersity index (0.132 ± 0.028) and a negative zeta potential (-17 ± 1.73 mV). This dispersion was added to an emulgel formulation. The rheological behavior of the emulgel containing copaiba oleoresin-loaded nanostructured lipid carriers showed non-Newtonian characteristics, with pseudoplastic behavior ($n < 1$), which means that with increasing shear rate, the viscosity decreased.

Macroscopic analysis

The retraction of the wound area was analyzed daily in each period of treatment, with observation of the best retraction in the groups treated with NeBa and NLC in the first 3 days of treatment and a decrease in local edema in these groups. According to the results obtained after 7 days of treatment, the animals treated with Col and NLC showed increases in wound retraction and no longer had fibrinous exudate compared to the other groups. After 14 days of treatment, there was a greater retraction of lesions in the groups treated with Col and NLC compared to all other treatments, in addition to complete reepithelialization of the wounds compared to the FST group (Figure 1).

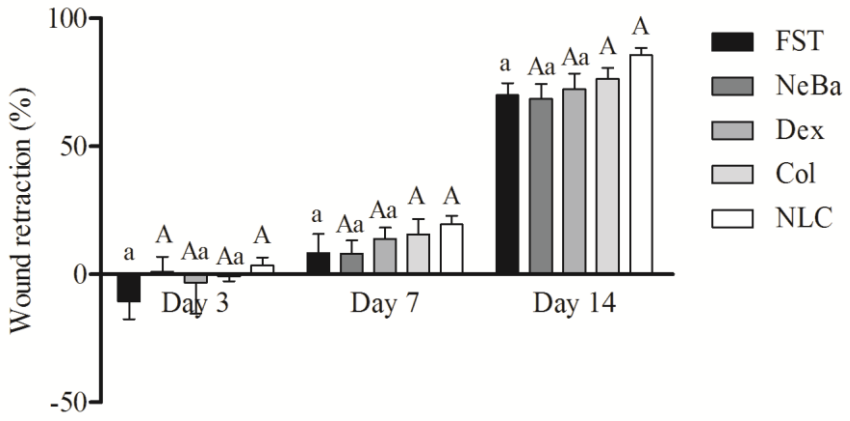

Figure 1. Wound retraction (%) in FST, NeBa, Dex, Col and NLC treatments during 3, 7 and 14 days. Different letters show statistical difference, according to two-way ANOVA followed by Bonferroni test (n = 5).

Histopathological analysis

The analysis of the total number of cells in the epidermis, border and center of the dermis showed that there were no differences among the wounded groups during days 3, 7 and 14 of treatment (Supplementary materials 1 to 4). Furthermore, there was a decrease in the thickness of the epidermis of the NeBa, Dex, Col and NLC treatments compared to the FST group during 14 days of treatment (Supplementary materials 2 and 5).

The results obtained from the quantification of blood vessels at the border of the lesions did not show any differences among the wounded groups but did show an increase compared to the control at 3 and 7 days (Supplementary materials 3 and 6). In the central region, there was no difference among the wounded groups and increased vascularization compared to the control at 3 and 14 days (Supplementary materials 4 and 6).

Analysis of the amount of collagen at the border of the dermis did not demonstrate any difference among wounded groups during any period of treatment (Figure 2 and Supplementary materials 7). At the center of the dermis, the FST group showed a decrease in collagen synthesis

compared to the NLC and control groups during the first 3 days of treatment (Figure 2 and Supplementary materials 8).

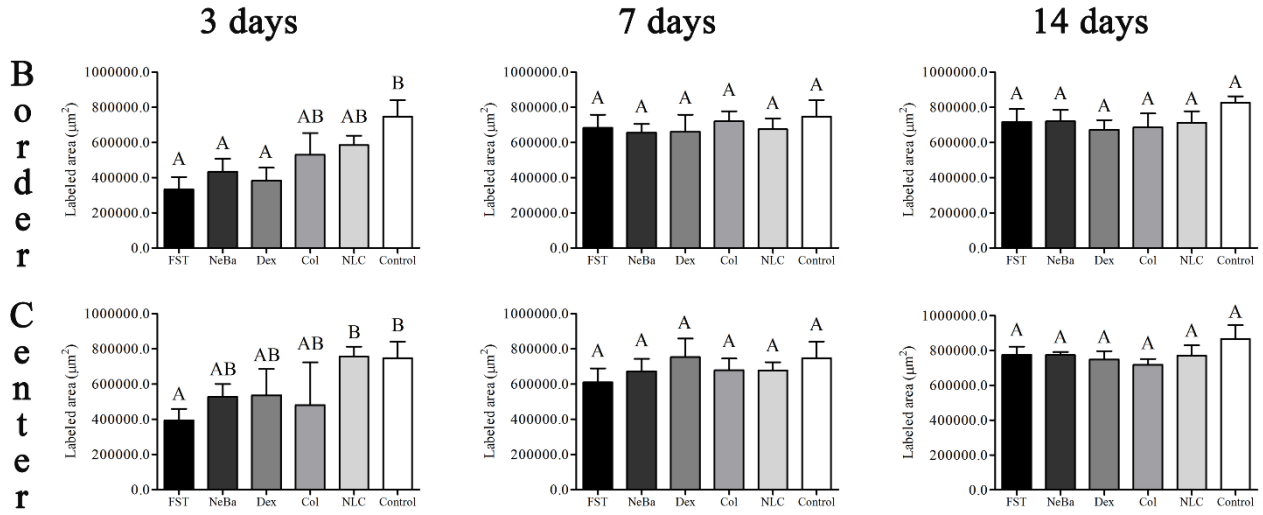

Figure 2. Quantification of collagen area (µm^2) at the edges and center of the dermis in wounds of FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days. Different letters show statistical difference, according to the Kruskal-Wallis test, followed by the Dunn post-test (n = 5).

Immunohistochemical analysis

The analysis of α-SMA demonstrated an increase in immunolabeled cells in the NeBa, Dex and NLC treatments at 3 and 7 days compared to the FST (Figures 3 and 4). The immunohistochemical analysis of desmoglein-3 showed increased immunolabeling in the keratinocytes of the FST group compared to NLCs during 3 and 7 days of treatment (Figures 3 and 5). With regard to laminin-γ2, after 3 days of treatment, it was possible to observe increased immunolabeling with the Col and NLC treatments compared to FST (Figures 3 and 6). The data on cell proliferation markers in the epidermis, border and center of the dermis did not demonstrate any difference among wounded groups in any period of treatment (Supplementary materials 9 to 12).

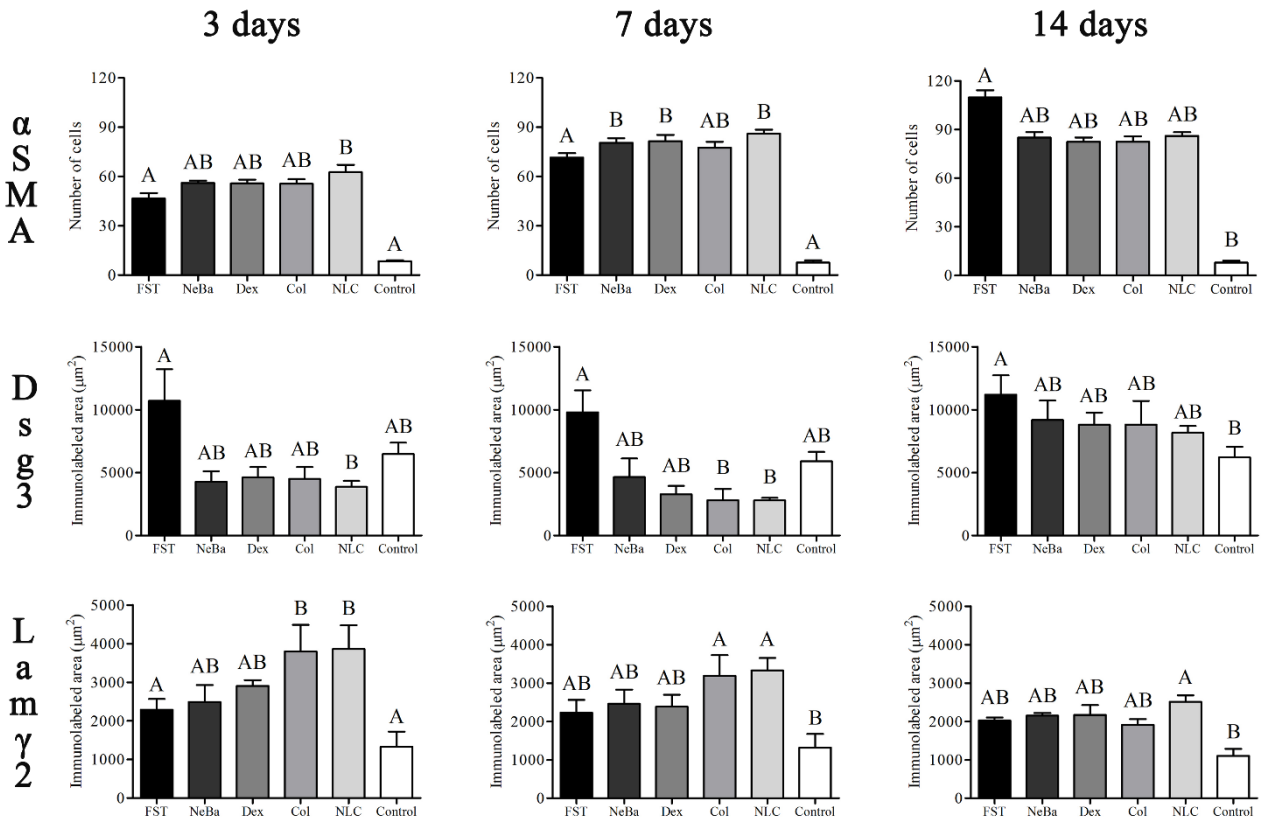

Figure 3. Immunolabeled area of desmoglein-3, lamminin-γ2 (µm^2) and number of positive α-SMA cells in the epidermis of wounds of FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days. Different letters show statistical difference, according to the Kruskal-Wallis test, followed by the Dunn post-test (n = 5).

