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Short communication

Combined therapy using low level laser and chitosan-policaju hydrogel for wound healing

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ABSTRACT

We have evaluated the effect of POLI-CHI hydrogel based on policaju (POLI) from cashew tree (*Anacardium occidentale* L.) gum and chitosan (CHI), associated or not with Low level laser therapy (LLLT), in wound healing. Sixty male Wistar rats were assigned into four groups: POLI-CHI hydrogel (H); LLLT (L); POLI-CHI with LLLT (HL) and saline control (C). Macroscopic evaluations were carried out using clinical observations and area measurements, as well as microscopic analysis by histological criteria. H and HL presented more esthetical scar tissue and larger wound contraction compared to C. Histopathological analyzes showed: stronger presence of fibrin-leukocyte crust in L and HL at day 3; stronger collagen presence in H, L and HL; weak presence of focal necrosis at 7 and 14 days in H; weak neutrophilic exudate in H, L and HL; regression of the vascular neoformation at 7 days in H, and modulation of the same in L and HL. These results demonstrated that POLI-CHI contributed to more efficient healing process and modulation of the inflammation; furthermore, the combined use with LLLT subtle potentiated this process.

1. Introduction

Skin wounds heal in four stages: hemostatic, inflammatory, proliferative and remodelative [1]. Polysaccharides have been used in wound healing. Policaju obtained from cashew tree (*Anacardium occidentale* L.) gum has showed potential application in wound healing [2]. Chitosan, a polysaccharide derived from the chitin by de-acetylation, also has presented biocompatibility, biodegradability, low toxicity, hemostatic, healing properties, and antimicrobial activity [3,4]. Both polysaccharides can form hydrogels or crystals in solutions [5].

Hydrogels are three-dimensional polymer nets capable of absorbing large amounts of water or biological fluid, being used to preserve cells, nutrients, drugs or proteins, and also represent a drug delivery system class [6]. Due to its physicochemical similarities with the extracellular matrix the hydrogels are generally biocompatible [7]. The combination of policaju (POLI) and chitosan (CHI) termed POLI-CHI has been previously characterized by our lab [8]. Evaluating hydrogels using different proportions the most attractive was that composed of POLI:CHI, 1:4. The FT-IR analyses confirmed the existence of physical interactions between the polysaccharides involved and rheological measurements showed an increase in complex viscosity with the increase of chitosan content.

Low level laser therapy (LLLT) acts causing several biological effects, such as: increasing proliferation and activation of lymphocytes, increasing the phagocytosis on macrophages; and the secretion of growth factors in fibroblasts, enhancing the uptake of fibrin and collagen through emission of radiation by stimulating the most external electric field [9].

The combination of laser therapy and sodium alginate/chitosan-based hydrogel film improved burn healing, apparently by modulating the epithelisation, blood vessels formation and collagenization processes [10].

The aim of this study was to evaluate the healing of skin wounds induced in Wistar rats treated with the POLI-CHI hydrogel combined or not with LLLT.

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2. Materials and methods

2.1. Materials

Polysaccharide from cashew tree (*Anacardium occidentale* L.) gum (POLI), collected from the south coast of Pernambuco, Brazil, was obtained according to Souza [11]. The chitosan (CHI) (deacetylation > 75%) was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Hydrogel preparation

The POLI-CHI hydrogel was made of policaju (POLI) and chitosan (CHI) in a ratio of 1:4 according to Soares [8]. Briefly, 50 mL of stock solutions of 10% (w/v) of policaju and 1% (w/v) of chitosan in 1% lactic acid (v/v) were prepared in advance. In a separated beaker, 15 mL of chitosan solution plus 200 μL of 0.1 M $CaCl_2$ was added and kept under stirring in Ultra-Turrax (IKA, USA) at 7000 rpm for 20 min. Then, using a 27 G syringe and a flow of 1 mL/min, was added 5 mL of policaju solution. The mixture was left under stirring (7000 rpm) for 20 min. The pH was adjusted to 5.0 with 1 M NaOH solution and called pre-gel. The pre-gel solution was distributed in petri dishes and kept in an oven at 40 °C for 16 h for drying and polymerization. The thin film obtained was hydrated with distilled water and termed POLI-CHI hydrogel, which was stored under refrigeration at 4 °C.

