UNIVERSIDADE TIRADENTES

CURSO DE ODONTOLOGIA

# Filmes bioativos de colágeno contendo própolis verde e vermelha como substituto dérmico para queimaduras cutâneas: avaliação *in vivo*

Trabalho de Conclusão de Curso apresentado à Coordenação do Curso de Odontologia da Universidade Tiradentes como parte dos requisitos para obtenção do grau de bacharel em Odontologia.

Ingrid Correia Prado
Ricardo Luiz Cavalcanti de Albuquerque Junior.

ARACAJU/SE
JUNHO/2010

**Ingrid Correia Prado**

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Aracaju,_____/_____/_____

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Ricardo Luiz Cavalcante de Albuquerque Júnior – orientador
(presidente)
UNIT

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– 1ª examinador
UNIT

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– 2ª examinador
UNIT

Determinação, coragem e auto confiança são fatores decisivos para o sucesso.
Se estamos possuídos por uma inabalável determinação conseguiremos superá-los.
Independentemente das circustâncias,devemos ser sempre humildes,recatados e despidos de orgulho.

Dalai Lama

# Agradecimentos

Para cada passo que dou, seja ele certo ou errado, agradeça a Deus sempre. E como não é pra ser diferente agradeço a ele por mais uma vitória alcançada e mais uma fase de minha vida que foi concluída.

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ARTIGO CIENTÍFICO

# Collagen-based films containing Brazilian red and green propolis as wound dressing for dermal burn healing: *in vivo* evaluation

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Resumo

Alguns estudos têm sido bem sucedidos na incorporação de produtos naturais em filmes bioativos de colágeno, a fim de melhorar a cicatrização de feridas e queimaduras. Além disso, foi demonstrado que a própolis verde brasileira apresenta uma gama de atividades biológicas, tais como propriedades cicatrizantes, mas não há relatos sobre a variedade vermelha brasileira. O objetivo deste estudo foi avaliar as propriedades cicatrizantes de filmes bioativos de colágeno contendo própolis brasileira verde e vermelha em queimaduras em roedores. Dispersões filmogênicas de colágeno foram obtidas de tendão bovino utilizando polietilenoglicol como plastificante, contendo extratos hidroalcoólicos de própolis verde e vermelha. Posteriormente, queimaduras de 1cm² foram realizadas no dorso de 125 ratos Wistar, divididos em cinco grupos (n = 25) de acordo com o substituto dérmico: CTR (controle), COL (filme bioativo de colágeno), GPa e GPb (filme bioativo de colágeno contendo 0,5 e 1,0% de própolis verde, respectivamente) e RP (filme bioativo de colágeno contendo 0,5% de própolis vermelha). Os animais foram eutanasiádos após 3, 7, 14, 21 e 30 dias, e a reação inflamatória, colagenização e diferenciação de miofibroblastos foram avaliados histologicamente. Os dados foram comparados com o teste ANOVA e post-hoc de Tukey. (α=5%). Em 7 e 14 dias, GPa, GPb e RP apresentaram redução do número de neutrófilos e da intensidade da reação inflamatória, respectivamente, mas apenas RP apresentaram formação de tecido de granulação rico em fibroblastos maduros. A epitelização foi mais avançada em GPa, GPb e RP em 7, 14 e 21 dias ($p < 0,05$), e a média de miofibroblastos foi maior em RP em 14 e 21 dias ($p < 0,05$). GPa, GPb e RP mostraram uma substituição mais rápida do colágeno tipo III por tipo I em 14 dias, e melhorou a densidade da colagenização em 21 e 30 dias. No entanto, apenas em RP, os feixes de colágeno se mostraram grosseiramente entrelaçados semelhantes ao arranjo dérmico normal. Concluiu-se que a utilização de filmes bioativos de colágeno contendo as duas variedades de própolis brasileira melhoraram o processo cicatricial, e que a própolis vermelha, proporcionou melhores resultados do que o verde.

Palavras – chave: própolis, colágeno, queimadura.

Abstract

Some studies have been successful in incorporating natural products into collagen-based films in order to improve wound and burn healing. Furthermore, it has been demonstrated that the Brazilian green propolis presents a wide range of biological activities, such as healing properties, but there are no reports regarding the red variety. The purpose of this study was to evaluate the healing properties of collagen-based

films containing Brazilian green and red propolis on burn wounds in rodents. Collagen-based filmogenic dispersions were obtained using polyethyleneglycol as plasticizer, containing hydroalcoholic extracts of red or green propolis. Subsequently, burn wounds of 1cm$^2$ were performed in the dorsum of 125 *Wistar* rats, assigned into five groups (n=25) according to the dressing used: CTR (burn wounds), COL (collagen-based dressing), GPa and GPb (collagen-based films containing 0.5 and 1.0% of green propolis, respectively) and RP (collagen-based films containing 0.5% of red propolis). The animals were euthanized after 3, 7, 14, 21 and 30 days, and the inflammatory reaction, collagenization and myofibroblasts differentiation were histologically assessed. Data were compared by using ANOVA and Tukey post-hoc test (α=5%). In 7 and 14 days, GPa, GPb and RP showed decrease of the neutrophils content and the inflammatory severity, respectively, but only RP presented formation of mature fibroblast-rich granulation tissue. Epithelization was advanced in GPa, GPb and RP in 7, 14 and 21 days ($p<0.05$), and the mean of myofibroblasts was higher in RP in 14 and 21 days ($p<0.05$). GPa, GPb and RP showed more rapid substitution of type-III for type-I collagen in 14 days, and improved the collagenization density in 21 and 30 days. However, only in RP, the collagen bundles were grossly interlaced resembling the normal dermal arrangement. We concluded that the use of collagen-based films containing both varieties of Brazilian propolis improved burn healing, and that the red propolis provided better results than the green one.

Keywords: propolis, collagen, burn healing.

## Introduction

The skin works as a barrier to the environment, which is responsible for protecting the organism from water loss and penetration of harmful substances. If this barrier is disturbed by mechanical and thermal action, the skin starts a complex repair mechanism known as wound healing (Fu et al, 2007). The analysis of the kinetic of this biological process in response to different forms of dermal substitution is important for the development of efficient therapeutic products capable of stimulating the wound healing (Alborova et al, 2007)

Despite the fact that there have been many recent advances in dermal substitution and wound healing research areas of medicine, neither the commercially available products nor the materials currently described in experimental studies are able to fully substitute for natural living skin (Shakespeare, 2001). However, healing of dermal wounds with macromolecular agents such as natural polymers is preferred to skin substitutes owing to many advantages such as biocompatibility, nonirritant and

nontoxic properties, and ease and safety of the application on dermis (Sezer et al, 2007).

