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Use of stem cells adipose tissue derived accelerates the healing process in third-degree burns

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ABSTRACT

Introduction: Burn can be defined as a traumatic injury, usually of thermal origin, that affects the epithelial and adjacent tissue and can be classified according to the depthreached. The complexity of tissue repair involved in this type of injury is a challenge for the methods both due to its severity and the multiplicity of complications. Regenerative medicine has focused on the use of low-level laser photobiomodulation therapy (LLLT) and adipose-derived stem cells (ADSC), especially in the early stages of the process, to promote better healing and shorter repair time. Therefore, aim of this study was to evaluate the action of LLLT (660 nm) and ADSC in the repair process of burned skin tissue; and investigate the association of techniques (LLLT and ADSC) in the treatment of full-thickness burns.

Materials and methods: An in vivo study was carried out using 96 rats (Wister) and a burn model by scalding with water at a temperature of 95°C and exposing the animal's back for 14 s. Animals were randomized into seven groups and three biopsy moments, five, 14 and 21 days: GC: Control group. ADSC-: Group treated with negative CD49d cells. ADSC+ : Group treated with positive CD49d cells. CULT: Group treated with conventional isolation cells. LLLT: Group treated only with LLLT Low Power Laser. ADSC-LLLT: Group treated with CD49d negative cells associated with LLLT. ADSC+LLLT: Group treated with positive CD49d cells associated with LLLT. The groups treated with LLLT (660 nm; 5 J/cm2) received irradiation three times a week, on alternate days for five, 14 and 21 days, according to the time of biopsy. ADSC-treated groups received one to three applications of the cells in a total volume of 1000 μL starting soon after the surgical debridement of the burn. Photographic

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monitoring was carried out at 5, 14 and 21 days after the beginning of the experiment to assess the degree of lesion contraction. Macroscopic, morphometric and histopathological analyzes were performed.

Results: This study showed significant re-epithelialization as well as an improvement in the healing process of the ADSC+ , LLLT and ADSC+LLLT groups. It was possible to observe effects in the reduction of the inflammatory phase, increase in angiogenesis, decrease in edema, greater collagen deposition, better organization of the extracellular matrix compared to the other treatments. Moreover, the immunomagnetic separation of ADSC cells through the expression of the CD49d protein proved to be a viable way to obtain a more homogeneous population of cells with a fundamental role in tissue regeneration compared to the ADSC- and CULT groups.

Conclusion: In conclusion, the association of ADSC+ with LLLT was effective in accelerating the burn repair process, in order to stimulate cell proliferation and help the formation of quality skin tissue and quality skin tissue

1. Introduction

Injuries caused by full-thickness burns are considered a severe form of damage to biological tissue, capable of destroying various structures and layers of the skin and adjacent tissues, causing local and systemic damage to the body [1,2]. Conventional treatment for a full-thickness burn wound aims to prevent infectious processes and remove necrotic tissue [3]. Although this type of treatment has proved to be efficient, there is also an increase in the number of individuals who start to present severe motor dysfunctions and psycho-emotional and social problems because of scar contractures and resulting aesthetic deformities [4]. Therefore, new therapeutic methods have been studied to promote a more adequate recovery and with better results in the treatment of tissues damaged by burns [5]. Currently, adipose tissue has been used in autologous grafts in the treatment of traumatic injuries, due to its endocrine and immunoregulatory activity [6]. The fractionation of cellular components of adipose tissue by enzymatic digestion and subsequent centrifugation of the material raised new possibilities for the use of this tissue in regenerative therapy and allowed the advance in the use of mesenchymal stem cells of name adipose-derived stem cells (ADSC) to obtain better results in repair of injured tissues [7–10]. However, traditional isolation methods are nonspecific, making the culture with 40–20% heterogeneity, which can compromise its proliferation and differentiation potential [9]. Use of stem cell populations in tissue engineering define that the ideal population is obtained as the well-standardized cell population, the selection of cells by means of specific surface proteins can allow isolation and expansion in vitro with a degree of higher specificity [6,7]. Therefore, studies have characterized the ADSC population according to its pattern of expression of adhesion molecules, being CD49d protein is a marker capable of differentiating ADSC from bone marrow-derived stem cells (BMMSC) [11–13]. In addition, the application of biostimulating agents with cell therapy has been a promising and less cost alternative for increasing the viability, proliferative potential, differentiation and morphofunctional organization of ADSC cells [14,15]. Low-level laser (LLLT) has been associated with the effectiveness of the progression of regenerative processes, as it seems to exert important modulatory effects on the inflammatory response in the early stages of tissue repair [1]. The use of LLLT as a biostimulating agent in association with mesenchymal stem cells (MSC) seems to provide better results in the healing of injured tissues, as they have a high capacity to induce cell proliferation and differentiation [5]. Therefore, the development of regenerative medicine, using ADSC associated with stimulation by LLLT, is a promising therapy, capable of improving the quality of healing of extensive lesions and with reduced cost, by reducing treatment time and lower risk of secondary contamination [16]. Considering the benefits that therapies can provide for the tissue repair process, further studies to better elucidate the risks and benefits of ADSC therapy alone and associated with LLLT in the treatment of burn wounds are necessary. Study aim was analyze the effects of using the culture of stem cells derived-adipose (ADSC) obtained by the traditional method and immunomagnetically separated by the expression of the CD49d protein, when applied alone or associated with low-level laser (LLLT) in the healing process in rat full-thickness burn wound models.

2. Material and methods

This study was submitted and approved for Ethics Committee on the use of animals (CEUA) of UFG. 105 female Wistar rats (Ratus norvegicus albinus) were used, weighing between 250 and 300 g, kept in three animals per cage, with food based on food and water. ad libitum. Bed changes were performed two to three times a week, using autoclaved shavings. The experimental design was completely randomized, with seven groups, consisting of five repetitions each, in which material was collected for microscopic analysis at three biopsy moments: 5, 14 and 21 days after induction of the lesion. The groups were organized as follows: 1. CG: control group; 2. ADSC+: group treated with injection of CD49d-positive adipose tissue-derived stem cells into the lesions; 3. ADSC-: group treated with injection of CD49d-negative ADSC into the lesions. 4. ADSC+LLLT: group treated with CD49 + ADSC injection and LLLT irradiation in the lesions; 5.

ADSC-LLLT: group treated with injection of ADSC CD49- and irradiation of LLLT in the lesions; 6. LLLT: group treated with LLLT irradiation in the lesions. 7. CULT: ADSC treated group isolated by conventional cultivation. According to the guidelines animals received pre-anesthetic, anesthetic solution and analgesic agent as described by Kawano et al. [17]. Induction of thermal injury was through contact with water at 95 °C for 14 s of one area of 1 cm [2] per animals' back epilated region (Figure S1).

After 24 h of lesion induction, surgical debridement was performed (Figure S1), and the animals was treated. Adipose-derived stem cells was obtained from adipose tissue the ventral flank region in six donor rats after euthanized with an overdose of Thiopental (100 mg/Kg). A part of the cell pellet obtained through enzymatic digestion with collagenase type I and cell centrifugation, according to Martins et al. [14] (2011) was used for cell separation by immunostaining with CD49d positive magnetic microbeads, using magnetic microbeads initially labeled with the primary antibody Rat CD49d (ITGA4 Monoclonal Antibody – ThermoFisher) and Anti-Mouse IgG1 MicroBeads (MACS®, Miltenyi Biotech) as a secondary antibody following the manufacturer's recommendations and cultured and expanded in 75 cm2 bottles with cell culture medium (Dulbecco's Modified Eagle Medium, 10% fetal bovine serum and 2% antibiotic and antimycotic solution ([10,000 IU/mL penicillin, 10,000 μg/mL streptomycin, 25 μg/mL amphotericin]) and a humidified oven at 5% CO2 at 37°C, until reaching the cellular confluence period of 75%. An aliquot of 1×10^6 cells from each bottle was destined for cultivation for cell differentiation in the chondrogenic, osteogenic and adipogenic lines to confirm the mesenchymal stem cell lineage. Another part of cells from adipose tissue was grown in a culture flask for stem cell separation by means of its adhesive properties to plastic, according to Atalay et al. [7] (2014).

Stem cells isolated, at a concentration of 1 × 106 cells/mL and diluted in PBS (phosphate buffer saline), were injected intradermally at four equidistant points around the lesions and directly onto the wound bed (0.2 mL per point). For LLLT applications, a Physiolux Dual P. 5040 laser device (660 nm wavelength, 30 mW power, BIOSET Equipment) was used. Applications of LLLT began 24 h after the induction of the lesions and were performed daily, in a punctual manner, in four equidistant applications of 1 cm at the edges of the wound and one application in the central bed of the wound, with 1 Joule/cm2 at each point, whose application time was approximately 3 s per point. Thus, the energy deposited in the bed and at each point of the wound edge was 1 Joule, totaling 5 Joules of energy with a fluency of 450 Joules/cm2 total deposited in the lesion.

