Article

Evaluation of mesenchymal cells (MSC) and dapsone for the treatment of dermonecrotic wounds caused by *Loxosceles laeta* venom in rabbits

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Abstract: We studied the efficacy of mesenchymal stem cells (MSC), either alone or associated with dapsone (DAP) in the treatment of dermonecrotic wounds caused by *Loxosceles laeta* spider venom. Twenty-five male adult rabbits were distributed into five groups, of which four groups received an intradermal injection of 20 µg of *L. laeta* venom and only one received ultrapure water (negative control). After 4 hours, each group that received venom, was treated with MSC, DAP, MSC + DAP and Phosphate-buffered saline – PBS (positive control). Photographic records were made for analysis of the wound area evolution by morphometry. Twelve days after treatment, the skin samples around the lesion were removed for subsequent histological analysis. Concerning the rate of wound contraction, we observed that DAP showed the best percentage of contraction at day 3. In the treatments using MSCs, a negative value of wound contraction was observed for the isolated MSCs, as well as a lower contraction value for the association of the MSC + DAP when compared to PBS group. Histopathological analysis showed diminished tissue lesion and less intense inflammation in MSCs and DAP groups. This could indicated potential use of stem cells in regenerative therapies after loxoscelic accidents.

Keywords: spider venom; wound repair; loxoscelism.

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1. Introduction

Loxoscelism is described in all the continents and corresponds to the most severe form of spider envenomation in Brazil [1, 2]. The clinical syndrome can develop into two distinct forms: the cutaneous form, characterized by local alterations, with a dermonecrotic wound of difficult healing, and the viscero-cutaneous form, presenting important systemic alterations such as acute renal failure, clotting disturbances, and risk of death, in addition to the local lesion [3, 4, 5, 6).

The cutaneous and systemic changes observed in loxoscelic accidents are due to multiple factors, involving direct tissue damage caused by venom components, secondary vascular injury, and enzimatic release by polymorphonuclear cells [3, 6). In spite of being a complex mixture of components, it is known that venom phospholipases D are mainly responsible for dermonecrosis. These enzymes are capable of interacting with cell membranes, initiating reactions involving the complement system, platelets, and leucocytes [4, 7].

Loxoscelic envenoming treatment is based on observed clinical signs and includes the use of dapsone, acetylsalycilic acid, broad spectrum antibiotics, corticosteroids, and specific antivenom, composed of heterologous antibodies developed in horses [4, 8, 9, 10]. Despite these treatment alternatives, tissue recovery after extensive dermonecrotic damage is slow and scar formation is difficult.

One therapeutic possibility for tissue repair is the use of stem cells [11]. Stem cells can be of embryonic, fetal, or mature tissue origin. Mesenchymal stem cells (MSC), from tissues such as mature bone marrow or adipose tissue, are being largely used in experimental assays of tissue regeneration due to their potential to differentiate into diverse cell types and ease in isolation and proliferation [12, 13]. They have the potential to differentiate in to cutaneous cells of the ectodermal, mesodermal and endodermal layers of the skin, with applications in regenerative medicine [14, 15].

Up until now, there have been no studies evaluating the use of stem cells in the treatment of dermonecrotic wounds caused by loxoscelism. Therefore, the main goal of this work is to assess the efficacy of mesenchymal stem cells, alone or associated with dapsone, for the treatment of dermonecrotic lesions caused by experimental inoculation of *Loxosceles laeta* venom in rabbits. The resulting wounds were monitored by the evaluation of tissue healing, tissue morphology by histological examination, collagen quantification, and immunohistochemistry.

2. Results

Characterization of stem cells derived from adipose tissue confirmed their identity as mesenchymal stem cells (MSC) as they attached to culture flasks, presented in fibroblastoid form, and were able to differentiate into bone and adipogenic tissues (Fig.1). Cell viability was further confirmed by MTT and alkaline phosphatase tests (data not shown). The cells were then used to treat dermonecrotic wounds induced by intradermal *L. laeta* venom injection.

After venom injection, macroscopic evaluation of the wounds was made daily to enable the follow-up of injury evolution and differentiate between distinct treatment groups. The amount of venom injected induced a typical dermonecrotic lesion [10, 18] that began with a hemorrhagic halo 4 hours after injection, associated with edema, erythema, and exacerbated sensitiveness. After 48 hours, the hemorrhagic area turned into a bluish necrotic area. Four days later, a crust was formed that was later detached from the skin (Fig.2).

