Stem cells from adipose tissue improve the time of wound healing in rats[1]

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ABSTRACT

PURPOSE: To evaluate the Adipose Stem Cells (ACS) therapy efficacy on the time and quality of wound healing process in rats.

METHODS: Nine male Wistar rats were randomly distributed into three groups I) 7 days of healing; II) 14 days of healing; III) 21 days of healing. Four incisions were made on the dorsal surface of each rat and then treated with intralesional ACS, meloxicam, and no treatment and ACS+meloxicam. Macroscopic evaluation was measured by percentage of healing and histopathological by hematoxylin-eosin was performed.

RESULTS: All groups have the wound reduced during the three weeks ($p<0.001$) and after 14 days of healing had greater reduction than others. Wounds treated with ASC had accelerated healing in relation to no treatment and only meloxicam ($p<0.001$), excepting the ASC+Meloxicam that was similar ($p=0.13$). There was no difference in histopathological analysis between lesions.

CONCLUSION: Adipose stem cell have benefits in reducing time of healing of experimental model of wound in rats, observed 7 days of after application.

Key words: Adipose Tissue. Wound Healing. Stem Cells. Rats.

Introduction

Adipose Stem Cells (ASCs) are pluripotent and heterogeneous population of cells which derivate from the adipose tissue[1]. Since 2001, this category of stem cells have been studied as an alternative to ethical issues of using embryonic stem cells and mesenchymal cells derived from bone marrow, which is associated with pain and morbidity limited amount in clinical practice[2].

Adipose tissue may be extracted as solid adipose tissue or by liposuction, being less invasive and safer than the extraction of bone marrow stem cells that is done by bone biopsies[3].

Wound healing is a complex process requiring cell migration, inflammation, re-epithelialization, granulation tissue formation, angiogenesis and extracellular matrix remodeling. Stem cells have an active role through this process, and studies with therapeutic application of ASCs have been shown that those cells enhance and improve wound-healing outcomes[4].

The involvement of stem cells in the wound-healing process is critical, mainly in wounds resulting from trauma, diabetes, vascular insufficiency, and other conditions. They have a role in the inflammatory, proliferative, and remodeling phases of wound healing, and their presence supports the physiologic process, culminating in a successful healing[5].

The literature is not clear about the beginning of efficacy of ASCs during the time in experimentation with rat model of cicatrization. Therefore, this study aimed to evaluate the ASCs therapy efficacy on the time and quality of wound healing process in rats.

Methods

This project was approved by the Ethics Committee of the Universidade Estadual do Pará.

Nine male Wistar rats (*Rattus norvegicus*) aged about four months and weighing between 300 and 350g, were randomly distributed into three groups according to the time between the injury and euthanasia, each one with three rats: I) 7 days of healing; II) 14 days of healing; III) 21 days of healing.

All animals were anesthetized with an intraperitoneal injection of 70 mg/kg of ketamine and 10 mg/kg xylazine. After adequate anesthesia and sedation of the animal, the epilation in the dorsal region (4x4cm) was done and antisepsis with iodine based solution was made. Then were made four incisions on the dorsal surface symmetrically with the aid of a sterile dermatological 6 mm punch, extending to the end of the dermis, to improve standardization of the size and shape of the wound. Immediately after preparation of the wounds, they were individually treated according to groups A, B, C and D (Figure 1).

- A) Intralesional injection of autologous ASCs (0.5ml);
- B) Intralesional injection of autologous ASCs (0.5ml) and meloxicam (1mg/0,1ml IM), single dose immediately after the punch;
- C) Control: no special treatment;
- D) Injection of meloxicam (1mg/0.1ml IM), single dose immediately after the punch.

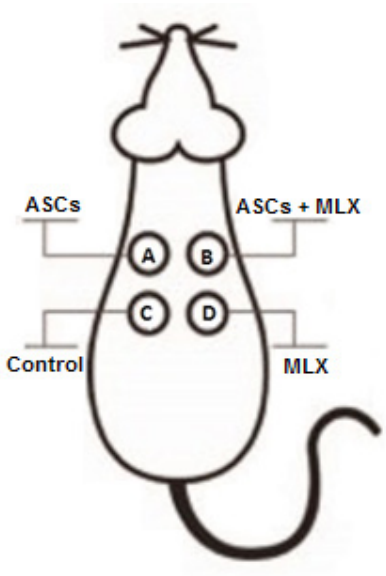

FIGURE 1 – Experimental groups.

Isolation, culture and expansion of ASC

For the collection of adipose tissue was performed anesthesia with ketamine (70mg/kg) and xilazyne (10mg/kg). A longitudinal median abdominal incision of 3cm was used to access the abdominal cavity, followed by resection of peri-epididymal adipose tissue bilaterally. Then the abdominal wound closure was performed with 4-0 nylon suture by plans.