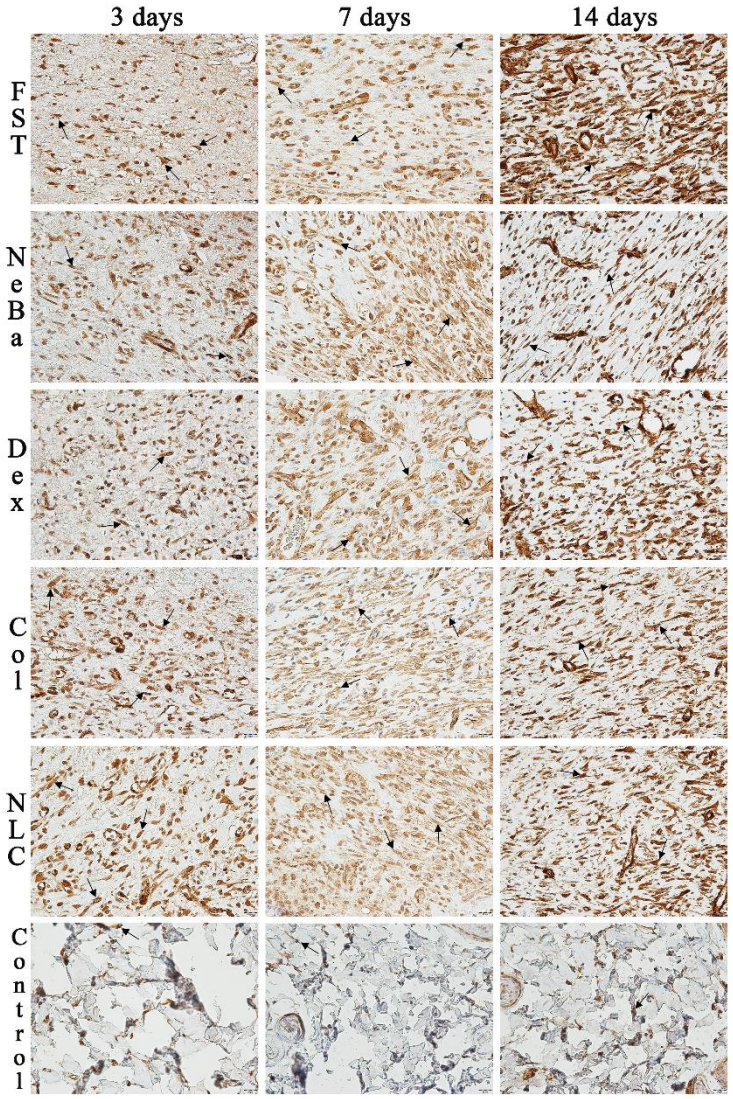

Figure 4. Photomicrographs of the immunolabeling of α-SMA in the dermis of wounds of FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days.

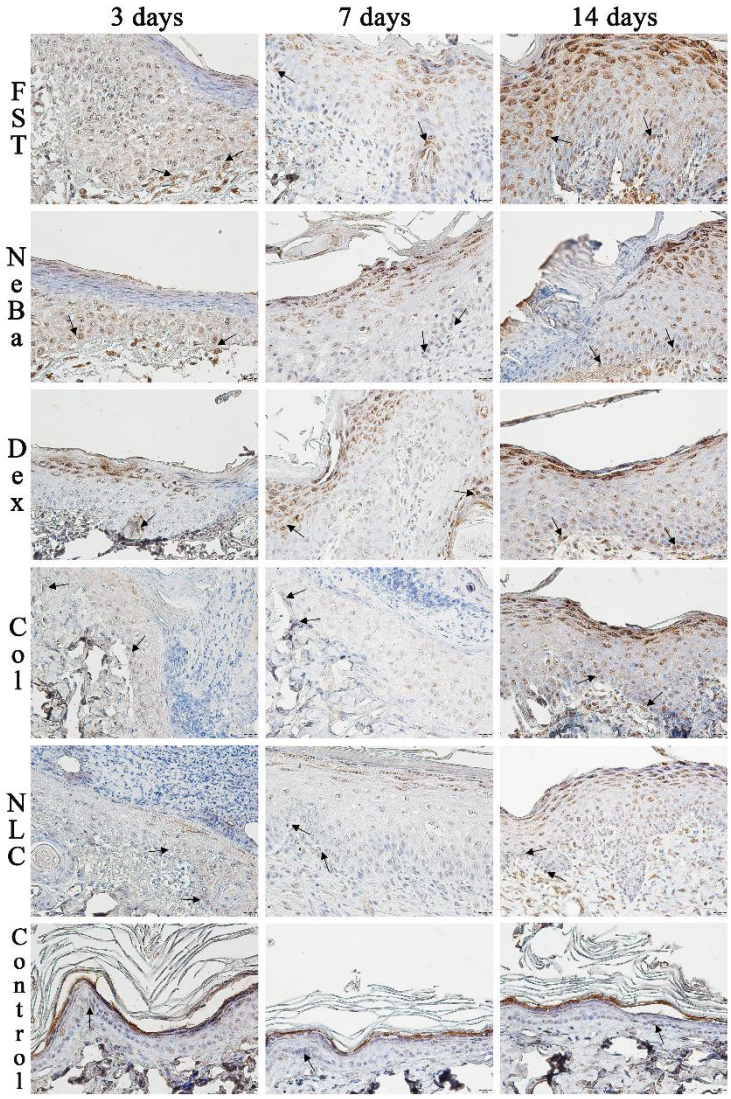

Figure 5. Photomicrographs of the immunolabeling of desmoglein-3 in the epidermis of wounds of FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days.

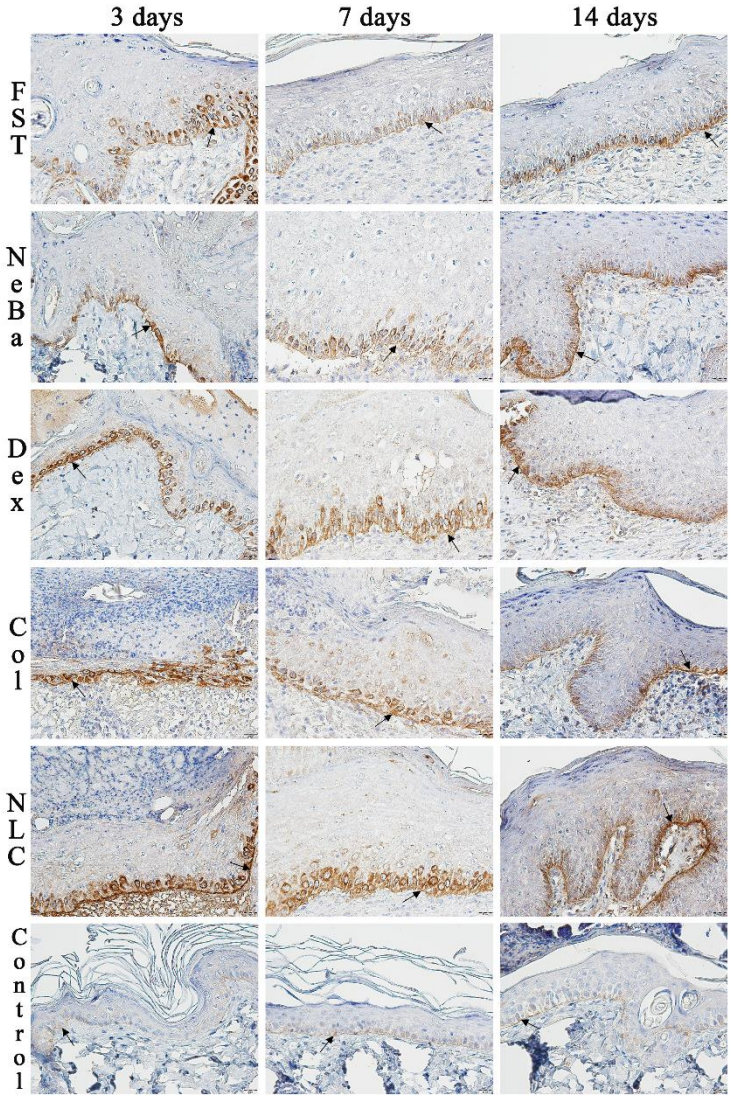

Figure 6. Photomicrographs of the immunolabeling of laminin-γ2 in the epidermis of wounds of FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days.

ELISA

The IFN-γ analyses obtained showed a decrease in the level of this proinflammatory cytokine in the NeBa, Dex, Col and NLC groups compared to the FST group after 7 and 14 days of treatment. The results for the proinflammatory cytokine IL-1β demonstrated a reduction in its level in animals treated with NeBa, Dex and NLC compared to FST for 3 and 7 days. With regard to IL-6, there was a decrease in the concentration of this interleukin in animals treated with NeBa, Dex and NLC compared to the FST group after 3 and 7 days. After 3 and 7

days of treatment, the levels of IL-10 in all groups in which the rats had surgically induced lesions were the same. However, at 14 days, NeBa, Dex, Col and NLC treatments increased the concentration of this anti-inflammatory interleukin compared to FST. The NeBa, Dex, and NLC groups showed reduced levels of TNF-α compared to the FST group during 14 days of treatment (Figure 7).

