Original Article

Application of *Solidago chilensis* and laser improved the repair of burns in diabetic rats

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ABSTRACT

Background: The repair of burns in diabetic patients is a clinical problem. It is relevant to study alternative therapies that can improve the healing process. Our aim was to investigate the effects of *Solidago chilensis* associated or not with laser on burns in diabetic rats.
Methods: The animals were divided in four groups (n = 30): C- without treatment; S– *S. chilensis* extract; L-laser irradiated; LS- laser and *S. chilensis*. In 7, 14 and 21 days samples were collected after the injury to structural, morphometric and molecular analysis.
Results: Our results demonstrate the association of *S. chilensis* and laser reduced the inflammatory infiltrate and favored the angiogenesis. In the groups treated only with laser or with the plant extract showed higher levels of VEGF. The low-level laser therapy (LLLT) promoted higher collagen I and reduction of collagen III. It was also observed higher MMP-2 activation and a decreasing of the active isoform of MMP-9 in the S, L and LS groups.
Conclusions: The treatments improved the repair of burns in diabetic rats, since it reduced the inflammatory infiltrate and favored the collagen organization presenting similar effects in the burn repair of the diabetics.

A number of complications are observed among diabetic patients highlighting the diabetic skin ulcer that can promote tissue amputation and mortality [1]. Studies in animal models show disorders during healing process of diabetic lesions. In cases the lesion are caused by burns the prognosis is even worse, because they carry several systemic changes such as increased metabolism, loss of fluid volume, high risk of infection and wound healing disorders, requiring more time for healing [2].

At a glance of commentary

Scientific background on the subject

The authors are specialists in wound repair looking for alternative methods, non-invasive and low-cost, to improve the quality of scar tissue. They are researchers in the line Pathophysiology of Tissue Repair, training human resources (teaching and researches) in the area.

What this study adds to the field

The study of alternative therapy, non-invasive and low-cost, in burn repair in diabetic patients, is relevant, and the LASER and Solidago chilensis reduced the inflammatory infiltrate and favored collagen organization, with similar effects in the repair of burns in rats diabetics.

The great potential of vegetable extracts to treat lesions and burns has been studied and used in the treatment of diabetes [3,4]. *S. chilensis* is known for its activities in the absorption of edema and wound healing, and it is considered medicinal for showing antiseptic, analgesic and healing properties [5]. The phytochemical analysis of *Solidago* showed that its flowers and leaves when isolated present monoterpenes, sesquiterpenes, diterpenes, flavonoids (quercitrin, quercetin and rutin), saponins and polyacetylenes, tannins, fatty acids, alkaloids, volatile oils, anthranoids and sesquiterpene lactones [6] considered potent compounds in wound healing with antioxidant and anti-inflammatory properties [7,8].

The low-level laser therapy (LLLT) has also been used to treat skin burns in animals [9,10]. The biostimulation is important in accelerating the repair process of injured tissue by promoting proliferation of fibroblasts, re-epithelialization, collagen deposition, triphosphate (ATP) synthesis and granulation tissue formation, as well as the influx of leukocytes, which increase the phagocytic activity of macrophages, angiogenesis, vasodilation, stimulation of mitochondria, increasing cell metabolism [11]. Among its many effects, LLLT has been shown to cause vasodilation [12] and facilitate the absorption of active compounds in herbal of simultaneous applications.

Considering the importance of the new treatments which improve the wound healing process of burns in diabetic patients, the aim of this study was to investigate the association of *S. chilensis* and laser therapy to benefit the wound healing in diabetic animal model.

Materials and methods

Plant material

The leaves of *S. chilensis* were collected in the campus of the University Center of Hermínio Ometto Foundation − FHO (Araras, São Paulo, Brazil), in the Medicinal Plants Garden, during the morning in the month of August (winter). In the Herbarium of School of Agriculture Luiz de Queiroz (ESA) USP/ ESALQ (Piracicaba/São Paulo, Brazil was deposited a voucher specimen (ESA114268).