Collagen films have been employed to improve the cicatricial repair of mechanical and chemical damages (Gopinath et al, 2005 ; Gopinath et al, 2004). Furthermore, in addition to the excellent biocompatibility properties presented by these films, it has been demonstrated that collagen matrices stimulate biological phenomena involved in the success of wound healing, such as myofibroblastic differentiation and fibroblastic proliferation (Helary et al, 2006).

Some studies have been carried out in order to incorporate bioactive compounds (synthetic or natural) within implantable materials, such as type-I collagen films (Gopinath et al, 2005 ; Gopinath et al, 2004). These modified films seem to provide controlled liberation of the incorporated product directly within the damaged tissue, and promote acceleration of the granulation tissue development and epithelization process (Helary et al, 2006).

Hydroalcoholic solutions of propolis, a resinous product produced by bees, have been currently employed in improving the cicatricial repair (Ramos & MIranda, 2007). Biological activity of propolis might be related to its antimicrobial, anti-inflammatory and immunomudalatory properties (Castaldo & Capasso, 2002). Research relating propolis and wound healing has been carried out by using different vehicles, such as sponges (Moura et al, 2009) and ointments (Sehn et al, 2009), but there is still no study employing collagen-based dressing films. Besides, the majority of the papers are focused in the Brazilian green propolis, so that only some few studies pointing at the biological effects of the Brazilian red variety have been reported (Marquele et al 2005; Ayres et al, 2007).

Therefore, the aims of this study were to investigate the suitability of the collagen-based films containing hydroalcoholic extracts of two different varieties of Brazilian propolis (green and red) on the dermal burn healing in rodent model.

## Material and Methods

*Assessment of the flavonoids content.* Flavonoids in propolis hydroalcoholic extracts were expressed as quercetine equivalent. Quercetine (Sigma, Germany) was used to make the calibration curve (standard solutions of 6.25, 12.5, 25.0, 50.0, 80.0 and 100.0 $g.mL^{-1}$ in 80% ethanol *(V/V)*. 0.5 mL of a product (ethanolic solutions of propolis) was mixed with 1.5 mL 95% ethanol *(V/V)*, 0.1 mL 10% aluminum chloride

*(m/V)*, 0.1 mL of 1 mol.$L^{-1}$ potassium acetate and 2.8 mL water. A volume of 10% (*m/V*) aluminum chloride was substituted by the same volume of distilled water in blank. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm.

*Preparation of the films containing red propolis.* Collagen was obtained from bovine tendon through treatment with NaCl, acetic acid and pepsin (Cardoso, 2005). Hydroalcoholic extracts of red propolis was solubilized in polyethylene glycol 400, which was employed as cosolvent and plasticizer. This solution was mixed to 1% collagen dispersion in acetic acid (0.5 mol/L) and the films were obtained by casting process. The final propolis concentration in the film was 0.5 and 1.0%. After solvent evaporation, the films were cut off in square shape (2x2cm), and sterilized using UV rays (20 min).

*Burning Procedurse and Groups Formation***.** A hundred twenty-five male *Wistar* rats (250 ± 50 g), supplied with food and water *ad libitum* in a temperature and humidity-controlled environment, were anesthetized with intraperitoneal ketamine-xylazine (100mg/kg - 5mg/kg). Second-degree burn wounds were performed in the back of the animals by the contact of a heated 1$cm^2$ standard-sized square-shaped copper plate with the skin for 10 s. Animals were handled in accordance with the principles of aseptic chain in order to avoid bacterial contamination. Subsequently, rats were randomly assigned into five groups (n=25): according to the dressing used: CTR (burn wounds), COL (collagen-based dressing), GPa and GPb (collagen-based films containing 0.5 and 1.0% of green propolis, respectively) and RP (collagen-based films containing 0.5% of green propolis). After 3, 7, 14, 21 and 30 days five animals of each group were euthanized in $CO_2$ chamber and the burned areas were removed, formalin-fixed and paraffin-embedded according to routine laboratorial techniques. Serial 5 µm thick histological sections were obtained and stained by histochemical and immunohistochemical techniques.

*Assessment of the inflammatory profile (IP) and epithelization rates (ER)*. The IP was classified as acute (predominance of polymorphonuclear cells) and chronic (predominance of mononuclear cells), and graded as mild/absent (+), moderate (++) or severe (+++). The ER was evaluated by measuring the epidermal migration from the normal wound margin to the point where the migrating epithelium stopped processing by using a morphometry software (ImageTool®). ER (%) was determined by the relation between the new epithelium present in the burn wound surface and total area of the burn wound surface.

*Determination of the mean of myofibroblasts count (MC).* Myofibroblastss were

detected by using a monoclonal antibody against the α-smooth muscle actin antigen (clone 1A4; 1:200, 12 h, Dako, Glostrup, Denmark). After washing in PBS, slides were incubated with biotin-labeled antimouse secondary antibodies (Vector Laboratories Inc., Burlingame, CA), then washed in PBS, and incubated with peroxidase-labeled streptavidin (DAKO). The reaction products were visualized by immersing the slides in freshly prepared diaminobenzidine (Dojindo, Kumamoto, Japan). Ten histological sections (x 40, 10 ocular, 0.739 $mm^2$ per field) were randomly selected and the mean of immunostained cells was assessed.

*Assessement of the collagen deposition*. Histological sections stained in picrosirius and analyzed under polarized light were used to the descriptive analysis of the collagen deposition. Collagen fibers were analyzed according their birefringence pattern (greenish/yellow-greenish or orange, orange-reddish), morphological appearance (wavy or stretched, thin or thick, short or long) and disposition (parallel-arranged or interlaced).

*Statistical analysis.* Statistical significance of the quantitative measurements was assessed by analysis of variance (one-way ANOVA) and Tukey test. Each time point was analyzed separately, and two-tailed α-levels of $P < 0.05$ were regarded as significant.

*Ethics aspects.* In accordance to the institution's guidelines outlined in "Guide for the Care and Use of Laboratory Animals", it is hereby assured that all animals received humane care during all the steps of the experimentation. Furthermore, the study protocols were approved by our National Research Council prior to the beginning of the experiments.

