Adipose-Derived Stromal Vascular Fraction Cells and Platelet-Rich Plasma: Basic and Clinical Evaluation for Cell-Based Therapies in Patients With Scars on the Face

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Background: Actually, autologous fat grafts have many clinical applications in breast surgery, facial rejuvenation, buttock augmentation, and Romberg syndrome as well as a treatment of liposuction sequelae.
Objective: The aim of this article was to describe the preparation and isolation procedures for stromal vascular fraction (SVF), the preparation of platelet-rich plasma (PRP), and the clinical application in the treatment of the scar on the face.
Methods: Ten patients with burns sequelae (n = 6) and post–traumatic scars (n = 4) were treated with SVF-enhanced autologous fat grafts obtained by the Celution System. Another 10 patients with burns sequelae (n = 5) and post–traumatic scars (n = 5) were treated with fat grafting based on the Coleman technique mixed with 0.5 mL of PRP. To assess the effects of their treatment, the authors compared their results with those of a control group consisting of 10 patients treated with centrifuged fat.
Results: In the patients treated with SVF-enhanced autologous fat grafts, we observed a 63% maintenance of contour restoring after 1 year compared with only 39% of the control group (n = 10) treated with centrifuged fat graft ($P < 0.0001$). In the patients treated with fat grafting and PRP, we observed a 69% maintenance of contour restoring after 1 year compared with that of the control group (n = 10).
Conclusions: Autologous fat grafting is a good method for the correction of scars on the face instead of the traditional scar surgical excision.

Key Words: Adipose-derived stem cell, enhanced stromal vascular fraction fat graft with platelet-rich plasma, platelet-rich plasma

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The stromal vascular cell fraction (SVF) of the adipose tissue has come more and more into the focus of stem cell research because this tissue compartment provides a rich source[1] of multipotent adipose tissue–derived stromal cells.

We decided to use the term *SVF* in this study as a compromise and only for cells that were (*a*) passaged several times, (*b*) shown to exert multipotential differentiation capacity, and/or (*c*) molecularly characterized by using a multipanel of mesenchymal differentiation markers.

The SVF can easily be isolated from human adipose tissue,[2,3] and it has the potential to differentiate into bone, cartilage, tendons, skeletal muscle, and fat when cultivated under lineage-specific conditions.[3,4] Tissue engineering of these mesenchymal organs is of major interest in human diseases, such as inherited, traumatic, or degenerative bone, joint, and soft tissue defects (skeletal regeneration and cartilage repair). Plastic tissue regeneration after oncoplastic surgery of the breast and other malignancies and reconstruction of muscle and adipose tissue defects after scars and burn injury do represent additional needs for cell-based therapies. Moreover, an initial effort has been made regarding the differentiation of SVF across the germ leaf-specific tissues into non–mesenchymal tissues (*cross-differentiation*), such as neurons or endocrine pancreatic cells.

The authors have already published the results obtained from using platelet-rich plasma (PRP) mixed with fat grafting in the treatment of chronic lower-extremity ulcers,[5–7] loss of substance on the lower limbs,[7] and application of enhanced SVF (e-SVF) in post–traumatic lower extremity ulcers.[8]

Their subsequent research suggests a new therapeutic plan: the use of enhanced SVF or fat graft mixed with PRP. The presentation of clinical cases has shown that the application of e-SVF can improve tissue healing and maintenance of fat graft volume. Improvement was reached through increased vascularization, the

secretion of growth factors improving tissue survival. In this study, e-SVF was extracted from the patient's adipose tissue at the bedside using the Celution System. With this technology, this cell population was 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 e-SVF.[9,10] The results achieved from this study also indicate the efficacy of the 2 treatments. The quality of the results was confirmed by the satisfaction of the patients. The new therapeutic plan differs from conservative treatment, which included skin grafting and local treatment (biosynthetic material), as well as from current available alternatives such as autologous skin (harvest, graft), skin from a bank (autologous, taken from cadaveric or living subjects), and engineered skin (autologous expanded in vitro).

MATERIALS AND METHODS

Patients

A total of 20 patients were treated at the Department of Plastic and Reconstructive Surgery of the University of Rome Tor Vergata. The analysis involved 20 patients with soft tissue defect and scar on the face.

