Clinical Treatment of Radiotherapy Tissue Damage by Lipoaspirate Transplant: A Healing Process Mediated by Adipose-Derived Adult Stem Cells

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Background: There is evidence that stem cells contribute to the restoration of tissue vascularization and organ function. The objective of this study was to assess the presence of adipose-derived adult stem cells left in their natural scaffold in the purified lipoaspirate and to assess the clinical effectiveness of lipoaspirate transplantation in the treatment of radiation side effects.

Methods: This study was designed beginning with surgical procedures in 2002 and envisaging a continuous patient follow-up to 31 months. Twenty consecutive patients undergoing therapy for side effects of radiation treatment with severe symptoms or irreversible function damage (LENT-SOMA scale grade 3 and 4) were enrolled. Purified autologous lipoaspirates (60 to 120 cc) taken from a healthy donor site were administered by repeated low-invasive computer-assisted injection. Therapy outcomes were assessed by symptoms classification according to the LENT-SOMA scale, cytofluorimetric characterization, and ultrastructural evaluation of targeted tissue.

Results: In the isolated stromal vascular fraction of 2 cc of human lipoaspirate, cells with mesenchymal stem cell physical properties and immunophenotype were in average 1.07 $\pm$ 0.5 percent ($n = 4$), with a clonogenic fraction of 0.139 percent. At least 1.02×10^3 colony-forming units–fibroblast were present in each lipoaspirate. Ultrastructure of target tissue systematically exhibited progressive regeneration, including neovessel formation and improved hydration. Clinical outcomes led to a systematic improvement or remission of symptoms in all evaluated patients, including otherwise untreatable patients exhibiting initial irreversible functional damage.

Conclusions: This surgical procedure is a low-invasive therapeutic approach for resolving the late side effects of radiotherapy. According to the proposed hypothesis of the ischemic nature of radiolesions, treatment with lipoaspirate transplantation is potentially extended to other forms of microangiopathies. (*Plast. Reconstr. Surg.* 119: 1409, 2007.)

An increasing amount of clinical evidence strongly supports the therapeutic potential of mesenchymal stem cells for ischemic tissue revascularization and restoration of function. The significant clinical results obtained by autologous transplantation of bone marrow–derived endothelial and hematopoietic stem cells in ischemic lesions in limbs,[1] myocardium[2] and retina[3] have been supported by in vitro studies and in animals, providing elucidation on stem cell–mediated mechanisms underlying neoangiogenesis. These appear to be based on the release of angiogenetic and antiapoptotic growth factors, which ultimately facilitates the recruitment of endothelial progenitor cells into newly sprouting vessels.[4]

Recent studies have demonstrated that the stromal-vascular cell fraction of adipose tissue represents a rich reservoir of regenerative precursor cells with proangiogenic capabilities comparable to those of bone marrow–derived stem cells.[5–7] In mice, adipose stromal cells have been proven to secrete angiogenic and antiapoptotic factors,[8] to differentiate into endothelial cells,

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and to incorporate into vessels,[9] thus promoting neovascularization in ischemic tissues.

We tested the therapeutic potential of adipose-derived adults stem cells within the framework of a clinical pilot study focused on the treatment of irradiation-induced lesions. These are well-known side effects of external oncologic radiation therapy, which affect irradiated healthy tissues and exhibit a several-year progression in a sort of self-maintaining pathologic condition.[10] The most frequent presentation is radiodermatitis with erythema, desquamation, and edema, which evolve over time in subcutaneous fibrosis and, in the most critical cases, toward radionecrosis. Hypotheses on the cause of radiolesions focused on vessel hyperpermeability and altered blood flow[11] were confirmed in this work by ultrastructural analysis, which revealed clear signs of ischemia, with capillary vessels reduced in number and exhibiting duplication of the basal membrane, ectatic lumen cytoplasmic activation of endothelial cells.

The chronic ischemic status of the irradiated tissue represented the rationale for the applicability of adipose-derived adult stem cell therapy, which was based on computer-assisted transplant of purified autologous lipoaspirates. The in vitro establishment and cytofluorimetric analysis were used to assess a priori presence and density of mesenchymal stem cell fraction in the transplanted tissue and to evaluate their differentiation potentialities. Ultrastructural analysis enlightened adipose-derived adult stem cell–mediated mechanisms of tissue neovascularization and regeneration during patient follow-up, in support of the observed and objectively assessed long-term clinical improvements.