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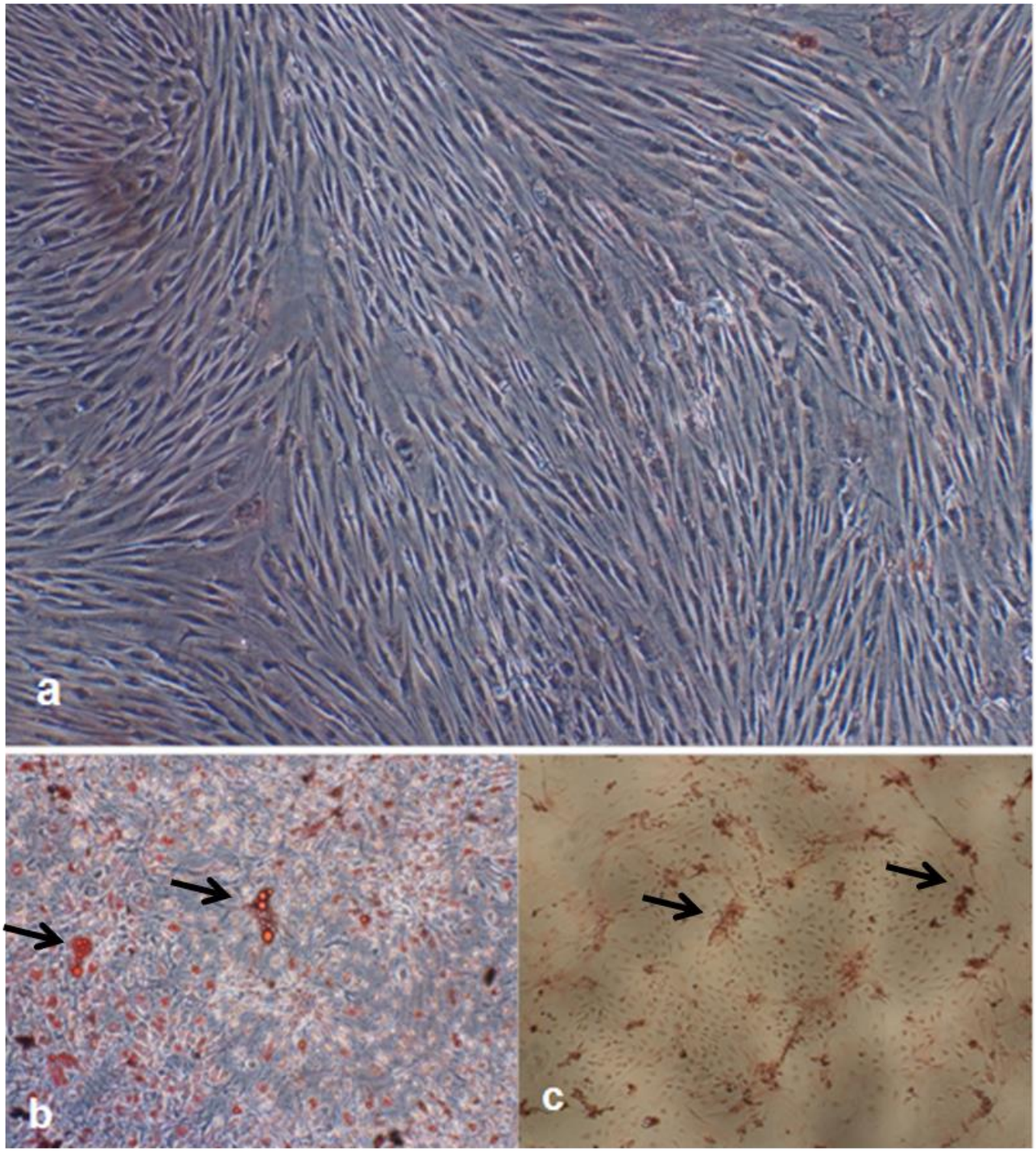

Figure 1: Photomicrographies from undifferentiated and differentiated mesenchymal stem cells (MSCs) derived from rabbit adipose tissue. **(a)** Undifferentiated MSCs cultured in unsupplemented media (400x). **(b)** MSCs cultured in specific media to induce adipogenic differentiation, stained with Oil Red (400x). Lipid droplets are marked red, demonstrating successful differentiation (arrows). **(c)** MSCs cultured in specific media to induce osteogenic differentiation, stained with von Kossa (400x). Mineralized matrix shows brownish coloration (arrows).

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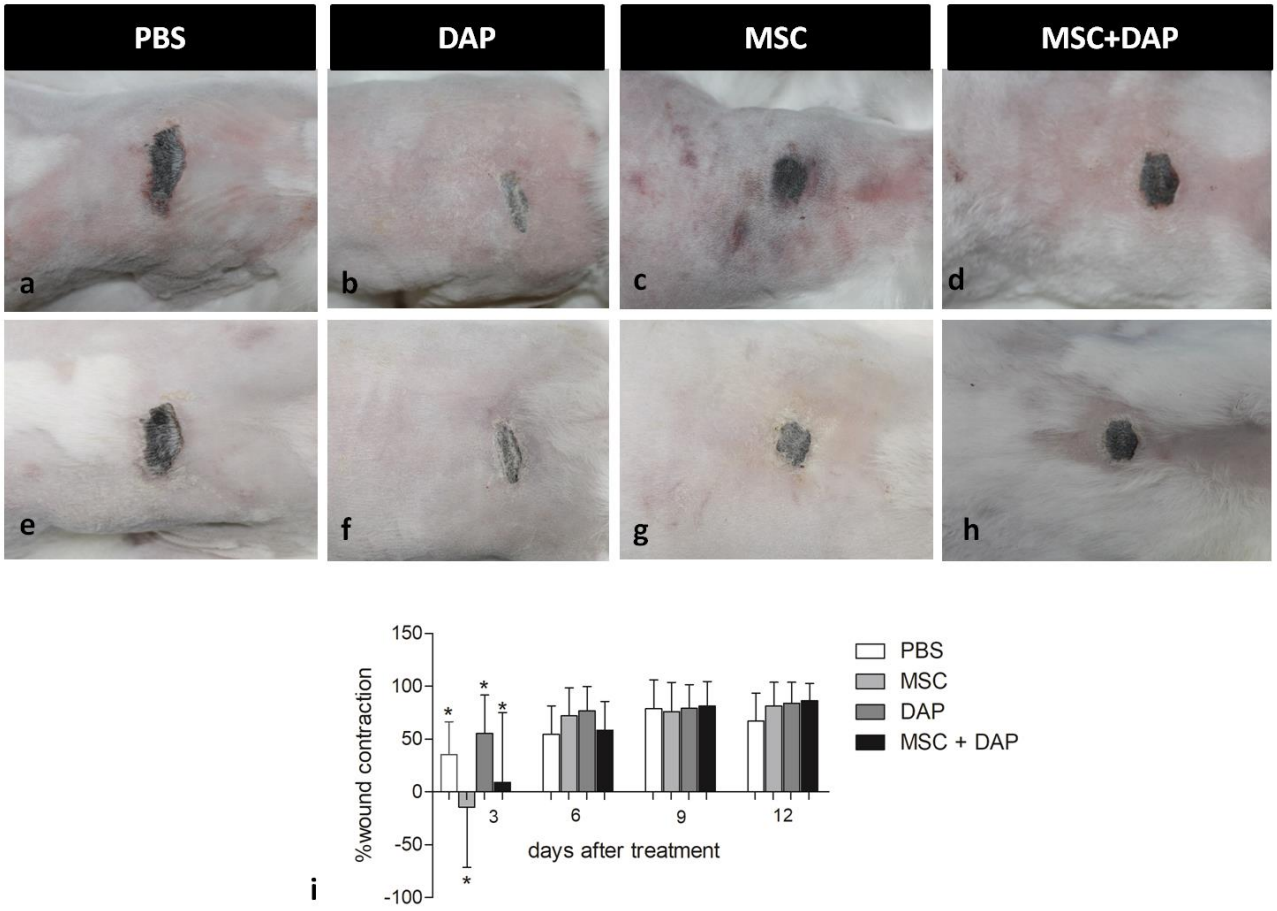