On the same days of LLLT applications, the lesions were macroscopically evaluated for: inflammation, necrosis, granulation tissue formation, degree of epithelium formation and scar evolution; and analyzed in scores from 0 to 3 (0-absent, 1- mild; 2- moderate; 3- severe), according to Lamaro et al. [18] (2019). To measure the area of the wounds, the macroscopic images of the lesions were analyzed using the semi-automatic software Image Processing and Analysis in Java, version 1.44 (Image J, NIH, USA). For microscopic evaluations, the groups underwent four biopsy moments: 5, 14, and 21 days after lesion induction. The material was evaluated by Hematoxylin and Eosin (HE) and Gomori's Trichomic (TG) techniques observing intensity of vascular and fibroblast proliferation, degree of tissue necrosis, crust formation, edema, type and intensity of cell infiltrate (polymorphonuclear or mononuclear), epithelium formation (evaluated regarding the extension, thickness of the epithelial layer and keratinization), formation of granulation tissue with the reorganization of the extracellular matrix of the dermis and the presence of hair follicles and sebaceous glands. The slides submitted to TG staining were evaluated for the presence and/or absence of collagen, formation of granulation tissue, reorganization of the extracellular matrix of the dermis with differentiation into superficial (loose connective tissue with light blue staining) and deep (connective tissue dense unpatterned with dark blue coloration) and the presence of hair follicles and sebaceous glands. For the microscopic parameters of HE and TG, scores from zero to four were established, as follows: score 0 (absent); score 1 (up to 25% of the fragment); score 2 (between 25% and 50% of the fragment); score 3 (between 50% and 75% of the fragment); and score 4 (from 75% to 100% of the fragment), according to Lamaro et al. (2019). Morphometric data were submitted to Tukey's test and analysis of variance (ANOVA). Histopathologically, the variables in HE was analyzed using the Kruskal-Wallis test (non-parametric). The TG histopathological variables were analyzed using the Tukey-Anova test and the T-Paired test. The significance level adopted for all analyzes was 5% significance ($p < 0.05$), using Sigma Stat 2.3 software Statistic.

3. Results

Macroscopically, the groups treated with LLLT, ADSC CD49d +, ADSC CD49d- and CULT showed a significant reduction ($p < 0.05$) in the lesion after 14 days, with emphasis on the ADSC CD49d+ group (98%) (Table 1). After 21 days injury induction, it was possible to observe that the CULT group (100%) had a significantly ($p = 0.017$) smaller lesion area (Fig. 1). These results were followed by the groups that received the application of LLLT associated with positive and negative ADSC CD49d, as well as only the application of LLLT (Figs. 1 and 2).

Greater epithelial thickening with wound retraction and epithelial formation as demonstrated after 14 days of injury induction in the group ADSC CD49d- associated with LLLT ($p < 0.01$) (Fig. 3A). Moreover, degree of re-epithelialization was significantly ($p < 0.01$) lower after 21 days on the group treated with stem cells isolated by the conventional method. LLLT group showed highest epithelium formation in the same period. Similarly, epithelium formation was observed from 14th day on in most treatments, except in the ADSC-LLLT and ADSC- groups. LLLT, ADSC+LLLT and ADSC+, presented greater layer thickness and epithelium extension in relation to the others groups in the remodeling phase (Fig. 3B).

Histopathologically (HE) results showed regarding necrosis which was a significant reduction throughout the healing process in the GC ($p = 0.040$), ADSC+ LLLT ($p = 0.040$) and ADSC- ($p = 0.024$) groups (Table 1).

Table 1 – Histopathological evaluations by HE staining of the control and treated groups on days 5, 14 and 21 after burn injury in rats.

		CONTROL	LLLT	ADSC+LLLT	ADSC-LLLT	ADSC-	ADSC+	CULT	p
NECROSIS	5 days	1,0 ± 0 (1)^{a}	0,33 ± 0,33 (0)	2 ± 0,58(0)^{a}	2,5 ± 0,50 (2)	1,75 ± 0,25 (2,5)^{a}	0,75 ± 0,75 (0)	2,0 ± 0,99 (1)	0113
	14 days	0,2 ± 0,20 (0)^{b}	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)^{b}	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)^{b}	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)	0757
	21 days	0 ± 0 (0)^{b}	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)^{b}	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)^{b}	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)	1000
	p	**0040** *	0791	**0040** *	0,0137	**0024** *	0848	0071	
CRUST	5 days	0,75 ± 0,75 (0)	0,33 ± 0,33(0)	2,37 ± 1,33(4)	0,5 ± 0,50 (0,5)	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)	1,67 ± 1,2 (1)	0231
	14 days	1,4 ± 0,68 (1)	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)	2,33 ± 1,2 (3)	2,0 ± 2,0 (2)	0,5 ± 0,5 (0,5)	0,0 ± 0,0 (0)	0204
	21 days	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)	1000
	p	0390	0791	0281	0294	0627	0651	0361	
EDEMA	5 days	3,0 ± 0,0 (3)^{Aba}	0,0 ± 0,0 (0)^{A}	2,0 ± 0,58 (2)^{Aba}	1,0 ± 1,0 (1)^{AB}	3,0 ± 0,58 (3)^{Aba}	1,5 ± 0,5 (1)^{AB}	3,33 ± 0,33^{B}	**0022** *
	14 days	1,4 ± 0,51 (1)^{ABb}	0,0 ± 0,0 (0)^{AB}	0,0 ± 0,0 (0)^{Ab}	1,67 ± 0,33 (2)^{B}	1,5 ± 0,5 (1)^{ABb}	1 ± 0,0 (1)^{AB}	0,33 ± 0,33^{AB}	**0026** *
	21 days	0,0 ± 0,0 (0)^{b}	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)^{b}	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)	0,25 ± 0,25 (0)	0,33 ± 0,33	0479
	p	**0007** **	1000	**0040** *	0105	**0011** *	0125	0071	
BLEEDING	5 days	3,25 ± 0,25 (3)^{a}	2,33 ± 0,67 (3)^{a}	1,67 ± 1,20 (1)	1,5 ± 0,5 (1,5)	1,75 ± 0,75 (2)	1,75 ± 0,48 (1,5)^{a}	1,33 ± 0,33 (1)	0373
	14 days	0,2 ± 0,2(0)^{b}	0,33 ± 0,33 (0)^{b}	0,0 ± 0,0 (0)	1,0 ± 0,0 (1)	0,5 ± 0,5 (0,5)	0,0 ± 0,0 (0)^{b}	0,33 ± 0,33 (0)	0152
	21 days	0,0 ± 0,0 (0)^{b}	0,0 ± 0,0 (0)^{b}	0,0 ± 0,0 (0)	1 ± 0,0 (1)	0,67 ± 0,33 (1)	0,0 ± 0,0 (0)^{b}	0,67 ± 0,33 (1)	0099
	p	**0009** **	**0007** **	0281	0457	0384	**0020** *	0178	
PMN INFILTRETE	5 days	0,0 ± 0,0 (0)^{A}	1,0 ± 0,0 (1)^{AB}	0,33 ± 0,33 (0)^{AB}	0,0 ± 0,0 (0)^{A}	0,25 ± 0,25 (0)^{AB}	1,5 ± 0,29 (1,5)^{Ba}	1,33 ± 0,33 (1)^{B}	**0010** *
	14 days	0,4 ± 0,24 (0)^{AB}	0,0 ± 0,0 (0)^{A}	0,0 ± 0,0 (0)^{A}	0,67 ± 0,33 (1)^{AB}	0,0 ± 0,0 (0)^{AB}	0,0 ± 0,0 (0)^{ABb}	1,0 ± 0,0 (1)^{B}	**0047** *
	21 days	1 ± 0,0 (1)	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)^{Ab}	0,33 ± 0,33 (0)	**0004** *
	p	0091	0050	0742	0346	0844	**0020** *	0254	
MN INFILTRETE	5 days	4,0 ± 0,0 (4)^{a}	4,0 ± 0,0 (4)^{a}	4,0 ± 0,0 (4)^{a}	4,0 ± 0,0 (4)^{a}	3,75 ± 0,25 (4)^{a}	3,75 ± 0,25 (4)^{a}	4,0 ± 0,0 (4)	0686
	14 days	3,0 ± 0,0 (3)^{ab}	2,67 ± 0,33 (3)^{ab}	1,5 ± 0,29 (1,5)^{ab}	3,0 ± 0,0 (3)^{ab}	2,5 ± 0,5 (2,5)^{ab}	3,0 ± 0,0 (3)^{a}	2,33 ± 0,88 (2)	0093
	21 days	2,0 ± 0,0 (2)^{ABb}	2,0 ± 0,41 (2)^{ABb}	1,0 ± 0,0 (1)^{Ab}	1,0 ± 0,0 (1)^{Ab}	2,33 ± 0,33 (0,88)^{Bb}	1,5 ± 0,29 (1,5)^{ABb}	2,67 ± 0,67 (2)	**0013** *
	p	**< 0001** *	**0011** *	**0018** *	**0005** *	**0033** *	**0001** *	0228	
FIBROBLAST	5 days	0,0 ± 0,0 (0)^{a}	1,0 ± 0,0 (1)^{a}	1,0 ± 0,58 (1)	1,0 ± 0,0 (1)^{a}	0,5 ± 0,29 (0,5)^{a}	1,0 ± 0,41^{a}	0,0 ± 0,0 (0)^{a}	0074
	14 days	1,6 ± 0,24 (2)^{ABab}	2,67 ± 0,33 (3)^{Aab}	2,75 ± 0,75 (3)^{Bb}	2,68 ± 0,35 (3)^{Bab}	0,0 ± 0,0 (0)^{Aa}	3,0 ± 0,0 (3)^{Bab}	2,68 ± 0,88 (3)^{Bb}	**0047** *
	21 days	2,67 ± 0,33 (3)^{Ab}	3,75 ± 0,25 (4)^{b}	4,0 ± 0,0 (4)^{B}	3,5 ± 0,29 (3,5)^{ABb}	3,68 ± 0,33 (4)^{ABb}	4,0 ± 0,0 (4)^{Bb}	2,33 ± 0,33 (2)^{Ab}	**0013** *
	p	**< 0001** *	**0004** *	0061	**0046** *	**0030** *	**0002** *	**0026** *	
GRANULATION TISSUE	5 days	0,0 ± 0,0 (0)^{a}	0,0 ± 0,0 (0)^{a}	0,0 ± 0,0 (0)^{a}	0,0 ± 0,0 (0)^{a}	0,0 ± 0,0 (0)	0,0 ± 0,0 (0)^{a}	0,0 ± 0,0 (0)	1000
	14 days	1,8 ± 0,58 (2)^{ABab}	0,33 ± 0,33 (0)^{Aa}	3,25 ± 0,48 (3)^{Bb}	0,0 ± 0,0 (0)^{Aa}	0,5 ± 0,5 (0,5)^{A}	2,0 ± 1,0 (2)^{ABab}	1,0 ± 0,58 (1)^{AB}	**0008** *
	21 days	3,67 ± 0,33 (4)^{ABb}	4,0 ± 0,0 (4)^{Bb}	3,5 ± 0,29 (3,5)^{ABb}	2,0 ± 0,71 (1,5)^{ABb}	2,0 ± 1,15 (2)^{AB}	4,0 ± 0,0 (4)^{Bb}	0,33 ± 0,33 (0)^{A}	**0019** *
	p	0060	**0013** *	**< 0001** *	**0024**	0294	**0002** *	0127	
ANGIOGENESIS	5 days	3,25 ± 0,75 (4)	4,0 ± 0,0 (4)^{a}	2,68 ± 0,88 (3)	4,0 ± 0,0 (4)^{a}	3,25 ± 0,75 (4)	3,5 ± 0,29 (3,5)^{a}	0,33 ± 0,33 (0)	0074
	14 days	3,2 ± 0,58 (4)^{B}	2,33 ± 0,33 (2)^{ABb}	0,75 ± 0,25 (1)^{A}	2,0 ± 0,58 (2)^{ABb}	3,0 ± 0,0 (3)^{B}	1,5 ± 0,5 (1)^{ABb}	1,33 ± 0,33 (0)^{AB}	**0049** *
	21 days	0,67 ± 0,33 (1)	1,0 ± 0,0 (1)^{b}	0,5 ± 0,29 (0,5)	0,75 ± 0,25 (1)^{b}	0,67 ± 0,33 (1)	0,75 ± 0,48 (0,5)^{b}	0,0 ± 0,0 (0)	0365
	p	0099	**0001** *	0135	**0004** *	0086	**0004** *	0100	
REPITELIZATION	5 days	0,0 ± 0,0 (0)^{a}	0,0 ± 0,0 (0)^{a}	0,0 ± 0,0 (0)^{a}	0,0 ± 0,0 (0)^{a}	0,0 ± 0,0 (0)^{a}	0,0 ± 0,0 (0)^{a}	0,0 ± 0,0 (0)	1000
	14 days	2,0 ± 0,63 (3)^{ABab}	0,33 ± 0,33 (0)^{Aab}	4,0 ± 0,0 (4)^{Bb}	0,0 ± 0,0 (0)^{Aa}	0,0 ± 0,0 (0)^{A}	2,0 ± 2,0 (2)^{ABab}	2,67 ± 1,33 (4)^{AB}	**0042** *
	21 days	3,67 ± 0,33 (4)^{ABb}	4,0 ± 0,0 (4)^{Bb}	4,0 ± 0,0 (4)^{Bb}	3,75 ± 0,25 (4)^{ABb}	3,33 ± 0,33 (3)^{AB}	4,0 ± 0,0 (4)^{Bb}	3,0 ± 0,0 (3)^{A}	**0025** *
	p	**0007** *	**0013** *	**0040** *	**0024** *	0051	0046	0168	