# Parecer Consubstanciado de Projeto de Pesquisa

**Título do Projeto: ESTUDO HISTOMORFOLÓGICO DO EFEITO DE FILMES BIOATIVOS DE COLÁGENO CONTENDO PRÓPOLIS VERDE E VERMELHA SOBRE O PROCESSO DE REPARO CICATRICIAL POR SEGUNDA INTENSÃO DE FERIDAS CIRURGICAS EM MODELOS MURINO**

**Pesquisador Responsável Ricardo Luiz Cavalcanti de Albuquerque Júnior**

**Data da Versão 05/03/2008** | **Cadastro 030308** | **Data do Parecer 06/05/2008**

**Grupo e Área Temática III - Projeto fora das áreas temáticas especiais**

**Objetivos do Projeto**

**Geral**

- **Analisar o efeito do tratamento com extrato hidroalcoólico de própolis verde incorporado a filmes bioativos de colágeno I sobre processo de reparo cicatricial por feridas cirúrgicas.**

**Específicos**

- **Avaliar clinicamente o processo de reparo cicatricial de feridas tratadas com filmes bioativos de colágeno acrescido de extrato hidroalcoólico de própolis verde.**
- **Analisar morfologicamente a influência do tratamento com filmes bioativos de colágeno acrescido de extrato hidroalcoólico de própolis verde sobre a intensidade da reação inflamatória durante o processo de reparo cicatricial de feridas.**
- **Analisar morfologicamente a influência do tratamento com filmes bioativos de colágeno acrescido de extrato hidroalcoólico de própolis verde sobre a dinâmica de deposição colagênica durante o processo de reparo cicatricial de feridas.**

**Sumário do Projeto**

A própolis é uma substância resinosa balsâmica de consistência viscosa e cor variada, fabricada por abelhas (Apis mellifera L.), cujas propriedades terapêuticas dependem da origem botânica, localização geográfica e procedência. Extratos hidroalcóolicos da própolis têm sido utilizados em diferentes situações como agentes antimicrobianos, antiinflamatórios e aos poucos vem sendo discutido sobre o seu potencial como agente cicatrizante. Assim, o objetivo deste projeto consiste em realizar um estudo histomorfológico do efeito de extratos de própolis verde sobre o processo de reparo cicatricial de feridas, em modelo murino. Para tanto, extratos hidroalcóolicos de própolis verde serão incorporados a filmes bioativos, preparados a partir de colágeno I extraído de tendão bovino, e aplicados sobre feridas padronizadas previamente confeccionadas em dorso de ratos. Os animais serão sacrificados 3,7 e 14 dias após esses procedimentos e a área de cicatrização será examinada e analisada microscopicamente. Tenciona-se, com isso, obter informações primárias acerca de um provável potencial cicatrizante dessa variedade da própolis. Os achados morfológicos a serem observados nesta pesquisa teriam, portanto, enorme potencial de aplicabilidade clínica, seja nas mais diversas áreas da odontologia (estomatologia, endodontia, cirurgia bucomaxilofacial, etc), seja em áreas afins das ciências biomédicas. Não se deve deixar de destacar, outrossim, que os resultados desta pesquisa, se favoráveis e em consonância com o esperado, poderão culminar na identificação de um importante viés comercial para a própolis verde.

| Itens Metodológicos e Éticos | Situação |
|---|---|
| Título | Adequado |
| Autores | Adequados |
| Local de Origem na Instituição | Adequado |
| Projeto elaborado por patrocinador | Não |
| Aprovação no país de origem | Não necessita |
| Local de Realização | Própria instituição |
| Outras instituições envolvidas | Não |
| Condições para realização | Adequadas |



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Comentários sobre os itens de Identificação

| | |
|---|---|
| Introdução | Adequada |

Comentários sobre a Introdução

| | |
|---|---|
| Objetivos | Adequados |

Comentários sobre os Objetivos

Devem ser melhor caracterizados: objetivos geral e específicos

| | |
|---|---|
| Pacientes e Métodos | |
| Delineamento | Adequado |
| Tamanho de amostra | Total Local |
| Cálculo do tamanho da amostra | Não Informado |
| Participantes pertencentes a grupos especiais | Não |
| Seleção eqüitativa dos indivíduos participantes | Adequada |
| Critérios de inclusão e exclusão | Adequados |
| Relação risco- benefício | Adequada |
| Uso de placebo | Não utiliza |
| Período de suspensão de uso de drogas (wash out) | Não utiliza |
| Monitoramento da segurança e dados | Adequado |
| Avaliação dos dados | Adequada - quantitativa |
| Privacidade e confidencialidade | Adequada |
| Termo de Consentimento | Adequado |
| Adequação às Normas e Diretrizes | Sim |

Comentários sobre os itens de Pacientes e Métodos

Todos as etapas foram devidamente esclarecidas.

| | |
|---|---|
| Cronograma | Adequado |
| Data de início prevista | |
| Data de término prevista | |
| Orçamento | Adequado |
| Fonte de financiamento externa | Não |

Comentários sobre o Cronograma e o Orçamento

| | |
|---|---|
| Referências Bibliográficas | Adequadas |

Comentários sobre as Referências Bibliográficas

Recomendação

**Aprovar**

Comentários Gerais sobre o Projeto

**Projeto de grande relevância.**

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Results

The yield of the hydroalcoholic extraction process of the green propolis was 41.43%, whereas the red propolis was 48.8%. On the basis of calibration curves for standard flavonoids, the concentration of these bioactive compounds in hydroalcoholic extracts of both propolis samples was assessed. The content of flavonoids of the green propolis was 0.95±0.44%, whereas the red propolis presented 1.87±0.26%.

On clinical evaluation, only CTR exhibited peripheral hyperemic halo surround the burn wounds until 7 days. The adaptation of the dressings was fully satisfactory. Besides, all films were utterly resorpted in 14 days.

As presented in table 1, in 3 days, the inflammatory infiltrate was moderate and limited to the margins of the burn wounds in all groups. Despite all groups showed severe inflammation in 7 days, an extensive infiltration of neutrophils was found in CTR and COL, whereas granulation tissue was seen in GPa, GPb and RP, particularly in the bottom of the burn wounds. In 14 and 21 days, there was a remarkable decrease in the severity of the inflammation content in the groups dressed with collagen-based films containing propolis. In 30 days, inflammation was inconspicuous in all groups. However, in RP it was possible to observe the progressive regeneration of cutaneous appendages (figure 1).

Despite in 3 days the epithelization process had been inconspicuous in all groups, the ER was significantly higher in GPa, GPb and RP than in CTR and COL in 7 ($p=0.000$), 14 ($p=0.000$), 21 ($p=0.005$) and 30 days ($p=0.015$). However there was no difference within the groups dressed with propolis-containing films or within CTR and COL ($p>0.05$) (figure 2).

The MC increased progressively from 3 to 14 days, and then started to slowly decrease until 21 days (figure 3). No significant difference was observed among the groups in 3, 7 and 30 days. Notwithstanding, the MC was significantly higher in RP in 14 ($p=0.00$) and 21 days ($p=0.04$).

