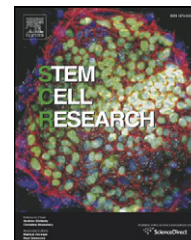




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REGULAR ARTICLE

Application of enhanced stromal vascular fraction and fat grafting mixed with PRP in post-traumatic lower extremity ulcers

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Abstract Background The authors presented their experience in regenerative surgery of post-traumatic lower extremity ulcers, evaluating the effects related to the use of Enhanced Stromal Vascular Fraction (e-SVF) and Fat Grafting with Platelet rich Plasma (PRP). The authors compared the results of two control groups.

Method The analysis involved 20 patients aged between 23 to 62 years affected by post-traumatic lower extremity ulcers. 10 patients managed with e-SVF and 10 patients managed with Fat grafting+PRP in the Plastic and Reconstructive Surgery Department at "Tor Vergata" University Rome. Patients in the first control group (n=10), were treated only with curettage and application of hyaluronic acid in the bed of ulcers. Patients in the second control group (n=10), were treated only with PRP. Results The authors showed that wounds treated with e-SVF healed better than those treated with hyaluronic acid. In fact, after 9.7 weeks, patients treated with e-SVF underwent $97.9\% \pm 1.5\%$ reepithelialisation compared to $87.8\% \pm 4.4\%$ of the first control group (only hyaluronic acid; $p < 0.05$). Patients treated with PRP and fat grafting also showed an improvement in reepithelialisation; in fact after 9.7 weeks, they underwent a $97.8\% \pm 1.5\%$ reepithelialisation compared to $89.1\% \pm 3.8\%$ of the second control group (only PRP; $p < 0.05$). As reported e-SVF and PRP mixed with fat grafting were the two treatments evidencing improvement in the healing of patients post-traumatic extremity ulcers.

Conclusions The results obtained proved the efficacy of these treatments, and the satisfaction of the patients confirmed the quality of the results.

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Introduction

The authors have already published the results obtained from using of Platelet Rich Plasma (PRP) mixed with fat grafting in the treatment of chronic-lower extremity ulcers [11,18] and loss of substance on the lower limbs [17]. Their subsequent research suggests a new therapeutic plan: the use of Enhanced Stromal Vascular Fraction (e-SVF). The presentation of clinical cases has shown that application of e-SVF can improve tissue healing. Improvement was reached through increased vascularization the secretion of growth factors improving tissue survival. e-SVF was extracted from patient's adipose tissue at the bedside using the Celution™ System. With this technology, this cell population were separated from adipocytes and extracellular matrix by enzymatic digestion and centrifugation. Consisting of a heterogeneous cell population they include endothelial cells, endothelial progenitor cells, smooth muscle cells, pericytes, macrophages and blood derived cells as well as multipotent Enhanced Stromal Vascular Fraction (e-SVF) [7,15]. The results achieved from this study also indicate the efficacy of the two treatments. The quality and potency of the results were confirmed by the patient satisfaction. The new therapeutic plan differs from conservative treatment that included elevation, skin grafting, local treatment (biosynthetic material), Vac (vacuum) therapy, from current available alternatives such as autologous skin (harvest, graft), skin from a bank (autologous, taken from cadaveric or living subjects), engineered skin (autologous expanded in vitro), and biosynthetic materials.

Results

Influence of e-SVF in tissue regeneration

The authors results showed that patients' wounds (Figs. 2A and 3A) treated with e-SVF healed better (Fig. 2B) than wounds treated with hyaluronic acid. In fact, after 9.7 weeks (Figs. 2C and 3B), the patients treated with e-SVF underwent a $97.9\% \pm 1.5\%$ reepithelialisation compared to $87.8\% \pm 4.4\%$ of 1st control group (hyaluronic acid only; $p < 0.05$). Patients treated with PRP and Lipostructure also showed an improvement in reepithelialisation; in fact after 9.7 weeks, they underwent a $97.8\% \pm 1.5\%$ reepithelialisation compared to $89.1\% \pm 3.8\%$ of 2nd control group (PRP only; $p < 0.05$). As reported e-SVF and PRP mixed with fat grafting were the two treatments that showed improvement in the healing process in post-traumatic extremity ulcers.