Ten patients (5 women and 5 men), aged between 23 and 67 years, with outcomes of burn (n = 6) and post–traumatic scars (n = 4) were treated with SVF-enhanced autologous fat grafts obtained by the Celution System (Fig 1F, G). The patients were subjected to additional wash and centrifugation cycles, after which 5 mL of the enhanced adipose-derived stem cells suspension was extracted from the system (Fig 1H). This quantity of 5 mL of enhanced adipose-derived stem cells was added to the tissue collection container with the liposuction (Fig 1I). Subsequent to the carrying out of a washing step, the e-SVF suspension was added and mixed with the washed fat graft. Using specific microcannulas for implantation, the SVF-enhanced fat graft was transferred into 10-mL syringes and aseptically re-injected into the subcutaneous area (Fig 1L).

Ten patients (5 women and 5 men), aged between 21 and 69 years, with outcomes of burn (n = 5) and post–traumatic scars (n = 5) were treated with fat grafting based on the Coleman technique mixed with 0.5 mL of PRP. The purified fat was obtained after centrifugation at 3000 rpm and placed in 1-mL syringes, therefore aseptically reinserted using specific microcannulas for implanting. The 1 mL of fat-centrifuged tissue was also mixed by the authors with 0.5 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 subcutaneous area of the scars. To implant the fat tissue, small tunnels were previously created, forcing the cannulas of 1.5 mm in 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.

To establish the effects of their treatment, the authors compared their results with those of a control group that consists of 10 patients treated with centrifuged fat. In each group (study and control), 1 operation was required in 6 cases, and 2 operations were required in 4 cases.

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

Control Groups

The control group consists of 10 patients (with the same characteristics of the groups of the study, number of people, age, and type of scars) and was treated with centrifuged fat in accordance with the Coleman procedure without PRP.

The authors considered exclusion criteria. The exclusion criteria were divided into 2 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 infection or diastasis. The authors did not consider tobacco use and genetic disorders as exclusion criteria.

Clinical Evaluation Methods

Through the analysis of preoperative (Fig 2A, B, E, F, and Fig 3A, C) and postoperative (Fig 2C, D, G, H and Fig 3B, D) photographs, the authors were able to evaluate the tissue regeneration. The sample photographs taken into account were of the same size and brightness and with the same contrast to facilitate comparison. In fact, the operators were able to calculate the percentage of fat reabsorption.

In addition, 3 methods for the evaluation of outcomes were used: (1) team evaluation, (2) magnetic resonance imaging (MRI) and ultrasound, and (3) patient self-evaluation. The team evaluation is an evaluation method based on clinical observation, using a scale of 6 values (excellent, good, discreet, enough, poor, inadequate). The patient-based self-evaluation uses the same 6 values mentioned previously. The factors/variables, which were taken into account, were pigmentation, vascularization, pliability, thickness, itching, and pain.

Finally, the mean between the patient and operator evaluation was made. The first group reunited the patients treated with e-SVF; the second also included together the patients treated with fat grafting mixed with PRP. The control group reunited the patients treated with centrifuged fat.

Preparation and Molecular Characterization of Adipose-Derived Stromal Fraction Cells

In this study, for the manual SVF extraction, the authors used the method published previously.[8]

The proliferation of SVF can be stimulated by fibroblast growth factor 2 (FGF-2) via the FGF-receptor-2,[11] by sphingosylphosphorylcholine via activation of c-Jun N-terminal kinase,[11] by platelet-derived growth factor via activation of c-jun N-terminal kinase,[12] and by oncostatin M via activation of the microtubule-associated protein kinase/extracellular signal-regulated kinase and the JAK3/STAT1 pathway.[13] In addition, SVFs do express an autocrine FGF-2 loop that maintains their self-renewal ability in vitro.[14] Because inhibition of microtubule-associated protein kinase 1 reduces the clonogenic potential of SVFs without affecting their differentiation potential, the extracellular signal-regulated kinase 1/2 signaling pathway seems to be involved in the FGF-2–mediated self-renewal.[15] In addition, the longevity of human SVF can be extended by overexpression of the catalytic subunit of the human *telomerase* gene.[16]

Stromal vascular cell fractions are known to secrete potent growth factors, such as vascular endothelial growth factor (VEGF), hepatocyte growth factor, and insulinlike growth factor 1.[17] Tumor necrosis factor can significantly increase the secretion of VEGF, hepatocyte growth factor, and insulinlike growth factor 1 from SVF by a p38 mitogen-activated, protein kinase–dependent mechanism.[17] The increasing knowledge on the molecular mechanisms regulating SVF proliferation might be useful for the improvement of isolation and culturing procedures.