In 3 days, all groups exhibited deposition of short thin delicate collagen fibrils, with greenish birefringence (type-III collagen) and reticular arrangement. The same pattern of collagenization was observed in CTR and COL in 7 days, whereas in GPa and GPb some few thicker yellowish and orange fibers (type-I collagen) were found. Besides, in RP, these Type-I collagen were abundant and parallel-arranged. In 14 days, there was almost full replacement of type-III for type-I collagen, but the arrangement of the fibers was still reticular in CTR, parallel in COL, GPa and GPb, and slightly interlaced in RP. In 21 days, the collagen deposition was quite dense in all groups, but the arrangement was parallel in CTR and COL and interlaced in the other groups. In 30

days, all groups exhibited dense deposition of thick gross interlaced type-I collagen bundles associated to some few thinner type-III collagen fibris in the papillary dermis. Furthermore, the density and highly interlaced appearance of the repaired dermal collagen observed in RP strongly resembled the histological features seen in the normal dermis of rats (figure 4).

## Discussion

The extraction yield was 41.43% for the green propolis and 48.8% for the red propolis, both values higher than the mimimum one (35%) specified in the Brazilian legislation (Brasil, 2001).

The concentration of flavonoids was assessed in both propolis samples collected from ten different localities in Brazil. The fact that the red propolis (collected from Sergipe - in Brazilian northeast) presented higher contents of flavonoids than the green propolis (collected from Minas Gerais - Brazilian southeast) suggests that the phytogeographic position of the hives might influence the physicochemical characteristics of propolis. However, the content of flavonoids of both samples were regarded as satisfactory, although they were lower than the values reported in previous studies (Volp et al, 2008). Flavonoids, one of the main groups of phenolic compounds in propolis, are the key compounds for estimation of propolis quality, and the content of flavonoids has been implied in the bio.ogical activities of propolis. The biochemical effects of flavonoids can be divided into four categories: (1) binding affinity to biological polymers; binding of heavy metal ions; catalysis of electron transport; and ability scavenge free radicals (Coneac et al, 2008). The ability to inhibit the incorporation of thymidine, uridine, and leucine into carcinoma cells, and block the DNA synthesis, presented by some flavonoids provides some antitumoral effects to the propolis. Besides, the flavonoids pinocembrin, galangin and pinobanksin, in addition to p-coumaric acid benzyl ester and caffeic acid phenethyl ester (CAPE), might probably be responsible for the antimicrobial activity of propolis (Havsteen, 2002).

The decrease of the inflammatory infiltrate observed in the groups treated with propolis-containing collagen-based films might be related to the wide range of immunomodulatory effects of the propolis, such as inhibition of prostaglandin synthesis and stimulation of phagocitary activity of macrophages (Russo et al, 2002; Orsolić & Basić, 2003; Ramos & Miranda, 2007). Therefore, the incorporation of propolis hydroalcoholic extracts into the films might provide reduction of prostaglandin-induced chemotactic stimuli and more rapid phagocytosis-associated microbial elimination,

which could consequently improve the healing process. Furthermore, the early development of granulation tissue suggests that the biological events that characterize the healing dynamics are taking place more rapidly in the groups treated with propolis-containing collagen-based films. The reason for the maintenance of intense inflammation in the group treated only with collagen-nased films lays on the fact that, although collagen presents plenty of biomodulatory effects, these molecules do not exhibit anti-inflammatory activity (Srivastava et al, 1990).

The epithelization process is important for wound healing, ensuring that the new formed epithelium binds to dermis and sticks to tissue, as stated in previous reports (Erdağ G Sheridan, 2004). In this study, the epithelization rates were highly improved by using propolis-containing collagen-based films. These data are supported by previous studies demonstrating that type-I collagen molecules may work as a matrix which orientates the migration of keratinocytes, add to endothelial cells, fibroblasts and leukocytes (Morimoto et al, 2005). In addition, caffeic acid esters and similar molecules present in propolis have been proved to stimulate proliferation of dermal keratinocytes (Brudzynski & Carlone, 2004). Recent studies also demonstrated that a wide variety of Brazilian propolis is able to induce macrophage activation under stress conditions (Missima & Sforcin, 2008). This immunomodulatory property could be indirectly related to the epithelization process, since activated macrophages release expressive amount of FGF (Logan et al, 1992), a cytokine involved in epithelial proliferation process (Werner & Grose , 2003). Furthermore studies have demonstrated intimate relation between epithelization and fibroplasia (fibroblastic proliferation and collagen deposition) (McDougall et al, 2006), demonstrating the relevance of the epithelial recovering for the success of the burn healing.

As also presented in the figure 1 (immunohistochemical stain) RP exhibited more evident myofibroblastic differentiation in 14 and 21 days. Myofibroblasts are fibroblasts rich in actin that present contratile properties and promote wound contraction (Ribeiro et al, 2009). Therefore, it is suggested that the use of collagen-based films containing hydroalcoholic extracts of red própolis could contribute to the formation of a more retracted final collagenic scar, and maybe minimize the risk of the development of hypertrophic scars or keloyds.

The dynamics of collagenization is extremely relevant to assess the success of the healing process (Ribeiro et al, 2009). It has been demonstrated that type-III collagen is initially produced by fibroblasts in order to orientate the proliferation and migration of fibroblasts and endothelial cells during the formation of the granulation

tissue (Ramos & Miranda, 2007; Fleischmajer et al, 1990 ; de Giacomo et al, 2005 ), and then it is gradually substituted for type-I collagen to provide tensile force and mechanical stability of the dermal fibrous connective fibrous tissue (Friedman et al, 1993). Based on this, the biological phenomena of collagen synthesis, deposition and maturation during wound healing seemed to be improved in GPa GPb and RP. Besides, the biological property of proliferative endothelial orientation during the granulation tissue formation can explain the fact that these type-III collagen was more abundant in CTR and COL in 7 days, when the granulation tissue was still in progress, and less distinguishable in propolis-containing collagen-based films dressed groups, where the well-developed granulation tissue had already installed, and endothelial growth orientation was no longer required.

Oppositely, type-I collagen deposition was progressing, once it is responsible for the tensile strength of the scar (Junqueira et al, 1983). In 14 days, all the groups presented absolute predominance of birefringent fibers consistent with type-I collagen. These data confirm *in vivo* findings on the healing phase of burn healing documented in previous studies (Provenzano et al, 2005). However, the collagenization was more advanced in the groups dressed with propolis-containing collagen-based films. These findings might be related to the stimulatory activity of some varieties of Brazilian propolis upon FGF synthesis, a cytokine involved in the dynamics of fibroblasts proliferation and collagen synthesis and deposition (Narine et al, 2006), but other studies are necessary to clarify this theory. Furthermore, the interlaced orientation of the collagen bundles found in RP since the 14$^{th}$ day is strongly suggestive that the collagenization was more advanced and better organized in this group, as long as this particular arrangement appears to mimic the architecture of the normal dermis (Junqueira et al, 1983; Ostrovskiĭ et al, 1992).