Preparation of the hydroalcoholic extract and phytochemical analysis

After collection, the fresh leaves (50 g) were selected, cleaned under running water to remove impurities and macerated dynamically with 300 mL of an ethanol aqueous solution (7:3, v/v) for 7 days at room temperature. This procedure was repeated three times with the same powder and the same solvent. After filtration, the solvent was completely evaporated under vacuum at 40 °C in a rotary evaporator, and the hydroalcoholic leaf extract of *S. chilensis* (S) was obtained after lyophilization. The yield of the lyophilized extract was 10%. The experimental procedures were carried out using dry crude extract dissolved in saline solution [6].

Animals

All surgical and experimental procedures used in this study were conducted according to the experimental requirements and biodiversity rights of the National Institute of Health for the Care and Use of Laboratory Animals (NIH Publication 80−23, reviewed in 1996). Studies have been done in accordance to the rules established by Arouca Law and approved by the Ethics Committee on Animal Use (CEUA) of University Center of Hermínio Ometto Foundation − FHO, under number 092/2011.

One hundred and twenty male Wistar rats were obtained from the Center of Animal Experimentation, University Center of Hermínio Ometto Foundation − FHO (Araras, São Paulo, Brazil) with 120 days and average weight 300 g. Throughout the experimental procedure, that did not promote stress, the animals were housed in individual cages, in ambient with humidity of 55%, temperature 23 ± 2 °C, 12/12-h light/dark cycle, and water and chow *ad libidum*.

Induction of experimental diabetes

The induction of experimental diabetes consisted of Alloxan solution [(Sigma®, Co., USA) (2,4,5,6 tetraoxohexahydropyrimidine) in single dose of 32 mg/kg body weight, diluted in citrate buffer pH 4.5 [13]. The characterization of the diabetic animal model was performed by measuring the peripheral blood glucose from tails of rats after seven and 30 days after the of diabetes induction, using reagent strips for readings in portable blood glucose meter (Accu-Chek Active®, AM Roche Diagnostics, EUA). The animals considered to the protocol had blood glucose ≥200 mg/Dl [13]. Before the euthanasia of rats (7, 14 and 21 days) was performed the measurement of glucose to check the stability of inducing diabetes.

Experimental procedures

The animals were anesthetized with peritoneal administration of xylazine hydrochloride (0.2 mg/kg) and ketamine hydrochloride (1 mg/kg) [14] and received analgesics (sodium dipyrone) [15]. The experimental second-degree burns were inflicted on the dorsal skin of all animals produced in the back of animals

with an aluminum plate measuring 2 cm in diameter connected to a temperature-controlling device that maintained a constant temperature of 120 °C. This plate was applied to the animal's skin for 20 s for the production of a second-degree burn. To ensure the same pattern of burns in all animals, we used a graduated support rod for sustaining the aluminum plate with the same pressure on the dorsal skin of the animals [14].

The four experimental groups randomly formed by 30 animals were: **C**, untreated; **S**, treated with the *S. chilensis* hydroalcoholic extract; **L**, irradiated with an LLLT; and **LS**, irradiated with an LLLT after treated with the *S. chilensis* hydroalcoholic extract. Lesion samples were collected from ten animals/group in each experimental period (7, 14, and 21 days after the burning procedure) for morphometric analysis (n = 5 animals) and for protein expression analysis by Western blotting, quantitative analysis of glycosaminoglycans, hydroxyproline, and zymography for metalloproteinases (n = 5 animals). The method of euthanasia of the animals was by an overdose of anesthesia and cervical dislocation.

LLLT was performed daily with a Physiolux Dual Bioset® - InGaP (Indium Gallium Phosphide) diode emitting a wavelength of 670 nm (visible red) with an output power of 30 mW, energy density of 4.93 J/cm^2, and total energy dose of 0.36 J, with the beam covering an area of 0.073 cm^2. Non-contact laser irradiation was performed punctually, in the continuous mode, at a distance of ±2 mm and an angle of 90° in relation to the wound surface. The time of laser application (12 s) was adjusted on the equipment in four points applied on the wound edges aimed to stimulate the healthy tissue from borders [6,14]. The calibration of the instrument was made by the manufacturer.