The authors reported clinical results in Tables 1 and 2 and observation of diameter lesion in Table 3.

Stromal Vascular Fraction nucleated cells from automatic and manual extraction

From adipose tissue, by manual extraction, we obtained about 250,000 ($\pm$) 34,782 nucleated cells/ml of fat tissue, instead by the automatic extractor, cell yield was about 50,000 ($\pm$) 6,956 nucleated cells/ ml of fat tissue ($p < 0.01$).

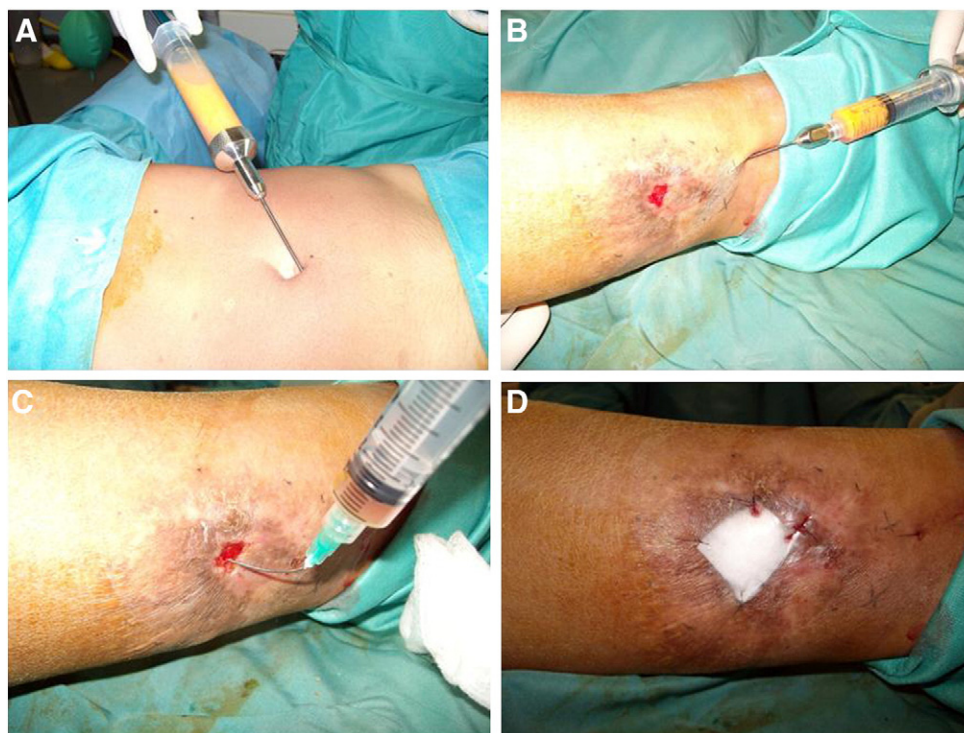


Figure 1 (A) Fat harvest; (B) intra-operative during peri-lesional injection (Fat + e-SVF); (C) surgical procedure of intra-lesional injection (only e-SVF); (D) medication with hyaluronic acid.



Figure 2 (A) pre-operative in lateral right; (B) post-operative -1 week- in lateral right after treatment based on perilesional injection of Fat+e-SVF and intra-lesional injection of only eSVF; (C) postoperative after 9.7 week, in lateral right.

Histopathological evaluation

Microscopic evaluation showed a progressive process of ulcer re-epithelialization, starting from a typical fibrin clot formation in biopsies taken at baseline (Fig. 4A), proceeding through the formation of dermal granulation tissue rich in newly deposited vessels with overlying early migration of keratinocytes at week 3 (Fig. 4B), followed by the appearance of reactive epidermal hyperplasia at week 7 (Fig. 4C), and finally resulting in complete re-epithelialization with newly deposited dermal collagen (Fig. 4D).

Discussion

New techniques in tissue regeneration are mostly explained in literature, but there are no articles concerning the possible use of e-SVF either by direct injection into the bed of the ulcers or injecting it in the perilesional area mixed fat graft.