No substantial difference was observed between GPa and GPb, suggesting that the difference in the concentration of the hydroalcoholic extract of green propolis incorporated into the collagen-based films did not modify the dynamics of the dermal cicatricial repair. However, the development of cutaneous appendages within the cicatricial area in RP, higher myofibroblastic differentiation and dense organization of the interlaced collagen scar, are indicative that the healing process, and consequently full recovery of the morphofunctional properties of the injured dermis, was more advanced in this group. The very reason for these results is still hardly justifiable, but it might be related to the content of flavonoids found in the red propolis extracts. Studies have suggested that the healing properties of propolis are the anti-inflammatory and antioxidant potential provided by the flavonoids (Marquele et al, 2004). Since the

content of flavonoid found in the red propolis extracts was higher than that observed in the green ones, the better results obtained with RP might be related to the concentration of these bioactive compounds.

Some steps of the healing process, such as collagenization and epithelization, were clearly more advanced in COL than in CTR, despite these differences had not been statistically significant. These findings appear to be related to mechanical protection of the wounds provided by the dressing films, which consequently would reduce bacterial contamination, and facilitate the development of the granulation tissue (Diegelmann & Evans, 2004).

Based on the findings reported in this study, it is suggested that collagen films containing Brazilian propolis was efficient in improving wound healing process when employed as wound dressing for dermal burn healing.

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References

Alborova A, Lademann J, Meye L,, Kramer A, Patzelt A, Sterry W, Antoniou C. Application of laser scanning microscopy for the characterization of wound healing. GMS Krankenhaushygiene Interdisziplinär. 2007;2(2):Doc37

Ayres D.C., Marcucci M. C., Giorgio S. Effects of Brazilian propolis on Leishmania amazonensis. Mem. Inst. Oswaldo Cruz . 2007 Mar;102(2):215-220.

Brasil 2001. Ministério da Agricultura. Instrução Normativa nº3 – Anexo VI – Regulamento técnico para fixação de identidade e qualidade de própolis. Diário Oficial da República Federativa do Brasil. Brasília, 19 Jan. 2001.

Brudzynski K, Carlone R. Stage-dependent modulation of limb regeneration by caffeic acid phenethyl ester (CAPE)--immunocytochemical evidence of a CAPE-evoked delay in mesenchyme formation and limb regeneration. J Exp Zoolog A Comp Exp Biol. 2004 May 1;301(5):389-400.

Cardoso JC. Modificação do colágeno pela reação de Maillard e avaliação de formulações na forma gel desenvolvidas a partir do material modificado. 2005. 152p. Tese de Doutorado, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Ribeirão

Preto, 2005.

Castaldo S, Capasso F. Propolis, an old remedy used in modern medicine. Fitoterapia. 2002 Nov;73(suppl 1): S1-S6.

Chang R, Piló-Veloso D, Morais SAL, Nascimento EA. Analysis of a Brazilian green propolis from Baccharis dracunculifolia by HPLC-APCI-MS and GC-MS. Rev. bras. farmacogn. 2008; 18(4): 549-556.

Coneac G, Gafiţanu E, Hădărugă DL, HădărugăNG, Pînzaru LA, Bandur G, Urşica L, Păunescu V, Gruia A. Flavonoid Contents of Propolis from the West Side of Romania and Correlation with the Antioxidant Activity. 53(67), 1-2, 2008.

Dantas Filho AM, Aguiar JL, Rocha LR, Azevedo IM, Ramalho E, Medeiros AC. Effects of the basic fibroblast growth factor and its anti-factor in the healing and collagen maturation of infected skin wound. Acta Cir Bras. 2007 Mar-Apr;22 Suppl 1:64-71.

de Giacomo C.C., Sennes LU, Saldiva PH, Tsuji DH, Ximenes Filho JA. Assessment of collagen deposits after implant of fascia lata and fat in the vocal folds of rabbits: histomorphometric study. Rev Bras Otorrinolaringol (Engl Ed). 2005 Nov-Dec;71(6):798-802.

Diegelmann RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. Front Biosci. 2004 Jan 1;9:283-9.

Erdağ G, Sheridan RL. Fibroblasts improve performance of cultured composite skin substitutes on athymic mice. 2004 Jun;30(4):322-8.

Fleischmajer R, Perlish JS, Burgeson RE, Shaikh-Bahai F, Timpl R. Type I and type III collagen interactions during fibrillogenesis. Ann N Y Acad Sci. 1990; 580:161-75.

Friedman DW, Boyd CD, Mackenzie JW, Norton P, Olson RM, Deak SB. Regulation of collagen gene expression in keloids and hypertrophic scars. J Surg Res. 1993 Aug;55(2):214-22.

Fu X, Fang L, Li H, Li X, Cheng B, Sheng Z. Adipose tissue extract enhances skin wound healing. Wound Repair Regen. 2007;15(4):540-8.

Gopinath D, Ahmed MR, Gomathi K, Chitra K, Sehgal PK, Jayakumar R...Dermal wound healing processes with curcumin incorporated collagen films. Biomaterials. 2004 May;25(10):1911-7.

Gopinath D, Kumar MS, Selvaraj D, Jayakumar R. Pexiganan-incorporated collagen matrices for infected wound-healing processes in rat. J Biomed Mater Res A. 2005 Jun 1;73(3):320-31.

Havsteen B.H., The Biochemistry and Medical Significance of the Flavonoids, Pharmacology & Therapeutics 2002, 96, 67– 202

Helary C, Ovtracht L, Coulomb B, Godeau G, Giraud-Guille MM. Dense fibrillar collagen matrices: a model to study of myofibroblast behaviour during wound healing. Biomaterials. 2006 Sep;27(25):4443-52.

Junqueira LC, Montes GS, Martins JE, Joazeiro PP Dermal collagen distribution. A histochemical and ultrastructural study. Histochemistry. 1983;79(3):397-403.

Logan A, Frautschy SA, Gonzalez AM, Baird A. A time course for the focal elevation of synthesis of basic fibroblast growth factor and one of its high-affinity receptors (flg) following a localized cortical brain injury. J Neurosci. 1992 Oct;12(10):3828-37.

Marquele FD, Di Mambro VM, Georgetti SR, Casagrande R, Valim YM, Fonseca MJ. Assessment of the antioxidant activities of Brazilian extracts of propolis alone and in topical pharmaceutical formulations. J. Pharm. Biomed. Anal. 2005 Sep;39(3/4):455-62.

McDougall S, Dallon J, Sherratt J, Maini P. Fibroblast migration and collagen deposition during dermal wound healing: mathematical modelling and clinical implications. Philos Transact A Math Phys Eng Sci. 2006 Jun 15;364(1843):1385-405.

Missima F, Sforcin JM. Green brazilian propolis action on macrophages and lymphoid organs of chronically stressed mice. Evid Based Complement Alternat Med. 2008 Mar;5(1):71-5.

Morimoto N, Saso Y, Tomihata K, Taira T, Takahashi Y, Ohta M, Suzuki S. Viability and function of autologous and allogeneic fibroblasts seeded in dermal substitutes after implantation. J Surg Res. 2005 May 1;125(1):56-67.