The dosimetry for application of *S. chilensis* hydroalcoholic extract was applied with a Pasteur pipette, dripping 1 mL over whole wound surface [6,15].

Morphometric analysis

Standardized samples (25 mm in diameter/10 mm in deep) were collected from the burned skin. The samples were fixed for structural and morphometric analysis [6,15]. The samples were cut into 6-μm longitudinal sections and stained with Dominici (detection of intracellular granules of inflammatory infiltrate), Mallory Trichrome (structural analysis of the epidermis and dermis) and with toluidine blue in McIlvaine buffer-pH 4.0 (measuring the number of fibroblasts and blood vessels). From each sample were captured the first 16 cuts, distributed in eight slide blades. The number of inflammatory infiltrate, newly formed blood vessels, fibroblasts (n/10^4 μm^2) in the repaired area were determined in longitudinal sections stained. Five samples of 10^{4}μm^2 were made for each section from the center of the specimen of each animal per group and for each method. Using a Leica DM2000 photomicroscope the images of sections were captured and digitized in bright field. For the morphometric analysis, samples were examined by one evaluator using the virtual Leica Image Measure™grid and the Sigma Scan Pro 6.0™program [16].

Western blotting and biochemical analysis

The densitometry values of TGF-β1, VEGF, Collagen type I and type III signals were developed according to a protocol developed by Ni et al. [17] and expressed relative to proteins stained with β-tubulin, which were taken as 100%.

Quantitative analysis of glycosaminoglycans

The glycosaminoglycans extraction of tissue fragments was made according to the DMMB method [18]. It was used one light visible spectrophotometer (526 nm) for the reading.

Quantification of hydroxyproline

Fragments of tissue were immersed in acetone (48 h) and then in chloroform: ethanol (2:1) for 48 h. The samples were hydrolyzed (HCl 6 N −1 mL for each 10 mg of tissue, 16 h, 110 °C) and neutralized (NaOH 6 N). The quantification of hydroxyproline was performed according to the method of Stegemann, & Stalder [19] with some modifications. Hydroxyproline concentrations between 0.2 and 6 μg/mL were used for the standard curve.

Zymography to metalloproteinases

The supernatant from each sample (50 μg protein) was used according to the protocol of Aro et al. [20] for the analysis of MMP-2 and MMP-9 activity. The intensity of the bands of different isoforms, for each group was determined by densitometry using Alpha 4.0.3.2 software (Scion Corporation, USA).

Statistical analysis

The results of the morphometric analysis, Western Bloting and quantitative of Hydroxyproline and Glycosaminoglycan (GAGs) were reported by mean and standard deviation (X ± SD) and the values were compared by ANOVA and Tukey's post-test (*p* < 0.05) using the GraphPadPrism® 3.0 software.

Table 1 Morphometric parameters evaluated in the wound healing process of second-degree burns in rats diabetics in different treatment groups and experimental periods.

Experimental periods		7 d	14 d	21 d
Parameters	Groups			
N of fibroblasts	C	13.2 ± 3.4	22.8 ± 3.8	27.4 ± 3.9
(n/10^4°μm^2)	S	12.1 ± 3.9	20.8 ± 4.2	27.9 ± 3.4
	L	23.2 ± 4.1[a]	31.1 ± 3.2[a]	37.2 ± 4.3[a]
	LS	24.2 ± 3.5[a]	31.2 ± 4.1[a]	37.4 ± 3.5[a]
N of inflammatory	C	20.6 ± 2.8	17.1 ± 2.1	7.7 ± 1.2
infiltrate (n/10^4 μm^2)	S	17.2 ± 1.7	13.7 ± 2.2	5.2 ± 1.3[a]
	L	17.1 ± 1.3	12.7 ± 1.8	5.1 ± 1.6[a]
	LS	16.2 ± 1.5	11.9 ± 2.2	5.2 ± 1.5[a]
N of vessels (n/10^4 μm^2)	C	1.4 ± 0.5	1.7 ± 0.6	1.8 ± 0.4
	S	1.7 ± 0.6	1.9 ± 0.8	2.0 ± 0.5
	L	1.8 ± 0.3	2.7 ± 0.3[a]	2.8 ± 0.5[a]
	LS	1.9 ± 0.3	2.8 ± 0.4[a]	2.9 ± 0.4[a]