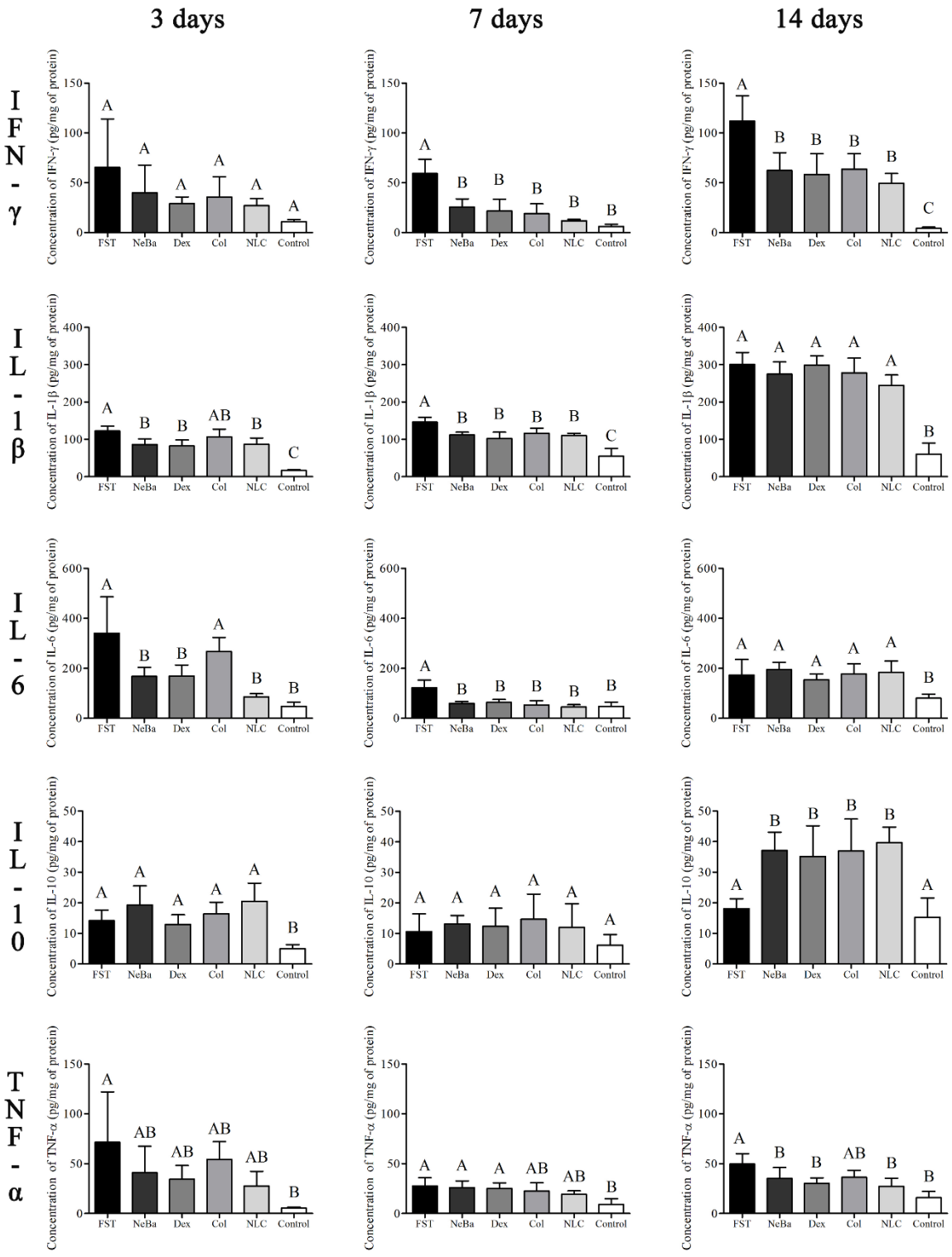

Figure 7. Concentrations of IFN-γ, IL-1β, IL-6, IL-10 and TNF-α (pg/mg protein) in skin wounds of rats treated for 3, 7 and 14 days. Different letters show statistical difference, according to one-way ANOVA followed by Tukey test (n = 5).

Oxidative stress assays

The activity of CAT in the Dex, Col and NLC groups was decreased compared to that in the FST group at 14 days of treatment. Regarding GPx activity, the NeBa, Col and NLC groups demonstrated higher enzyme activity than the FST, Dex and Control groups at 7 days of treatment. There was no difference among wounded groups in the quantification of GSH during any period of treatment. The SOD activity of the NLC group was decreased compared to that of the FST, NeBa, Dex and Col groups during 7 days of treatment (Figure 8).

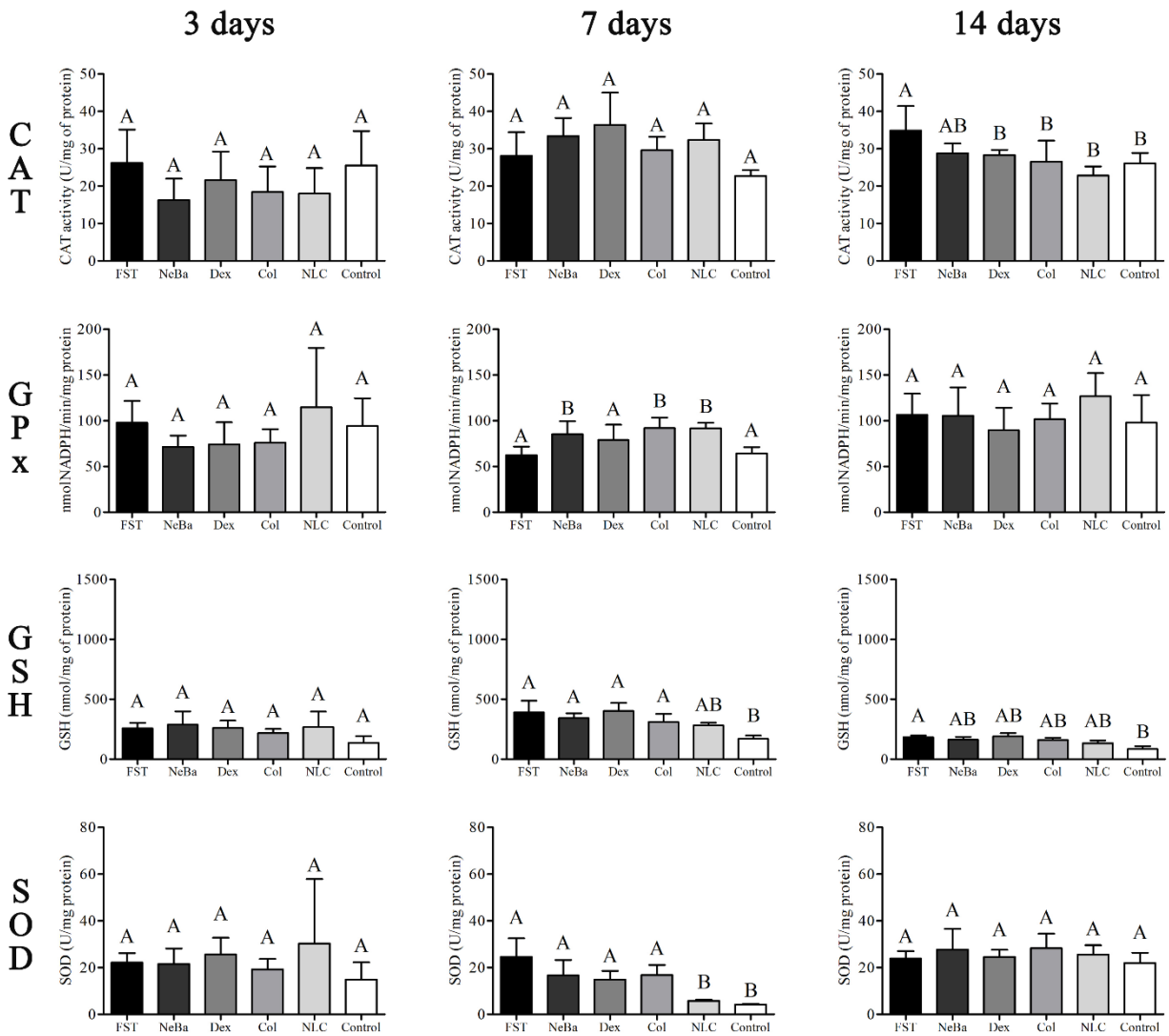

Figure 8. Concentration and enzyme activities of CAT, GPx, GSH and SOD in skin wounds of rats treated for 3, 7 and 14 days. Different letters show statistical difference, according to one-way ANOVA followed by Tukey test (n = 5).

Toxicological analysis

The results of the systemic toxicity evaluation of liver enzymes (AST, ALT and γ-GT) and kidney proteins (creatinine and urea) did not show differences among all treatments and normal parameters (Control) (Table 1).

Table 1. Systemic toxicity analysis data for liver (AST, ALT, γ-GT) and renal (creatinine, urea) parameters in the serum of rats treated for 14 days.

Groups	AST (IU/L)	ALT (IU/L)	γ-GT (IU/L)	Creatinine (mg/dL)	Urea (mg/dL)
FST	143 ± 28	62 ± 12	1.2 ± 0.4	0.30 ± 0.03	44 ± 2.1
NeBa	138 ± 17	65 ± 7.7	1.1 ± 0.3	0.31 ± 0.04	44 ± 6.2
Dex	150 ± 28	80 ± 17	0.9 ± 0.3	0.27 ± 0.03	43 ± 5.6
Col	153 ± 29	63 ± 12	1.0 ± 0.2	0.30 ± 0.05	45 ± 4.6
NLC	147 ± 49	67 ± 13	1.0 ± 0.2	0.27 ± 0.04	45 ± 5.5
Control	147 ± 25	65 ± 9.1	1.0 ± 0.2	0.30 ± 0.02	44 ± 4.9

Discussion

In previous studies, our group demonstrated the healing potential of copaiba oleoresin in cream formulations[8]. However, cream formulations have low patient acceptance and less physical and chemical stability than emulgel formulations. Furthermore, nanostructured lipid carriers also increase the bioavailability and physical-chemical stability of drugs[9,10]. Therefore, our group synthesized an emulgel formulation containing nanostructured lipid carriers with 1% copaiba oleoresin and confirmed the efficacy of the treatment in a rat skin excision wound model and the mechanisms of wound healing. Furthermore, the formulation of NLC presented

promising results compared to three reference drugs because although NeBa (antimicrobial)[2], Dex (cell proliferation)[3] and Col (extracellular matrix remodeling)[4] affect one mechanism of wound healing, NLC treatment improves anti-inflammatory, reepithelialization, wound retraction and remodeling mechanisms.