2.3. Animals and treatment groups

Sixty male Wistar rats (*Rattus norvegicus*), 90-120 day-old, weighing 250–300 g were anesthetized intraperitoneally with 2% (w/v) of xylazine hydrochloride and 10% (w/v) ketamine hydrochloride at 1:1 ratio. A circular surgical wound (Ø = 0.8 cm) were made in the skin of the dorsal region of each animal using a biopsy punch and a scalpel blade No. 15. After surgery the animals were randomly divided into four groups, according to treatment (n = 15): (H) 0.1 mL of POLI-CHI hydrogel; (L) LLLT; (HL) 0.1 mL of POLI-CHI hydrogel plus LLLT and (C) 0.1 mL of 0.9% (w/v) NaCl as Control. The irradiation was carried out in a punctually way starting from the center of the wound at 2 mm from the skin using Therapy XT (DMC medical, USA). The parameters used were: λ = 660 nm, A = 1 cm2, ED = 4 J/cm2, P = 100 mW, F = 50 Hz. This treatment was carried out after surgery and at a 48 h interval until the euthanasia time. All animal procedures were in accordance with the Colégio Brasileiro de Experimentação Animal (COBEA) and the Animal Ethical Committee/UFPE No. 23076.050933/2012–10.

2.4. Macroscopic evaluation

The specimens were clinical daily evaluated according to the presence of the following criteria: edema, hyperemia, presence of exudate, crust, detachment and epithelialization. Images were generate using a photographic camera (Alpha 3000 K/B – SONY) and a tripod (Viv-Tr75 – Vivitar). The wound area image was processed using the ImageJ software (version 1.45) and the area (pixels) was applied in the contraction of the wound formula: [(initial area – area on the day of measurement)/initial area] × 100 = percentage of contraction on the day of measurement [12].

2.5. Euthanasia and histological processing

Five animals from each group were sacrificed after 3, 7 and 14 days following the surgical procedure using lethal doses of sodium thiopental (200 mg kg^{-1}). Skin fragments were collected with a wide margin (± 1 cm) from the original lesion and storaged in 10% (v/v) formalin [13]. The histological specimens were included in paraffin and after microtome cut, the sections were stained using hematoxylin-eosin (HE), for cellular observation, and picrosirius (PS) for collagen fibers.

2.6. Light microscopic evaluation

The microscopy slides were analyzed according to presence and intensity (absent, weak, moderate, and strongly present) of the following histological findings: Fibrin-leukocyte crust, Collagen, Focal necrosis, Fibrin deposits, Neutrophilic exudates, Edema, Eosinophilic exudates, Mononuclear infiltrate, Macrophage infiltrate, Granuloma, Neovascularization, Fibroblast proliferation and Fibrosis.

2.7. Statistical analysis

The statistical evaluation was carried out using the analysis of variance (ANOVA) method and Bonferroni's multiple comparison test. The statistical significance was 5% ($p < 0.05$) and the software used for data entry and processing was the Graphpad Prism for Windows, version 5.0 from Graphpad Software, Inc.

3. Results and discussion

3.1. Macroscopic evaluation

The clinical findings are shown in Fig. 1. Edema, Hyperemia, Exudate and Crust are present from day 1 to 3 after surgery in all groups, although was found a thicker Crust without Exudate in L and HL groups in comparison to other ones. From day 4 to 6, all groups still present Crust being Edema and Hyperemia absents. At day 6, the HL group started to lose its crust (Detatchment). From day 7 to 9, all groups presented a similar pattern with Crust, Detachment and Reepithelization. From day 10 to 12 presented Detachment and Reepithelization, and from day 13 to 14, just reepithelization.

These results demonstrated that in all experimental groups the scar tissue after the surgical procedure was much reduced compared to control, wich was more evident at day 14. They are in accordance with Moravvaej [14] and Avci [15] studying the reduction of hypertrophic scars in human patients under LLLT. In this study H group presented the same pattern than HL group, which may suggest a hydrogel benefit. This advantage has been reported in rosacea skin disease treatment by sulfated anionic polysaccharide [18].

In this study soon after the surgical procedures, the animals of L and HL groups started to feed and drink, while in other groups it was only after 12 h. This indicates that the LLLT may act as an analgesic factor corroboranting the findings of Pozza [16]. According to Soon & Acton [17] animals subjected to stress and pain had a poor wound healing process.

The wound contraction expressed in percentage is displayed in Fig. 2. At day 3, the H group presented statistically higher arithmetical mean (56.21 ± 4.31) followed by HL (48.86 ± 11.53), compared to control (28.57 ± 14.59), being H also significant in comparison to L (33.68 ± 7.43).

Observing day 7, H (84.22 ± 3.51), L (69.99 ± 9.52) and HL (84.46 ± 4.42) were statistically significant in comparison to control (39.83 ± 14.58). At 14 days after surgery there was not significance in comparison to control (H: 94.15 ± 1.17; HL: 96.13 ± 1.46; L: 94.97 ± 1.63; C: 84.09 ± 3.86). The experimental groups presented a similar trend for repair. These findings corroborate with studies with chitosan [19,20], policaju [2,21] and LLLT [15,22] as healing agents.