Role of adipose-derived stem cells in chronic cutaneous wound healing

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AIM: *Adipose tissue represent an alternative source of multipotent stem cells with characteristics similar to bone marrow-derived mesenchymal stem cells (BMSCs), easier to isolate and effective in wound healing enhancement.*
MATERIAL OF STUDY: *Each patient being considered for stem cells graft was subjected to a conventional liposuction procedure (local anaesthesia, aspirated volume about 80cc), in order to isolate a pellet of adipose-derived stem cells (ASCs), which was then mixed with the platelet-rich plasma (PRP) previously collected, in order to obtain an enhanced-ASCs-PRP (e-PRP), now ready for grafting in the context of skin edges as well as at the bottom of the lesion itself.*
RESULTS: *Flow cytometric analysis performed on the pellet obtained with our isolation process, showed that a mean of 5 x 10^5 ASCs (range: 4,0-6,0 x 10^5, SD: ± 1 x 10^5) were collected from 80 ml of adipose tissue, harvested with standard wet liposuction procedure. It represented the 5% of all sample cells (1 x 10^7), while the others 95% were mostly being blood-derived and endothelial cells.*
DISCUSSION: *By now, the most used isolation protocols take about two hours due to the complex isolation procedure, requiring both animal-derived reagents and collagenase. The amount of ASCs obtained with our isolation process is sufficient to be directly engrafted in the wound without the need of in vitro expansion but, neither serum nor animal-derived reagents are used, and it takes only 15' minutes.*
CONCLUSION: *ASCs application is an innovative, effective approach in the treatment of chronic wounds.*

KEY WORDS: Adipose-derived stem cells, Chronic cutaneous ulcers, Regenerative medicine

Introduction

The treatment of skin ulcers requires a world annual financial commitment of 7 billion euro, affecting approx. 1% of adults and 3.6% of people aged over 65 years and are responsible for 85% of all non-traumatic lower limb amputations [1]. Venous ulcers alone account for 70% of all leg ulcers, while arterial, diabetic and pressure ulcers represent the other most common etiologies[1]. Over 60% of the patients affected by chronic lower limb ischemia shows at least one leg ulcer [1].
Regenerative medicine frontier in hard-to-heal wounds is represented by cytokines, growth factor and autologous stem cells [2-3].
The aim of this study is to prove not only the effectiveness of combined adipose-derived stem cells (PRP) with autologous adipose-derived stem cells (ASCs) in wound healing but also to show an easier and faster way to gain a ready-to-use ASCs amount.
PRP, which derives from whole blood through double-spin centrifugation, contains multiple growth factors and

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adhesion molecules in α-granules [4]. Usually, the normal adult human platelet count ranges between 150,000 and 350,000/μL, with an average of 200,000/μL. The working definition for PRP coincides with the concentration of 1 million platelets per μL in a 5-ml volume of plasma since it has been shown that this is the concentration that produces clinical benefits [5-6]. Lesser concentration cannot be relied upon to enhance wound healing and greater concentrations have not yet been shown to further enhance wound healing [7]. Platelets contain two basic granules: α-granules and dense granules. At least seven fundamental protein growth factors have been proven to exist within α-granules for initiating wound healing. These growth factors include the three isomers of platelet-derived growth factor (PDGF-AA, PDGF-BB, and PDGF-AB), transforming growth factor-β (TGF-β1 and TGF-β2), vascular endothelial growth factor (VEGF), and epithelial growth factor (EGF)[5]. The α-granules also contain three proteins known to act as cell adhesion molecules: fibrinogen, fibronectin, and vitronectin[8]. In addition to the seven basic growth factors, scientists have also found other growth factors such as the insulin-like growth factor (IGF-I, IGF-II), fibroblast growth factor (FGF), endothelial cell growth factor (ECGF), and platelet-derived angiogenesis factor (PDAF) [4-7]. Bioactive factors are also contained in dense granules, including serotonin, histamine, dopamine, calcium, and adenosine, which are also involved in wound healing [7]. These bioactive factors promote matrix stem cells migration and proliferation enhancing the wound healing; they inhibit macrophages cytokines release, improve the wound bed and the granulation tissue by promoting neo-angiogenesis and re-epithelialization. PRP platelets can also release chemokines for macrophages, interleukines from the leukocyte fraction and anti-microbial proteins, leading to an effective anti-microbial action. In literature, it has been described PRP application in different area: periodontal and oral surgery, maxillofacial surgery, orthopaedics and traumatology, plastic and aesthetic surgery, vertebral surgery and cardiac surgery [9-10]. To our knowledge, due to its recent introduction in the clinical use only few study have been carried out yet reporting its effectiveness.