Moura SAL, Negri G, Salatino A, Lima LDC, Dourado LP, Mendes, Andrade SP, et al. Aqueous extract of Brazilian green propolis: primary components, evaluation of inflammation and wound healing by using subcutaneous implanted sponges.eCAM 2009 Aug 18; 10.1093/ecam/nep112:1-9.

Narine K, De Wever O, Van Valckenborgh D, Francois K, Bracke M, DeSmet S, Mareel M, Van Nooten G. Growth factor modulation of fibroblast proliferation, differentiation, and invasion: implications for tissue valve engineering. Tissue Eng. 2006

Oct;12(10):2707-16.

Orsolić N, Basić I. Immunomodulation by water-soluble derivative of propolis: a factor of antitumor reactivity. J Ethnopharmacol. 2003 Feb;84(2-3):265-73.

Ostrovskiĭ AA, Levé OI, Shatrova VO. The development of the interfollicular epidermis in rats following autotransplantation. Morfologiia. 1992 Jun;102(6):105-12.

Park YK, Ikegari M, Abreu JAS, Alcici NMF. Estudo da preparação dos extratos de própolis e suas aplicações. Ciênc. Tecnol. Aliment.. 1998 Aug;18(3):313-318.

Provenzano PP, Alejandro-Osorio AL, Valhmu WB, Jensen KT, Vanderby R. Intrinsic fibroblast-mediated remodeling of damaged collagenous matrices in vivo. Matrix Biol. 2005;23:543Y555.

Ramos AFN, Miranda JL. Propolis: a review of its anti-inflammatory and healing actions. J. Venom. Anim. Toxins incl. Trop. Dis. 2007 Nov;13 (4):698-710.

Ribeiro MA, Albuquerque RL, Ramalho LM, Pinheiro AL, Bonjardim LR, Da Cunha SS. Immunohistochemical Assessment of Myofibroblasts and Lymphoid Cells During Wound Healing in Rats Subjected to Laser Photobiomodulation at 660 nm. .Photomed Laser Surg.27(1):49-55.2009

Russo A, Longo R, Vanella A. Antioxidant activity of propolis: role of caffeic acid phenethyl ester and galangin. Fitoterapia. 2002 Nov;73 Suppl 1:S21-9.

Sehn E., Hernandes L., Franco S.L., Gonçalves C.C.M., Baesso M.L., Dynamics of reepithelialisation and penetration rate of a bee propolis formulation during cutaneous wounds healing. Analytica Chimica Acta 2009 (635): 10.1016/j.aca.2009.01.019: 115–120.

Sezer AD, Hatipoğlu F, Cevher E, Oğurtan Z, Baş AL, Akbuğa J. Chitosan film containing fucoidan as a wound dressing for dermal burn healing: preparation and in vitro/in vivo evaluation. AAPS PharmSciTech. 2007 May 24;8(2):Article 39.

Shakespeare P. Burn wound healing and skin substitutes. Burns. 2001;27:517Y522.

Silva BB, Rosalen PL, Cury JA, Ikegaki M, Souza VC, Esteves A, Alencar SM. Chemical Composition and Botanical Origin of Red Propolis, a New Type of Brazilian Propolis. Evid Based Complement Alternat Med. 2008 Sep;5(3):313-316.

Srivastava S, Gorham SD, French DA, Shivas AA, Courtney JM. In vivo evaluation and comparison of collagen, acetylated collagen and collagen/glycosaminoglycan

composite films and sponges as candidate biomaterials. Biomaterials. 1990 Apr;11(3):155-61.

Volp ACR,Renhe IRT, Barra K, Stringueta PC. Flavonoids anthocyanins: characteristics and properties in nutrition and health.Rev Bras Nutr Clin 2008;23(2):141-9.

Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiol Rev. 2003 Jul;83(3):835-70.

Table 1. Semiquantitative assessment of the inflammatory infiltrate within the burn wounds in the experimental groups.

| Time (days) | Animals | Experimental groups | | | | |
|---|---|---|---|---|---|---|
| | | CTR | COL | GPa | GPb | RP |
| 3* | R1 | 2a | 2a | 1a | 2a | 2a |
| | R2 | 1a | 2a | 2 a | 1a | 2a |
| | R3 | 1a | 1a | 1a | 1a | 1a |
| | R4 | 1a | 1a | 1a | 2a | 2a |
| | R5 | 2a | 1a | 2a | 1a | 1a |
| 7 | R1 | 3a | 3sa | 3sa | 3c | 3c |
| | R2 | 3a | 3sa | 3sa | 3c | 3c |
| | R3 | 3a | 3a | 3c | 3c | 3sa |
| | R4 | 3a | 3sa | 3c | 3sa | 3c |
| | R5 | 3a | 3a | 3c | 3c | 3c |
| 14 | R1 | 3c | 3c | 3c | 2c | 2c |
| | R2 | 3sa | 3c | 2c | 2c | 2c |
| | R3 | 3c | 2c | 2c | 2c | 2c |
| | R4 | 3c | 3c | 2c | 2c | 2c |
| | R5 | 3sa | 3c | 3c | 2c | 2c |
| 21 | R1 | 2c | 2c | 1c | 2c | 1c |
| | R2 | 2c | 2c | 2c | 1c | 1c |
| | R3 | 2c | 2c | 1c | 1c | 2c |

| | | | | | | |
|---|---|---|---|---|---|---|
| | R4 | 2c | 2c | 1c | 2c | 1c |
| | R5 | 2c | 2c | 1c | 2c | 1c |
| | R1 | 1c | 1c | 1c | 0 | 0 |
| | R2 | 1c | 1c | 0 | 1c | 1c |
| 30 | R3 | 1c | 1c | 1c | 0 | 0 |
| | R4 | 1c | 1c | 0 | 1c | 0 |
| | R5 | 1c | 1c | 0 | 1c | 0 |

**[*]** –infiltrate limited to the matgins of the burn wounds; **[3]** – Intense infiltrate (more than 50% of inflammatory cells); **[2]** moderate infiltrate (between 10 and 50% of inflammatory cells); **[1]** – mild infiltrate (less than 10% of inflammatory cells); **[0]** – lack of infiltrate/ **[a]** – acute infiltrate (neutrophils-rich); **[sa]** – subacute infiltrate (composed of plymorphonuclear and mononuclear inflammatory cells); **[c]** – chronic infiltrate (predominantly rich in lymphocytes/plasma cells).