Note. Mode, median and standard error values of the mean scores (0–4): 0: absent; score 1: up to 25%; score 2: between 25% and 50%; score 3: between 51% and 75%; and score 4: from 76% to 100%. PNM: Polymorphonuclear; MN: Mononuclear. Different lowercase letters in the same column, for the same parameter, indicate the existence of a significant difference between groups by the Kruskal-wallis test ($p < 0.05$)*. Different capital letters in the same line indicate the existence of a significant difference between the days in the group, by the Kruskal-wallis test ($p < 0.05$).

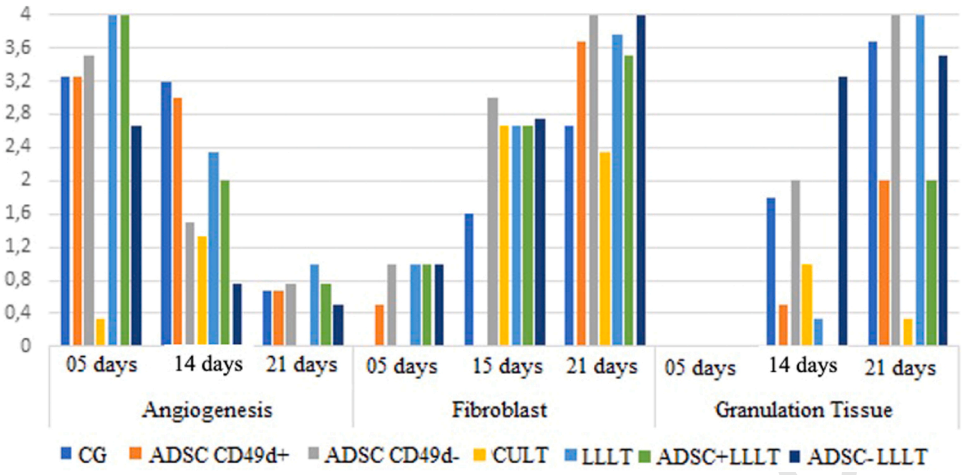

Fig. 1 – Area size of full-thickness burn wounds. Percentage decrease in area size of full-thickness burn wounds in rats at different stages of the healing process (5, 15 and 21 days). *CULT group had a significantly (p = 0.017) smaller lesion area of time 21 days in relation to the other treatments. ADSC-: CD49d Cell Group Negative; ADSC+ : CD49d Positive cell group; CULT: Group of cells without immunomagnetic isolation; LLLT: group treated with Low Power Laser.

LLLT group showed a significant swelling decrease after 5 days of injury (p = 0.022) follow by ADSC+LLLT and CULT (p = 0.026). On the 21st day after the injury, only the ADSC+ and CULT groups still showed some edema (Fig. 4). Inflammatory infiltrates presented a significant reduction polymorphonuclear (PMN) in the number of cells in most groups (5 days: p = 0.010; 15 days: p = 0.047; 21 days: p = 0.004). During the proliferative phase, at 14 days, there was a significant reduction (p = 0.020) of this inflammatory infiltrate in the ADSC+ group, and GC group showed a progressive increase in the number of polymorphonuclear cells until the wound remodeling phase, suggesting a possible modulation of PNM by the different treatments. Moreover, significantly reduction quantity of mononuclear (MN) infiltrate was observed in all groups throughout the healing process, however, at 21 days, there was a significant decrease (p = 0.004) in the ADSC+LLLT and ADSC-LLLT groups (Fig. 5).

Fibroblasts' pattern was similar in all groups, with a significant increase in the number of cells after the fifth day of injury, except for the ADSC- group. On the 14th day the groups treated with CD49 + cells (ADSC+ and ADSC+LLLT) has greater number of fibroblasts. Progressively, at 21 days, both groups maintained an increase in the number of fibroblasts, while there was a reduction in this number in the CULT group (Fig. 6). Granulation tissue, there was a progressive and significant increase in most groups, except in GC and CULT. After 14 days of injury induction, granulation tissue was significantly more developed in the ADSC+LLLT and ADSC+ groups, while at 21 days the LLLT group was more expressive (Fig. 6). Moreover, angiogenesis there was an increase in the formation of new vessels until the fifth day, mainly in the LLLT, ADSC-LLLT and ADSC+ groups, with a significant decrease in these groups after this period. On the 14th day after the injury, the CG still showed a significant amount of newly formed vessels (Fig. 6).

Microscopically the groups that received laser irradiation showed greater angiogenesis, decreased edema, greater infiltration of mononuclear cells and increased number of fibroblasts at the beginning of tissue repair, in addition to the connective tissue showing better organization and formation of epithelial attachments at 21 days after injury (Fig. 8 - G to I).