Figure 2: Dermonecrosis evolution of New Zealand rabbits after *Loxosceles laeta* venom (20 μg) inoculation and treated with phosphate buffered saline (PBS), dapsone (DAP), mesenchymal stem cells (MSC) or in association of both MSC and DAP (MSC+DAP). (**a,b,c,d)** Dermonecrotic lesion in rabbits four days after starting treatment. **(e,f,g,h)** Dermonecrotic lesion in rabbits 12 days after starting treatment. (**i)** Mean values of percentage of wound retraction and standard deviation using a mixed linear model approach of SAS, using first-order autocorrelation covariate structure. Animals were considered as a random factor, with each animal nested within treatments, with repeated measurements over time. Significant day versus treatment interactions were examined using the PDIFF procedure with preplanned comparisons. The significance level was set as $P<0.05$. After *L. laeta* venom inoculation, groups were treated with either PBS (C+), mesenchymal stem cells (MSC), dapsone (DAP), or in association of both MSC and DAP (MSC+DAP). Measurements were taken 3, 6, 9, and 12 days after treatment.

Through the evaluation of the mean values of wound contraction, statistical significance was observed at day 3 in all treatments, and dapsone showed the best percentage of wound contraction at that time. In the treatments using MSCs, a negative value of wound contraction was observed for

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the isolated MSCs, as well as a lower contraction value for the association of the MSC + DAP when compared to PBS group (Fig. 2i).

When analyzing histopathological changes in HE staining of wounded skin sections, animals from the PBS group (C+) showed larger necrotic areas, intense neutrophilic infiltrate, intradermic hemorrhage, and mineralization spots (Fig. 3b), confirmed by von Kossa staining (Fig. 3d) [30, 31]. Other studies also describe early eosinophilic infiltration (30, 32], which was not observed in our study, probably due to the time elapsed from the beginning of treatment and sample collection.

Animals from the DAP group showed less significant lesions, with mineralization and angiogenesis areas, as was also seen in the MSC group (Fig. 3c). Lesions in the MSC + DAP group were considerably more discrete and the inflammatory infiltrate was also less intense. Animals from the negative control group showed no changes (Fig. 3a).

Two different methods were used to assess the deposition of collagen fibers during the wound healing process: total collagen identification by Massom's trichrome staining, identification, and quantification of mature and immature fibers by Picro-Sirius Red staining (Fig. 4 and 5). These collagen analyses are important to assess the scarring stage, since that is when collagenic organization and gradual substitution of immature by mature fibers can be observed [39].

Animals submitted to treatment with DAP and MSC showed a higher number of total collagen fibers compared to other groups ($P>0.05$). The MSC group was the only that presented significant difference when compared to healthy tissue ($P<0.05$). What may represent a greater stimulus for collagen deposition in this treatment (Fig 6).

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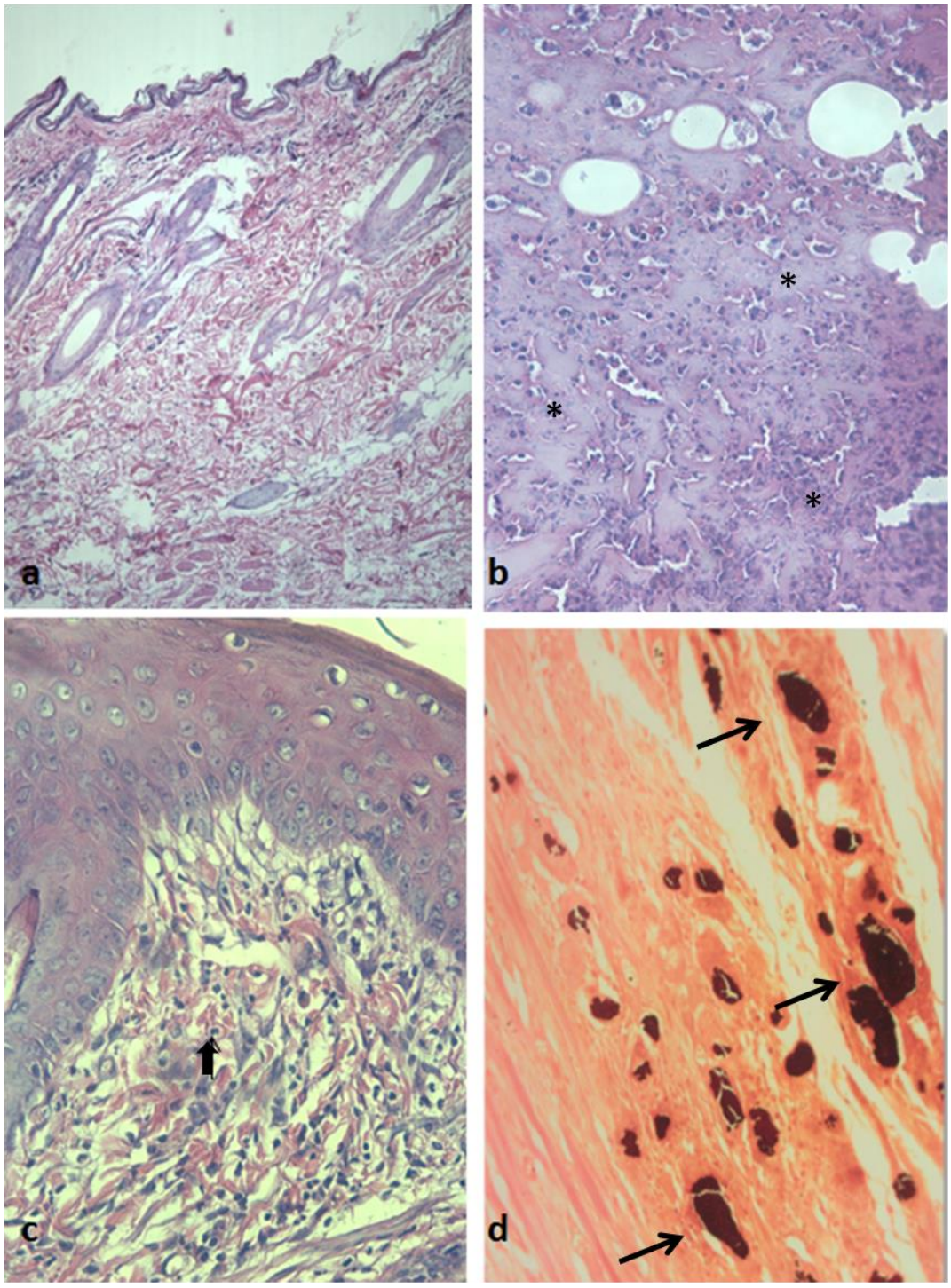