Rat adipose tissue was enzymatically dissociated for 30 min at 37°C by 0.1% (w/v) collagenase type I (Sigma-Aldrich). After centrifugation, the stromal cell pellet was resuspended in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (FBS) (Cultilab, Campinas-SP, Brazil), 100 U/ml penicillin (SigmaAldrich) and 0.1 mg/ml streptomycin (Sigma-Aldrich). In the next day, the culture medium was exchanged to

remove the nonadherent cells. This process was repeated 3 to 3 days until the concentration of viable cells get $3x10^6$ cells/ml.

After this process, it was added trypsin-EDTA (0.25 % trypsin and 0.01 % EDTA in HB- CMFHBSS) in sufficient volume to cover the cell layer and incubated at 37°C for 5-10 minutes. Then, the cells were placed into complete culture medium (twice the original volume) to inactivate trypsin, and all the material was centrifuged at 3000rpm for 10 minutes. The supernatant was discarded and the cells were resuspended to be injected in the lesions.

Differentiation assays was done according to the method described by Suartz *et al*.[6].

Macroscopic analysis

The lesions were photographed at the moment of the wound were produced and in the day of euthanasia, using a camera Sony Cyber Shot DSC- H20 on a standardized height. The lesion area was measured with the ImageJ© Software program.

Microscopic analysis

After the euthanasia with overdose of ketamine and xylazine a dorsal incision (4x4cm) was made. The skin and subcutaneous tissue were fixed in 10% formaldehyde. After the fixation, the tissues were submitted to dehydration in ethanol solutions in growing order of graduation (70, 96 and 100%), following by fast baths of xylol and impregnation in paraffin. The longitudinal histological cuts were accomplished to 4µm thickness. The staining, of the tissues was made with hematoxylin and eosin. The glasses were mounted with Etellan.

Histopathological analysis of the slides was made with light microscopy. It was based on vascular proliferation, fibroblast proliferation, inflammatory infiltration, granulation tissue and epithelialization. Therefore, they assigned three scores: 0 absent, 1 moderate and 2 severe. For epithelialization: 0 absent, 1 partial and 2 complete.

Statistical analysis

Statistical analysis of the macroscopic data was performed by Two-Way ANOVA and evaluation of the difference between the means by T-Test, with significance level of $p < 0.05$. The microscopic data were analyzed by non-parametric method of Friedman. All the tests were performed with the Software BioEstat© 5.0.

Results

The data of lesions size reduction among the treatments performed in each group and time of healing are shown on the Table 1. There was statistical difference between the time of healing ($p<0.0001$) and the treatments comparing the groups ($p=0.0008$). Regarding the time, there was statistical difference between all the groups ($p<0.001$) and the group with 14 days of healing had greater reduction than others.

TABLE 1 - Lesions size reduction (%) among the treatments and time of healing.

Groups	Time of healing		
	7 days[a,b]	**14 days[a,c]**	**21 days[b,c]**
ASCs[d,e]	53.25	82.82	76.26
ASCs + Meloxicam[f,g]	56.54	88.12	78.51
Control[d,f]	41.02	75.39	62.33
Meloxicam[e,g]	35.07	75.01	58.56

a. $p<0.001$
b. $p<0.001$
c. $p<0.001$
d. $p=0.0018$
e. $p<0.001$
f. $p<0.001$
g. $p<0.001$

In relation to the treatments, the percentage of lesion treated with ASC have major reduction than all groups, except when compared the treatment with ASCs and the ASCs + meloxicam ($p=0.1354$). Wounds treated with meloxicam did not differ from control ($p=0.1593$). There was no statistical difference in the microscopic analysis ($p>0.05$) (Figures 2 to 4).

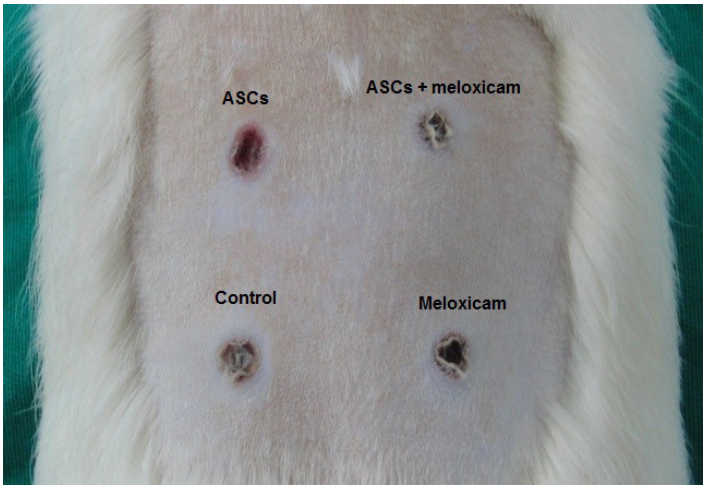

FIGURE 2 – Evolution at the 7th day.