The retraction of wounds is mediated by myofibroblasts, which are cells that differentiate from fibroblasts stimulated by TGF-β1, acquiring a contractile phenotype due to intracellular proteins such as α-smooth muscle actin. Myofibroblasts bind to contractile proteins and multiple points of attachment, contracting them and reducing the area of the wounds[20,21]. Thus, we demonstrate the influence of the NLC formulation on the retraction mechanism, with better results compared to untreated lesions and reference drugs, improving wound retraction in all periods of treatment. The retraction mechanism was mediated by the α-SMA pathway, increasing the retraction of the wounds due to increased numbers of immunolabeled myofibroblasts.

With the synthesis of a fibrin clot by an organism after formation of a skin lesion, the cells release molecules that stimulate the migration of leukocytes to the wound (IFN-γ, IL-1β, TNF-α and IL-6), initiating the inflammatory mechanism. First, neutrophils migrate to the region to promote the debridement of necrotic tissue and the phagocytosis of possible pathogens. After a few hours, macrophages migrate to the lesion, assisting in tissue debridement, antigen phagocytosis and the synthesis of cytokines and growth factors that will influence other healing mechanisms[22]. However, chronic leukocyte activity and the increase in proinflammatory cytokines increase the production of ROS in the region, with the consequent perpetuation of local oxidative stress and chronic inflammation, causing errors in healing, such as fibrosis, hypertrophic scars or even nonhealing wounds[23]. Therefore, the balance of antioxidant mechanisms and pro- and anti-inflammatory mediators is necessary for continuity

of the healing process, and understanding the role of these mediators in healing can lead to more efficient treatments for chronic injuries[24].

Among the mediators, there are molecules involved in oxidative stress and antioxidant mechanisms. The death of cells in the lesion is accompanied by the formation of reactive oxygen species such as superoxide radicals, highly reactive molecules that can lead to mutations in genetic material and cell death[25]. To inhibit the deleterious effects of superoxide radicals, cells in the region produce the enzyme SOD, converting superoxide radicals into hydrogen peroxide. However, H_2O_2 also has a cytotoxic effect. Therefore, local cells synthesize the enzymes GPx and catalase to transform H_2O_2 into nontoxic compounds in the cell, such as H_2O and O_2[16,18,26]. Our results obtained from oxidative stress evaluation demonstrated that NLC and the reference drugs used did not significantly interfere with the oxidative mechanism of skin wounds, showing similar data compared to the physiological parameters of the control group.

Moreover, analyzing the balance among pro- and anti-inflammatory mediators, several cytokines, such as IFN-γ, IL-1β, IL-6 and TNF-α, are synthesized by macrophages, neutrophils and lymphocytes and have multiple functions. These cytokines promote the differentiation, migration and activation of macrophages, neutrophils and NK cells and act on the synthesis of collagen by fibroblasts[27,28]. Therefore, the maintenance of proinflammatory cytokines at reduced levels demonstrates the anti-inflammatory potential of NLCs, with reduced concentrations of TNF-α, IL-1β, IL-6 and IFN-γ compared to the untreated group. Another result that proves the anti-inflammatory effect of NLC formulations is the concentration of IL-10, which was much higher in NLC-treated animals than in untreated animals. This is because IL-10 is an anti-inflammatory interleukin that inhibits the synthesis of proinflammatory mediators, preventing chronic inflammation and fibrosis during healing[27].

Reconstruction of the vascular networks of lesions is essential for the correct healing process, providing oxygen and nutrients necessary for the proliferation and migration of cells.

However, an increase in the amount of blood vessels over a long period of time can be considered one of the markers of tissue fibrosis, and after a period of time, endothelial cells undergo apoptosis, reducing vascularization to normal levels[23]. The data obtained by counting the number of blood vessels in the border and center of wounds did not show the effects of NLCs or reference drugs on the angiogenesis mechanism, with no differences among the treatments and FST groups.

Another important mechanism of skin wound healing, reepithelialization, occurs with the proliferation of keratinocytes at the edges of the lesions, the dissolution of adhesion molecules and the synthesis of anchoring proteins to assist in migration through the extracellular matrix[29]. Desmoglein-3 is a transmembrane adhesion molecule present in desmosomes that maintains keratinocyte attachment and is degraded in migratory keratinocytes during the reepithelialization mechanism[29]. Laminin-γ2 is another important protein with a role in reepithelialization. It is synthesized by migratory keratinocytes from wound edges in the dermo-epidermal junction and assists in keratinocyte migration, anchoring the cells through the extracellular matrix[30]. However, the excessive proliferation of keratinocytes – as a result of chronic inflammation – can lead to an increase in the thickness of the epidermis. The keratinocytes from wound edges acquire a hyperproliferative, hyperkeratotic and parakeratotic phenotype, with a delay in the migratory potential of these cells and impairment of the reepithelialization mechanism, resulting in pathologic scars or nonhealed wounds[31]. For this reason, it is necessary to control the proliferation and migration of keratinocytes for the correct reepithelialization of the injury. Therefore, our immunohistochemistry results for Ki-67, desmoglein-3 and laminin-γ2 demonstrated the stimulation of treatments with NLC formulation for reepithelialization, increasing the number of proliferating keratinocytes, decreasing the amount intercellular adhesion protein (desmoglein-3) and increasing the anchoring protein (laminin-γ2) levels during migration. Furthermore, we showed that although NLC treatment

increased the proliferation and migration of keratinocytes, there was no increase in the thickness of the epidermis as a sign of pathologic wound healing.

The last stage of skin wound healing is remodeling of the extracellular matrix. In this mechanism, myofibroblasts, endothelial cells and excess fibroblasts undergo apoptosis, type III collagen is degraded by extracellular matrix metalloproteinases, and the resistant and elastic permanent extracellular matrix is synthesized and rich in collagen I and elastin[32]. However, remodeling of the extracellular matrix must be carefully controlled, and the chronic production of collagen I and mediators such as α-SMA and TGF-β1 can lead to chronic fibrosis and trigger the formation of hypertrophic scars. Thus, microscopic analysis of Masson's trichrome showed that NLC formulation influenced the synthesis of collagen in the early period of remodeling, anticipating the synthesis of collagen and the remodeling mechanism compared to untreated animals. Moreover, we showed a decrease in α-SMA in reference drugs and NLCs at 14 days, proving the absence of fibrosis and hypertrophic scars as a consequence of the retraction mechanism.

Conclusion

With our results, we demonstrated that a nanostructured lipid carrier containing oleoresin of *Copaifera langsdorffii* at 1% improved skin wound healing in a rat excision model through anti-inflammatory activity, retraction of the lesion mediated by myofibroblasts, reepithelialization and extracellular matrix remodeling. Moreover, we showed the promising potential of the new formulation compared with three different commercial drugs used to treat wounds, with the improvement of several key mechanisms of wound healing by NLC instead of only one with the reference drugs. Therefore, we believe that this formulation has great potential in the treatment of skin wounds in the future with further investigation.

Acknowledgments

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Declaration of Competing Interest

None.