Figure 3: Microscopy of New Zealand rabbits' skin inoculated with *Loxosceles laeta* venom (C+) and treated with dapsone (DAP), mesenchymal stem cells (MSC) or in association of both MSC and DAP (MSC+DAP). **(a)** Normal skin showing integrity of epidermal and dermal cells (C-) (HE, x100). **(b)** Presence of necrotic crust on the skin of envenomed rabbits that received PBS (C+) (*) (HE, x200). **(c)** Presence of mild inflammatory polymorphonuclear infiltrate (arrow) in animals treated with dapsone (DAP) (HE, x 200). **(d)** Multiple dark spots can be seen in the dermis, a sign of tissue calcification in the skin of animals treated with PBS (arrows) (Von Kossa, 100x).

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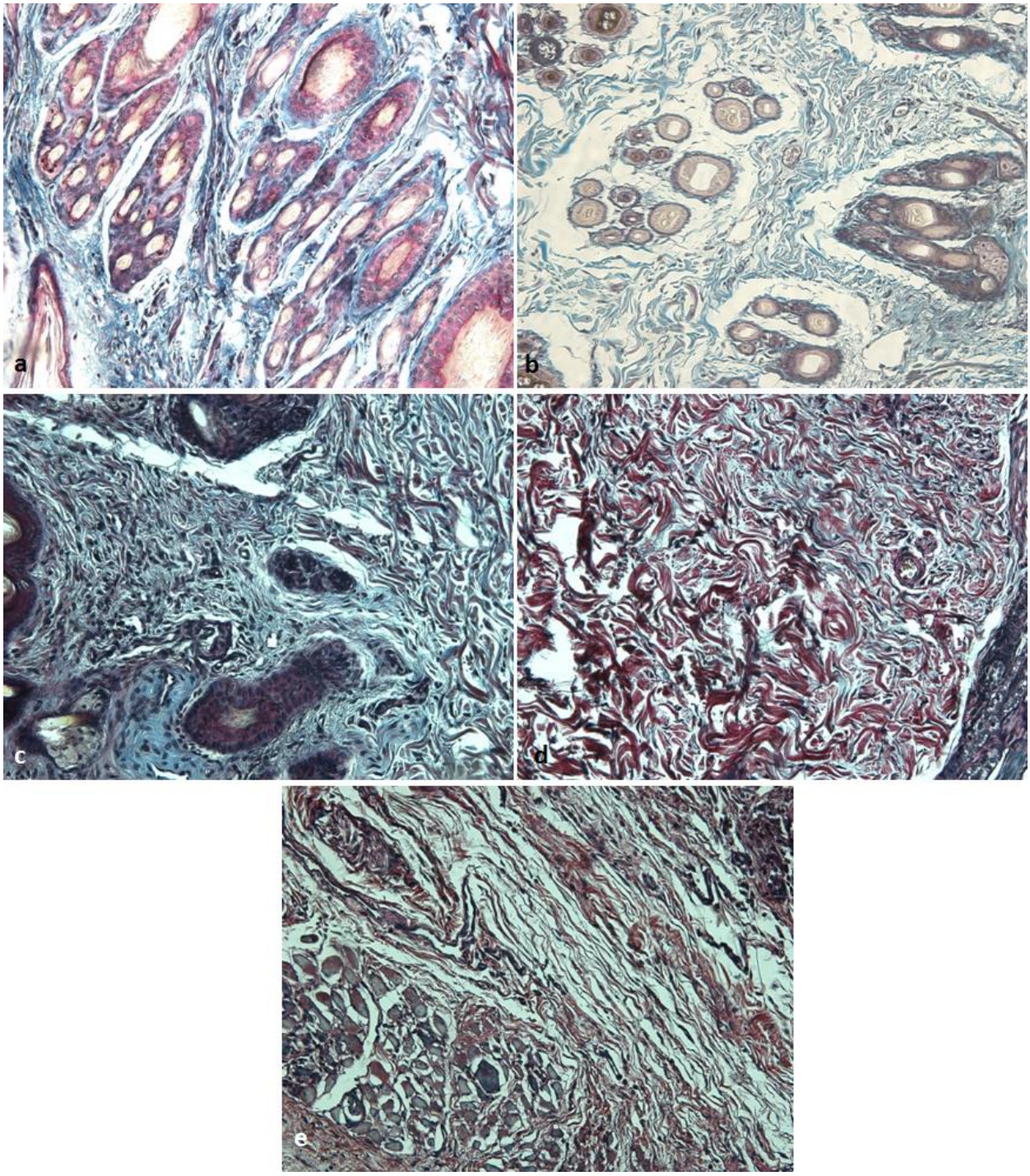

Figure 4: Collagen deposition analysis through Massom's trichome of New Zealand rabbits' skin inoculated with *Loxosceles laeta* venom and treated with dapsone (DAP), mesenchymal stem cells (MSC) or in association of both MSC and DAP (MSC+DAP). **(a)** Sample from a control group skin (C-) (200x). **(b)** Pattern of collagen deposition following *Loxosceles laeta* envenomation (C+) (*) (200x). **(c)** Collagen deposition in envenomed rabbits treated with dapsone (DAP) (*) (200x). **(d)** Collagen deposition in envenomed rabbits treated with mesenchymal stem cells (MSC) (*) (200x). **(e)** Collagen deposition in envenomed rabbits treated with DAP and MSC (DAP-MSC) (*) (200x).