ASCs are similar to bone marrow-derived stem cells (BMSCs) in that they are capable of maturing toward multiple mesodermal tissue types, and show similar surface protein marker expression [11]. ASCs are unique from BMSCs because they are easily obtained by a standard wet liposuction procedure under local anaesthesia, without the need of expansion in culture [12]. For these reasons ASCs are appealing for cell-based therapies in tissue repair and regeneration. Stem cells isolated from the lipoaspirate have demonstrated a broad in vitro adipogenic, chondrogenic, osteogenic and myogenic lineage commitment [13-14] and also differentiation toward pancreatic cells, hepatocytes and neurogenic lineage, as our former studies have shown [15,16].

ASCs are supposed to be in the so-called stromal vascular fraction (SVF) [17] of the adipose tissue, together with a heterogeneous population of many other cell types, including preadipocytes, endothelial cells and pericytes, hematopoietic-lineage cells and fibroblasts.

Madonna *et al* [18] demonstrated the SVF ability to differentiate into mature endothelial cells, promoting neoangiogenesis and suggesting its topical application in ischemic tissues. As a matter of fact, SVF cells show endothelial phenotype markers CD31, CD144 (VE-cadherin) and von Willebrand factor. DiMuzzo *et al.* [19] described differentiated endothelial cells isolated from adipose tissue and their potential use in vascular graft development. The Makarov *et al.* also described SVF grafting in rats' corpora cavernosa, suggesting its application in erectile dysfunction disease [14]. Further studies showed *in vitro* differentiation of SVF toward endothe-

Fig. 1: The harvested adipose tissue was first collocated in the vibrating shaker and straight after centrifuged. Both the devices were in the operating room inside a laminar air flow cabinet.

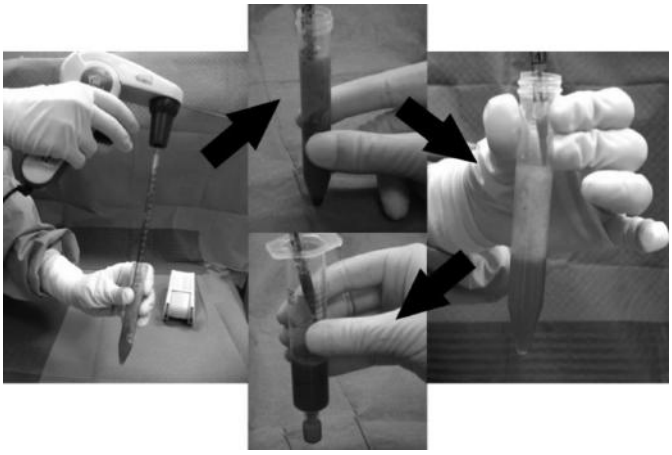

Fig. 2: The pellet at the bottom of each tube was collected by means of an automated pipetting system and poured together with 5 ml of previously collected autologous PRP.

lial lineage: Planat-Bernard *et al.* [20] founded CD31 and von Willebrand factor expression in CD13-CD34-SVF harvested in Matrigel and observed the formation of three-dimensional networks of branches consistent with the formation of vascular structures. Miranville *et al.*[21] obtained the same result, analysing CD31-CD34-SVF cells. In other related works, Cao et al.[22] founded endothelial markers expression under VEGF presence in CD31-CD34-CD106-flk1 cells isolated from adipose tissue. *In vivo* studies obtained the same results: an ischemic tissue model [23] in rat showed that CD31 cells graft was able to significantly improve angiogenesis and perfusion homeostasis of the damaged tissues. Regenerative features of SVF include also its paracrine effect: VEGF, HGF and TGF-β are secreted by SVF cells under different stimuli (hypoxia, growth factor) [24,25] and influence strongly differentiation of niche stem cells, promoting angiogenesis and wound healing, potentially aiding new tissue growth and development.