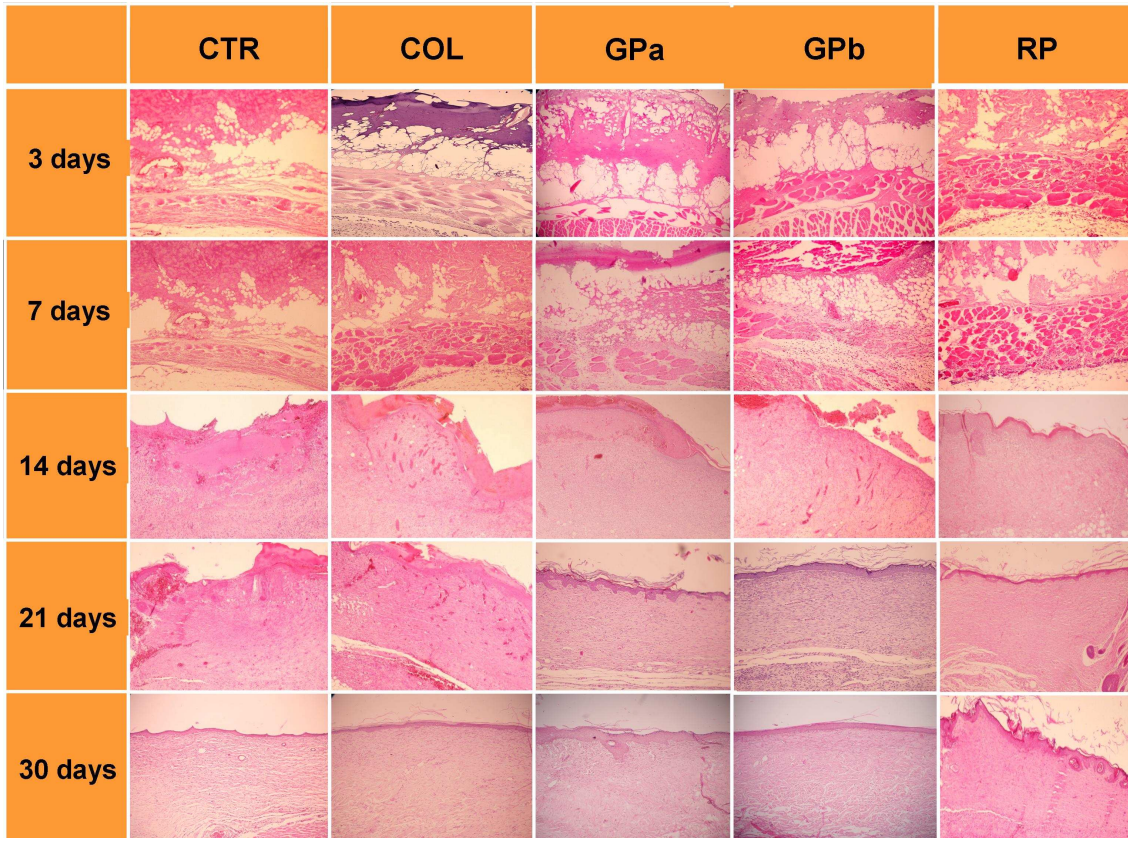


Figure 1. Inflammatory dynamics in the experimental groups. Acute infiltrate was mild in three days but expressive in seven. Chronic infiltrate was associated to immature (blood capillaries-rich) granulation tissue in CTR and COL, in opposition to the mature (fibroblasts-rich) granulation tissue seen in GPa, GPb and RP in 14 days. In 21 days, there was still a mature granulation tissue in CTR and COL, whereas GPa, GPb and RP presented early fibrous scar

with delicate appearance. In 30 days, a gross well-formed fibrous scar was seen in GPa, GPb and RP, but only in the latter there was the development of cutaneous appendages in the center of the scar (HE, 100x).

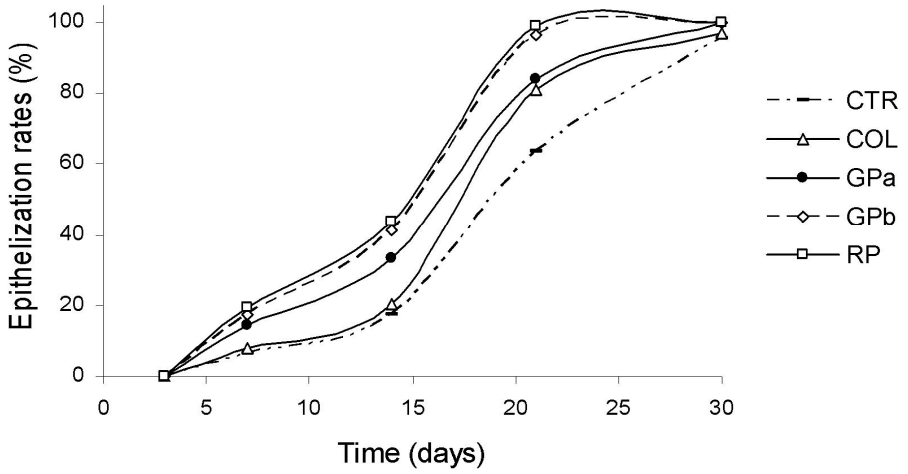


Figura 2. Percentual epithelization rates of the burn wounds along the time. Observe ethat GPa, GPb and RP exhibited higher rates than CTR and COL.

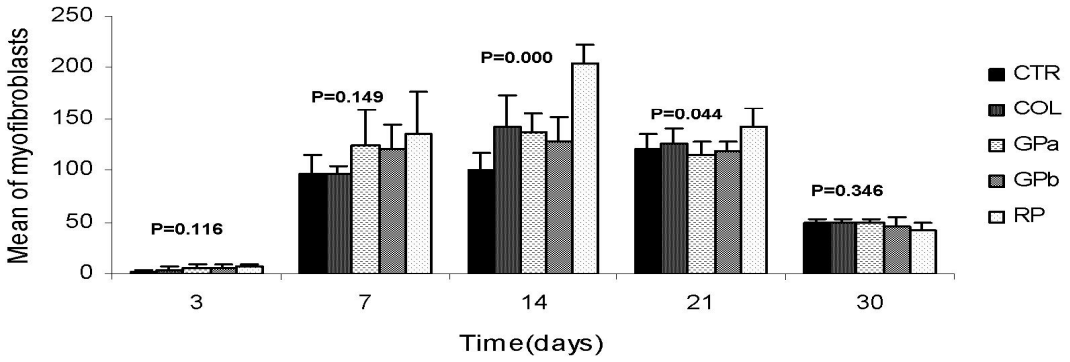


Figure 3. Quantitative assessment of the mean of myofibroblasts count in the experimental groups along the time.