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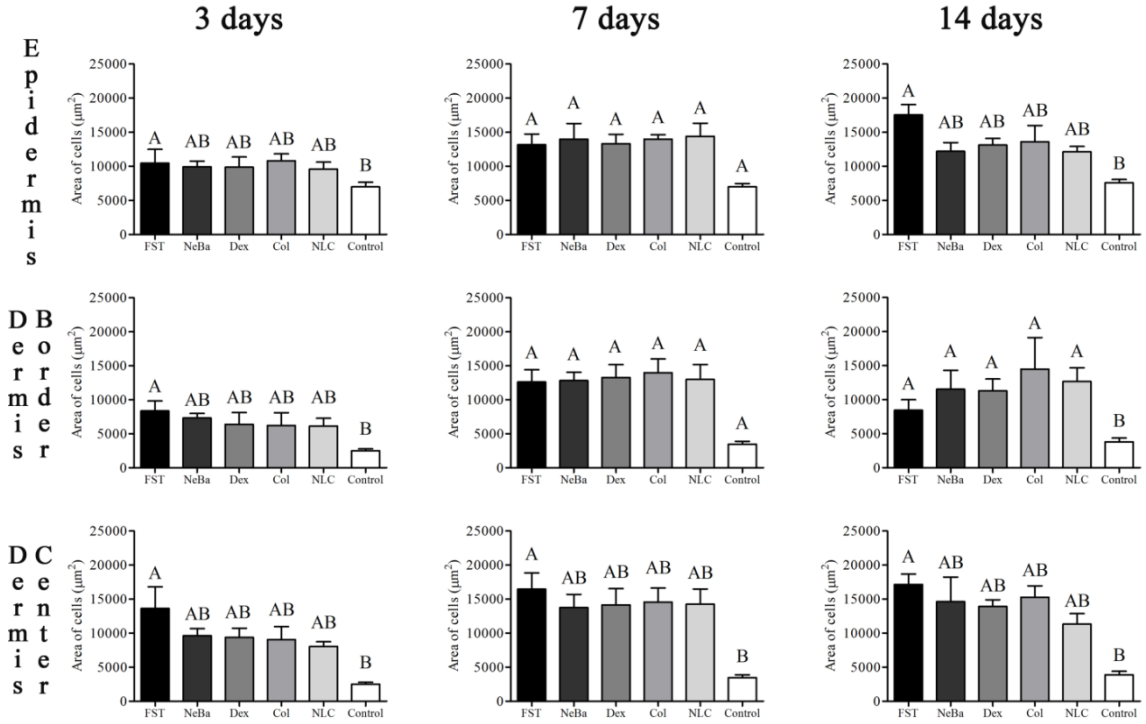
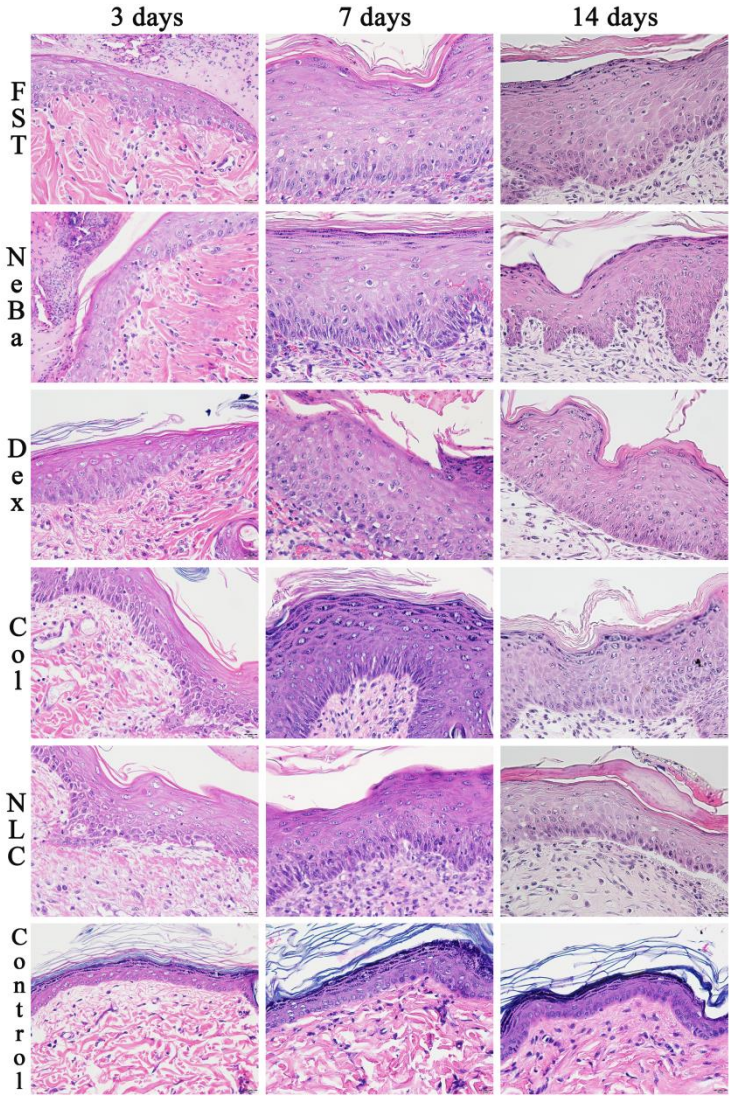
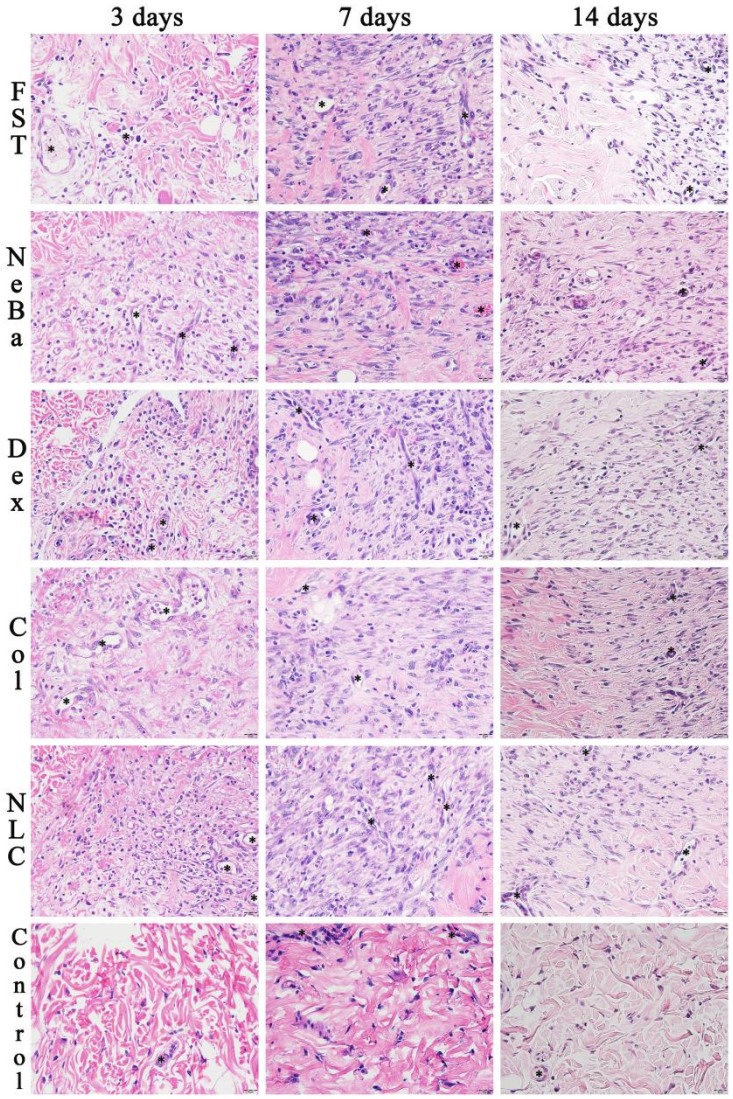
Supplementary materials



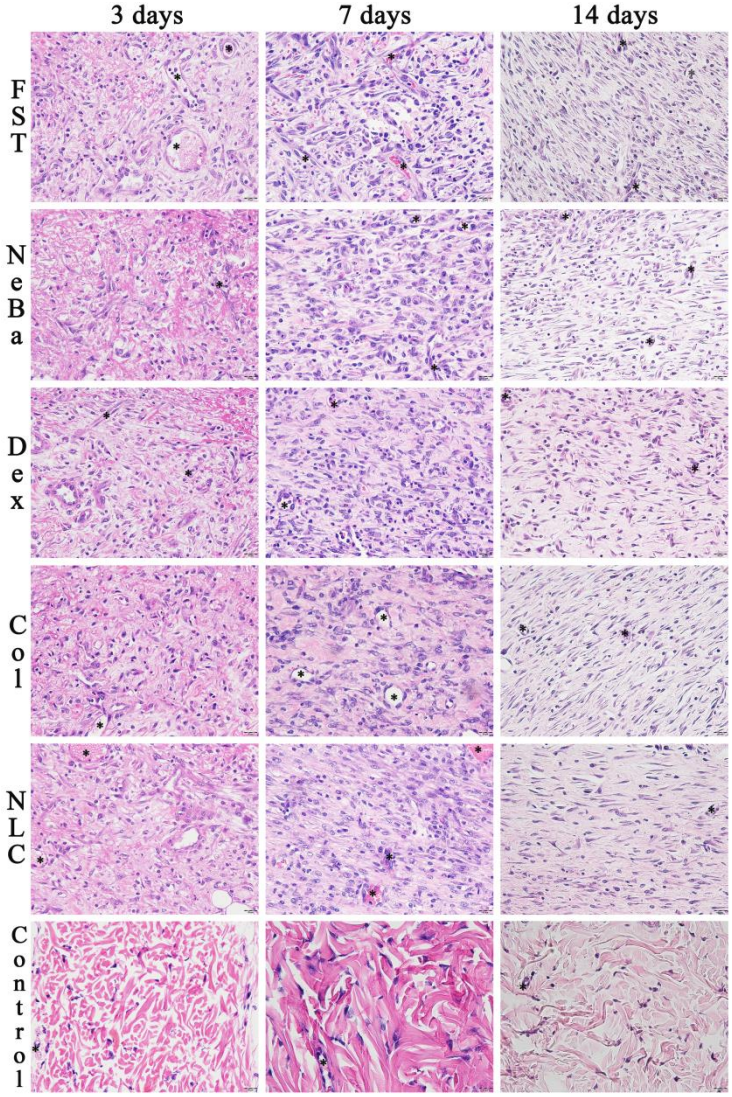
Supplementary materials 1. Quantification of the area (µm^2) of cells in the epidermis, border and center of the dermis in wounds of FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days. Different letters show statistical difference, according to the Kruskal-Wallis test, followed by Dunn post-test (n = 5).



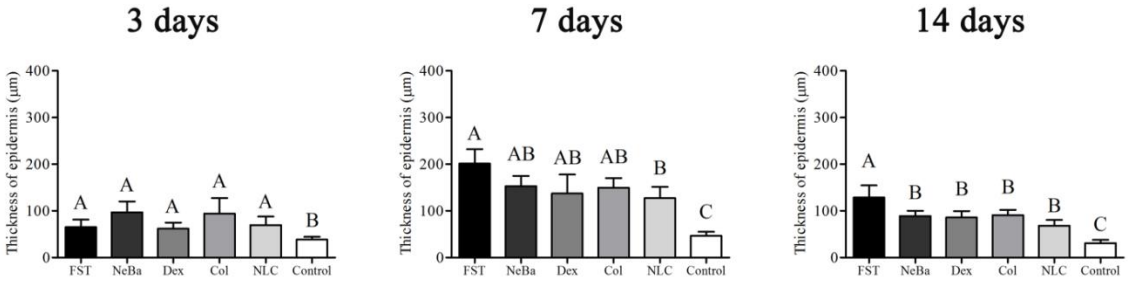
Supplementary materials 2. HE photomicrographs of the epidermis in FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days.