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has proposed, most recently, a minimal set of 4 criteria to define human mesenchymal stem cells (MSC)[18]:

1. MSC have to be plastic-adherent when maintained under standard culture conditions.

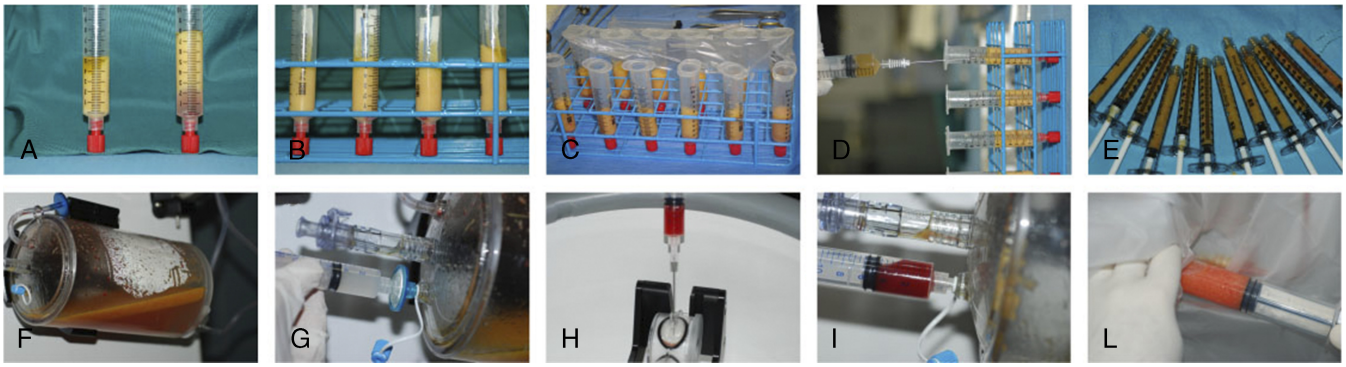

FIGURE 1. Method of preparation of fat graft enhanced with PRP: image of syringes with fat tissue and fluid fat portion separated for the serous bloody part (A), syringes with purified fat tissue after drain of fluid and the serous bloody part (B), syringes with purified fat tissue ready to PRP addition (C), image of PRP addition (D), 1-mL syringes with purified fat tissue + PRP (E). F, Method of preparation of SVF-enhanced fat graft: image of a wash cycle. G, The Celase 835/CRS Reagent was added to enzymatically digest the tissue that released SVF. H, Four to 5 mL of the SVF suspension was extracted from the system. I, Stromal vascular cell fraction suspension was added and mixed with the washed fat graft. L, The SVF-enhanced fat tissue for grafting.

2. MSC must have the ability for osteogenic, adipogenic, and chondrogenic differentiation.
3. MSC must express CD73, CD90, and CD105.
4. MSC must lack expression of the hematopoietic lineage markers c-kit, CD14, CD11b, CD34, CD45, CD19, CD79, and human leukocyte antigen (HLA)-DR.

The transcriptional and molecular events triggering the lineage-specific mesodermal differentiation into adipocytes,[9,19,20] myocytes,[10,21,22] osteocytes,[23,24] or chondrocytes[23] are well known, and several reviews focus on that point. In the case of adipocyte differentiation, although several transcriptional key events regulating the differentiation of pre-adipocytes into mature adipocytes have been identified in the last decade, master genes committing the multipotent mesenchymal stem cell to adipoblasts are still awaiting discovery. Recently, transcriptional coactivator with PDZ-binding motif (TAZ) was identified as an early "molecular rheostat" modulating mesenchymal stem cell differentiation.[25–27] Whereas runx-2, the key osteogenic transcription factor, triggers MSC to an osteogenic differentiation program, adipogenic differentiation is mainly