When comparing the ADSC+ and ADSC- groups (Fig. 7 – D to I) with ADSC+LLLT and ADSC-LLLT (Fig. 8 – D to I), it is observed that the first two presented a slower healing process, so that at 21 days it was still possible to observe granulation tissue, as well as MN inflammatory infiltrate. In addition, the groups treated with ADSC+ and ADSC- cells that received LLLT irradiation demonstrate a significant increase in angiogenesis and MN cells during the inflammatory phase (Fig. 7 – D to I). Similarly, the CULT group showed efficient wound repair with a high rate of angiogenesis and re-epithelialization from 14 days after injury induction (Fig. 7 - J, K, L). In relation to granulation tissue, a significant expression ($p < 0.001$) was initially observed in the LLLT and ADSC-LLLT groups, but with a statistically significant decrease ($p < 0.05$) in most treatments at 14 and 21 days. Likewise, a better matrix organization was presented at 5 days after lesion induction, by the LLLT, ADSC+LLLT and ADSC+ groups (p = 0.010). This result remained statistically significant at 21 days, although there was a progressive improvement in matrix organization in all groups, after lesion induction, these results were significantly more evident in the LLLT, ADSC+LLLT and ADSC+ groups (Table 2).

Furthermore, the analysis of slides submitted to the Trichrome of Gomori (TG) staining technique showed differences between the treated and control groups (Table 2). Although there was no statistical difference between the groups in relation to the number of fibroblasts at the beginning of the healing process, at 5 days after the injury it is possible to observe an increase in these cells in the LLLT and ADSC- groups followed by a significant decrease ($p < 0.05$). Organization of extracellular matrix as well as the formation and maturation of collagen (Figs. 9 and 10) was observed. Therefore, it was observed in the control group (CG) at 5 days after induction of the lesion (Fig. 9 A) intense edema with disorganized extracellular matrix and a large amount of infiltrate. In this same group, at 14 days there was little evolution in the healing process, in which the epithelium was not intact, as well as the edema and granulation tissue

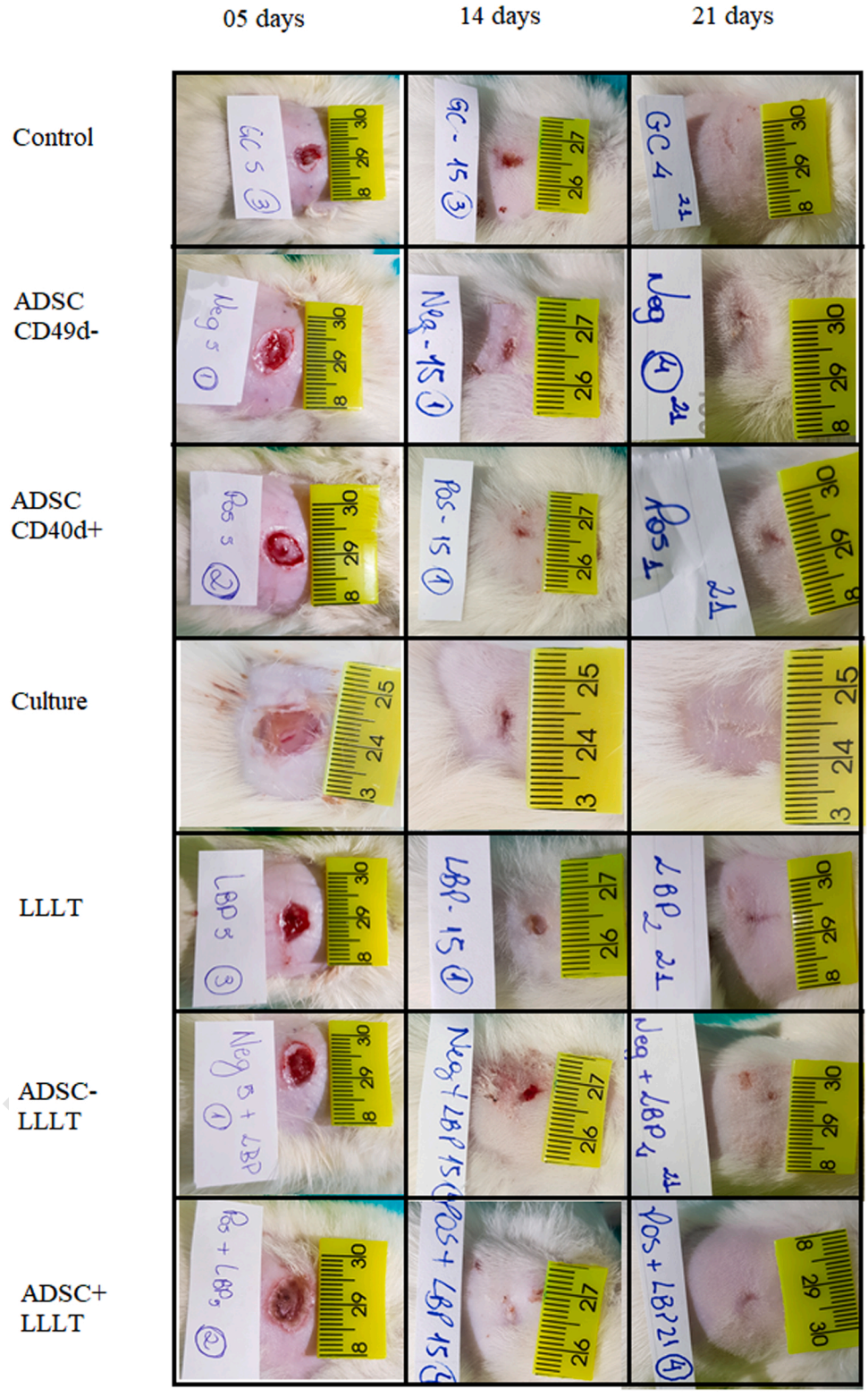

Fig. 2 – Full-thickness burn lesions on the day of the biopsy in each group. A, B and C) Control group moment of biopsy 05, 14 and 21 days after the injury. D, E and F) Group treated with negative CD49d cells on the days of lesion induction, biopsy moment 05, 14 and days after the lesion. G, H and I) Group treated with positive CD49d cells on the days of lesion induction, biopsy moment 05, 14 and days after the lesion. J, K and L) Group treated with conventional isolation cells on the days of lesion induction, biopsy moment 05, 14 and days after the lesion. M, N and O) Group treated only with Low Power Laser (LLLT) on the days of lesion induction, biopsy moment 05, 14 and days after the lesion. P, Q and R) Group treated with CD49d negative cells associated with LLLT on the days of lesion induction, biopsy moment 05, 14 and days after the lesion. S, T and U) Group treated with positive CD49d cells associated with LLLT on the days of lesion induction, biopsy moment 05, 14 and days after the lesion.

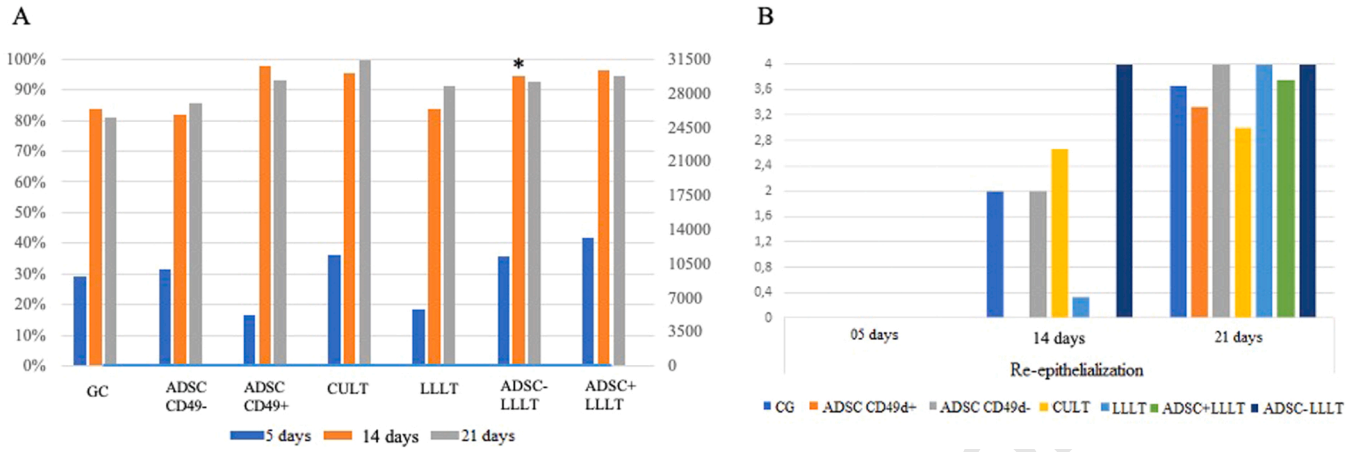

Fig. 3 – Wound retraction and re-epithelialization. A) Percentage of decrease in the size of the area of third-degree burn injuries in rats at different stages of the healing process (5, 15 and 21 days); *epithelial formation as demonstrated after 15 days of injury induction in the group ADSC CD49d- associated with LLLT ($p < 0.01$). B) Mean of histological re-epithelialization scores evaluated by HE staining in third-degree burn injuries in rats. Scores from 0 to 4 being: score 0: absent; score 1: up to 25%; score 2: between 25% and 50%; score 3: between 51% and 75%; and score 4: from 76% to 100%. Group control; NEG: Negative CD49d cell group; POS: Positive CD49d cell group; CULT: Group of cells without immunomagnetic isolation; LLLT: group treated with Low Power Laser.

remained intense (Fig. 9 B). After 21 days of injury, it was possible to observe complete re-epithelialization of the wound (Fig. 9 C). However, the deposition of collagen fibers was still immature, and the granulation tissue was not very evident (Fig. 9 C).