The potential benefit of e-SVF supplementation could be explained by the ability of cells, which exist within the e-SVF population [2], to secrete various growth factors that improve survival and increased vascularization [3,4] leading



Figure 3 (A) pre-operative in back projection; (B) post-operative -9.7 week- in back projection after treatment based on perilesional injection of Fat+e-SVF and intra-lesional injection of only e-SVF.

Table 1 A Patients, affected by post-traumatic lower-extremity ulcers treated with SVF-enhanced autologous fat grafts, obtained by Celution System; 1B Patients, affected by post-traumatic lower-extremity ulcers treated with fat grafting based on Coleman technique mixed with PRP

Pz	Age	Co-morbidity	Patology	Old treatment	Treatment	Complication
A						
1	42	No	Post-traumatic lower extremity ulcers	Wound complex	Fat graft (Celution)	No
2	34	No	Post-traumatic lower extremity ulcers	Toilette+wound complex	Fat graft (Celution)	No
3	40	No	Post-traumatic lower extremity ulcers	Wound complex	Fat graft (Celution)	No
4	29	No	Post-traumatic lower extremity ulcer	Amputation	Fat graft (Celution)	ematoma
5	37	Dislipidemy	Post-traumatic lower extremity ulcers	Toilette+wound complex	Fat graft (Celution)	No
6	39	No	Post-traumatic lower extremity ulcers	Wound complex	Fat graft (Celution)	No
7	35	Ipertension	Post-traumatic lower extremity ulcers	Toilette+wound complex	Fat graft (Celution)	No
8	56	No	Post-traumatic lower extremity ulcers	Wound complex	Fat graft (Celution)	No
9	51	Cardiological disease	Post-traumatic lower extremity ulcers	Toilette+wound complex	Fat graft (Celution)	No
10	60	No	Post-traumatic lower extremity ulcers	Wound complex	Fat graft (Celution)	No
B						
1	46	No	Skin depression+post-traumatic wound	No	Lipostructure+PRP	No
2	23	No	Post-traumatic ulcers	No	Lipostructure+PRP	No
3	41	No	Post-traumatic foot ulcers	No	Lipostructure+PRP	No
4	62	No	Skin depression	No	Lipostructure+PRP	No
5	37	Ipertension	Post-traumatic lower extremity wound	No	Lipostructure+PRP	No
6	32	No	Post-traumatic wound	No	Lipostructure+PRP	No
7	34	No	Post-traumatic wound	No	Lipostructure+PRP	No
8	42	Ipertension	Post-traumatic skin depression+Ulcers	No	Lipostructure+PRP	No
9	36	No	Post-traumatic lower extremity ulcers	No	Lipostructure+PRP	No
10	35	No	Post-traumatic lower extremity ulcers	No	Lipostructure+PRP	No

to an increased survival of the graft as shown by a study on a rodent [5]. In this work we analyzed the SVF cellular content from the automatic system, since we previously did it for the manual extraction used in our *in vitro* studies (Cervelli et al 2009). Our results documented that the SVF cell yield from the manual system was much more efficient than the automatic system. It would be necessary to have further informations about automatic system operation to explain this finding. We hypothesize that the mechanism of regeneration of the tissue is the following: targeting of damaged areas, release of angiogenic and antiapoptotic factors followed by formation of new vessels and oxygenation.

Implanted adipose tissue must survive by a simple diffusion mechanism until an active blood supply is re-established. Thus survival of the graft, particularly of a larger volume graft, is balanced between this process and hypoxia-induced cell death [1,6]. Pro-survival factors may therefore promote.

long term retention and consequently durability of the graft. In an animal study, this effect was achieved by using gene therapy to deliver Vascular Endothelial Growth Factor (VEGF; a potent pro-angiogenic factor) to the graft. This resulted in increased blood vessel density within the graft and a significant improvement in graft retention at 15 weeks [7]. Neo-angiogenesis was also confirmed from an histopathologically point of view highlighting

the abundance in capillaries sprout within the healing tissue, leading to a complete re-epithelialization of the ulcers.