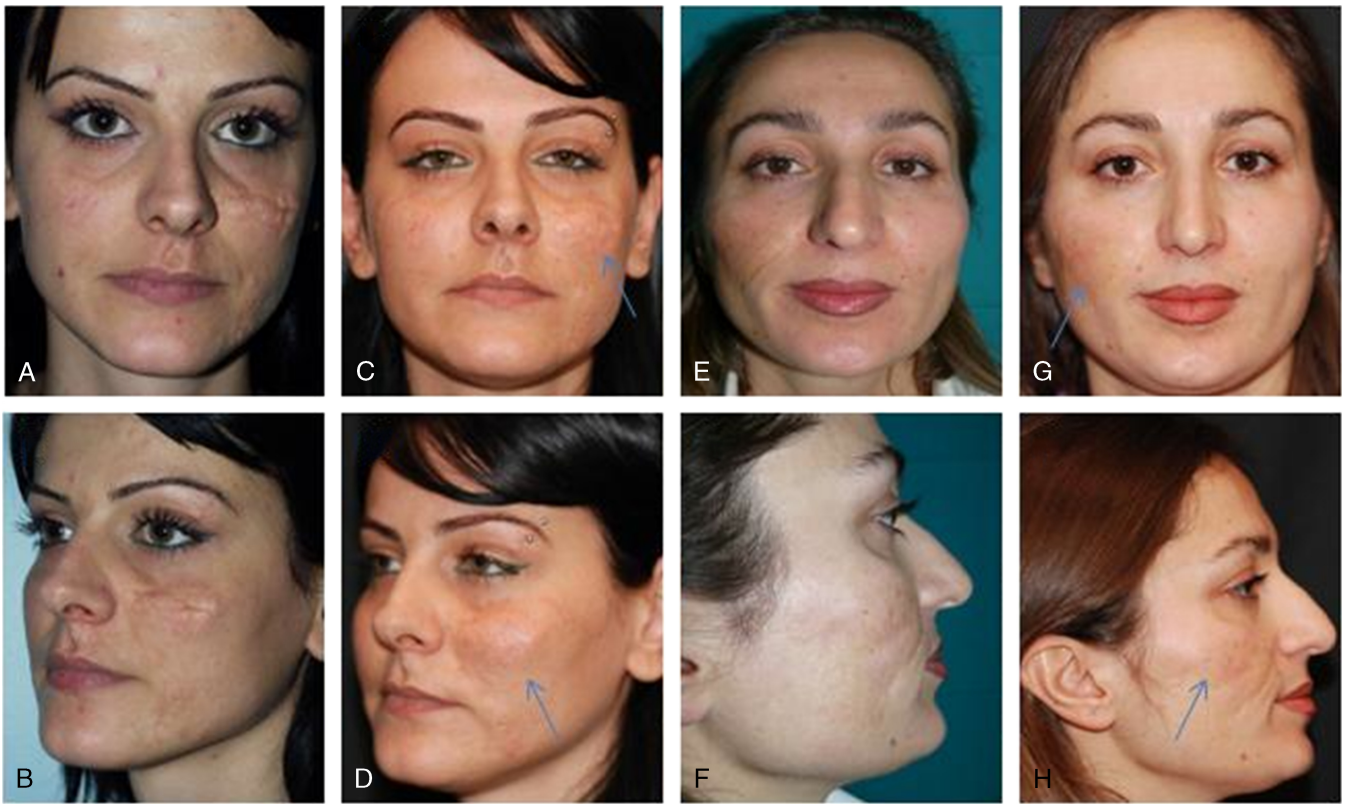

FIGURE 2. A, Preoperative situation of a patient with soft tissue defect and posttraumatic scar in frontal projection. B, Preoperative situation in three-fourths left projection. C, Postoperative situation after 30 months and after 1 treatment based on the use of e-SVF in frontal projection. D, Postoperative situation in three-fourths left projection. E, Preoperative situation of a patient with soft tissue defect and post–burn scar in frontal projection. F, Preoperative situation in lateral right projection. G, Postoperative situation after 50 months and after 1 treatment based on the use of fat grafting mixed with PRP in frontal projection. H, Postoperative situation in lateral right projection.

