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Adipose-derived stem cells improve full-thickness skin grafts in a rat model

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ABSTRACT

To investigate the effects of heterologous adipose-derived stem cells (ADSCs) on autologous full-thickness skin grafts, we designed a first-intention healing model using Wistar rats. We harvested and sutured two full-thickness skin grafts in the dorsal recipient beds of 15 rats, randomized into three groups. In the treatment group, 1×10^6 ADSCs resuspended in saline solution (200 µL) were administered subcutaneously to the skin graft. The control group received only saline solution subcutaneously, whereas the negative control group did not receive any treatment. Compressive dressings were maintained until postoperative day 5. The grafts were assessed by two observers, who checked for the presence of epidermolysis on day 14. Planimetry showed the relative areas of normal skin, redness, ulceration, and contraction. Graft samples were obtained on day 14 and stained with hematoxylin and eosin and Masson's trichrome. Epidermal analysis evaluated thickening, keratosis, acanthosis, hydropic degeneration, and inflammatory infiltrate. Dermal evaluation investigated the absence of hair follicles, granulation tissue formation, presence of inflammatory infiltrate, and collagen deposition. Immunohistochemistry was performed for dermal anti-VEGF and epidermal anti-Ki-67 staining. The ADSC group presented better macroscopic aspects, lower incidence of epidermolysis, and less loss of hair follicles. In addition, the ADSC group presented the lowest frequency of histopathological changes in the dermis and epidermis, as well as the largest subcutaneous and granulation tissue VEGF averages and the weakest Ki-67 staining of the epidermal basal layer. Subcutaneous administration of ADSCs may improve the integration of skin grafts, reducing the deleterious effects of ischemia and reperfusion injury.

1. Introduction

Full-thickness skin grafts are avascular segments of skin, composed of epidermis and dermis (Valencia et al., 2000; MacFarlane, 2006; Mesimeris, 2006; Pavletic, 2010; MacPhail, 2013), detached completely from their beds (Valencia et al., 2000; Mesimeris, 2006; Pavletic, 2010; MacPhail, 2013). They are used to reconstruct skin defects caused by tumor excision, trauma, congenital defects, and burns, or by diabetic, irradiated, and chronic wounds (Valencia et al., 2000; Zografou et al., 2011). Partial or complete necrosis of the grafted tissue is a common complication (Valencia et al., 2000; MacFarlane, 2006; Zografou et al., 2011; Pavletic, 2010; MacPhail, 2013), just as excessive contraction, which leads to distortion of the surrounding tissues, with associated cosmetic deformity and limitation of joint mobility (MacFarlane, 2006). In the case of necrosis, the tissues suffer ischemic injury – hypoxia, edema, arterial vasospasm, arterial or venous occlusion, congestion and dehiscence, or infection – and a reperfusion injury syndrome when circulation is reestablished after prolonged hypoxia (Siemionow and Arslan, 2004; Mesimeris, 2006; Reichenberger et al., 2012a; Kern and Sucher, 2013).

Adipose-derived stem cells (ADSCs) are easy to isolate, multipotent, and play a regenerative, therapeutic, and immunomodulatory role in skin healing. They have been used in human trials, due to their positive influence on wound healing, which include higher rate of

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epithelialization, increase in granulation tissue formation and in angiogenesis, and decrease in inflammatory infiltrate, besides immune response modulation (Chen et al., 2012; Jackson et al., 2012; Maxson et al., 2012; Kim et al., 2013).

Studies indicate the possibility of using ADSCs to protect axial flaps from ischemia and reperfusion injury (IRI) with satisfactory results similar to those obtained in procedures without ischemia, due to an increase in angiogenesis and in blood perfusion (Ichioka et al., 2004; Simman et al., 2005; Lu et al., 2008; Uysal et al., 2010; Gao et al., 2011; Reichenberger et al., 2012a,b; Suartz et al., 2014); and through the same mechanisms, autologous ADSCs can be used to increase the survival of full-thickness skin grafts (Zografou et al., 2011). However, the use of autologous cells may limit the amount of cells to be transplanted or transplantation time because expansion time is needed to aquire a sufficient amount of cells to treatment. Considering of their immunological characteristics, ADSCs can be used for non-autologous applications (Niemeyer et al., 2010; Larocca et al., 2013; Le Blanc et al., 2003), being a safe and fast way of applying cell therapy in the clinical setting (Nishihori et al., 2010; Kyriakou et al., 2010). Thus, the aim of this study is to test subcutaneous administration of heterologous ADSCs into full-thickness autologous skin grafts. It is expected that ADSCs-treated grafts will have a better macroscopic appearance and lower incidence of histopathological findings.

2. Material and methods

2.1. Experimental animals

This study was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre (process no. 13-0414). All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health; Brazilian Law no. 11.794/2008; the Brazilian Guidelines for the Care and Use of Laboratory Animals; and the Guidelines for the Euthanasia of Animals. The animals were housed individually and received food and water *ad libitum* in an environment with controlled temperature (22 ± 1 °C), humidity (65–70%), and light-dark cycle (12/12 h). The procedures were carried out exclusively by veterinarians experienced in the handling of laboratory animals. All surgical procedures were conducted under sterile conditions with minimal variation in performance.