e-SVF can favour neo-angiogenic vascularization and fibrogenic activity of fibroblasts that favour adipose tissue survival and three-dimensional organization. Compared to traditional fat grafting, the survival of the graft is more probable and fat necrosis is potentially reduced due to improved vascular development in the implanted area. Results of this study offer an *in vivo* tissue-engineering approach that provides an optimized microenvironment, supporting the correct architectural adipocyte distribution, better cell-to-cell interaction, adipose tissue survival, and maybe limited differentiation from e-SVF; this could offer early protection from surrounding inflammatory events. The early establishment of new micro capillary networks and the proper deliver of nutrients and oxygen to the implant may contribute to the improved outcomes observed [8,9].

Our clinical study clearly documented that e-SVF improved skin ulcer reepithelialisation in patients who underwent regenerative surgery. Moreover, e-SVF improved maintenance and function of adipose tissue graft. The authors, having a significant experience with Platelet Rich Plasma mixed fat grafting, compared the results of the clinical study. In fact, platelet-rich plasma was used for lower extremity ulcers, demonstrating that the use of PRP

Table 2 A The first control group treated with curettage and hyaluronic acid; 2B the 2nd control group treated with only PRP

Pz	Age	Co-morbidity	Patology	Old treatment	Treatment	Complication
A						
1	59	Dislipidemy	Post-traumatic Foot ulcer	Toilette	Wound complex	Edema, Infection
2	47	No	Post-traumatic Foot ulcer	No	Wound complex	No
3	43	No	Post-traumatic Foot ulcer	Toilette	Wound complex	No
4	54	Dislipidemy	Post traumatic Foot ulcer	Toilette	Wound complex	Infection
5	62	Ipertension	Post-traumatic Lower extremity ulcers	Toilette	Wound complex	No
6	49	Cardiological disease	Post-traumatic Lower extremity ulcer	Amputation	Wound complex	No
7	54	Cardiological disease	Post-traumatic Lower extremity ulcer	Toilette	Wound complex	Edema, Ematoma, Infection
8	44	No	Post traumatic Foot ulcer	No	Wound complex	No
9	31	Arteriopatya lower extremity	Post traumatic Foot ulcer	No	Wound complex	No
10	23	Trauma	Post traumatic Foot Ulcer	N° 1 Vacuum therapy	Wound complex	No
B						
1	45	Ipertension	Post-traumatic Foot ulcer	Toilette	PRP	Edema
2	33	No	Post-traumatic Foot ulcer	No	PRP	No
3	37	Dislipidemy	Post-traumatic Foot ulcer	Toilette	PRP	No
4	60	Trauma	Post traumatic Foot ulcer	Toilette	PRP	Ematoma
5	61	No	Post-traumatic Lower extremity ulcers	Toilette	PRP	No
6	29	Arteriopatya lower extremity	Post-traumatic Lower extremity ulcer	Amputation	PRP	No
7	35	Cardiological disease	Post-traumatic Lower extremity ulcer	Toilette	PRP	Edema, Ematoma
8	40	No	Post traumatic Foot ulcer	No	PRP	No
9	43	No	Post traumatic Foot ulcer	No	PRP	No
10	23	No	Post traumatic Foot Ulcer	N° 1 Vacuum therapy	PRP	No

combined with fat tissue resulted in numerous advantages [10,11]. The authors in the treatment with PRP used an average of 0.3-0.5 ml of activated platelet gel combined with 1 ml of centrifuged fat tissue. Platelet gel is a mixture of autologous proteins [12,13] that contains from 300,000 to 350,000 platelets, which once injected stimulate skin fibroblast.

This mixture favour tissue growth by a new synthesis of collagen. During the wound-healing process (4 phases: haemostasis, inflammation, proliferation, and re-modelling), platelet growth factors serve as messengers to regulate a well-orchestrated and complex series of events involving cell-cell and cell-matrix interactions that promote the proliferation of mesenchymal and other stem cells at the wound site [14]. The authors, on the basis of this concept, have added fat grafting to PRP. Autologous platelet-derived wound-healing factors were proposed to regulate wound healing of chronic ulcers by promoting the formation of granulation tissue in the early healing phase. Alternatively, the authors used conservative treatment that included elevation, skin grafting, local treatment (biosynthetic material), and Vac (vacuum) therapy.