In summary, the groups treated with LLLT and ADSC+ associated or isolated showed a decrease in edema and bleeding. During the initial phase of the healing process (5 days) they showed an increase in inflammatory cells that were not seen in the late phase (21 days). During the proliferation phase (14 days) this groups had a greater number of fibroblasts and better granulation tissue, which contributed to a better organization of the extracellular matrix.

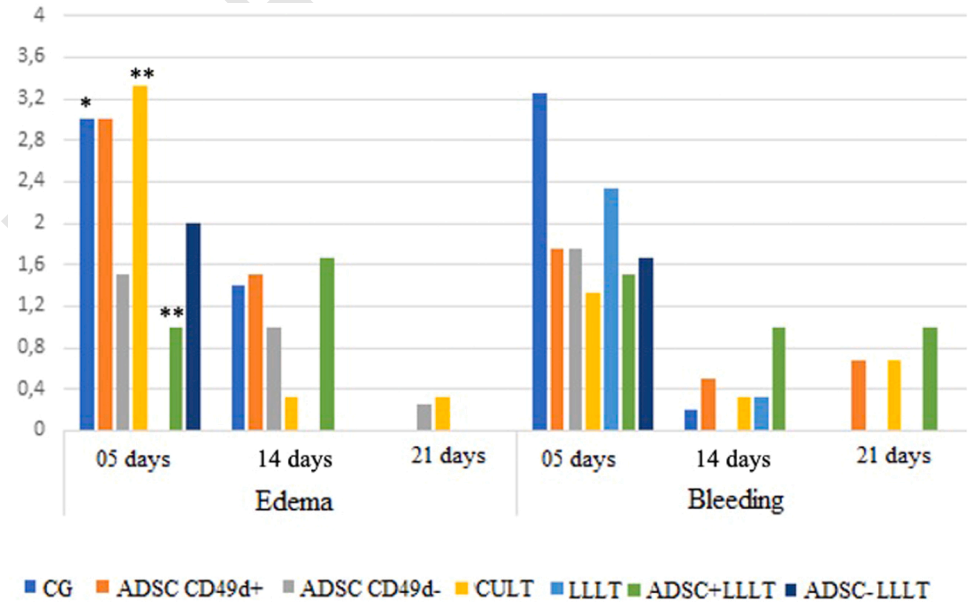

Fig. 4 – Mean histological swelling and bleeding scores assessed by HE staining in full-thickness burn injuries in rats.*LLLT showed a significant swelling decrease after 5 days of injury ($p = 0.022$) follow by **ADSC+LLLT and CULT ($p = 0.026$). Scores from 0 to 4 being: score 0: absent; score 1: up to 25%; score 2: between 25% and 50%; score 3: between 51% and 75%; and score 4: from 76% to 100%. Group control; NEG: Negative CD49d cell group; POS: Positive CD49d cell group; CULT: Group of cells without immunomagnetic isolation; LLLT: group treated with Low Power Laser.

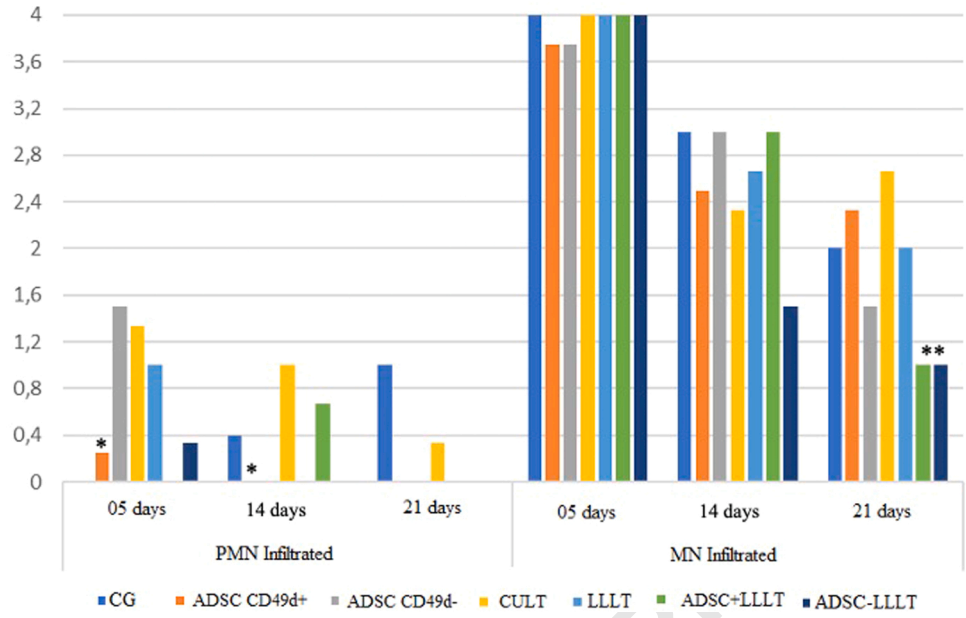

Fig. 5 – Mean histological scores of polymorphonuclear (PNM) and mononuclear (MN) infiltrates assessed by HE staining in full-thickness burn injuries in rats. * 15 days, there was a significant reduction (p = 0.020) of this inflammatory infiltrate PMN in the ADSC+ group. * *21 days, there was a significant decrease (p = 0.004) in the ADSC+LLLT and ADSC-LLLT groups for MN inflammatory cells. Scores from 0 to 4 being: score 0: absent; score 1: up to 25%; score 2: between 25% and 50%; score 3: between 51% and 75%; and score 4: from 76% to 100%. Group control; NEG: Negative CD49d cell group; POS: Positive CD49d cell group; CULT: Group of cells without immunomagnetic isolation; LLLT: group treated with Low Power Laser.

4. Discussion

Several studies demonstrate that laser photobiomodulation can alter cell metabolism, stimulating cell proliferation, making the healing process much more efficient [12,13,17]. In the present study, was observed that LLLT irradiation groups treated showed improvement in swollen and bleeding in the initial phase of the healing process, as well as an increase in polymorphonuclear inflammatory cells and angiogenesis, important elements during this phase. Similarly, Bayat et al. [18] and Novaes et al. [19] demonstrated that laser therapy can accelerate the initial phase of healing by promoting increased neovascularization in the injured tissue and inflammatory cells necessary for the release of growth factors and phagocytosis of cell debris. When checking the formation of scabs in the wounds, the results showed no difference between groups and days, probably due to

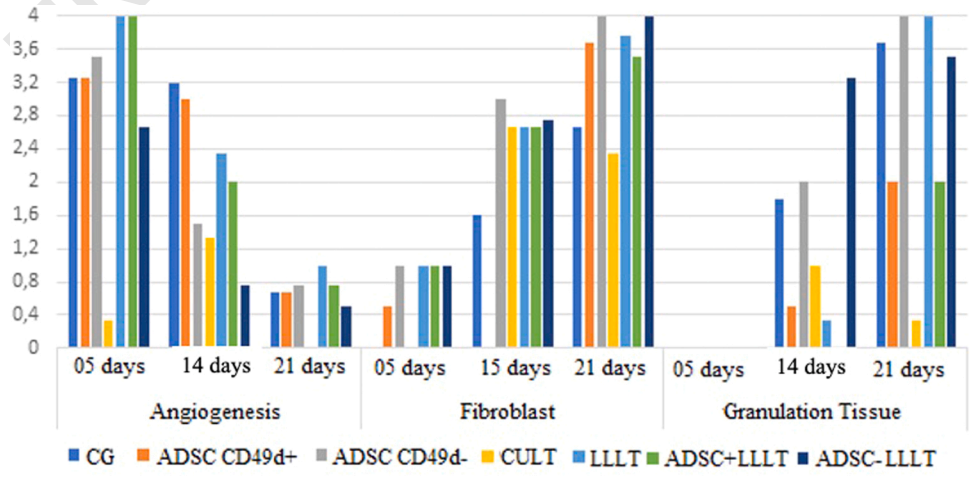

Fig. 6 – Mean histological scores of angiogenesis, fibroblasts, and granulation tissue assessed by HE staining in full-thickness burn injuries in rats. Scores from 0–4 being: score 0: absent; score 1: up to 25%; score 2: between 25% and 50%; score 3: between 51% and 75%; and score 4: from 76% to 100%. Group control; NEG: Negative CD49d cell group; POS: Positive CD49d cell group; CULT: Group of cells without immunomagnetic isolation; LLLT: group treated with Low Power Laser.