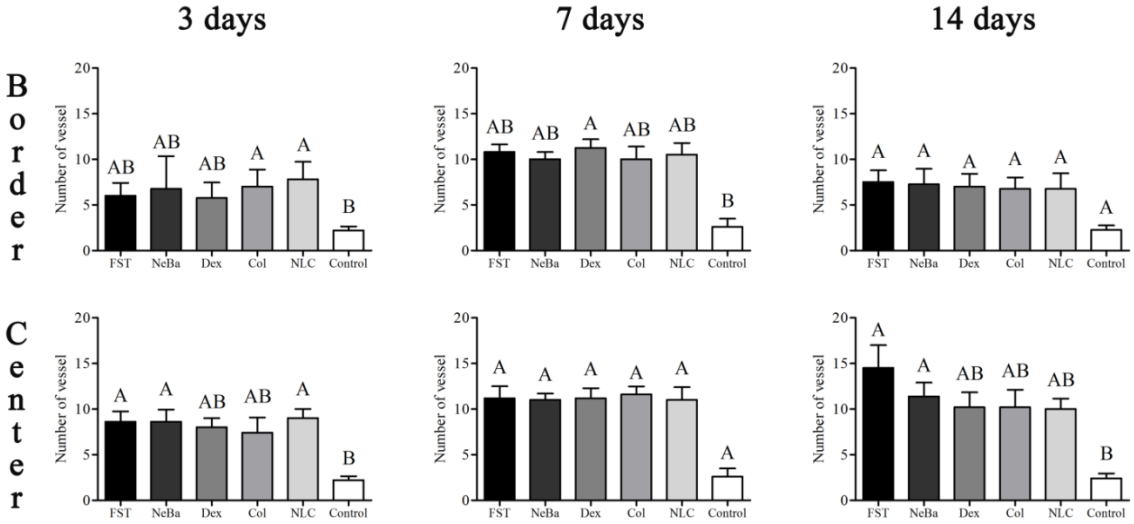
Supplementary materials 3. HE photomicrographs of the border of the wounds in the dermis of FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days. Asterisks indicate the blood vessels.



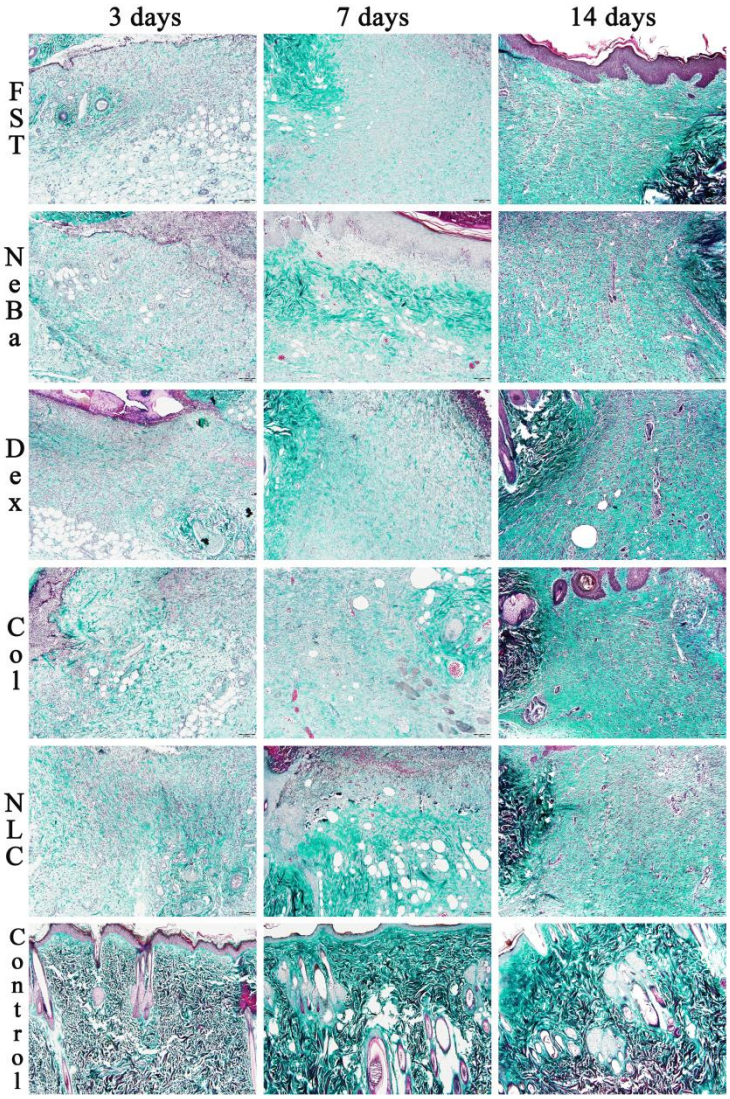
Supplementary materials 4. HE photomicrographs of the center of wounds in the dermis of FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days. Asterisks indicate the blood vessels.



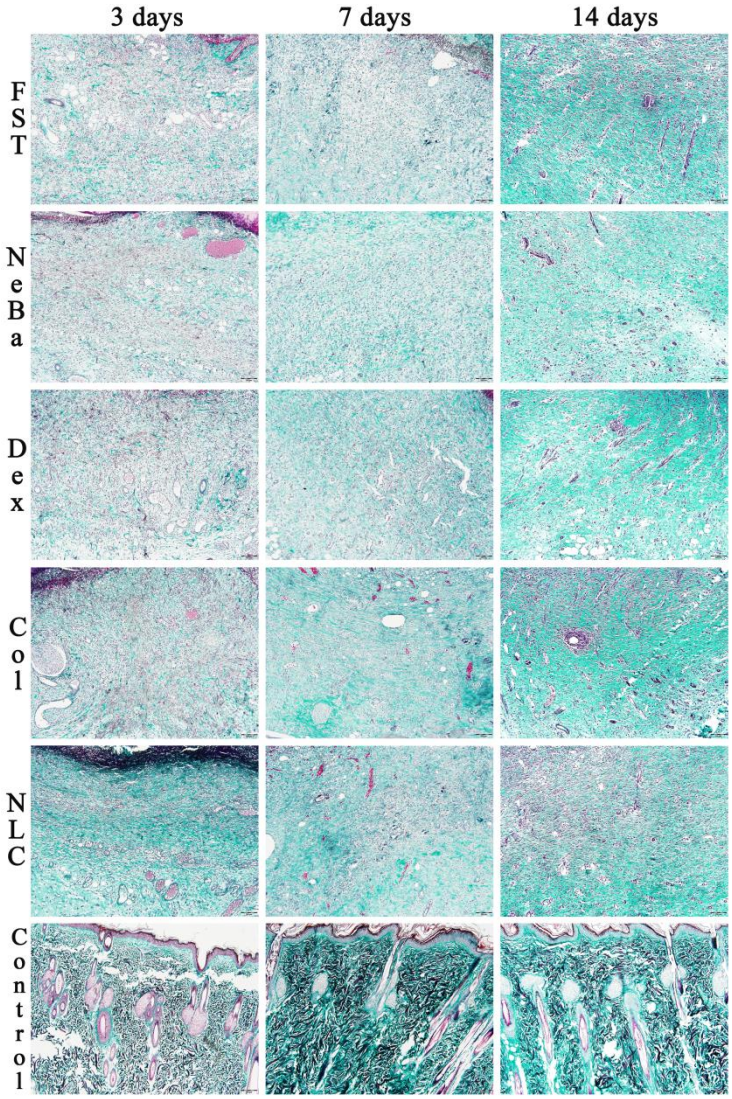
Supplementary materials 5. Epidermis thickness (µm) of FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days. Different letters show statistical difference, according to the Kruskal-Wallis test, followed by the Dunn post-test (n = 5).



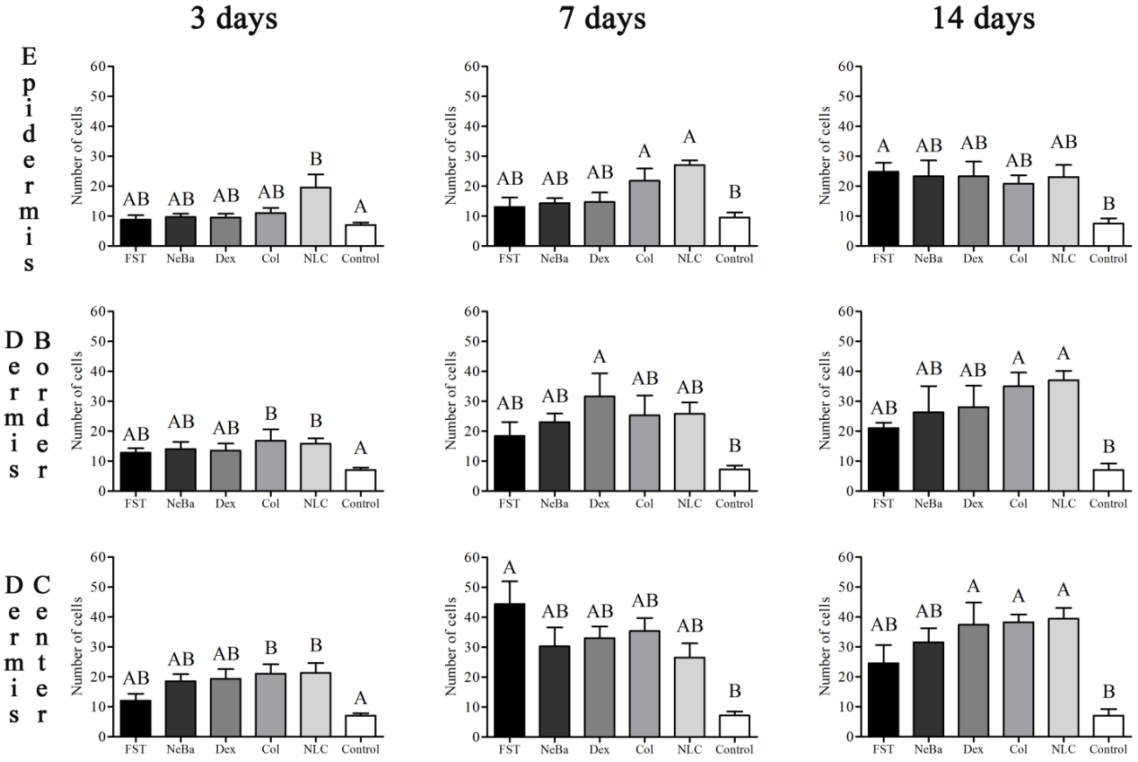
Supplementary materials 6. Number of blood vessels in the border and center of the dermis in wounds of FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days. Different letters show statistical difference, according to the Kruskal-Wallis test, followed by the Dunn post-test (n = 5).