The current available alternatives for treating ulcers are autologous skin (harvest, graft), skin from a bank (autologous, taken from cadaveric or living subjects), engineered skin (autologous expanded in vitro), and biosynthetic materials.

The results proved the efficacy of these two new treatments (e-SVF and PRP).

Material and methods

Patients

A total of 20 patients were treated at the Plastic and Reconstructive Surgery Department of "Tor Vergata" University, Rome.

10 patients (5 female and 5 male), aged between 29 and 60 years (Table 1A), affected by post-traumatic lower-extremity ulcers were treated with SVF-enhanced autologous fat grafts, obtained by Celution System. The patients were subjected to additional wash and centrifugation cycles, after which 5 ml of the e-SVF suspension was extracted from the system. The medical process continued by dividing the 5 ml of e-SVF into two equal parts. The first half of e-SVF (2,5 ml) was added to the tissue collection container with the liposuction. Subsequent to the carrying out of a washing step, the e-SVF suspension was added and mixed with the washed fat graft. Using specific micro-cannulas for implantation the SVF-enhanced fat graft was transferred into 10 ml syringes and aseptically re-injected into the perilesional area. The remaining part was injected into the bed of ulcer after curettage.

Table 3 A Diameter of the lesion in the patients treated with e-SVF; 3B diameter of the lesion in the patients treated with fat grafting mixed with PRP; 3C diameter of the lesion in the patients treated with hyaluronic acid; 3D diameter of the lesion in the patients treated with PRP

Diameter of the lesion	Time 0	Time 1° week	Time 3° week	Time 5° week	Time 7,1° week	Time 9,7° week	Time 16° week
A							
Pz 1	4,7	4,1	3,8	3,0	2,7	0	0
Pz 2	7,3	6,3	5,8	5,1	4,6	0,5	0
Pz 3	3,8	3,5	3,0	2,8	2,2	0	0
Pz 4	5,6	5,3	5,0	4,8	4,1	0,77	0
Pz 5	4,1	3,5	3,1	2,0	1,1	0	0
Pz 6	4,3	3,7	3,3	2,4	1,5	0	0
Pz 7	4,3	3,9	3,4	2,8	2,0	0	0
Pz 8	7,5	7,2	6,8	5,9	5,0	0	0
Pz 9	5,1	4,7	4,2	3,4	2,4	0	0
Pz 10	6,2	5,8	5,3	4,5	3,7	0	0
B							
Pz 1	7,1	6,9	6,2	4,1	1,1	0,6	0
Pz 2	3,6	3,3	2,7	1,6	0	0	0
Pz 3	5,9	5,5	4,9	2,7	0	0	0
Pz 4	7,2	6,9	6,4	5,3	1,4	0	0
Pz 5	4,5	4,3	3,9	2,1	0	0	0
Pz 6	6,3	6,1	5,8	2,6	0	0	0
Pz 7	3,3	3,0	2,7	1,4	0	0	0
Pz 8	4,2	3,9	2,3	1,5	0	0	0
Pz 9	5,1	5,0	4,5	4,2	2,1	0,7	0
Pz 10	3,8	3,6	3,1	2,6	1,4	0	0
C							
Pz 1	6,8	6,8	6,5	5,8	4,0	2,8	1,2
Pz 2	3,9	3,9	3,5	2,7	0,9	0	0
Pz 3	6,2	6,2	5,9	5,0	3,4	1,3	0,2
Pz 4	7,0	7,0	6,7	5,9	3,6	1,6	0,4
Pz 5	4,9	4,9	4,5	3,6	1,2	0	0
Pz 6	6,0	6,0	5,7	4,9	2,5	0,8	0
Pz 7	3,0	3,0	2,7	1,9	0,4	0	0
Pz 8	3,9	3,9	3,5	2,7	1,0	0	0
Pz 9	4,8	4,8	4,5	3,7	2,1	0,9	0
Pz 10	4,0	4,0	3,6	2,8	1,2	0,2	0
D							
Pz 1	5,4	5,4	5,0	4,6	2,3	1,0	0
Pz 2	4,3	4,3	3,9	3,4	1,4	0	0
Pz 3	5,1	5,1	4,7	4,3	2,1	0,3	0
Pz 4	6,3	6,3	5,9	5,5	3,4	1,9	0,3
Pz 5	5,1	5,1	4,7	4,3	2,3	1,1	0
Pz 6	6,5	6,5	6,1	5,4	3,3	1,7	0,2
Pz 7	3,7	3,7	3,3	2,9	1,0	0	0
Pz 8	4,5	4,5	4,1	3,8	1,9	0,3	0
Pz 9	4,1	4,1	3,7	3,4	1,3	0	0
Pz 10	4,9	4,9	4,5	4,1	2,0	0	0