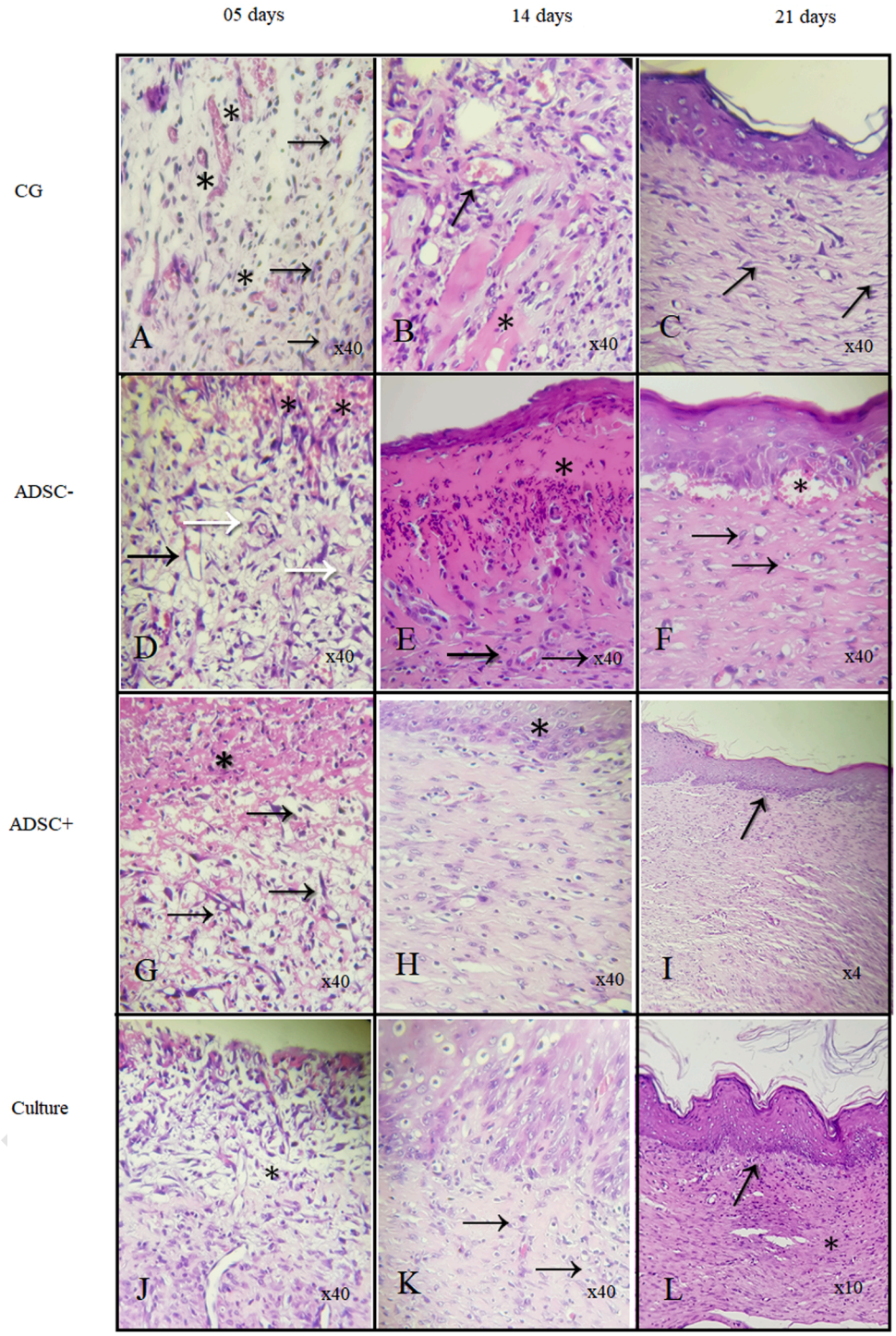

Fig. 7 – Photomicrograph of full-thickness burn injuries in rats by HE staining. A: GC 5 days, presence of neovessels (asterisk) and inflammatory infiltrate (arrows), 40x; B:GC 14 days, presence of blood vessels (arrow) and area of necrosis (asterisk), 40x; C: GC 21 days, presence of fibroblasts (arrow), 40x; D: ADSC- 5 days, there is an area with hemorrhage (asterisk), presence of angiogenesis (black arrows) and intense inflammatory infiltrate (white arrows), 40x; E:ADSC- 14 days, presence of crust (asterisk) and intense amount of inflammatory cells (arrows), 40x; F: ADSC group- 21 days, angiogenesis (asterisk) and intense infiltrate (arrows), 40x; G: ADSC+ 5 days, cells with a fusiform appearance (arrows) and moderate amount of inflammatory infiltrate (asterisk), 40x; H and I: ADSC+ 14 and 21 days with complete re-epithelialization (arrows), 40x and 4x respectively. J and K: CULT 5 and 14 days, moderate presence of PMN and MN is observed (asterisk and arrows), 40x; L: CULT 21 days, re-epithelialization (arrow), 10x.

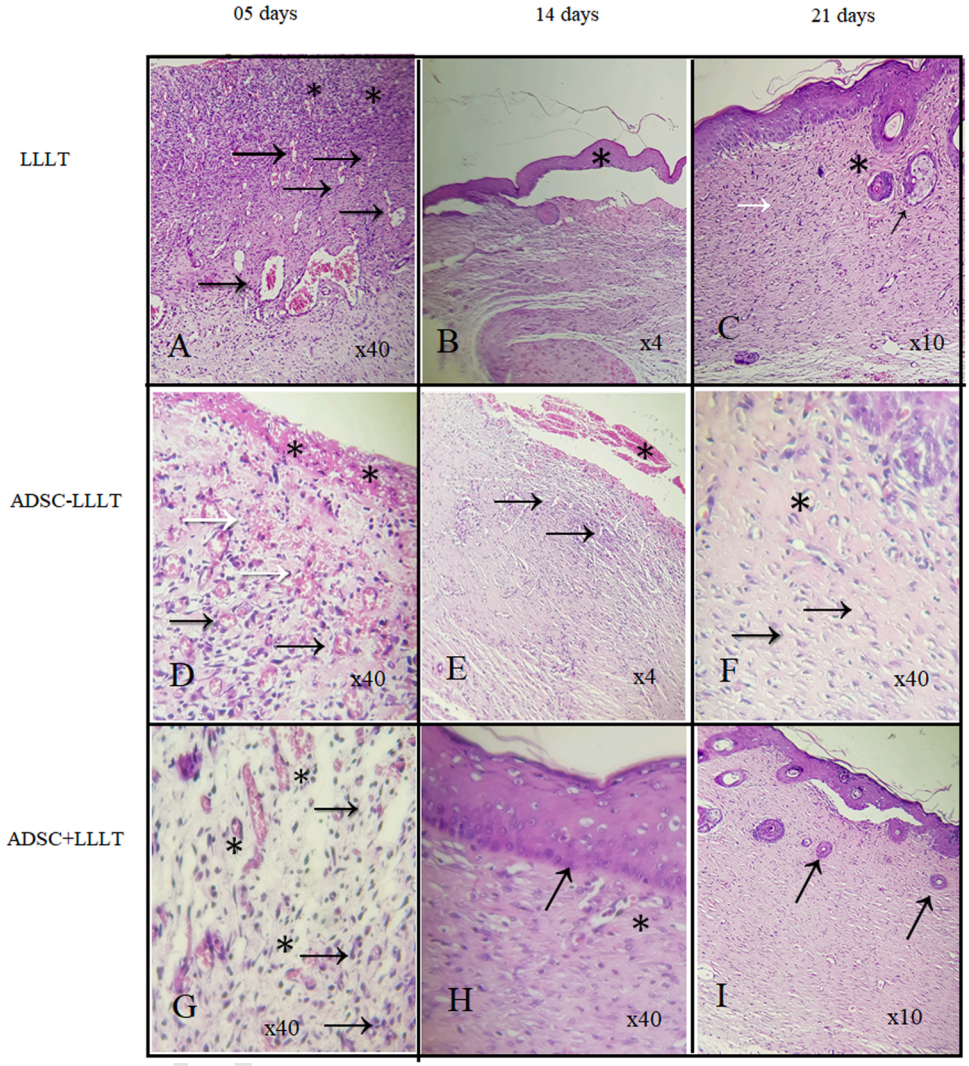

Fig. 8 – Photomicrograph of full-thickness burn injuries in rats by HE staining (continued). A: LLLT 5 days, presence of neovessels (arrows) and intense inflammatory infiltrate (asterisk), 40x; B: LLLT 14 days, complete re-epithelialization (asterisk), 4x; C: LLLT 21 days, presence of epithelial attachments (black arrow), good organization of the extracellular matrix (asterisk) and intense presence of fibroblasts (white arrow), 40x; D: ADSC-LLLT 5 days, area with hemorrhage (white arrows), crust (asterisk) and presence of angiogenesis (black arrows), 40x; E:ADSC-LLLT 14 days, presence of crust (asterisk) and edema (arrows), 4x; F: ADSC-LLLT group 21 days, moderate presence of fibroblasts (arrows) moderate organization of connective tissue (asterisk), 40x; G: ADSC+LLLT 5 days, intense angiogenesis (asterisks) and moderate amount of inflammatory infiltrate (arrows), 40x; H: ADSC+LLLT 14 days with complete re-epithelialization (arrow) and presence of neovessels (asterisk), 40x; I: ADSC+LLLT 21 days, the presence of epithelial attachments is observed, 10x.

the use of the Tegaderm™ dressing, which prevented dryness and formation of scabs in the wound during healing. Furthermore, as the formation of crust and edema is influenced by cellular exudative processes, a histologically greater formation of these events was observed in the groups that did not receive laser induction. This result may indicate that LLLT reduces cellular exudative processes, favoring the reduction of edema, combined with the fact of greater local angiogenesis. The increase in angiogenesis, in addition to anticipating the phases of the initial phase of inflammation, allows for greater tissue drainage and, consequently, a greater reduction in edema, in addition to contributing to local nutrition and oxygenation, favoring cell proliferation. Corroborating this, Brassolatti et al. [12], when evaluating the effects of LLLT (660 nm) at fluences of 12.5 J/cm^2 and 25 J/cm^2 (per point of application) in full-thickness burns in rats, reported that the laser was capable of to reduce the inflammatory phase and stimulate angiogenesis, thus restoring local microcirculation, essential for cell migration and proliferation. Furthermore, during the proliferative phase, a greater amount of granulation tissue followed by a more effective re-epithelialization was observed in the groups treated with LLLT alone or in association with stem cells. Some authors suggest that laser energy of a certain wavelength is absorbed by components of the mitochondrial respiratory chain, leading to an increase in reactive oxygen species (ROS) levels and ATP levels. This increases the expression of growth factors and cytokines,

Table 2 – Histopathological evaluations by Gomori's Trichrome staining of control and LLLT groups on days 5, 14 and 21 after burn injury in rats.