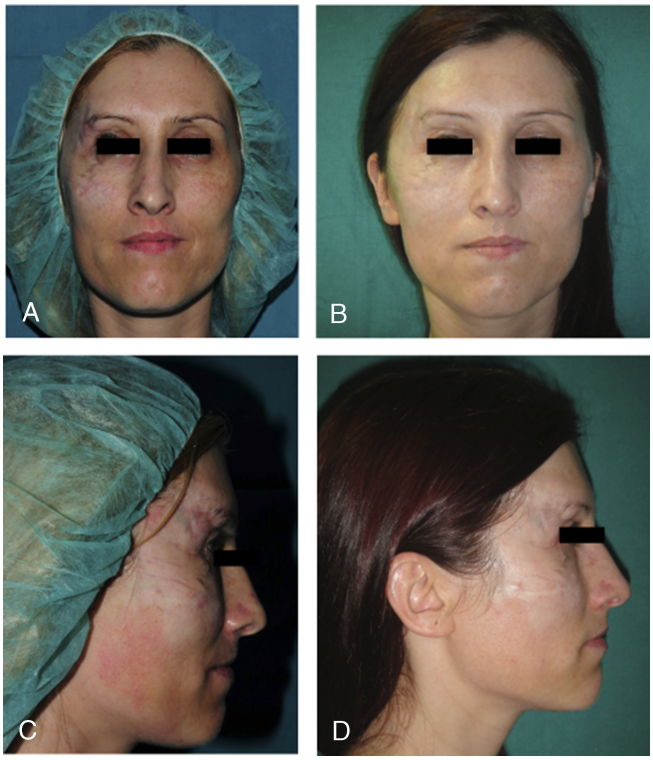

FIGURE 3. A, Preoperative situation of a patient with soft tissue defect and post–traumatic scar in frontal projection. B, Postoperative situation after 60 months and after 2 treatments based on the use of fat grafting mixed with PRP in frontal projection. C, Preoperative situation in lateral right projection. G, Postoperative situation after 60 months and after 2 treatments in lateral right projection.

promoted by PPAR. It is mainly of interest how these 2 transcription factors are regulated to determine these alternative cell fates. Hong and Yaffe[26] demonstrated that TAZ coactivates runx-2–dependent gene transcription and inhibits PPAR-dependent gene transcription. As a net result, osteogenic differentiation is favored. By modulating TAZ expression in cell lines, mouse embryonic fibroblasts, primary MSC in culture, and in zebrafish in vivo, Hong and Yaffe[26] were successful in triggering osteogenic versus adipogenic differentiation. These results indicate that TAZ functions as a real molecular rheostat that allocates MSC to either osteogenic or adipogenic differentiation. In this context, catenin signaling and WNT3A are important mediators in reducing the osteogenic differentiation in human adipose tissue–derived MSC.[28]

Platelet-Rich Plasma and Fat Preparation Based on Current Regulation

This study was conducted with the informed consent of the patients and at the presence of a physician of transfusional service, in accordance with the current European regulation. Briefly, the process of preparing PRP consists of 4 phases: blood collection, centrifugation for platelet concentration, induction of gelation (if the PRP is to be used in gel form), and activation. In general, most systems, whether large or small in volume, do not concentrate the plasma proteins of the coagulation cascade.

Therefore, the set of rules must be shaped on products that maintain the quality standards of biopharmaceutical drugs. Accordingly, the regulations are linked to the related laws, which describe a complex pathway for authorization. Reference is made to the Regulation number 1394/2007 of the European Parliament for advanced therapies, where the definition of "bioprocess engineering products" is given. Here, it is specifically said that this definition excludes those products that contain, or are made exclusively of, cells and non–vital human or animal tissues and that do not have pharmacological, immunologic, or metabolic action. Included among the advanced therapy pharmaceutical products are those used for gene and somatic cell therapy (Directive 2001/83/[European Parliament] European Community, Annex I). Cells and tissues are to be considered products of bioprocess engineering if they undergo "considerable manipulation." The same regulation defines the difference between extensive and minimum manipulation and lists, which are considered relevant or not.

Manipulations that are not considered as bioprocess engineering are as follows: cutting, grinding, shaping, sterilization, centrifugation, soaking in antibiotic or antimicrobial solutions, sterilization, irradiation, separation, concentration or purification, filtration, lyophilization, freezing, cryopreservation, and nitrification.