10 patients (Table 1B) 5 female and 5 male aged between 23 and 62 years affected by post-traumatic lower extremity ulcers were treated with fat grafting based on Coleman technique mixed with PRP. The purified fat was obtained after centrifugation at 3000 RPM and placed in 1 ml syringes, therefore aseptically reinserted using specific micro – cannulas for implanting. The 1 ml of fat centrifuged tissue was also mixed by the authors with 0.3-0.5 average ml of PRP. The selection of location destined to receive the implant was determined taking into account the diversity in the lesions. The harvested material was implanted into the lower extremity ulcers of the perilesional area. In order to implant the fat tissue, small tunnels were previously created forcing the cannulas of 1.5 mm diameter with accurate and controlled movements. Once the fat tissue had been implanted at different levels the access incisions were closed using 5-0 nylon stitches and no compressive bandage was applied.

This study is part of a research project approved by Tor Vergata.

Control groups

In order to establish the effects of their treatment, the authors compared their results with two control groups each made up of 10 patients.

The authors considered exclusion criteria. Exclusion criteria were divided in two types: local and systemic. The systemic criteria include: platelet disorders, thrombocytopenia, anti aggregating therapy, bone marrow aplasia, uncompensated diabetes, sepsis and cancer. The local criteria include: osteomyelitis, loss of substance of more than 50% of the segments. The authors was not considered exclusion criteria tobacco use and genetic disorders.

The first control group (Table 2A) comprised 5 females and 5 males aged between 23 and 62 years all affected by post-traumatic lower extremity ulcers. This sample group was treated with curettage and application of hyaluronic acid in the bed of the ulcers. The medication based on hyaluronic acid where removed from the authors after 15 days.

The second control group (Table 2B) comprised the same characteristics of the first (number of people, age and type of ulcers), however patients underwent a different treatment. They received only PRP.

The PRP was obtained using 18 ml of blood from a peripheral venous access collected in two sterile 9-ml tubes. The blood was extracted using the Cascade Kit and subjected to centrifuging for 10 min at 1,100 g [10,11].

After centrifugation, the two tubes of inactivated PRP were switched with two 10-ml tubes containing CaCl [10,11].

From this point, after the addition of CaCl (which determined the activation of PRP), the two tubes obtained were centrifuged for 15 min at 1,100 g. A product with increased consistency was obtained.

This sample group was treated with curettage and it was applied to it only PRP obtained in the bed of the ulcers. The bed of the ulcer contained the PRP solid gel fixed with nylon.