		CG	LLLT	ADSC+LLLT	ADSC-LLLT	ADSC-	ADSC+	CULT	p
Fibroblast	5 days	3,5 ± 0,5 (4)^{b}	4,0 ± 0,0 (4)^{b}	3,33 ± 0,68 (4)^{b}	2,0 ± 0,0 (2)^{ab}	4,0 ± 0,0 (4)^{b}	3,5 ± 0029^{b}	2,0 ± 0,0 (2)	0058
	14 days	2,4 ± 0,24 (2)^{ab}	2,0 ± 0,0 (2)^{ab}	2,33 ± 0,33 (2)^{ab}	2,67 ± 0,33 (3)^{b}	2,5 ± 0,5 (2,5)^{ab}	1,0 ± 0,0 (1)^{a}	2,0 ± 0,0 (2)	0109
	21 days	1,0 ± 0,0 (1)^{a}	1,0 ± 0,0 (1)^{a}	1,0 ± 0,0 (1)^{a}	1,25 ± 0,25 (1)^{a}	1,0 ± 0,0 (1)^{a}	1,0 ± 0,0 (1)^{a}	1,33 ± 0,33 (1)	0552
	p	**0021** *	**0011** *	**0003** *	**0017** *	**0005** *	**0020** *	0361	
Granulation Tissue	5 days	2,5 ± 0,64 (2,5)$^{\Lambda B}$	4,0 ± 0,0 (4)^{Ba}	3,68 ± 0,33 (4)^{Ba}	4,0 ± 0,0 (4)^{B}	3,5 ± 0,29 (3,5)^{Ba}	3,5 ± 0,29 (3,5)^{Ba}	1,0 ± 0,0 (1)$^{\Lambda}$	< 0001 *
	14 days	2,2 ± 0,20 (2)	2,33 ± 0,33 (2)^{ab}	2,68 ± 0,33 (3)^{ab}	1,68 ± 0,33 (2)	2,0 ± 0,0 (2)^{ab}	2,5 ± 0,5 (2,5)^{ab}	1,33 ± 0,33 (1)	0141
	21 days	1,0 ± 0,0 (1)	1,25 ± 0,25 (1)^{b}	1,8 ± 0,2 (2)^{b}	1,25 ± 0,63 (1)	1,0 ± 0,58 (1)^{b}	0,5 ± 0,29 (2)^{b}	2,0 ± 0,58 (2)	0227
	p	0180	**0004** *	**0012** *	**0064** *	**0008** *	**0004** *	0252	
Organization of the extracellular matrix	5 days	1,0 ± 0,0 (1)$^{\Lambda Ba}$	2,33 ± 0,33 (2)^{Ba}	2,0 ± 0,58 (2)^{Ba}	1,0 ± 0,0 (1)$^{\Lambda ba}$	0,5 ± 0,29 (0,5)$^{\Lambda a}$	2,0 ± 0,0 (2)^{Ba}	1,0 ± 0,0 (1)$^{\Lambda ba}$	**0010** *
	14 days	2,8 ± 0,2 (3)^{b}	3,67 ± 0,33 (4)^{b}	3,33 ± 0,33 (3)^{ab}	2,67 ± 0,33 (3)^{ab}	2,0 ± 0,0 (2)^{ab}	3,0 ± 0,0 (3)^{ab}	2,33 ± 0,33 (2)^{b}	0066
	21 days	2,5 ± 0,5 (2,5)$^{\Lambda ab}$	4,0 ± 0,0 (4)^{Bb}	3,8 ± 0,20 (4)^{Bb}	3,25 ± 0,25 (3)$^{\Lambda Bb}$	3,33 ± 0,33 (3)$^{\Lambda Bb}$	3,75 ± 0,25 (4)^{Bb}	2,0 ± 0,0 (2)$^{\Lambda ab}$	< 0001 *
	p	**0009** *	**0005** *	**0017** *	**0013** *	**0005** *	**0004** *	**0049** *	

Note. Median, standard error and mode values of scores (0–4): 0: absent; score 1: up to 25%; score 2: between 25% and 50%; score 3: between 51% and 75%; and score 4: from 76% to 100%. Different lowercase letters in the same column, for the same parameter, indicate the existence of a significant difference between groups by the Kruskal-wallis test ($p < 0.05$)*. Different capital letters in the same line indicate the existence of a significant difference between the days in the group, by the Kruskal-wallis test ($p < 0.05$)*

leading to cell proliferation and, consequently, greater formation of granulation tissue [20–22].

Histopathological analyzes showed that there was a significant reduction in necrosis scores up to 5 days after injury, while morphometric and macroscopic evaluations indicate more accelerated contraction of wounds in the group treated with conventionally grown ADSC (CULT) when observed Fig. 1. These data corroborate those of Singer et al.24, who, when using stem cells in the treatment of full-thickness burn injuries in rats, observed a significant reduction in necrosis in the unburned interspaces in a comb model. Therefore, studies have shown that adipose tissue has been used in the treatment of traumatic injuries, as it presents stem cell activity together with the synthesis of growth factors and cytokines [5,10,11].

In addition, the results presented demonstrate that the groups treated with ADSC in general showed better capacity for tissue regeneration, with a greater number of fibroblasts, granulation tissue and better re-epithelialization during the proliferative phase.

The mechanism of action by which ADSC modulate proliferation seems to be related to paracrine signaling, especially VEGF, which is the main mediator of angiogenesis and contributes greatly to tissue repair [23]. Lim and Yoo [15], for example, showed that the application of stem cells derived from adipose tissue in experimental cutaneous wound models in rats can accelerate wound closure, promote partial re-epithelialization and increase granulation tissue deposition. Similarly, Martins et al. [14] also demonstrated that ADSC have tissue repair potential equivalent to the regenerative capacity of dermal fibroblasts in the treatment of cutaneous wounds in rats [11].

In addition to the signaling activity of ADSC, studies indicate that the association with LLLT for the healing of skin lesions in athymic rats is capable of potentiating the healing process, increasing neovascularization, improving the regeneration of dermal annexes, reducing the number of apoptosis in the wound bed and increase the production of growth factors [19,25]. Effect of LLLT on several cultured cell lines, including ADSC, and reported that laser irradiation is capable of promoting an increase in cell proliferation, according to most studies. Similarly, in this study, the groups treated with LLLT associated with ADSC+ and ADSC- showed more satisfactory healing parameters compared to the other groups. When comparing the ADSC+ and ADSC-groups with ADSC+LLLT and ADSC-LLLT, the first two showed a slower healing process, so that, at 21 days, it was still possible to observe tissue granulation, as well as inflammatory infiltrate MN. In addition, ADSC+ and ADSC- treated groups that received LLLT irradiation demonstrate a significant increase in angiogenesis and MN cells during the inflammatory phase. These findings show that wounds treated with ADSC plus laser were at a more advanced stage of the tissue repair process compared to GC and other treatments.

Morphometrically, it was possible to observe a significant reduction in the lesion area in the groups treated with LLLT and ADSC, as well as a greater degree of re-epithelialization both at 14 and 21 days after lesion induction. In addition, microscopic analyzes showed that the LLLT and ADSC+LLLT groups presented greater angiogenesis, PNM and MN