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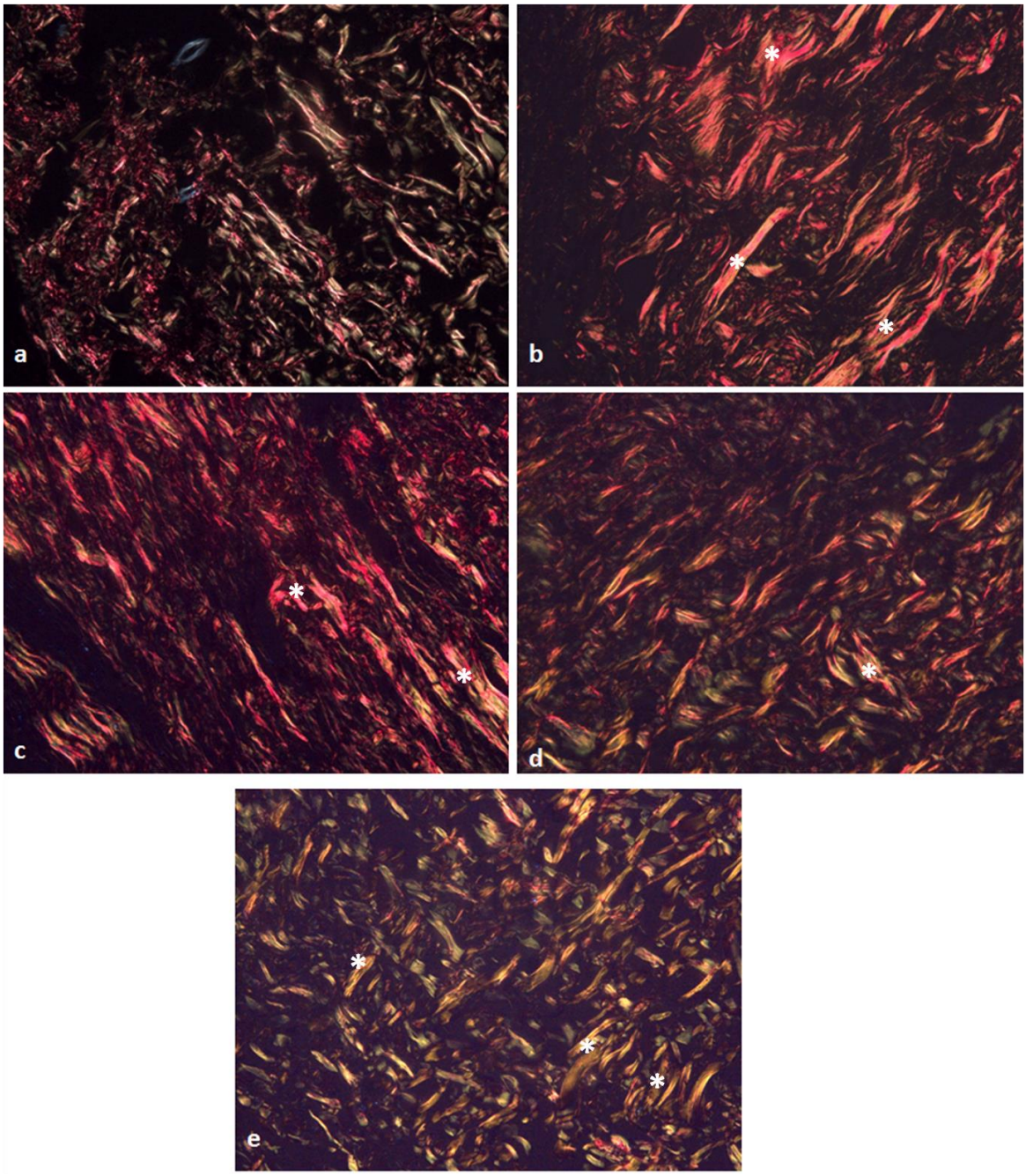

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152 **Figure 5:** Collagen deposition analysis through Prico-sirius stain of New Zealand rabbits' skin
153 inoculated with *Loxosceles laeta* venom and treated with dapsone (DAP), mesenchymal stem cells
154 (MSC) or in association of both MSC and DAP (MSC+DAP). **(a)** Sample from a control group skin
155 (C-) (200x), **(b)** Pattern of collagen deposition following *Loxosceles laeta* envenomation (C+) (*) (200x),
156 **(c)** Collagen deposition in envenomed rabbits treated with dapsone (DAP) (*) (200x), **(d)** Collagen
157 deposition in envenomed rabbits treated with mesenchymal stem cells (MSC) (*) (200x), **(e)** Collagen
158 deposition in envenomed rabbits treated with DAP and MSC (DAP-MSC) (*) (200x).

