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Titolo: Clinical-sperimental characterization and assessment of application of Mesenchymal stem cells isolated from lipaspirate + PRP in chronic skin wound therapy.

Nome autore: Guido Libondi

Istituzione: Università degli Studi di Parma, UO di Chirurgia della Cute e degli Annessi, Mininvasiva, Rigenerativa e Plastica, Direttore Prof. E.Raposio

Summary

Adipose-derived stem cells (ASCs) hold great promise for regenerative medicine applications, due to their ability to promote the healing process through *in situ* differentiation and secretion of paracrine factor. The aim of this study is to present a clinical adjunct for chronic skin wound therapy based on ASCs added to platelet-rich plasma (PRP), to obtain an enhanced-PRP (*e*-PRP).

For 18 months, 24 control group patients (31 chronic skin ulcers) were treated with standard wound care; while 16 experimental group patients (21 chronic skin ulcers) were treated with standard wound care and e-PRP injection once. The patients were randomly assigned to the control or experimental group. Outpatients had weekly follow up visits where they were subjected to standard treatment and the wound healing process was assessed.

Control and experimental groups had similar healing rates but wound closure rates were significantly different (p =0.0257): 0,0890 cm^2 x day and 0,2287 cm^2 x day respectively. No side effects were reported.

In our experience, *e*-PRP significantly enhanced wound closure rates when compared to standard wound care, without causing any serious complications. This finding highlights *e*-PRP as a valuable resource for chronic wound treatment.

Introduction

Adipose-derived stem cells (ASCs) are particularly promising for regenerative therapies as they can be easily harvested through standard liposuction procedures, with minimal donor site morbidity.[1] ASCs have a differentiation potential similar to other mesenchymal stem cells as well as a higher yield upon isolation and a greater proliferative rate in culture when compared to bone marrow-derived stem cells.[2,3]

Moreover, as ASCs do not necessitate extensive manipulation before application, compliance with "cell manufacturing" in accordance with current Good Manufacturing Practice Guidelines is not required.[4] In effect, these restrictions do not apply in cases of minimal manipulation [Regulation (EC) No 1394/2007 of the European Parliament and of the Council].

Platelet-rich plasma (PRP) is defined as platelet concentration above baseline normal platelet count in a small volume of plasma; it derives from whole fresh blood through double-spin centrifugation and has healing anti-inflammatory and pro-regenerative properties.[5,6]

The aim of this study was to describe a combined application of autologous ASCs and PRP, in order to obtain an enhanced-PRP (*e*-PRP) for the treatment of chronic skin ulcers.

Materials and Methods

Surgical Technique

Within the same day-surgery procedure, ASC isolation and *e*-PRP injections were performed. On the morning of surgery, a 54 cc blood sample was taken from each patient in order to obtain the PRP, and fractionated using centrifugation (3200 rpm for 15 minutes), with

anticoagulant citrate dextrose-A (ACD-A, 6 mL in 60 mL syringe). The platelets were concentrated in the PRP at levels approximately 7 times the baseline level, as it has been shown that a concentration of approximately four to seven times more than the usual baseline platelet count, produces clinical benefits, while higher concentrations do not appear to result in a greater effect.[6] The resulting PRP and platelet-poor plasma (10 cc) were stored at room temperature until needed (about 40 minutes).

A vibrating shaker and centrifuge were both used in the operating room and placed in a laminar airflow bench in order to isolate the ASC pellet, as previously described.[7] In brief, the abdominal subcutaneous adipose tissue was harvested using a standard liposuction procedure by means of 400-ml of Klein solution as tumescent volumes, 3.0 blunt-tipped cannula and 10-ml Luer-Lock syringes. Eighty milliliters of adipose tissue were usually harvested, which were subsequently positioned in the vibrating shaker at 600 vbr/min for 6 minutes and centrifuged at 1600 rpm/min for another 6 minutes. The ASCs obtained were poured into a 10-mL Luer-Lock syringe containing 5 mL of previously collected autologous PRP: the *e*-PRP was ready to be injected into the skin edge as well as at the bottom of the lesion itself.

The wound dressing was made with the remaining 5 ml of PRP activated by adding autologous thrombin (at a ratio of 10:1) produced by mixing the autologous PRP with a calcium chloride solution ($CaCl_2$ 1 ml 10%; 1000 units: 1 ml; ratio *e*-PRP/PPP:10/1) in order to obtain the platelet-gel that was inserted into the surgical field as needed, and kept till the 5^{th} postoperative day. Total procedural time: 45 minutes.

Patients Enrolled

The informed consent was obtained by each patient enrolled with chronic (venous, diabetic, ischemic) ulcers. Exclusion criteria were :

- Patients on chemotherapeutic agents;

- Patients with the following abnormal laboratory test levels: hemoglobin <10.5 g/dl, platelet count <100 x 10^3/μl , serum albumin level < 2.5 g/gl;
- Wounds due to malignancy;
- Wounds with active clinically diagnosed infection.

In order to assess the effectiveness of the *e*-PRP, 40 patients were enrolled (mean age: 70 years; 21 males, 19 females) and randomly divided into two groups, both of which were treated with the same advanced dressings, but only the experimental group were treated with *e*-PRP once.

The control group population included 24 patients (mean age. 74.5 years; 10 males and 14 females); there were 31 chronic skin ulcers, 42% of which were of arterial etiology, 45% were consequent to venous insufficiency, 10% were diabetes-related and 3% were post-traumatic; mean wound size was 11.24 cm^2 (SD= 17.22) at baseline. The chronic wound had onset on average by 14.5 months (SD=17.22).

The *e*-PRP group population included 16 patients (mean age: 70.75 years; 11 males and 5 females); there were 21 chronic skin ulcers, mean wound size was 25.18 cm^2 (SD= 32.6) at baseline. The chronic wound had onset on average by 26.57 months (SD=46.39).