Values are the mean and standard deviation of each group and were compared by ANOVA with Tukey's post-test (*p* < 0,05).
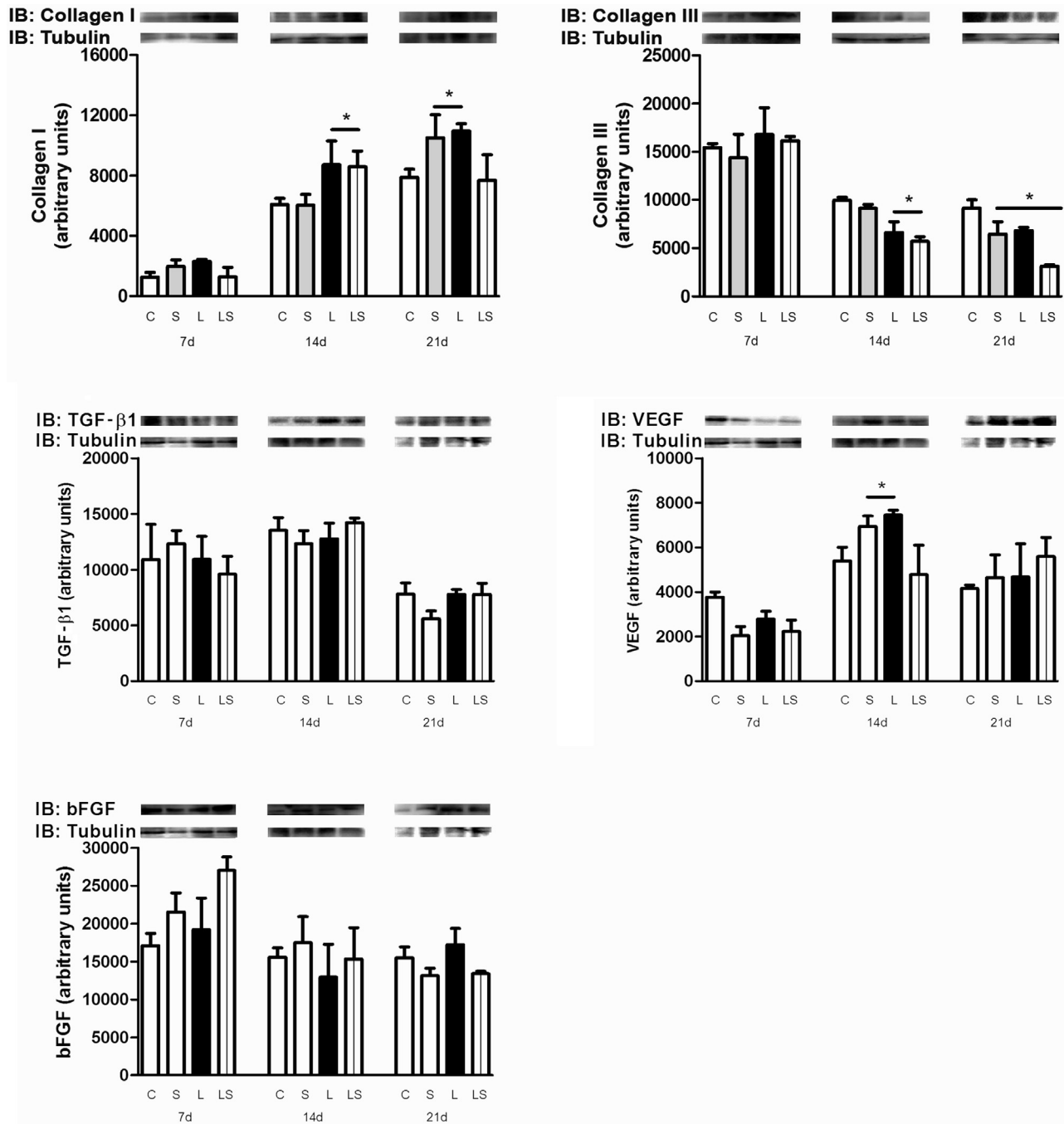[a] Significant difference.