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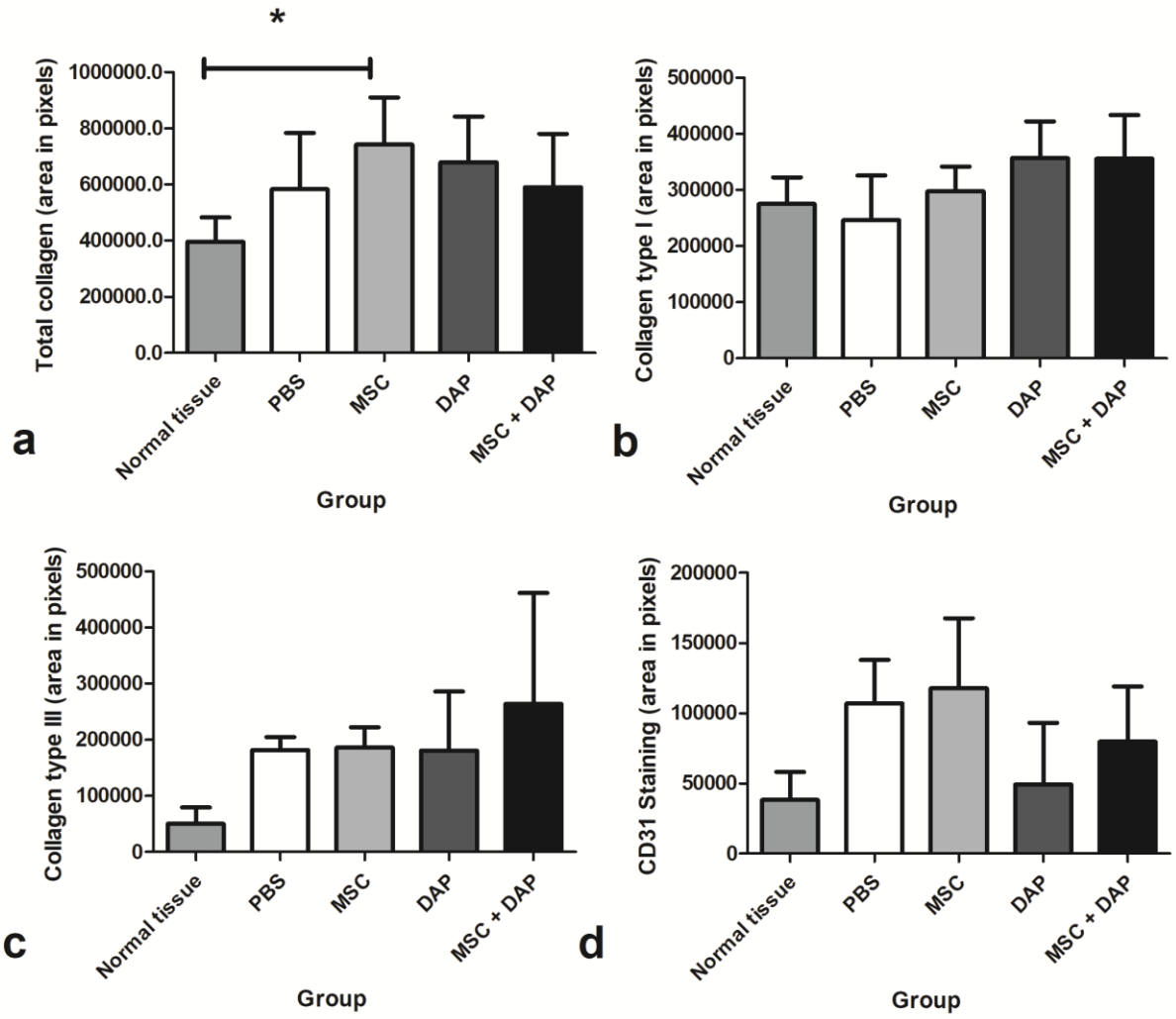

Figure 6: Collagen deposition analysis of New Zealand rabbits' skin inoculated with *Loxosceles laeta* venom and treated with phosphate buffered saline (PBS), dapsone (DAP), mesenchymal stem cells (MSC) or in association of both MSC and DAP (MSC+DAP). **(a)** Means and standard deviation of the collagen deposition areas in *Loxosceles laeta* envenomed rabbit skin, submitted to different treatments after Massom's trichrome stain. **(b)** Means and standard deviation of areas of deposition of type I (mature) or type III **(c)** (immature) collagen fibers in *L. laeta* envenomed rabbits skin, submitted to different treatments. **(d)** Means of the blood vessels' areas in pixels, after CD-31 marking. Data was statistically analyzed using the software SAS v.9.0. The normality of the data was evaluated using a Shapiro-Wilk and Kolmogorov-Smirnov tests. Tukey's test was employed for the comparison of total collagen fibers, type III collagen, and angiogenesis area. Logarithmic transformation of type I collagen values was performed prior to Tukey's test. The significance level was set as $P<0.05$.

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3. Discussion

3.1. Isolation and characterization of mesenchymal stem cells from adipose tissues

The MSCs used throughout this work were obtained from interscapular adipose tissue from donor rabbits to avoid stressful handling of the animals posteriorly envenomed and treated, which could affect wound healing and the correct evaluation of employed treatments. Allogeneic MSCs are a promising option because of their low immunogenicity and immunosuppressive and tissue repair capabilities [16]. Furthermore, auto-MSC extraction is time-consuming, making it difficult to use them promptly to treat acute diseases such spider envenomation. MSCs derived from adipose tissue are gaining importance and being more frequently used in experiments, due to their easy isolation, good proliferative capacity, and the possibility of obtaining excellent cell quantity in a single procedure. This enables the use of fewer donor animals, when compared to the isolation of MSCs from bone marrow [17].

3.2. Wound evaluation

This typical lesion observed, confirmed the use of rabbits as good animal models for cutaneous loxoscelism [4, 19], unlike mice and rats, which do not develop dermonecrosis. It must be pointed out, however, that wound development in rabbits occurs faster than in humans [2, 4, 5]. This can be explained by structural differences between these two animals or the fact that experimentally produced wounds tend to be more controlled.

3.2.1. Morphometric evaluation and wound contraction rate

This fact can be explained by the increase in initial inflammation after the application of stem cells, that may occur in the first days, because MSCs secrete a broad spectrum of bioactive molecules such as cytokines and growth factors that favor a regeneration microenvironment [20, 21]. No statistical difference was observed in the others days, however, it was observed that the treatments that received stem cells and dapsone were superior to the PBS group already on the sixth day ($P > 0.05$). This may suggest a positive influence on wound healing, since inflammatory factors and inflammatory cells start tissue regeneration by replenishment of cells and extracellular components [22]. MSC can contribute to a faster and more effective wound healing, due to reepithelization, fibroblast proliferation, and neovascularization stimuli [23, 24]. These stimuli occur mainly by paracrine effect, through the production of soluble factors such as bioactive molecules and growth factors that promote a tissue repair microenvironment [20, 21].

Clinical assays using dapsone as treatment for wound repair show contrasting results. The benefits of dapsone in the treatment of dermonecrosis caused by loxoscelism have already been observed by Rees et al. [25], who used dapsone with or without precocious surgical tissue excision to treat patients. Barret et al. [26] also successfully used dapsone in an experimental model using guinea pigs. Conversely, Phillips et al. [27] and Elston et al. [19] did not observe statistical differences in wound retraction when using dapsone for treating rabbits.