The wound was arterial in 67% of cases, venous in 19% and of diabetes-related etiology in 14%.

Methods

The study had an overall time duration of 18 months.

All the patients were weekly subjected to standard wound treatment, which consisted of disinfection, debridement when needed, application of advanced wound dressing and bandage.

Pictures of the lesions were taken at time 0, and then at 1, 3, 6, 12 and 18 months. Wound areas were measured by digital planimetry using the VistaMetrix 1,33 (SkillCrest, USA)

software. Firstly the wound sizes at T_0 were analyzed, then the wound closure rate, which was obtained by dividing the ulcer size at T_0 by the number of days needed for healing (cm^2/day).

Data are expressed as mean ± SD. Statistical comparisons between the groups were performed using Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

It has been studied both the platelet count in the blood and in the PRP, as well as the number of ASCs isolated on average for each procedure, so as to be able to correlate the results obtained with the effectiveness of the technique.

Flow cytometry assay

The isolated cells were characterized by flow cytometric analysis of specific surface antigens**.**

The monoclonal antibodies (MoAb) used were:

CD31 FITC (852.561.010, Diaclone)

CD34 APC (345804, Becton Dickinson)

CD45 APC-Cy7 (348815, Becton Dickinson)

CD73 PE (550257, Becton Dickinson)

ADSCs were stained with saturating concentrations of each antibody according to manufacturer's instructions for 20 min at room temperature in the dark, washed with Ca2+ and Mg2+ free Phosphate-Buffered Saline, and were resuspended in 500 µl FACS buffer.

The samples were acquired on a Becton Dickinson eight-color flow cytometry (**FACSCanto II)** equipped with 2 laser: blue (488 nm 520 mW) and red (633 nm, 17 mW HeNe) able to detect up to 8 fluorescent.

The analysys was performed with FACS™ Diva software

The dye 7-AAD was added to assess viability of the cells.

At least 20.000 viable events (7-AAD negative) were acquired.

Data were expressed as a percentage of the aviable cells acquired.

Results

At the end of the study, the chronic skin wounds of 68% (n=21) of the control group and 71% (n=15) of the *e*-PRP group (Fig. 1-2) healed completely.

The analysis between the two groups were performed on all data collected but no statistical significance was found; hence, only the healed patients of both groups were assessed.

The mean wound onset values were 10.19 months (SD=10.37) for the control group and 14.53 months (SD=1.859) for the *e*-PRP group, distance between means was 4,343 ± 3,501; no statistically significant differences emerged (P value=0.2233).

Mean wound sizes were: 8.519 cm^2 (SD=2.902) for the control group and 29.59 cm^2 (SD=9.752) for the *e*-PRP groups, distance between means was 0.1397 ± 0.05987; *t* test highlighted a statistically significant difference (p =0.0236).

The wound closure rate of the control group was 0.0890 cm^2/day (SD=0.01871), while that of the *e*-PRP group was 0.2287 cm^2/day (SD=0.06617). The *t*-test confirmed that the difference was statistically significant (p =0.0257).

After the procedure some patients complained of discomfort in the abdominal region for a few days (no longer than one week), which is a common side effect of liposuction, but no further side effects were reported.

The mean baseline platelet count was 244 x 10^3/μl while the PRP platelet concentration was 137 x 10^4/μl. Starting from 80 ml of adipose tissue, 5 x 10^5 ASCs (5% of the total number of sample cells), were collected on average, with a cell survival rate of 97%; while the remaining 95% cells were mostly blood-derived and endothelial cells.

The flow cytrometric analysis confirms the presence of a conspicuous number of cell population characterized by ASC markers[4] CD45-/CD34+/CD31- and mesenchymal stem

cell (MSC) marker CD90+. The number of ASC isolated in 80ml adipose tissue is the 5% (5 x 10^5 of the total number of cells available (10 x 10^7).

Discussion

ASCs have diverse clinical applications, including non-healing wounds[8-11], while PRP is an advanced wound therapy which has been used in hard-to-heal acute and chronic wounds for over 20 years.[5,12]

Our clinical experience with the use of *e*-PRP shows not only the effectiveness of the procedure but also its feasibility; in effect, it takes only 45 minutes to be performed, with minimal patient discomfort. Moreover, the safety of e-PRP is guaranteed by the use of only autologous components.

PRP not only works as a vehicle for ASCs but may also act synergistically with stem cells through its growth factor, influencing both resident and newly-brought cells. However, it must be specified that a major bias in our study was the impossibility of determining the exact, single contribution to the healing process of ASCs and PRP when injected individually. Further studies are needed to assess whether such a co-administration synergistically improves the effects of ASCs and PRP alone.

Chronic wounds and their treatment pose a great medical challenge for patients as well as for the health care system. Regenerative medicine and cell-based therapy have recently tried to respond to the need for new methods of enhancing the healing process in order to achieve optimal outcomes. In this field, ASCs are the stem cell of choice due to their abundance, the minimally-invasive harvesting procedure, high yield upon isolation and easy clinical application, with no prior need to be manipulated. We have herein described our *e*-PRP therapy for chronic wounds; since preliminary results are promising and on account of the

therapy's effectiveness and safety, we believe we have advanced a new therapeutic approach for hard-to-heal wounds.

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Legends

Fig. 1: A 79-year-old male patient with chronic venous wound of the right lower limb, lasting from 18 months, who completely healed in 2 months after *e*-PRP therapy, shown preoperatively (a) and postoperatively (b).

Fig .2 A 76-year-old male patient with chronic arterial wound of the right lower limb, lasting from 12 months, completely healed in 40 days after *e*-PRP therapy, shown preoperatively (a) and postoperatively (b).