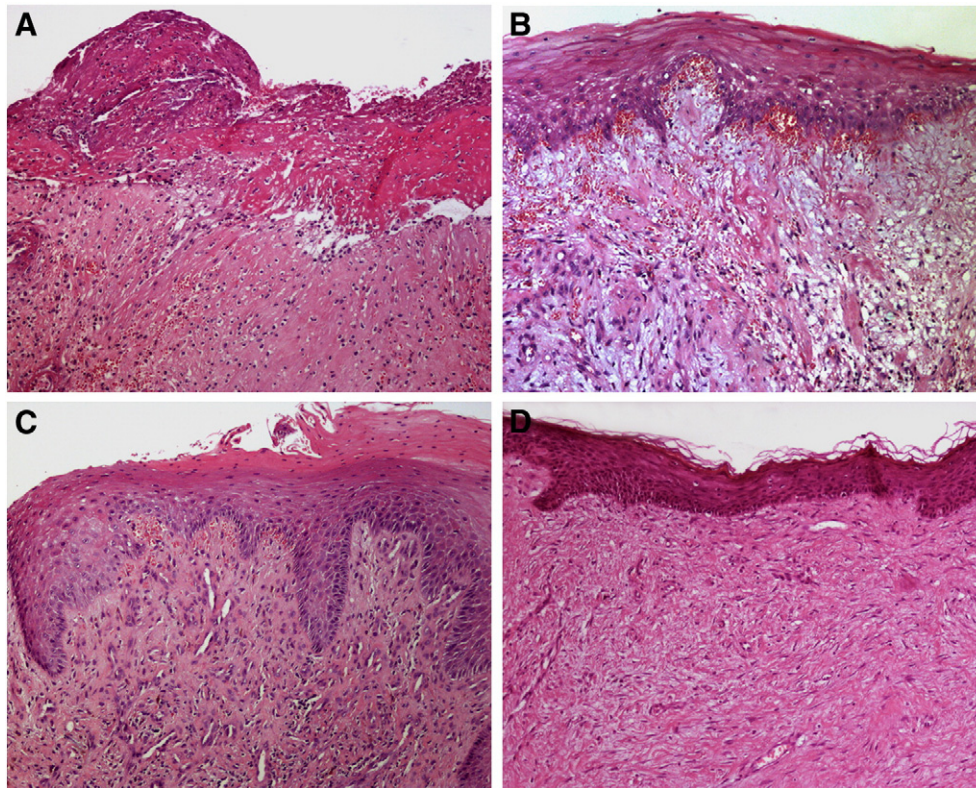


Figure 4 Histopathological findings of leg chronic ulcer before, during and after treatment by e-SVF. (A) Baseline evaluation of a skin punch biopsy revealing the typical fibrin clot with cellular debris and dermal inflammatory infiltrate rich in neutrophils; (B) At week 3, formation of dermal granulation tissue, rich in newly formed vessels, along with chronic inflammatory infiltrate, with overlying initial migration of keratinocytes starting to re-epithelialize the defect; (C) At week 7, regenerative skin with reactive epidermal hyperplasia with underlying chronic lymphomonocytic infiltrate, abundant capillaries sprout and fibroblasts; (D) After treatment, at week 16, complete healing with re-epithelialization and newly deposited dermal collagen. (Hematoxylin and eosin stain. Original magnification, A-D: x100).

Harvest region and preparation of the SVF-enhanced autologous fat graft

A solution based on 1 fl of adrenalin in 500 cc of cold physiological solution was injected into the abdominal region of the patients treated with e-SVF. The cell and tissue preparation procedure was a two phases process. In first phase the abdominal region of each using 3 mm cannulas of an average of 400 ml of liposuction (Fig. 1 A). Maintaining on aseptic technique, the plunger of the 60 ml-syringe was removed and the tip was closed with a cap. Half of the liposuction (200 ml average) was placed into the tissue collection container of the Celution™ 800/CRS System (Cytori Therapeutics Inc., USA). A wash cycle removed blood and free lipid from the tissue and the Celase™ 835/CRS Reagent was added in order to enzymatically digest the tissue which released e-SVF. After additional wash and centrifugation cycles, 5 ml of the e-SVF suspension was extracted from the system.

Having completed the first steps the treatment, the authors divided e-SVF suspension in to two equal parts. In phase one of the study, the first aliquot of suspension containing e-SVF (2.5 ml) was injected directly into the bed of the ulcer (Fig. 1C).

In the second phase, the remaining 200 ml of liposuction was added to the tissue collection container and a washing step was carried out. Once completed, the remaining aliquot of the previous division of e-SVF suspension was added and mixed with the washed fat graft, resulting in approximately 170 ml of SVF-enhanced fat tissue for grafting. This newly processed cell-enhanced fat graft typically consisting of 25%-30% of water which will be reabsorbed by the body in the post-operative period. This overall process is controlled through automated sensors and processing algorithms ensuring standard handling of the tissue and cells the process is completed within 160 minutes. The SVF-enhanced fat graft was transferred into 10 ml syringes and aseptically re-injected in the perilesional area (Fig. 1B) using specific-micro-cannulas for implantation.