Morphometric analysis for obtaining the percentage of wound retraction during the scarring process has shown to be an efficient and accessible method [28, 29]. The evaluation of wound retraction rate, instead of the wound area, eliminates the differences caused by wounds of different initial sizes that can be formed, even when injecting the exact same amount of venom.

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214 *3.2.2. Histopathological evaluation*

215 Tissue neutrophilic infiltration is considered one of the main causes of dermonecrotic lesions
216 observed in loxoscelism (4, 33). This fact was confirmed in the PBS (C+) group, which showed
217 intense inflammatory infiltrate with neutrophil predominance, in addition to crust formation and
218 damage to the epidermis and superficial and deep dermis (Fig. 3b).
219 In contrast, neutrophilic infiltrates were also more mild in groups that showed less intense
220 wounds, as occurred in the group treated with DAP, MSC and MSC + DAP. This fact can be
221 explained by dapsone's anti-leukocyte aggregation effect, which has already been described by some
222 authors [25, 26, 34, 35]. In addition, MSCs shows immunomodulatory effect that can attenuate
223 inflammation and reprogram the local immune system, favoring tissue repair and inhibiting
224 exuberant fibrosis [36, 37].
225 The observations made in the groups that received MSC probably occurred due to positive
226 stimulus on the local cells, such as keratinocytes and progenitor cells [21]. MSCs have the ability to
227 interact with the local microenvironment, attracting progenitor cells, differentiating in other tissues
228 and producing a series of soluble factors, such as cytokines and growth factors that positively
229 influence the scarring of chronic wounds, as the ones observed in cutaneous loxoscelism [21, 38].
230 Furthermore, MSCs have an immunomodulatory effect on skin local inflammation, suggesting it can
231 be used in patients with hard-to-heal wounds [24].

232 *3.2.3. Collagen fiber evaluation*

233 Animals submitted to treatment with DAP and MSC showed a higher number of total collagen
234 fibers compared to other groups ($P>0.05$). The MSC group was the only that presented significant
235 difference when compared to healthy tissue ($P<0.05$). What may represent a greater stimulus for
236 collagen deposition in this treatment (Fig 6). This overall increase can be explained by the specific
237 increase of type I collagen, representing mature collagen, but it was not statistically significant.

238 *3.2.4. Immunohistochemical evaluation of angiogenesis*

239 There was no noticeable statistical difference in the area occupied by blood vessels between the
240 control group and all the tested treatments (Fig. 6). Angiogenesis is a process of the proliferative
241 phase of scarring and very important in wound healing evolution, occurring from intact or newly
242 damaged capillaries. This event is modulated by the vascular endothelial growth factor (VEGF-A),
243 produced by endothelial cells, macrophages, and active epidermal cells [40]. It is generally accepted
244 that MSC stimulates angiogenesis through macrophage chemotaxis and endothelial cells stimulation
245 by pro-angiogenic factors, such as VEGF-A. The fact that there was no observable difference in
246 neovascularization between the different groups can be explained by the sample collection timing. A
247 study concerning wounds in dogs after treatment with MSCs corroborates this hypothesis. In this
248 study, no difference in the number of blood vessels was observed 14 days after stem-cell treatment,
249 but on day 21, a statistical difference in the treated groups could be seen [24].
250

251 **4. Conclusions**

252 The amount of *L. laeta* venom used in this study (20 μg) was capable of inducing a significant
253 dermonecrotic lesion in rabbits. MSC may increase initial inflammation following application in the
254 first few days, however as the time progresses it may contribute to wound healing.

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Histopathological analysis showed diminished tissue lesion and less intense inflammation in DAP e MSCs groups. This could indicated some potential use of stem cells in regenerative therapies after loxoscelic accidents. Although few parameters presented statistical significance, treatment with DAP, MSC and DAP+MSC revealed tendencies to improvement. Additional studies could provide more evidence and are needed to define MSC and it is association with dapsone as potential treatment options for cutaneous lesions of *Loxosceles*.

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5. Materials and Methods

5.1. Mesenchymal stem cells (MSC) isolation

MSCs were obtained from the adipose tissue extracted from interscapular region of two donor outbred New Zealand rabbits, under general anesthesia with propofol (6 mg/kg). The collected tissue was washed in PBS and underwent enzymatic digestion with type II collagenase for 1 hour. Afterwards, collagenase activity was inhibited by adding fresh Dulbeco's Modified Eagle Medium (DMEM) to the cells and centrifuging at 23°C, 1200 rpm for 10 minutes. The resulting pellet was resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) and plated in T75 sterile culture flasks. Cell cultures were maintained at humid atmosphere, at 37°C, 5% CO_2 until 90% confluence. Cells were then detached with 0.5 ml trypsin-EDTA 0.25%, diluted at 1:3 ratio, and transferred to another T75 flask. Isolation of MSCs was based on their ability to adhere to the flasks [17].

5.1.1. MSC characterization

Between the 3rd and 5th passages, MSCs were phenotypically characterized by differentiation induction into adipogenic or osteogenic cells to confirm their pluripotency.

For adipogenic differentiation, 5 x 10^4 cells/well were plated in 6-well plates, with DMEM supplemented with 10% FBS, dexamethasone (0.5 μM), insulin (1 μM), indomethacin (60 μM), and isobuthylmethylxantine (0.5 μM) [12]. Culture media were replaced every three days and culture was maintained for 21 days. Afterwards, cells were stained with Oil Red to evince lipid droplets in the cytoplasm, thus confirming differentiation into adipocytes.

For osteogenic differentiation, 5 x 10^4 cells/well were plated in six-well plates and cultured for 21 days in DMEM supplemented with 10% FBS, ascorbic acid (50 μg/ml), β-glycerophosphate (10 mM), and dexamethasone (0,1 μM). Media were replaced every 3 days [12].