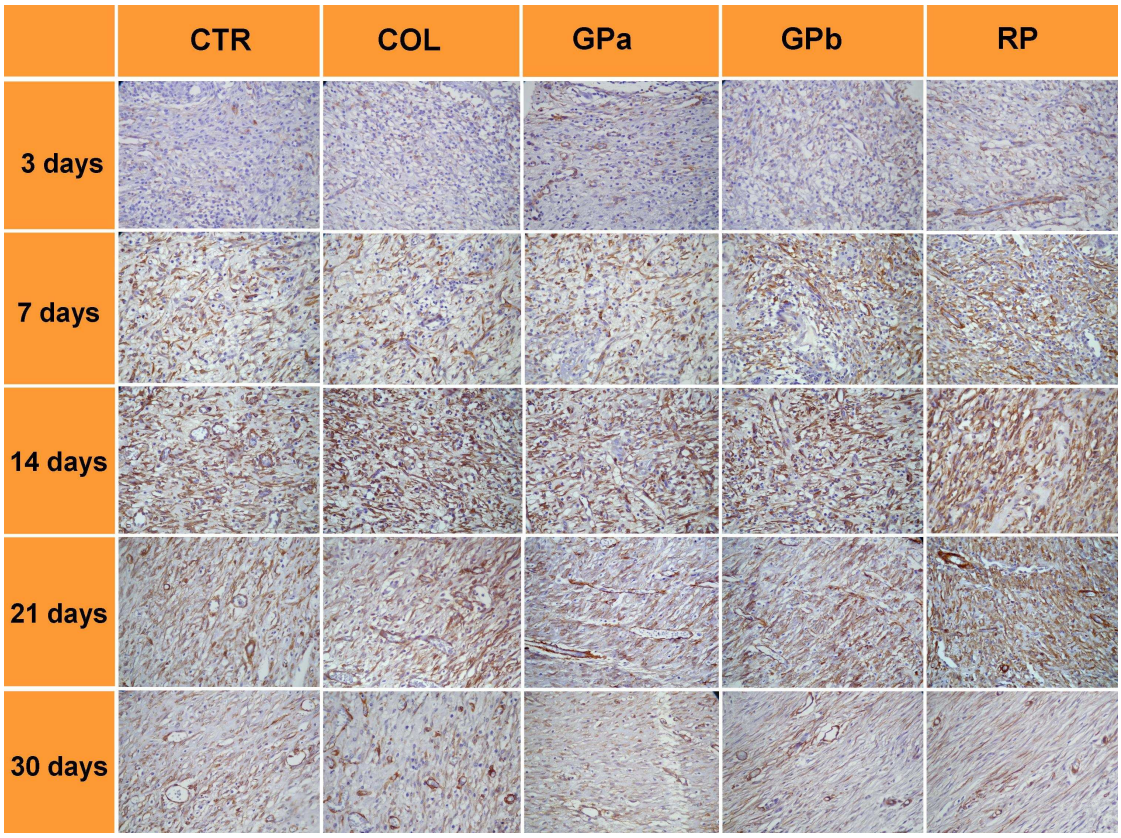


Figure 4. Dynamics of myofiblastic differentiation in burn healing. Whereas a scanty immunomarking was found in the third day in all groups, a remarkable increase in the myofibroblasts differentiation was observed In 7 and 14 days. Observe that the labeled cells were diffusely arranged within the burn wounds. In 21 days, there was a strong tendency to a parallel arrangement of the myofifibroblasts. In 30 days, a clear decrease in the count of immunostained cells was verified in all groups (Clone 1A4, LSAB, 200x).

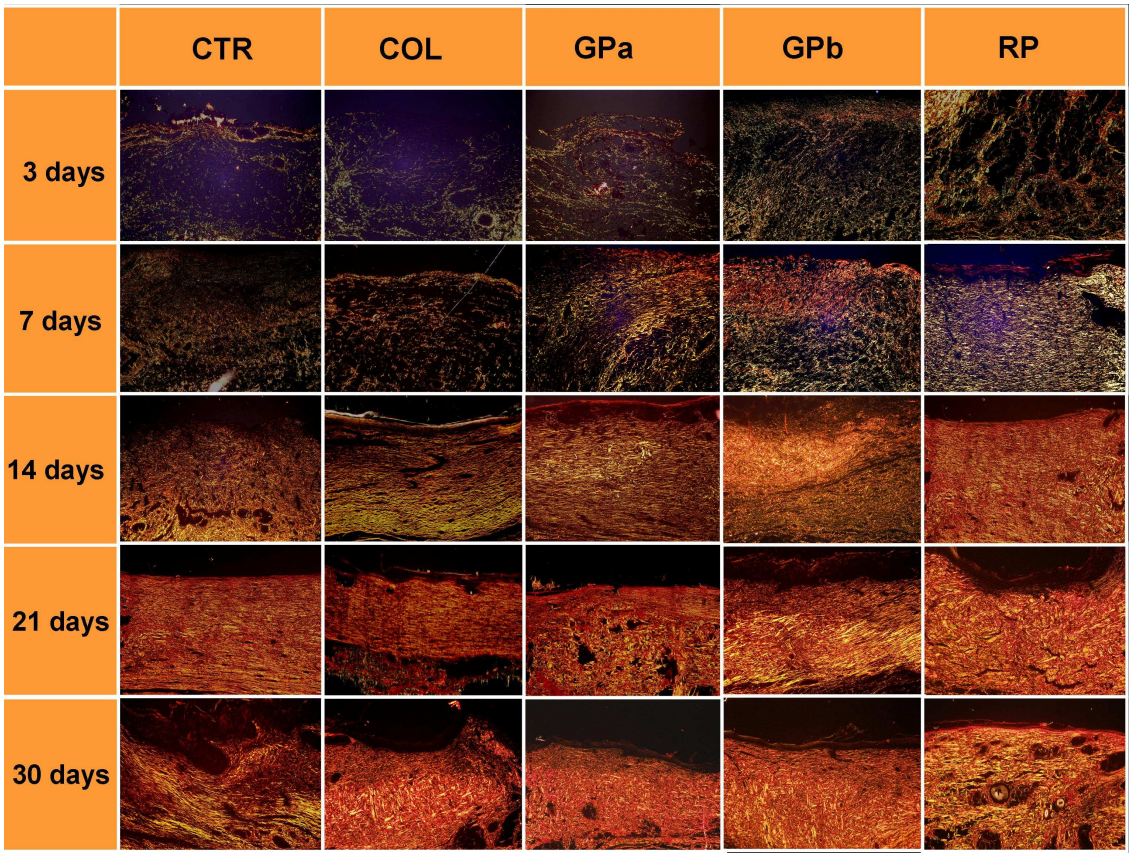


Figure 5. Dynamics of collagen deposition in the burn wounds. Observe the gradative replacement of the reticularly arranged type-III collagen (greenish) for the type-I collagen (orange), initially in a parallel arrangement and subsequently interlacedly disposed, along the time. Note the architectural appearance of the collagenization pattern resembling the normal dermis in RP in 30 days (Sirius red/polarized light, 100x).