Fig. 1 *Imunoblotting* analysis for Collagen I and III, TGF-β1, VEGF and bFGF in the wound healing process in C, S, L and LS groups in 7 (7 d), 14 (14 d) and 21 (21 d) days after injury. Typical blots are shown above average densitometry results. Values are the mean and standard deviation of each group were compared by ANOVA wich Tukey's post-test (*p* < 0.05) (*) significant difference.

Results

After 7 days of diabetes induction by intravenous Alloxan, animals presented an average glucose of 347.48 mg dL-1 that was maintained during the 30 experimental days.

The morphometric analysis showed that the total number of fibroblasts increased in the L and LS groups in relation to the others. The amount of inflammatory infiltrate decreased in the treated groups compared to the group C, on the 21st day after experimental protocol. It was observed that the number of blood vessels increased in the L and LS groups on the 14th and 21st day [Table 1].

Collagen I analysis showed higher amount during the experimental period with a difference on the 14th day in L and LS groups compared to the other groups, and on the 21st day in the S and L groups. On the other hand, Collagen III reduced during the experimental period, with difference on the 14th day

Table 2 Biochemical parameters evaluated in the wound healing process of second-degree burns in rats diabetics in different treatment groups and experimental periods.

Experimental periods		7 d	14 d	21 d
Parameters	Groups			
Glycosaminoglycans (μg/mg of dry tissue)	C	1.26 ± 0.14	1.39 ± 0.17	1.67 ± 0.14
	S	1.29 ± 0.16	1.45 ± 0.16	1.65 ± 0.15
	L	1.79 ± 0.13[a]	1.93 ± 0.13[a]	1.96 ± 0.13[a]
	LS	1.78 ± 0.16[a]	1.92 ± 0.14[a]	1.93 ± 0.14[a]
Hydroxyproline (μg/mg of dry tissue)	C	108.9 ± 14.1	93.1 ± 17.2	89.8 ± 14.2
	S	117.2 ± 16.2	96.4 ± 17.1	98.6 ± 15.4
	L	116.4 ± 17.2	91.6 ± 13.4	89.4 ± 18.3
	LS	112.9 ± 16.4	92.7 ± 14.6	92.2 ± 17.1

Values are the mean and standard deviation of each group and were compared by ANOVA with Tukey's post-test ($p < 0.05$).
[a] Significant difference.

in the L and LS groups, and on the 21st day in all treated groups, especially in the LS group [Fig. 1]. The results showed no differences for the TGF-β1 level between the experimental groups. The VEGF level was increased at day 14th in the groups treated with S and L. The quantification of bFGF had no differences throughout the study period in all experimental groups [Fig. 1].

Regarding to GAGs, there was a gradual increase between groups during the experimental period, particularly in the L and SL groups which had higher values in relation to the others [Table 2]. Considering the collagenesis, by hydroxyproline quantification, which infers the total collagen content in the tissue, no difference between groups was observed in all experimental periods [Table 2].

The active MMP-9 form was detected in all groups and periods, with the exception of S, L and LS, on the 21st day, where there was a marked decrease in MMP-9 activity in relation the control [Fig. 2, Table 3]. In the zymography analysis for MMP-2, the intermediate isoform was detected in all groups and experimental periods, except for the C group on the 21st day. Higher values were observed for catalytic activity of this isoform in the S group 14th and 21st days [Fig. 2, Table 3].