To evaluate cell viability of MSCs, MTT (3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide) metabolism assay was performed. Cells/m^2 (5 x 10^4) were plated in 24-well plates and incubated with 170 μl of MTT (5mg/ml; Sigma-Aldrich, USA), at 5% CO_2, 37ºC), for 2 hours. Formed formazan crystals were observed in inverted light microscope and then solubilized by adding dodecyl sodium sulfate (SDS) in 10% HCl. Optical density was quantified at 595 nm, in automated plate reader.

5.1.2. Alkaline phosphatase activity

Considering that differentiated osteoblasts are able to secrete alkaline phosphatase [41], its activity was measured in plated cells to ensure differentiation, using similar parameters described in the section above. BCIP-NBT solution was added to each well and incubated for 2 hours. Purple

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precipitates were solubilized with SDS 10% HCl and optical density was quantified at 595 nm, in automated plate reader.

5.2. Animals

Twenty-five male New Zealand one-year old rabbits, weighting 1.5 kg, were used throughout this study. They were placed in individual metal cages (75 cm x 30 cm), receiving water and food *ad libitum.* All procedures were conducted following ethical principles, respecting the animals' welfare and minimizing eventual discomfort. The experiment was approved by the Ethics Committee in Animal Use (CEUA) of Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil (Protocol No. 83/2013).

5.2.1. Experimental groups and treatments

After a five-day adaptation period, the animals were randomly distributed into five groups of five animals. Negative control group (C-) was inoculated only with ultrapure water. All the other four groups were intradermally inoculated with 20 μg of *L. laeta* venom diluted in 0.2 ml of phosphate buffered saline (PBS). All treatments began four hours after venom injection with the intention of simulating early identification of the lesion and maximizing the effect of the proposed therapies [27]. Positive control group (C+) was treated only with PBS (0.5 ml), injected intradermally in four points equidistant from the center of the pre-formed lesion. The dapsone group (DAP) received a dose of 2 mg/kg of dapsone diluted in ethanol and ultrapure water (4:6 ratio) by nasoesophageal catheter every 24 hours for four days. The mesenchymal stem cells group (MSC) was treated with 1.25 x 10^6 cells suspended in 0.5 ml of PBS, injected intradermally in four points equidistant from the center of the pre-formed lesion, totaling 5 x 10^6 cells injected per animal. The 5th group was treated with a mixture of dapsone and mesenchymal stem cells (DAP+MSC), according to the procedures described above for each treatment.

5.2.2. Wound area evaluation

Wounds were measured and recorded daily to verify their evolution according to treatment protocol. Photographic records were taken with digital camera (Canon Rebel XSI EOS zoom 24 mm), kept at a constant distance of 50 cm from the wound. The collected data were evaluated with IMAGE PRO software. For wound contraction analysis, measurements were taken 3, 6, 9, and 12 days after treatment. Wound contraction percentage was calculated using the mathematical model proposed by Oliveira et al. [28] and Wu et al. [29]:

$$\left[\frac{\text{area at day 1} - \text{area at day X}}{\text{area at day 1}}\right] * 100$$

5.2.3. Histopathological evaluation

The animals were euthanized twelve days after treatment by propofol anesthesia with a dose greater than 10 mg/kg. Skin fragments (5 cm x 6 cm) were removed and fixed in paraformol for 48 hours. Fixed tissues were then processed, included in paraffin, and 4-μm thick histological slices were taken for further analysis.

After hematoxylin-eosin staining [42], fixed skin tissue images were obtained with an Accu Scope microscope, coupled with TCS pro 500 software. Each slide was blindly described by a pathologist. Changes in epidermis, dermis, and muscle morphological structure, as well as the

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intensity and composition of inflammatory infiltrate and other specific findings such as tissue mineralization, were described.

To confirm tissue calcification observed on slides, von Kossa staining was performed [42]. Massom's trichrome staining was used to evaluate the total number of collagen fibers [42]. Photographic registry (Accu Scope microscope) was used with TCS pro 500 software to capture images. Collagen fibers were quantified using software ImageJ software. Picro-sirus Red staining was performed to evaluate collagen fiber differentiation [42]. In this method, type I collagen fibers are stained red and type III collagen fibers are greenish. Pictures were taken with Leica Core microscope coupled with Leica Application Suite Core software. Six pictures of random fields of the lesion area of each slide were taken, and stained fibers were quantified using ImageJ software. Final evaluation was made considering each group's mean and standard deviation.

5.2.4. Immunohistochemistry

Immunohistochemistry was used to evaluate angiogenesis and vascular damage of affected skin tissues. Monoclonal CD31 antibody (clone JC70A, Dako) was used at 1:20 dilution, with 16 hours of incubation, to mark endothelial cells. To quantify the area marked by the used antibody, ten random fields of the lesion from each animal were captured using the Accu Scope microscope (400x) and the image capture software TCS pro 500. Images were analyzed by ImageJ software. The mean for each group was calculated for statistical evaluation.

5.3. Statistical analysis

We review the statistical design as follows: "Data was statistically analyzed using the software SAS v.9.0. The normality of the data was evaluated using a Shapiro-Wilk and Kolmogorov-Smirnov tests. Tukey's test was employed for the comparison of total collagen fibers, type III collagen, and angiogenesis area. Logarithmic transformation of type I collagen values was performed prior to Tukey's test. Statistical analysis of wound contraction percentage was carried out using a mixed linear model approach of SAS, using first-order autocorrelation covariate structure. Animals were considered as a random factor, with each animal nested within treatments, with repeated measurements over time. Significant day versus treatment interactions were examined using the PDIFF procedure with preplanned comparisons. The significance level was set as $P<0.05$".

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Author Contributions: Marilia Melo and Benito Soto-Blanco conceived and designed the experiments; Guilherme Martins, Maira Oliveira, Ana Flávia Botelho and Conrado Gamba performed the experiments; Marilia Melo and Benito Soto-Blanco analyzed the data; Geovanni Cassali, Carlos Chávez-Olórtegui, Adriane Bicalho and Alfredo Goes contributed reagents/materials/analysis tools; Guilherme Martins, Clara Duarte, Maira Oliveira and Ana Flávia Botelho wrote the paper.

Conflicts of Interest:

The authors declare no conflict of interest.

The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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