PATIENTS AND METHODS

Study Population

At the Second Division of Plastic and Reconstructive Surgery, Ospedale Civile Maggiore (Verona, Italy), patients suffering from progressive lesions after radiation therapy are screened and objectively assessed according to the LENT-SOMA scale.[12,13] Starting in 2002, the therapeutic option based on autologous transplantation of purified lipoaspirate was explained to all patients, none of whom had a medical history of connective, metabolic, or skin disease. Until now, a total of 120 patients have given their informed consent and have been treated accordingly. Among this patient population, the first 20 consecutive patients with LENT-SOMA grade 3 (severe symptoms) or grade 4 (irreversible functional damage) were selected for this specific pilot study, which required ultrastructural and cytofluorimetric characterization of the lipoaspirate and repeated ultrastructural analysis of tissue samples from the treated area during patient follow-up. The rationale and aims of these additional procedures were clearly explained, and all patients gave their informed consent. The mean age of this patient subgroup was 50.9 years, ranging from 37 to 71 years. Targeted areas included the supraclavicular region, the anterior chest wall after mastectomy with or without breast prosthesis,[14] and breast after quadrantectomy. Patients had undergone radiotherapy treatment with a prescribed total dose ranging from 45 and 55 Gy administered in 20 to 25 irradiations (2.00 to 2.25 Gy per session). The supraclavicular area received 40 Gy, administered in 20 sessions of 2.0 Gy. Table 1 reports patient classification based on objective clinical evaluation of the severity of symptoms caused by radiolesions.

Surgical Technique and Tissue Purification Protocol

The areas eligible to be adipose tissue donor sites were the medial area of the knee, the abdominal region, and the trochanteric region. The selected region was infiltrated with a cold saline solution with the addition of 15 cc of adrenaline and 20 to 30 cc of 0.5% lidocaine per 500 cc. Adipose tissue was removed using a 2-mm-diameter cannula and a 2-cc syringe.

The lipoaspirate purification procedure was designed to remove a large part of the triglyceride stored in the tissue and to cause lesion in the thin cytoplasmic sheets of the mature adipocytes, as a way to favor their rapid clearance after injection. Purification was obtained by centrifuging the syringes (IEC Medispin-6; Krackeler Scientific Inc., Albany, N.Y.) at 2700 rpm for 15 minutes to separate the tissue from its water content and from the oil produced by the destruction of damaged adipocytes. The layer of oil and residual liquid were discarded. The lack of cell culture ensured the reduction of the risk of microorganism contamination. In addition, stem cells were not isolated but maintained in their natural three-dimensional scaffold, which was described to favor the reconstruction of a microvascular bed.[15] The number of cell therapy fractions was established as a function of the initial clinical picture. Radiation injuries had been present within a time span ranging from 1 to 30 years (median ± quartile, 4.5 ± 8). The number of procedures was one in five

Table 1. Summary of Patient Clinical Assessment before Treatment

No. of Patients	LENT-SOMA Grade	Area Involved	Symptoms	Notes
4	4	Chest wall (mastectomy) without prosthetic implant	Fibrosis, atrophy, retraction, ulcers, telangiectasia	Osteoradionecrotic rib exposure (1 case)
4	4	Chest wall (mastectomy) with prosthetic implant	Fibrosis, atrophy, retraction, ulcers with implant exposure	Extended telangiectasia (>4 cm^2) (1 case)
2	4	Breast (quadrantectomy)	Fibrosis, atrophy, retraction, ulcers	
1	4	Supraclavicular region	Fibrosis, atrophy, retraction, telangiectasia, itching, hypersensitivity	
4	3	Chest wall (mastectomy) without prosthetic implant	Fibrosis, atrophy, retraction	Pain (grade 3)
4	3	Chest wall (mastectomy) with prosthetic implant	Fibrosis, atrophy, retraction	Telangiectasia (grade 2)
1	3	Breast (quadrantectomy)	Fibrosis, atrophy, retraction	

patients, two in eight patients, three in six patients, and six in one patient. The average quantity of injected purified lipoaspirate varied between 60 and 80 cc at each fraction. All patients underwent the routine surgical and pharmacologic procedures designed for necrotic ulcers. Clinical results after treatment with lipoaspirates were assessed by means of LENT-SOMA scoring. The nonparametric *t* test equivalent sign test for dependent samples was applied for statistical evaluation.

In Vitro Characterization of Adipose-Derived Adult Stem Cells

Isolation of Stromal-Vascular Fraction

To assess the presence of mesenchymal stem cells in human lipoaspirates and to evaluate their multilineage properties, we cultured and characterized by flow cytometry the stromal-vascular fraction derived from adipose tissue. The harvesting was performed on the first five patients. Consistent results suggested that we avoid continuing the cytofluorimetric characterization on the entire patient population. According to the current methodologies, the isolation of the stromal-vascular fraction was performed on 40 cc of lipoaspirates, which were extensively washed with sterile Hank's balanced salt solution. Extracellular matrix was digested at 37°C in Hank's balanced salt solution with 1 mg/ml collagenase type I and 2% bovine serum albumin. After incubation, digestion enzyme activity was neutralized with Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and centrifuged at 1200 rpm for 10 minutes to obtain high-density stromal-vascular fraction pellet. This was then resuspended in 160 mM ammonium chloride and incubated at room temperature for 10 minutes to lyse contaminating red blood cells. The stromal-vascular fraction was collected by centrifugation and filtered through a 70-μm nylon mesh to remove cell debris.