Discussion

Study of experimental phytotherapy is important to verify its efficacy to the healing of diabetic lesions, since the use of different extracts in the treatment of skin lesions is a common popular practice [21,22]. The orientation for use of the species S.
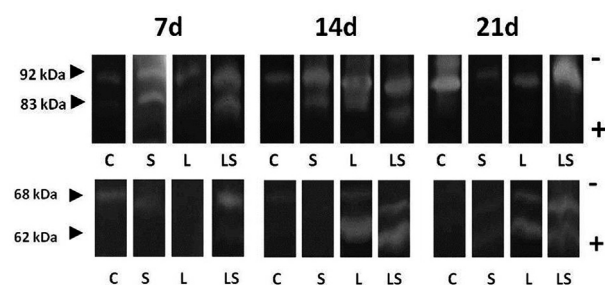

Fig. 2 Zymogram for analyzing the isoforms latent (92 kDa) and active (83 kDa) of MMP-9; and the intermediate isoforms (68 kDa) and active (62 kDa) of MMP-2 in C, S, L and LS groups 7 (7 d), 14 (14 d) and 21 (21 d) days after lesions.

chilensis and its products is only externally [23]. The plant has no toxicity for external use, which was used in this research and was included in the list of medicinal plants of SUS ("Sistema Único de Saúde - Brazil) interest for use by the population. In phytochemical analysis of the constituents of the extract of S. chilensis leaves was identified the following classes of chemical compounds: flavonoids, saponins, tannins, fatty acids, alkaloids, volatile oils and anthranoids [6]. Especially flavonoids, tannins and alkaloids, considered potent compounds in wound healing, can influence the migration of fibroblasts, collagen stabilization and in the release of VEGF [24].

The LLLT has also been widely used to solve or minimize burn lesions [25]. The choice of the InGaP 670 nm laser occurred due to its performance in the healing of second-degree burns as was found in previous studies of our group and other reports in the literature [6,9,10,14,15,26,27]. Disorders in tissue repair are a feature of Diabetes mellitus where the synthesis and deposition of collagen fibers, fibroplasia, epithelialization, angiogenesis and inflammatory phase are compromised [28].

The analysis of inflammatory infiltrate indicates that the extract and laser, isolated or in association, have been effective in reducing inflammatory phase during the studied period improved the tissue repair. It is known that the persistence of inflammation is detrimental to tissue repair in both burned and diabetic patients [6,28]. According to Tamura et al. [29] the anti-inflammatory activity of this extract was attributed mainly to the flavonoids and tannins present in the leaves and flowers of this species. The anti-inflammatory property is characteristic of arnicas [30] and this effect has also been shown in other studies [5,31]. Besides that, it is known that the use of LLLT in skin burned treatment of healthy and diabetic rats has anti-inflammatory action [32,33]. Data of our group showed that the use of laser on burned lesion in non-diabetic rats favorably modulated the initial inflammatory process [6]. It is important to mention that both treatments used in our study, isolated or in association, reduced the inflammatory infiltrate.

The analysis of VEGF demonstrated that the treatments favored the angiogenesis, an important process for the repair of tissue, because it provides oxygen and energy substrate. It is also known that VEGF and bFGF are growth factors that have pro-angiogenic properties and the activities of these are essential for an appropriated tissue repair [34]. bFGF is a potent angiogenic agent that acts as a chemoattractant in

Table 3 Densitometry of the bands (pixels) corresponding the respective isoforms in the lesion area of second-degree burns in diabetics rats in different treatment groups and experimental periods.