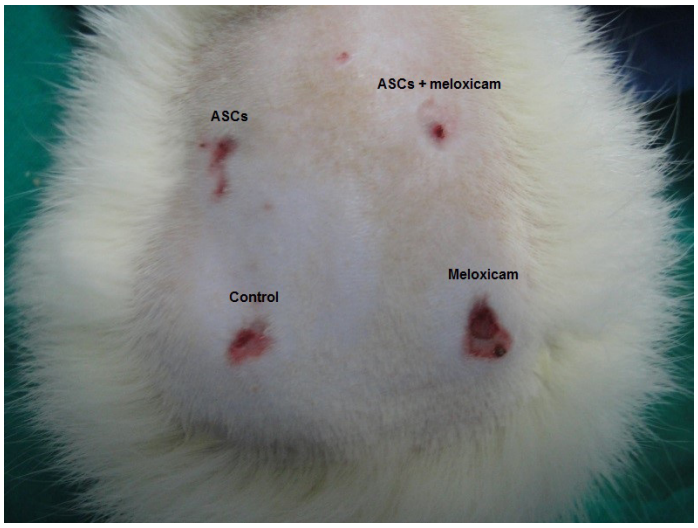

FIGURE 3 – Evolution the 14th day.

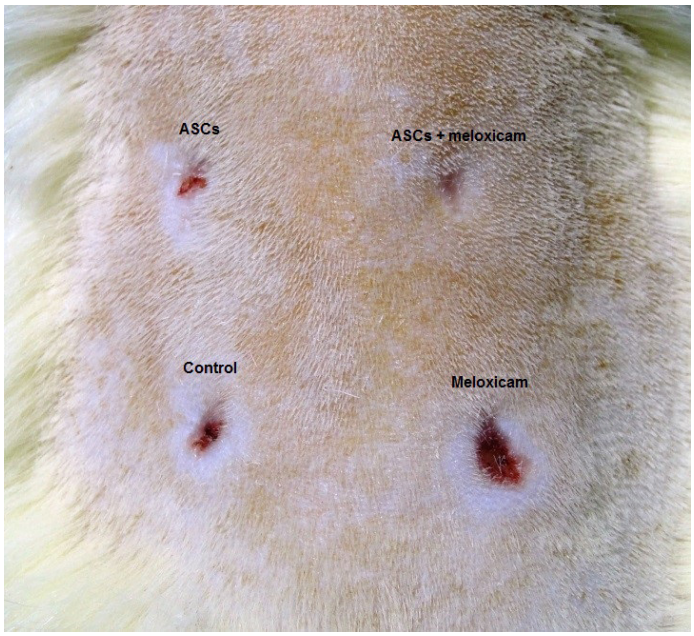

FIGURE 4 – Evolution at the 21st day.

Discussion

Wound healing is a complex process that involves coordinated efforts of many types of cells and cytokines released from them Topical application of ASC showed viability and positive results in relation to others groups with interesting findings in order to accelerate skin healing time. We note that after 7 days, the acute lesions have ameliorated by ASCs and others postoperative days this is maintained, what is agreed with literature findings[7,8].

The rat was used due to the wide use of rodents in this kind of research, 90% of this kind of research is performed in these animals[9]. The protocol to obtain adult adipose stem cell was proposed by Rodbell *et al.*[10] a classic and consagrated study of adipocytes isolation in rat. The meloxicam was used as a drug therapy control to diminish inflammatory process and improve the healing, however, when associated with ASCs there was no increase of healing. This supports that only ASC may have acted to better results of ASCs+Meloxicam group.

In contrast with the expected and found in other studies[8,11], there was no difference in microscopic evaluation, the inflammatory process was not changed. Probably in immunohistochemistry evaluation the results would be more satisfying.

ASCs are a promising for wound human repair due to its property of promoting endothelial dif. Rates of isolation and efficiency are 100%[9,12]. ASCs have similar outcomes of Mesenchymal Stem Cell (MSC) derived from bone marrow, but the surgery is less invasive. Moreover, in clinical situations, the ASC is adequate due to it amount availability, mainly if daily application is necessary or during the burn trauma that ASCs is not suppressed, in contrast with MSC derived from marrow[13].

Adipose-derived stem cells is already used for healing of oncology lesions from radiotherapy in humans, ameliorate skin flap viability and chronic skin ulcers with diabetic mice[8,11,14,15]. The focus of this study was to evaluate in acute wounds.

The ASCs mechanism of action in wound healing is multifactorial. There is a dependent of fibroblast growth factor, autocrine and paracrine secretion of substances as tipe I collagen and neoangiogenesis[8,16].