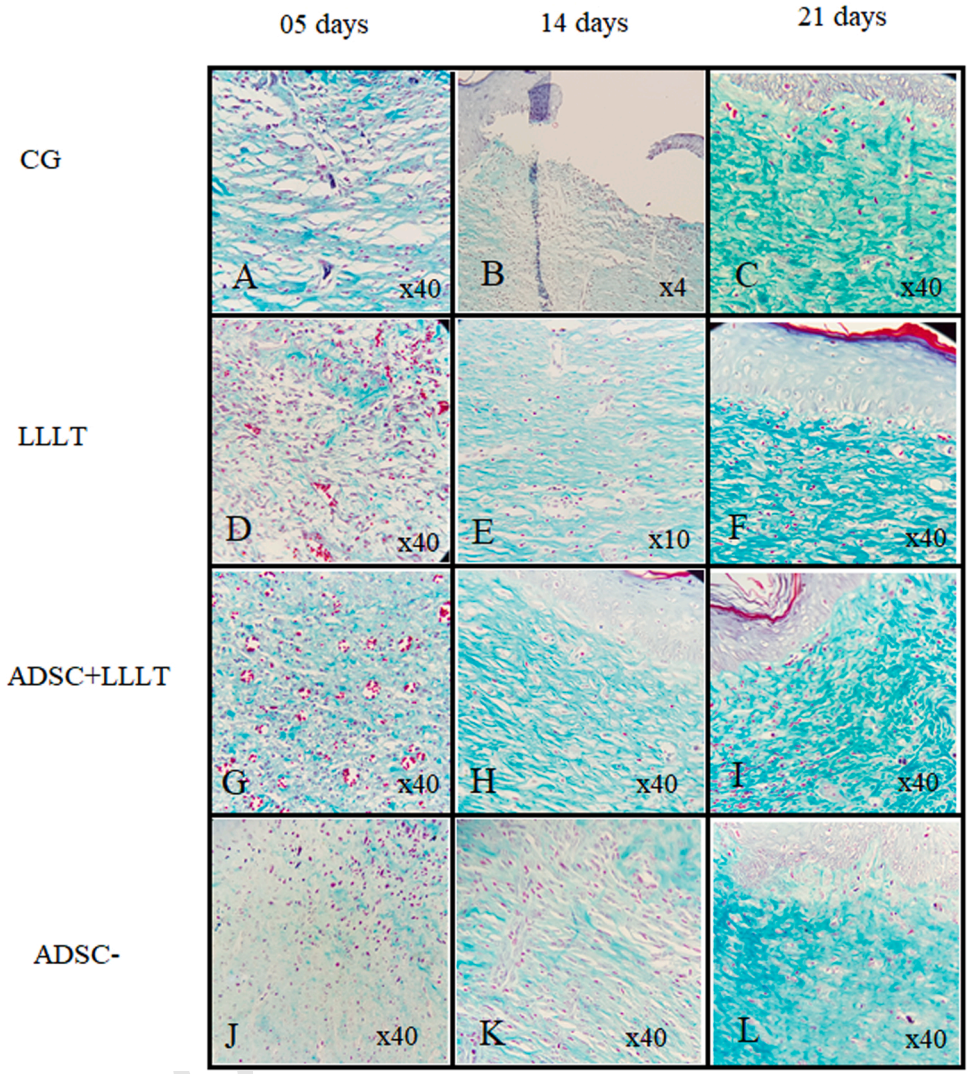

Fig. 9 – Histopathological photomicrograph of full-thickness burn injuries in rats, Staining: Gomori's Trichrome. A and B: GC 5 and 14 days, there is intense presence of edema (asterisk and arrow, respectively), 40x and 4x; C: CG 21 days, presence of inflammatory cells (arrow) and disorganized collagen deposition (asterisk), 40x; D: LLLT 5 days, intense area with angiogenesis (arrow), 40x; E:LLLT 14 days, little presence of inflammatory cells (arrow), 40x; F: LLLT group 21 days, complete re-epithelialization (arrow) and extracellular matrix with organized fibers (asterisk), 40x; G: ADSC+LLLT 5 days, intense angiogenesis is observed (arrows), 40x; H: ADSC+LLLT 14 days shows intense amount of collagen fibers (asterisk) and moderate amount of fibroblasts (arrow), 40x; I: ADSC+LLLT 21 days with complete re-epithelialization and presence of keratin (arrow) and mature collagen fibers with onset of superficial and deep dermis differentiation (asterisk), 40x. J and K: ADSC- 5 and 14 days, there is moderate presence of PMN and MN (arrow) with little collagen deposition and disorganized extracellular matrix (asterisk), 40x; L: ADSC- 21 days, complete re-epithelialization (arrow) with disorganized extracellular matrix, 10x.

inflammatory infiltrates and number of fibroblasts at the beginning of the healing process, evolving with an increase in the amount of granulation tissue at 14 days compared to the other treatments. A significantly progressive reduction in the number of inflammatory cells was observed in all groups throughout the healing process, however, at 21 days, there was a significant decrease in the LLLT, ADSC+LLLT and ADSC-LLLT groups, indicating a positive interaction between LLLT and ADSC in reducing the inflammatory process and improving healing. Corroborating this, several authors point out that the photobiomodulation of LLLT in the inflammatory process of skin burns in rats [14,27], reduces the number of infiltrates, contributing to cell proliferation, collagen deposition, reduction of mast cells in the late stages and accelerating the inflammation period. In addition, it was observed in the LLLT, ADSC+ and ADSC+LLLT groups, greater proliferation of fibroblasts and greater angiogenesis, which contributed to a better organization of the extracellular matrix with greater collagen deposition in these animals during the remodeling phase, as also observed by trichrome staining. from Gomori. A study suggests that low-intensity laser induces mitotic activity in fibroblasts by stimulating the synthesis of basic fibroblast growth factor (FGFb), a multifunctional polypeptide capable of inducing proliferation and

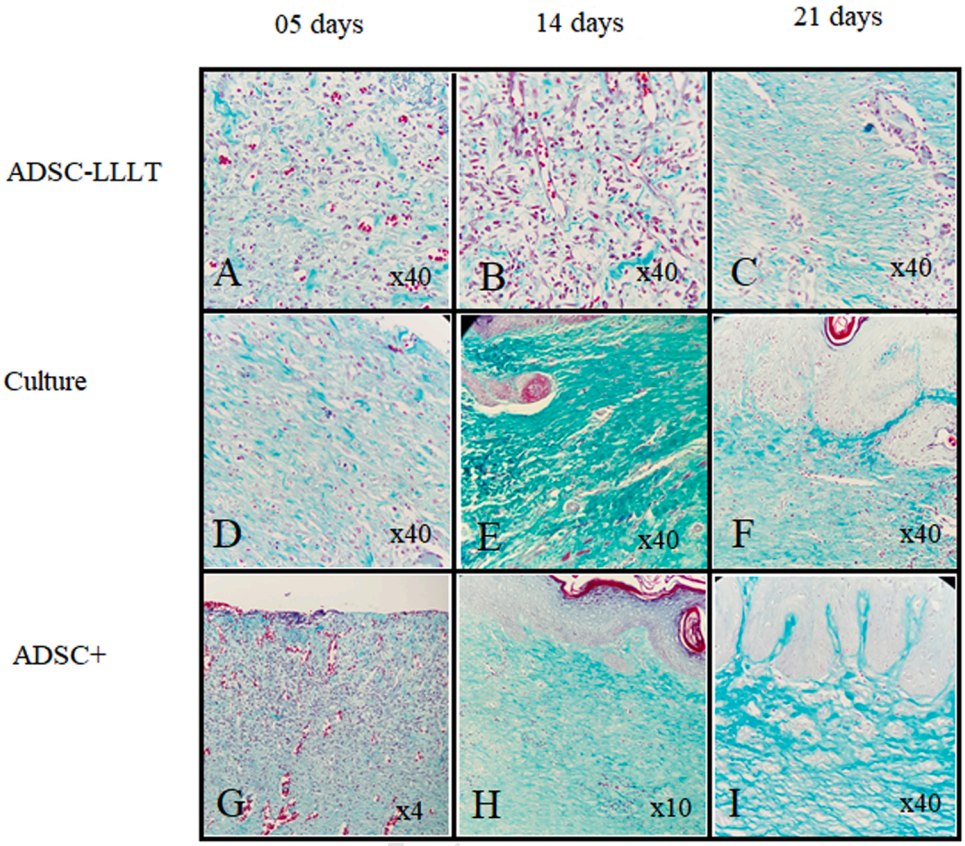

Fig. 10 – Histopathological photomicrograph of full-thickness burn injuries in rats. Staining: Gomori's Trichrome (continued). A: ADSC-LLLT 5 days, the presence of neovessels is observed (arrows), 40x; B: 14-day LLLT, angiogenesis (asterisk) and intense inflammatory infiltrate (arrow), 40x; C: ADSC-LLLT 21 days, presence of moderately organized collagen fibers (asterisk) and presence of inflammatory cells (arrow), 40x; D: CULT 5 days, moderate presence of PMN and MN (arrows), 40x; E: CULT 14 days, demonstrates intense deposition of disorganized collagen fibers (asterisk), 40x; F: CULT group 21 days, intense presence of inflammatory cells is observed (arrows), 40x; G: ADSC+ 5 days, intense angiogenesis is observed (arrows), 4x; H: ADSC+ 14 days with complete re-epithelialization (arrow), 10x; I: ADSC+ 21 days, there is presence of papillae in the epidermis and extracellular matrix with dense collagen fibers (asterisk), 40x.

differentiation of fibroblasts, affecting cells that secrete cytokines and other regulatory factors of the fibroblast growth28. This fact may explain the better results found in groups with LLLT and ADSC+ interaction. In summary, treatments with ADSC+ and LLLT were shown to significantly influence the healing process, so that the application of LLLT associated with ADSC+ promoted greater angiogenesis, decreased edema, greater infiltration of mononuclear cells and increased number of fibroblasts at the beginning of repair tissue, in addition to promoting re-epithelialization in a more organized way and with the formation of epithelial attachments at 21 days after the injury. These results demonstrate that the association between ADSC and LLLT cells can accelerate the healing process of cutaneous wounds caused by third-degree burns, as well as improving the scar formed.

5. Conclusion

Therefore, we can conclude that the use of LLLT as a biostimulating agent for immunomagnetically isolated ADSC seems to provide better results in the repair of injured tissues, as it has a high capacity for inducing angiogenesis, cell proliferation and differentiation.

Declaration of Competing Interest

M.R. has received research grants through National Council for Scientific and Technological Development (CNPq) scholarship from Brazil.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.burns.2023.08.018.

Uncited references

[24,26,28,29,30,31,32].

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