As an accepted model for studies on skin healing (Wanda and Dorsett-Martin, 2004; Wong et al., 2011), 15 young adult male Wistar rats (*Rattus norvegicus* sp.), weighing 300–350 g, from the same animal facility, were used in this study. All surgeries and procedures were performed under general anesthesia with inhaled isoflurane (Instituto Bioquímico Indústria Farmacêutica LTDA, Brazil), vaporized in 100% oxygen, with 5% isoflurane used for anesthetic induction and 2% as maintenance dose (in 0.5 L/min), and all the animals received tramadol chloride (Laboratório Teuto Brasileiro S.A., Brazil) (5 mg·kg^{-1}) intraperitoneally before lesion induction and every 12 h on the two subsequent days. They received a pre-surgical antibiotic therapy with sodium ampicillin (20 mg·kg^{-1}) subcutaneously (Laboratório Teuto Brasileiro S.A., Brazil).

2.2. ADSCs extraction and culture

ADSCs were isolated from inguinal fat of four Wistar rats and processed according to Terraciano et al. Shortly afterwards, adipose tissue was minced and then subjected to enzymatic digestion with collagenase type IV (GIBCO, Brazil). After centrifugation, the cell pellet was cultured with Dulbelcco's modified Eagle's medium (DMEM) - low glucose (Sigma-Aldrich, Brazil), supplemented with 20% fetal bovine serum (FBS) (GIBCO, Brazil) and 1% penicillin/streptomycin (GIBCO, Brazil). Cells were maintained in a humidified incubator at 37 °C, with 5% CO_2. When cells were 80–90% confluent, adherent cells were harvested with 0.25% trypsin solution (GIBCO, Brazil) for passage.

2.3. ADSCs immunophenotype and differentiation

To verify whether ADSCs met the minimum criteria for identification, cell characterization and differentiation tests were conducted according to the International Society for Cellular Therapy (Dominici et al., 2006). Procedures in this study were performed using ADSCs from the fourth passage. Before experimental use, cell characterization was carried out to confirm the presence of specific markers using flow cytometry with anti-rat CD90, CD73, CD29, CD45, and CD11b monoclonal antibodies (all from BD Biosciences, USA) on a FACSCalibur flow cytometer (BD Biosciences, USA), using the cell QUEST software (BD Biosciences, USA). The expression of cell surface markers was determined by comparison with an isotype control.

According to Terraciano et al. (2014), the ability of ADSCs to differentiate *in vitro* into adipocytes, chondrocytes, and osteocytes was assessed with the use of the appropriate media for 21 days (20) and subsequent staining of cells to estimate whether the cells were differentiated (20). Oil-Red O, Alizarin Red, and Alcian Blue were used for adipogenic, osteogenic, and chondrogenic cell line staining, respectively.

2.4. Experimental groups

Fifteen rats were randomly distributed into three groups: ADSC, SS, and NC (two grafts in each rat and five rats in each group). Each graft in the ADSC group received 1 × 10^6 ADSCs in 200 µL 0.9% saline solution (SS) during the surgery. The control group, called SS, received 200 µL 0.9% saline solution in each graft during the surgery to assess the physical effect of injecting 200 µL of liquid into the grafts. The negative control group (NC) was grafted, but it did not receive any substance in the subcutaneous layer.

2.5. Full-thickness skin graft model

After anesthetic induction, the operative field was scrubbed with a 2% chlorhexidine gluconate solution (Vida Animal Farmácia Veterinária Ltda., Brazil). Two 12-mm-diameter skin fragments were removed from the midline of the back including the *panniculus carnosus* (Fig. 1). Two full-thickness skin grafts were created by removing the *panniculus carnosus* tissue with a surgical microscope (Leica M651, Germany) and by administering one dose of ADSCs (1 × 10^6 ADSCs resuspended in 200 µL SS) into the subcutaneous layer. The graft was then reattached to the underlying fascial layer with 6-0 mononylon suture (Brasuture Indústria, Comércio Importação e Exportação LTDA, Brazil).

The grafts received tie-over pressure dressings (Fig. 1) sutured to the skin with mononylon 4-0 (Brasuture Indústria, Comércio Importação e Exportação LTDA, Brazil). A Solid Vaseline (Rioquímica Indústria Farmacêutica LTDA, Brazil) layer separated the tie-over dressing from the graft. A secondary dressing was applied over the tie-over using a hypoallergenic tape (Micropore, 3 M do Brasil, Brazil). The dressings were maintained until postoperative day 5 in all groups.

2.6. Clinical evaluations

The rats were weighed on the day of the surgery (d_0) and on postoperative days 5 (d_5) and 14 (d_{14}). On d_5 and d_{14}, the grafts were assessed by two observers, who checked for the presence of epidermolysis and of secretions on the borders of recipient beds. The observers were blinded to the treatments.

2.7. Planimetry

For planimetry, the grafts were traced on transparent film applied