The definition of medicines for advanced therapy excludes non–repetitive preparations carried out under supervision of a physician, running a personal prescription for a product specifically designed for that particular patient, without, of course, violating the relevant rules relating to quality and safety.

In the experience of the authors, PRP was prepared from a small volume of blood (18 mL) in accordance with the method of Cascade-Selphyl-Aesthetic Factors system, with modifications. Briefly, to prepare PRP, blood was taken from a peripheral vein using sodium citrate as an anticoagulant. The traditional preparation of PRP consisted of a slow centrifugation, which allows the platelets to remain suspended in the plasma, whereas the leukocytes and erythrocytes are displaced to the bottom of the tube.

The current systems for preparing platelet concentrations use various centrifuges (the authors use 3300 rpm for 10 min). The final aim was to obtain a platelet pellet, although the preparation is not selective and includes leukocytes. The secretion of growth factor begins with platelet activation.

The PRP protocol uses Ca^{2+} to induce platelet activation and exocytosis of the α granules. Before proceeding to the activation of PRP, under general anesthesia, we harvested fat tissue from the abdominal region using some specific cannulas. Maintaining asepsis, we took the plunger of syringes; after closing with cap, we positioned them flatly in the sterile centrifuge. The syringes were processed for 3 minutes at 3000 rpm in accordance with the Coleman technique (Fig 1A–C). The authors mixed 0.5 mL of PRP with 1 mL of centrifuged fat tissue (Fig 1D). The implanting location identified to receive the implant was selected through an accurate study of the necessary corrections. On this basis, the harvested material was implanted for facial scar in the zygomatic region, cheek, buccal rime, upper and lower eyelid, temporal area, as well as orbital area. Fat tissue was implanted at different levels in small tunnels, previously created by forcing the cannulas (1.5-mm diameter) with accurate and controlled movements (Fig 1E). Small quantities of fat cells were laid, 1 to 2 at a time, in the exiting movement of the cannulas to create a large grid to facilitate a correct vascular development around each fat cell.

RESULTS

Clinical Observation

In the patients treated with SVF-enhanced autologous fat grafts, we observed a 63% maintenance of contour restoring and three-dimensional volume after 1 year compared with only 39% of the control group (n = 10) treated with centrifuged fat graft ($P < 0.0001$). In the patients treated with fat grafting mixed with PRP, we observed a 69% maintenance of contour restoring and three-dimensional volume after 1 year compared with that of the control group (n = 10). Transplanted fat tissue reabsorbing was analyzed with instrumental imaging (MRI-ultrasound) (Fig 4). In the patients

with scars on the face, reconstruction with SVF-enhanced fat tissue and fat graft + PRP showed a lower fat reabsorbing. All the patients were satisfied with the resulting texture, softness, as well as contour, and MRI confirmed absence of cyst formation and microcalcification. There were no complications in any patient and the results were lasting in all cases (the mean follow-up period was of 60 mo). As reported, e-SVF and PRP mixed with fat grafting were the 2 treatments evidencing improvement in the maintenance of fat volume.

Stromal Vascular Fraction Nucleated Cells From Automatic and Manual Extraction

From adipose tissue, by manual extraction, we obtained approximately 250,000 (±) 34,782 nucleated cells per milliliter of fat tissue; on the other hand, by the automatic extractor, the cell yield was approximately 50,000 (±) 6,956 nucleated cells per milliliter of fat tissue ($P < 0.01$).

Platelet-Rich Plasma Increases Adipose Tissue Stem Cells Number in Vitro

As reported previously, PRP induced an increase of ASC number without any morphologic changes compared with the control group. There was a statistically significant increase, by approximately 4-fold, at 4 and 6 days, when cells were preconfluent ($P < 0.02$). After 8 days, at confluency, there was a 3-fold increase of adipose ASC number in PRP cultures compared with the control group. Oil Red O staining did not reveal any significant difference in intracytoplasmic lipid accumulation compared with the PRP-treated and control ASCs.

Statistical Analysis

Values as mean plus standard error or standard deviation were analyzed by means of the Student's *t*-test, and differences considered statistically significant for $P < 0.05$. For 3 or more groups of univariate data, single-factor analysis of variation was used to obtain *P* values.