Groups		C	S	L	LS
Isoforms	Periods				
MMP-9	7 d	17.94 ± 6.12	56.33 ± 7.23[a]	32.87 ± 6.39[a]	58.97 ± 8.42[a]
latent	14 d	49.77 ± 9.93	52.38 ± 7.44	51.78 ± 8.99	49.85 ± 9.40
92 kDa	21 d	42.12 ± 5.73[a]	28.92 ± 7.08	35.94 ± 8.22	48.86 ± 9.55[a]
MMP-9	7 d	9.67 ± 2.32	48.22 ± 8.11[a]	31.87 ± 8.82[a]	56.43 ± 9.77[a]
active	14 d	6.21 ± 2.21	39.73 ± 8.69[a]	37.89 ± 5.72[a]	1.0 ± 0.00
83 kDa	21 d	5.33 ± 1.93[a]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
MMP-2	7 d	18.42 ± 5.32	14.84 ± 4.85	10.94 ± 2.94	44.75 ± 5.35[a]
intermediate	14 d	17.91 ± 3,75	15.27 ± 2.92	48.48 ± 9.14[a]	58.11 ± 6.22[a]
68 kDa	21 d	0.00 ± 0.00	13.31 ± 6.22[a]	51.92 ± 5.89[a]	55.91 ± 6.55[a]
MMP-2	7 d	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Active	14 d	0.00 ± 0.00	0.00 ± 0.00	59.24 ± 7.47[a]	61.38 ± 8.94[a]
62 kDa	21 d	0.00 ± 0.00	12.92 ± 6.88[a]	64.31 ± 7.11[a]	59.89 ± 7.92[a]

Values are the mean and standard deviation of each group and were compared by ANOVA with Tukey's post-test ($p < 0.05$).
[a] Significant difference.

different cell types and influence the proliferation and differentiation of endothelial cells and fibroblasts [35].

Increased numbers of fibroblasts was observed in the groups treated with laser in all periods. The LLLT stimulates the fibroblast proliferation in diabetic lesions [36]. Catarino et al. [6] found similar results in non-diabetic rats using the same experimental model used in this study.

The hydroxyproline is an indicator of the collagen concentration in the tissue, which is organized in fibers bundles [37]. No difference in the hydroxyproline concentration was observed between the groups in all periods, but considering the collagen I and III, some differences were detected. The laser isolated or in association with the extract, stimulated the collagen I synthesis, and contributed to decreasing of collagen III. Study by Novaes et al. [38] observed a gradual reduction of the fibers of collagen type III and accumulation of fibers of collagen type I in both groups treated with low-level laser (dose 3 J/cm^2 and 30 J/cm^2) in excisional lesions in the skin of the rats. Although we do not have structural data, the increase of collagen I and the decrease of collagen III, might indicate more quality to tissue during remodeling phase [39].

Corroborating with the hypothesis of greater tissue reorganization, our results showed an increase in active MMP-2 in the treated groups. Several studies correlate the greater activity of MMP-2 with greater tissue organization, proving its action in the remodeling of collagen fibers [20,39–41]. It's important to emphasize that the smaller activity of MMP-9 in the 21st observed in treated groups is also indicative of accelerated repair process considering its intense tissue remodeling process [39].

This study showed also increase in the amount of GAGs in all experimental period in the groups treated with laser. Novaes et al. [38] also reported increase in deposition of GAGs in the tissue repair of excisional injuries in rats treated with laser GaAs 30 J/cm^2. The GAGs deposition participates in the tissue repair process, contributing to the stabilization of collagen fibers controlling the final alignment and thickness [42].

Although we have obtained good results in the cicatrization of burns with the topical use of S. chilensis, it was observed that the association of the laser with the phytotherapic was more effective. Since LLLT activates cellular,

molecular mechanisms, cell proliferation and, by photochemical reaction, changes the permeability of the cell membrane, it can be deduced that this therapy assisted in the permeation and activity of S. chilenses in the repair tissue. The application on the laser stimulates mitochondria, and chromophores of the protein components of mitochondrial respiratory chain that seem to absorb red and infrared light of the laser, triggering a series of biochemical events such as an increase in enzyme activity, protein synthesis, cell proliferation, adenosine ATP production, and collagen organization [12,43–46], thus contributing to tissue repair in association with the phytotherapic.

Conclusions

Our results show that the treatment with LLLT (670 nm InGaP laser) and with the extrat of S. chilensis improved the tissue repair in burns of diabetic rats with decrease in the inflammatory infiltrate and increase in the collagen I, MMP-2 activity and amount of GAGs. Therefore, they can be used as an alternative therapy in the treatment of this type of lesion.

Conflicts of interest

We certify that there is no actual or potential conflicts of interest related to this article exist.

Acknowledgments

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