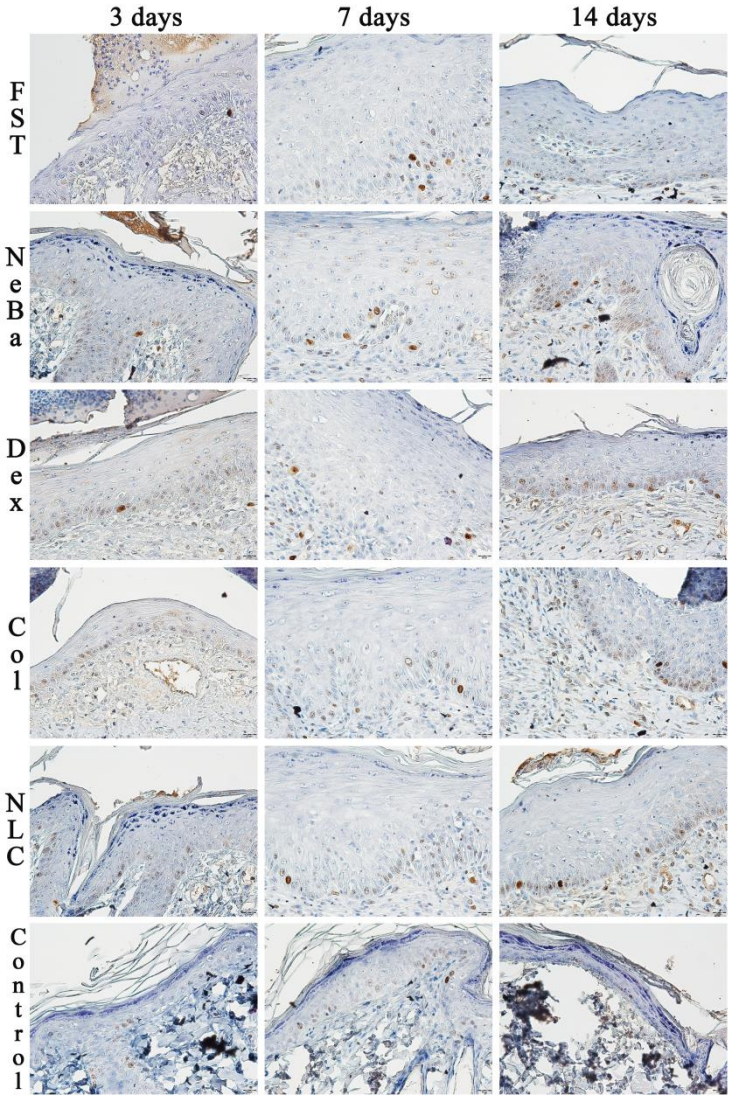
Supplementary materials 7. Masson's trichrome photomicrographs of the border of the wounds in the dermis of FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days.



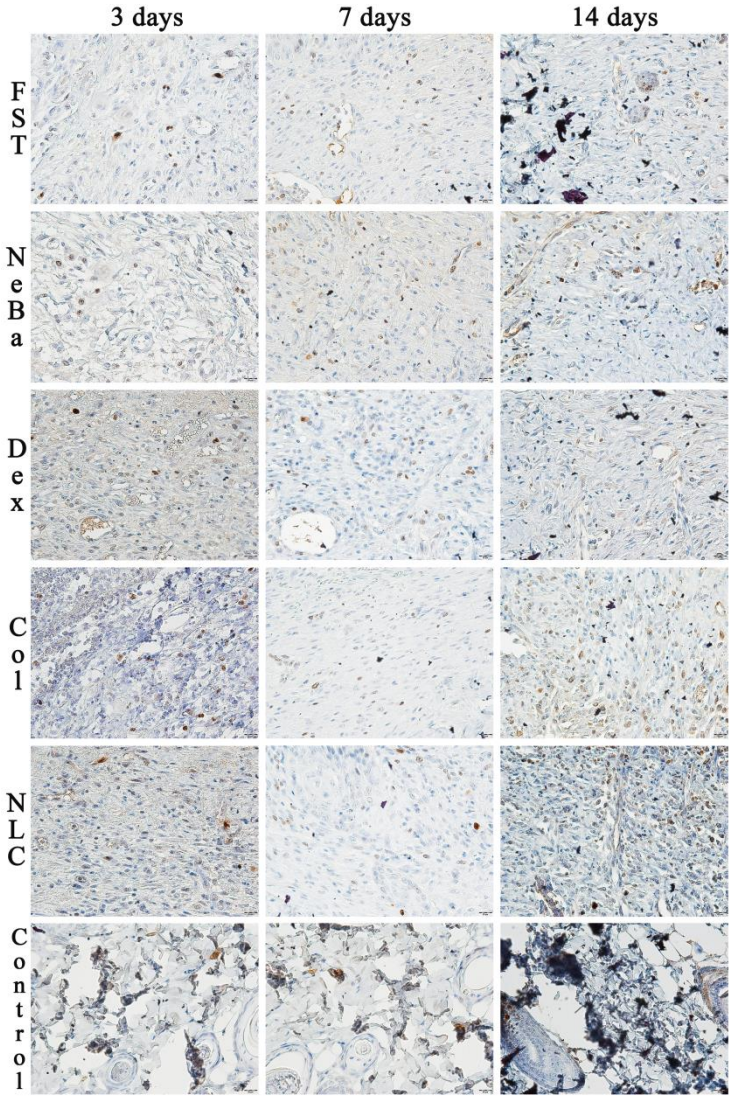
Supplementary materials 8. Masson's trichrome photomicrographs of the center of the wounds in the dermis of FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days.



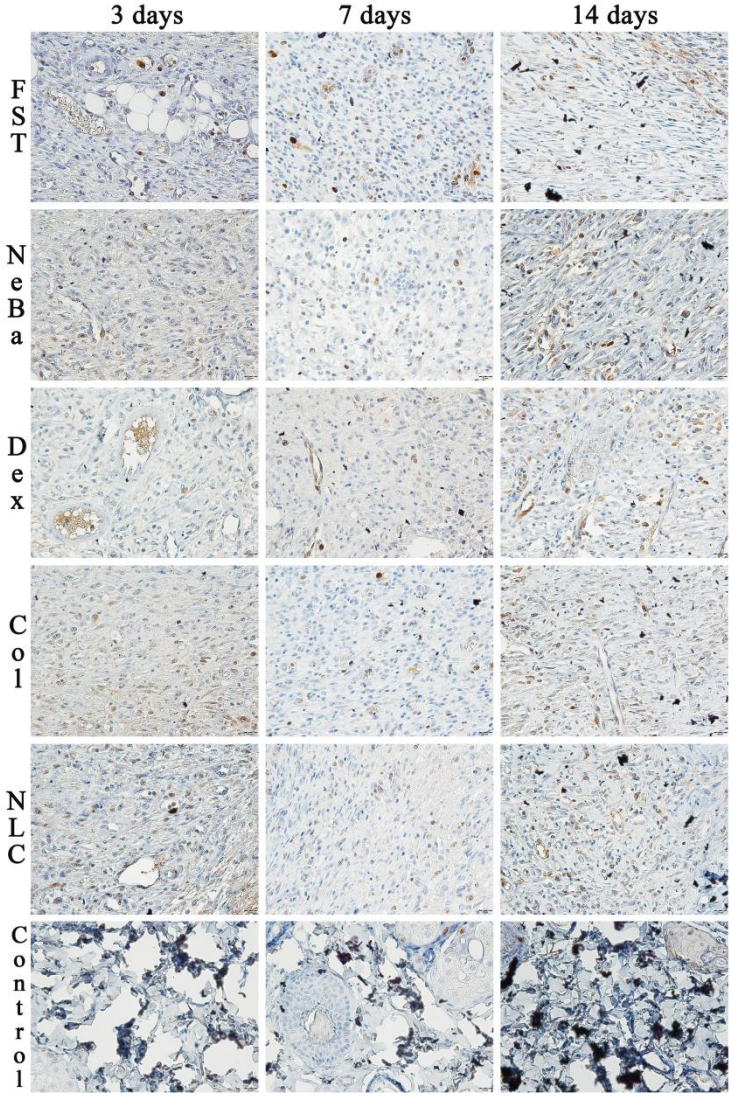
Supplementary materials 9. Ki-67 immunolabeling quantification of proliferating cells in the epidermis, border and center of the dermis in wounds of FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days. Different letters show statistical difference, according to the Kruskal-Wallis test, followed by the Dunn post-test (n = 5).



Supplementary materials 10. Photomicrographs of the immunolabeling of Ki-67 in the epidermis of wounds of FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days.



Supplementary materials 11. Photomicrographs of the immunolabeling of Ki-67 of the border of the wounds in the dermis of wounds from FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days.



Supplementary materials 12. Photomicrographs of the immunolabeling of Ki-67 of the center of the wounds in the dermis of wounds from FST, NeBa, Dex, Col, NLC and Control groups during 3, 7 and 14 days.