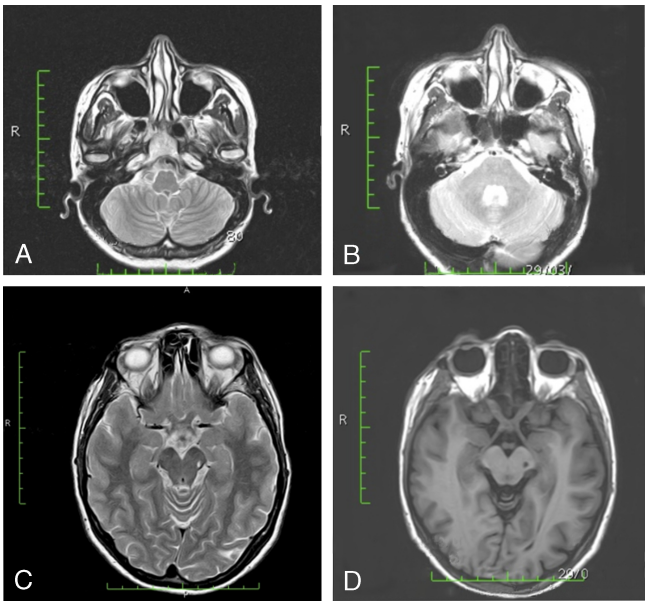

FIGURE 4. Magnetic resonance images of a patient shown in Figure 4. A, T2 imaging of the preoperative situation in the zygomatic region. B, T2 imaging of the postoperative situation after 60 months and after 2 treatments based on the use of fat grafting mixed with PRP in frontal projection. C, T2 imaging of the preoperative situation in the temporal region. D, T2 imaging of the postoperative situation after 60 months and after 2 treatments.

DISCUSSION

Our results clearly documented that the use of PRP during fat grafting improves adipose tissue maintenance and survival. Moreover, our in vitro data are in accordance with the hypothesis that PRP stimulates adipose tissue regeneration, as demonstrated in controlled animal studies for both soft and hard tissues.[29] Results of the present in vivo tissue-engineering approach suggest 4 fundamental points. First, PRP sustains an optimal microenvironment that allows correct architectural adipocytes distribution, better cell-to-cell interaction, adipose tissue growth, and differentiation from ASCs; the latter offers early protection from surrounding inflammatory events. Second, PRP-induced early development of neoangiogenetic microcapillary network facilitates the delivery of proper nutrient and oxygen levels to grafted cells.[30,31] Third, e-SVF can favor neoangiogenic vascularization and fibrogenic activity of fibroblasts that favor adipose tissue survival and three-dimensional organization. Fourth, e-SVF and PRP improved fat graft maintenance in patients who underwent regenerative surgery.

About the use of PRP, new techniques in tissue regeneration are mostly explained in the literature, but there are no articles concerning the possible use of PRP mixed with fat graft and e-SVF fat graft in the treatment of outcomes scar and soft tissue defects. In addition, there are no articles concerning the use of these procedures compared with traditional lipofilling. Our results documented that the SVF cell yield from the manual system was much more efficient than that from the automatic system. It would be necessary to have further information about automatic system operation to explain this finding.

Also, the ASC extraction must comply with 2 fundamental concepts: traceability and qualification.[32]

We hypothesized 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 through a simple diffusion mechanism until an active blood supply is reestablished. Prosurvival 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 VEGF (a potent proangiogenic factor) to the graft. This resulted in increased blood vessel density within the graft and a significant improvement in graft retention at 15 weeks.[33] Neoangiogenesis was also confirmed from a histopathological point of view highlighting the abundance in capillaries sprout within the healing tissue, leading to a complete re-epithelialization of the ulcers.

Recently, Bourin et al[25] identified, in the SVF, the cells phenotypically by the following markers: CD45-CD235a-CD31-CD34þ.

The 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. Moreover, e-SVF improved the maintenance and function of adipose tissue graft. The current available alternatives for treating outcomes scar are autologous skin (harvest, graft), skin from a bank (autologous, taken from cadaveric or living subjects), engineered skin (autologous expanded in vitro), biosynthetic materials, and traditional lipofilling.

The results proved the efficacy of these 2 new treatments: e-SVF and PRP.

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