Materials and Methods

Exclusion criteria were: patients under chemotherapy, with Hb <10.5 /dL, platelet <100 x 10^9/L, serum albumin <2.5 g/dL, neoplastic or infected cutaneous ulcers.
Every patient underwent the following Day-Surgery procedure: eighty ml of adipose tissue were harvested by conventional wet liposuction procedure, performed with eight 10-ml luer lock syringes and blunt-tipped cannula. Then, in the same operating room, the adipose tissue was immediately processed under a laminar airflow cabinet (1200 FLO, FIMS, Concorezzo, Italy) where a vibrating shaker (Multi Reax, Heidolph, Schwabach, Germany) and a centrifuge (MPW 223, Johnson & Johnson Medical, New Jersey, USA) were positioned. The content of each syringe was collected in an equal number of 10 ml plastic test tubes and positioned in the vibrating shaker at 1600 vib/min x 6 min in order to tear out the stem cells from the surrounding adipose tissue. This step was followed by the centrifugation at 1600rpm x 6 min, so that the previously detached ASCs joined the stem cell of the fluid portion at the bottom of each tube. Once the isolation process was over, always under the same laminar flow cabinet, the pellet at the bottom of each tube was collected by means of an automated pipetting system and poured together into a 10-ml Luer Lock syringe containing 5 ml of previously collected autologous PRP. The enhanced-ASCs-PRP (e-PRP) obtained, was now ready for grafting in the context of skin edges as well as at the bottom of the lesion itself.
In order to assess the effectiveness of our procedure, we characterized the isolated cells with flow cytometric analysis. The monoclonal antibodies used were: CD31, CD34, CD45, CD73, CD90 and CD105.
Moreover, we assessed the viability of the cells with the dye 7-Amino-Actinomycin D (AAD).

Results

Flow cytometric analysis, performed over the isolated cells, confirmed the presence of a stem cell population characterized by the following markers: $CD31^-$, $CD45^-$, $CD34^+$, $CD73^+$, $CD90^+$, $CD105^+$.
While, as far as it concern the viability of the cells, it resulted that 95% of all the cells were viable and this rate rose up to 97%, considering only the ASCs.
Starting from 80ml of adipose tissue, we routinely collected a mean of 5 x 10^5 of ASCs (range: 4,0-6,0 x 10^5, SD: ± 1 x 10^5), 5% of the total number of sample cells (1 x 10^7), while the majority of other cells (95%) were blood-derived and endothelial cells.

Discussion and Comments

As far as we know, most isolation process relies on time-consuming procedures that need expensive and complex mechanical devices and involve the use of serum and animal derived reagents, hypothetically capable of transmitting prions related diseases. Moreover, these procedures allow only to isolate ASCs from the cellular portion of the lipoaspirate, wasting stem cells from the fluid portion of it [22-23].
Our isolation process takes about 15min, doesn't require high grade technologically devices, no collagenase, serum and animal-derived reagents are needed, and it yields to the collection of a conspicuous amount of ASCs which are useful for a faster and safer cell-based therapy [26,27].
Stem cells obtained from lipoaspirate significantly increase the healing process and tissue regeneration, patients experience significantly less pain and any subsequent skin grafts positioned to the same area show better engraftment.

Conclusions

The use of ASCs has proven to be a valuable resource for the treatment of chronic skin ulcers, both individually and combined with other therapeutic options such as skin grafts. It represents an innovative and effective approach in the treatment of chronic wounds. For these reasons, ASCs are appealing for cell-based therapies in tissue repair and regeneration.

Riassunto

Le ulcere cutanee croniche colpiscono circa l'1% della popolazione adulta e il 3.6% della popolazione con più di sessantacinque anni di età. Per quanto riguarda l'impegno finanziario per la terapia delle ulcere cutanee, questo è pari a sette miliardi di euro annui a livello mondiale.
Le cellule staminali mesenchimali prelevate da lipoaspirato (ASCs) rappresentano una delle frontiere più pro-

mettenti della medicina rigenerativa. Diversi studi hanno, infatti, dimostrato l'efficacia terapeutica di questa popolazione di cellule staminali nel trattamento delle ulcere cutanee croniche. Le ASCs hanno caratteristiche morfologiche e funzionali paragonabili alle cellule staminali del midollo osseo (BMSCs), tuttavia se ne differenziano per la maggiore facilità di prelievo e isolamento, e per il loro più cospicuo numero. È, infatti, sufficiente eseguire una liposuzione tradizionale e in seguito utilizzare metodiche d'isolamento appropriate per ottenere un pellet di ASCs pronte per essere innestate nel fondo e nel margine dell'ulcera cutanea, al fine di promuoverne la guarigione. Diversi sono i protocolli d'isolamento; i più diffusi prevedono complicati e lunghi procedimenti, caratterizzati dal susseguirsi di differenti prodotti sia chimici sia biologici, i quali sono non solo costosi ma anche potenzialmente pericolosi. Il nostro protocollo d'isolamento richiede solo 15' e prevede che il lipoaspirato sia prima sistemato in uno shaker cellulare e successivamente in una centrifuga così da ottenere le ASCs richieste per la terapia.
L'uso delle ASCs si è rivelato una risorsa preziosa per la terapia delle ulcere cutanee croniche, accelerando significativamente il processo di guarigione e la rigenerazione dei tessuti; con protocolli d'isolamento efficaci e semplificati, il loro utilizzo routinario risulta essere facilitato.

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