Our results support literature data and can aid in validation of human application of ASCs as a complementary treatment of acute wound. Further studies are necessary, we included only histopathological and macroscopic analysis, without exploring immunohistochemical markers and electronic microscopy, which could clarify microscopic differences between groups. Perhaps, a major sample could elucidate better the statistical tendency that was found between groups ASC and ASC+ meloxicam.

Conclusion

Adipose stem cell have benefits in reducing time of healing of experimental model of wound in rats, observed 7 days of after application.

References

1. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001 Apr;7(2): 211-28. doi: 10.1089/107632701300062859.
2. Zuk PA, Zhu M, Ashjian P, Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a

source of multipotent stem cells. Mol Biol Cell. 2002, Dec; 13(12): 4279–95. doi: 10.1091/mbc.E02-02-0105

3. Fromm-Dornieden C, Koenen P. Adipose-derived stem cells in wound healing: recent results in vitro and in vivo. OA Mol Cell Biol. 2013 Dec 20;1(1):8. PMID: 1403602403.
4. Lee DE, Ayoub N, Agrawal DK. Mesenchymal stem cells and cutaneous wound healing: novel methods to increase cell delivery and therapeutic efficacy. Stem Cell Res Ther. 2016 Mar;7(37):1-8. doi: 10.1186/s13287-016-0303-6.
5. Maxson S, Lopez EA, Yoo D, Danilkovitch-Miagkova A, Leroux MA. Concise review: role of mesenchymal stem cells in wound repair. Stem Cells Transl Med. 2012 Feb;1(2):142-9. doi: 10.5966/sctm.2011-0018.
6. Suartz CV, Gaiba S, França JP, Aloise AC, Ferreira LM. Adipose-derived stem cells (ADSC) in the viability of a random pattern dorsal skin flap in rats. Acta Cir Bras. 2014 29(Suppl. 3). doi: 10.1590/S0102-86502014001700001.
7. Nakagawa H, Akita S, Fukui M, Fujii T, Akino K. Human mesenchymal stem cells successfully improve skin-substitute wound healing. Br J Dermatol. 2005 Jul;153:29–36. doi: 10.1111/j.1365-2133.2005.06554.x.
8. Kuo YR, Wang CT, Cheng JT, Kao GS, Chiang YC, Wang CJ. Adipose-derived stem cells accelerate diabetic wound healing through the induction of autocrine and paracrine effects. Cell Transplant. 2016 Apr;25(1):71-81. doi: 10.3727/096368915X687921.
9. Zuttion MSSR, Wenceslau CV, Lemos PA; Takimura C; Kerkis I. Células-tronco de tecido adiposo e a importância da padronização de um modelo animal para experimentação pré-clínica. Rev Bras Cardiol Invasiva. 2013;21(3):281-7. doi: 10.1590/S2179-83972013000300015.
10. Rodbell M. Localization of lipoprotein lipase in fat cells of rat adipose tissue. J Biol Chem. 1964 Mar;239:753-5. PMID: 14154450.
11. Nambu M, Kishimoto S, Nakamura S, Mizuno H, Yanagibayashi S, Yamamoto N, Azuma R, Nakamura S, Kiyosawa T, Ishihara M, Kanatani Y. Accelerated wound healing in healing-impaired db/db mice by autologous adipose tissue-derived stromal cells combined with atelocollagen matrix. Ann Plast Surg. 2009 Mar;62(3):317-21. doi: 10.1097/SAP.0b013e31817f01b6.
12. Gimble J, Guilak F. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. Cytotherapy. 2003;5(5):362-9. doi: 10.1080/14653240310003026.
13. Mizuno H. Adipose-derived stem cells for tissue repair and regeneration: ten years of research and a literature review. J Nippon Med Sch. 2009 Apr;76(2):56-66. PMID: 19443990.
14. Rigotti G, Marchi A, Galiè M, Baroni G, Benati D, Krampera M, Pasini A, Sbarbati A. Clinical treatment of radiotherapy tissue damage by lipoaspirate transplant: a healing process mediated by adipose-derived adult stem cells. Plast Reconstr Surg. 2007 Apr 15;119(5):1409-22. doi: 10.1097/01.prs.0000256047.47909.71.
15. Suartz CV, Gaiba S, França JP, Aloise AC, Ferreira LM. Adipose-derived stem cells (ADSC) in the viability of random skin flap in rats. Acta Cir Bras. 2014;29(Suppl. 2):6-9. doi: 10.1590/S0102-86502014001400002.
16. Kim WS, Park BS, Sung JH, Yang JM, Park SB, Kwak SJ, Park JS. Wound healing effect of adipose-derived stem cells: a critical role of secretory factors on human dermal fibroblasts. J Dermatol Sci. 2007 Oct;48(1):15-24. doi: 10.1016/j.jdermsci.2007.05.018.

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