Surgical technique and location of implantation

The authors injected the SVF-enhanced adipose tissue using specific micro-cannulas (1-2 mm in diameter) for implantation.

The area destined to receive the graft was determined based on the necessary selection of a perilesional graft or an intra-lesional application or both. Subsequently, the

authors applied to the post-traumatic lower extremity ulcers the first quantity (2.5 ml) of the e-SVF suspension directly into the ulcer bed. A medication based on hyaluronic acid was then fixed applying a 3-0 nylon at the margins and a superior silicon substrate (Fig. 1D). The other remaining aliquot of e-SVF suspension, was mixed with fat graft and injected into the perilesional area at the subcutaneous tissue level. The substrate was removed after 8 days, the medication was removed after 16 days. After pre-tunnel, fat tissue was implanted at different levels using a delivery cannula (1-2 mm in diameter) with precise, controlled movements. While using the cannula small quantities of e-SVF enhanced fat graft were injected to create a large grid, thus increasing the probability of transplanted tissue survival. Several layers were laid down to increase the contact surface between the receiving tissue and the implant. This technique is of fundamental importance: it allows each layer deposited to survive by diffusion during the few days necessary for growth of blood vessels which will nourish the implant permanently. The incisions were closed with 5-0 nylon suture and no compressive bandage was applied.

Clinical evaluation methods

Through the analysis of pre-operative and post-operative photos, the authors were able to evaluate the tissue regeneration. The sample photos taken into account were of the same size, brightness and with the same contrast to facilitate comparison. In fact, operators were able to calculate the percentage of wound healed. Finally, the mean between patient and operator evaluation was made. To evaluate the healing rate of skin chronic ulcer used the method of Kazakos [16] with minor modifications. This process comprises a further division of the patients in four groups each of which differentiate in treatment. The first group reunited the patients treated with e-SVF, the second included also together the patients treated with fat grafting mixed with PRP. The third group reunited the patients treated with hyaluronic acid and the fourth included the patients treated with PRP. Finally, the change of wound surface area (2 cm) was calculated on a weekly basis.

Stromal Vascular Fraction nucleated cells isolation and counting

For manual SVF extraction, liposuction aspirates were washed three times with phosphate-buffered saline (PBS) and suspended in an equal volume of PBS and 0.1% collagenase type I (C130; Sigma-Aldrich, Milan, Italy) pre-warmed to 37 °C. Adipose tissue was placed in a shaking water bath at 37 °C with continuous agitation for 60 min and centrifuged for 10 min at 600 g at room temperature. The supernatant, containing mature adipocytes, was aspirated. The SVF pellet was resuspended in erythrocyte lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA) and incubated for 5 min at room temperature. After centrifugation at 1100RPM for 5 min, the pellet was resuspended in few micro-litres of growth medium and passed through a 100-µm Falcon strainer (Becton and Dickinson, Sunnyvale, CA) and

cellular population was counted using hemocytometer. For automatic SVF extraction the Celution® System was used. After centrifugation of fat inserted in the system for two hours a 5 ml ringer solution was obtained containing Stromal Vascular Fraction Cells. SVF pellet was resuspended in erythrocyte lysis buffer and cellular population was counted as described above. After centrifugation at 1100RPM for 5 min, the pellet was resuspended in a few micro liters of growth medium and cellular population was counted using hemocytometer.

Histopathological evaluation

Incisional punch biopsies (3 mm in diameter) at the level of the ulcers were obtained from a small sample of patients at baseline, week 3, week 7 and week 16. Microscopic evaluation of routine hematoxylin-eosin stained paraffin-derived sections was performed to morphologically verify the healing process of the ulcers treated by e-SVF.

Statistical analysis

Values are shown as mean plus standard error of mean (s.e.m). Results were analyzed by means of Students *t*-test, and differences considered statistically significant for *p* < 0.05.

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