Conclusões gerais

Capítulo IV

Conclusões gerais

A análise dos resultados obtidos durante o projeto de doutorado permite concluir que:

- Os fármacos testados à base de β-cariofileno e carreadores lipídicos nanoestruturados contendo óleo-resina de copaíba nas concentrações de 1% estimularam a cicatrização de feridas cutâneas *in vivo* em modelo de excisão circular;
- Ambos os fármacos testados aceleraram o mecanismo de retração macroscópica das lesões nos três períodos estudados, sendo mediada pelo aumento dos miofibroblastos na fase inicial da cicatrização (aumento da imunomarcação para α-actina de músculo liso);
- As duas formulações testadas apresentaram atividade anti-inflamatória local verificada pela diminuição das citocinas pró-inflamatórias IFN-γ, IL-1β, IL-6 e TNF-α e aumento da citocina anti-inflamatória IL-10;
- Houve a redução do estresse oxidativo local pelos dois fármacos do projeto, sendo a atividade antioxidante mediada pelo aumento da enzima GPx;
- Os resultados imunohistoquímicos confirmam a aceleração do mecanismo de reepitelização das feridas por ambas as formulações testadas, mediada pela redução da desmogleína-3 e aumento de laminina-γ2;
- Ambos os fármacos testados no projeto estimularam a síntese de colágeno nos três primeiros dias da cicatrização, acelerando o remodelamento de matriz extracelular no início do processo;
- Tanto o emulgel contendo β-cariofileno a 1%, quanto a formulação de carreadores lipídicos nanoestruturados contendo 1% de óleo-resina de copaíba apresentaram resultados semelhantes ou melhores que os três medicamentos comerciais em todos os mecanismos estudados.

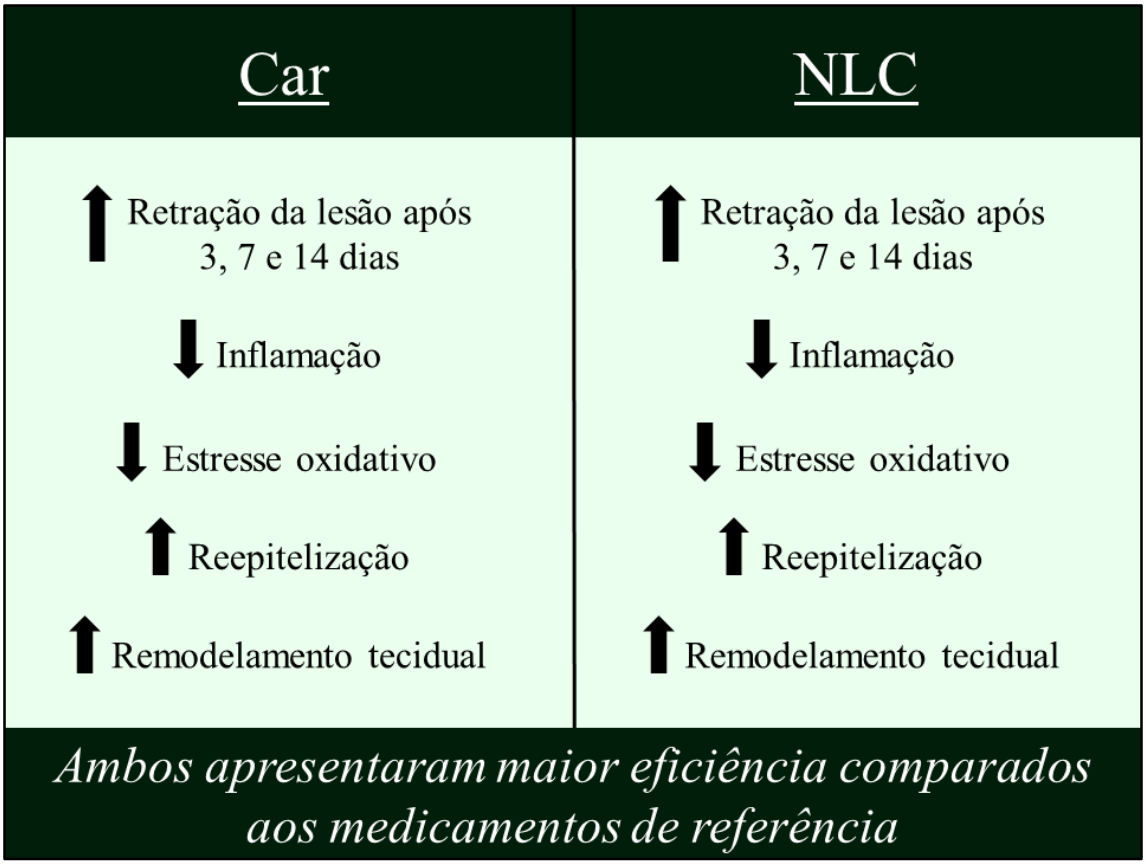
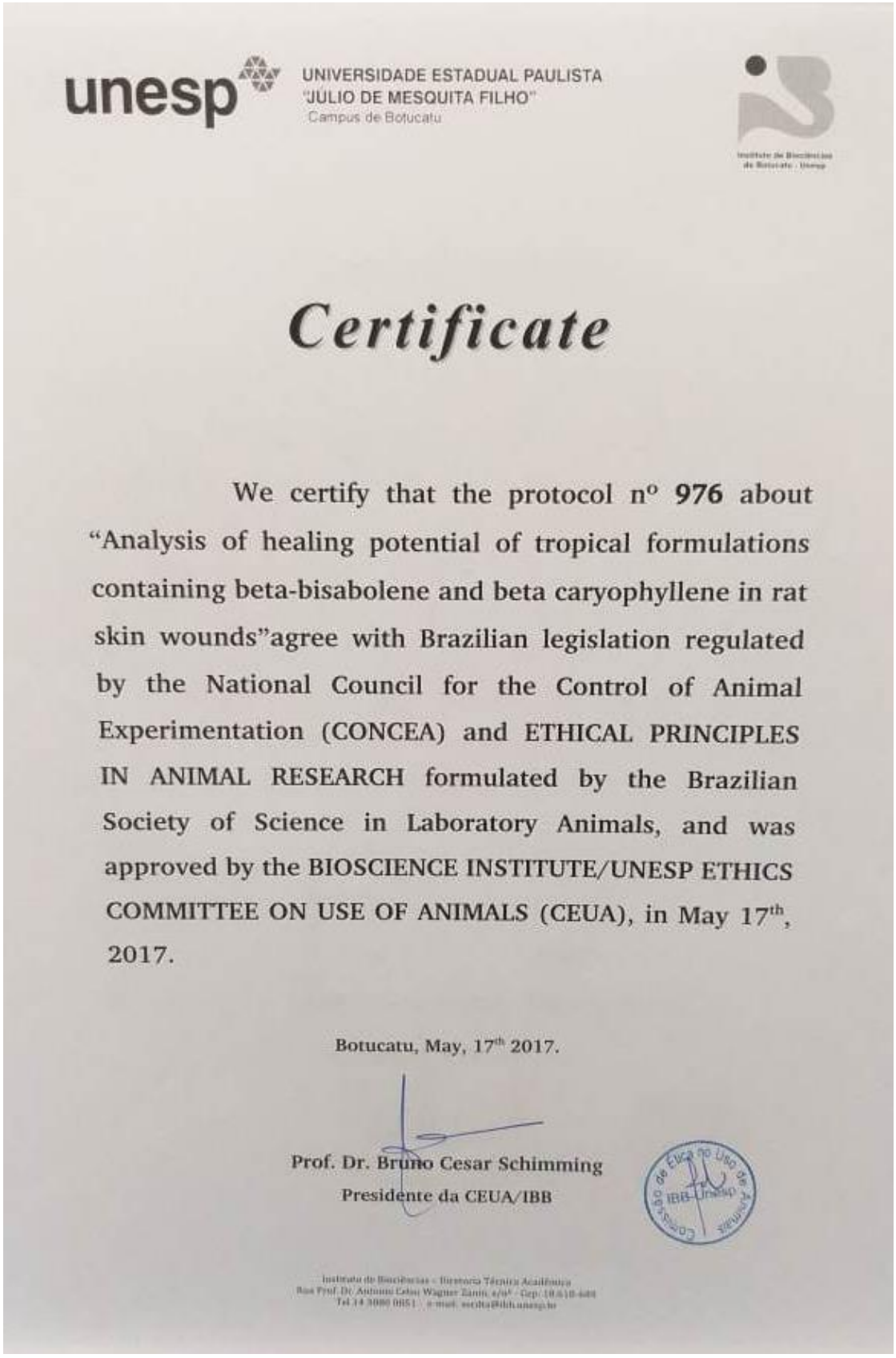


Figura 1. Mecanismos de ação dos fármacos Car e NLC em lesões cutâneas *in vivo*.

Anexo A - Certificado de aprovação do estudo pelo Comitê de Ética no Uso de Animais



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"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

Instituto de Biociências de Botucatu - Unesp

Certificate

We certify that the protocol nº **976** about "Analysis of healing potential of tropical formulations containing beta-bisabolene and beta caryophyllene in rat skin wounds"agree with Brazilian legislation regulated by the National Council for the Control of Animal Experimentation (CONCEA) and ETHICAL PRINCIPLES IN ANIMAL RESEARCH formulated by the Brazilian Society of Science in Laboratory Animals, and was approved by the BIOSCIENCE INSTITUTE/UNESP ETHICS COMMITTEE ON USE OF ANIMALS (CEUA), in May 17th, 2017.

Botucatu, May, 17th 2017.

Prof. Dr. Bruno Cesar Schimming
Presidente da CEUA/IBB

Comissão de Ética no Uso de Animais IBB-Unesp

Instituto de Biociências - Diretoria Técnica Acadêmica
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Tel 14 3880 0051 - e-mail: secdta@ibb.unesp.br