Mesenchymal Stem Cell Expansion

Stromal-vascular fractions were cultured in 25-cc flasks (BD Falcon; Becton Dickinson, Milan, Italy) at a concentration of 1×10^5 cells/cm^2 using Dulbecco's Modified Eagle Medium with high glucose concentration, GLUTAMAX I, 15% heat-inactivated fetal calf serum, 100 U/ml penicillin,m and 100 μg/ml streptomycin (all from Gibco BRL/Life Technologies, Milan, Italy). Cultures were incubated at 37°C in a 5% carbon dioxide atmosphere. After 72 hours, nonadherent cells were removed. When 70 to 80 percent confluent, adherent cells were trypsinized (0.05% trypsin at 37°C for 5 minutes; Gibco BRL/Life Technologies), harvested and washed with medium to remove trypsin, and expanded in larger flasks. A homogenous cell population was obtained after 2 to 3 weeks of culture.

Clonogenic Assay

Colony-forming units–fibroblast (CFU-F) assay was used to evaluate the frequency of mesenchymal stem cells in the stromal-vascular fraction, by culturing with the same medium 10^4 cells in a 10-cm Petri dish and then counting the number of fibroblastic colonies with more than 50 cells 10 days later.

Mesenchymal Stem Cell Immunophenotype

Mesenchymal stem cells were recognized by immunophenotype using monoclonal antibodies specific for CD105 (endoglin), CD73, CD106 (VCAM-1), CD29, CD44, and CD90. In addition,

we assessed the lack of endothelial cell (with anti-CD31 antibodies) and hematopoietic (with anti-CD45, anti-CD14, anti-CD11c, and anti-CD34 antibodies) marker expression. All antibodies were purchased from Pharmingen/Becton Dickinson (Milan, Italy). For immunophenotypic analysis, mesenchymal stem cells were detached using trypsin/ethylenediamine tetraacetic acid for 5 minutes, immediately washed with phosphate-buffered saline to remove trypsin, and resuspended at 10^6 cells/ml. One hundred microliters of cell suspension was incubated at 4°C for 10 minutes with 15% fetal calf serum, followed by incubation with the specific antibody at 4°C for 30 minutes. Cells were washed with phosphate-buffered saline. At least 10,000 events were analyzed by flow cytometry (FACScalibur; Becton Dickinson) using Cell Quest software. In addition, the enumeration of CD105+ cells with mesenchymal stem cell physical properties (high fetal calf serum and saline sodium citrate values) was carried out in the collected stromal-vascular fraction to compare the results obtained by the CFU-F assay.

Mesenchymal Stem Cell Differentiation Assay

Mesenchymal stem cells were tested for their ability to differentiate into adipocytes, osteoblasts, and chondrocytes, as previously described.[16–18] Adipocyte differentiation was achieved after 2 weeks' culture of mesenchymal stem cells with adipogenic medium, containing 10^{-6} M dexamethasone, 10 μg/ml insulin, and 100 μg/ml 3-isobutyl-1-methylxantine (all from Sigma Immunochemicals, Milan, Italy). Osteoblast differentiation was achieved after 2 weeks' culture with osteogenic medium containing 10^{-7} M dexamethasone, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate (Sigma Immunochemicals). Chondrocyte differentiation was achieved after 2 weeks'culture with chondrogenic medium containing 10^{-7} M dexamethasone and 10 ng/ml transforming growth factor-β (Sigma Immunochemicals). Oil Red O, von Kossa, and toluidine blue dyes were used to identify adipocytes, osteoblasts, and chondrocytes, respectively. More than 90 percent of the cells differentiated, depending on the time left in culture with the differentiating agent. Four different trials of mesenchymal stem cell differentiation were performed. Results are reported as mean ± SD.

Computerized Model for Injection

The adipose tissue was implanted in single tunnels using an injection cannula 1 mm in diameter. Entry points and direction of tissue injection tunnels were planned at the computer by means of a multidimensional, unconstrained, nonlinear minimization[19] based on multiple two-dimensional representations of the patient-specific area of intervention. The goal was to provide the surgeon with an interactive intraoperative guidance, to achieve maximum uniformity of distribution and to limit significant overlaps and gaps in tissue deposition. The starting point is represented by the acquisition of a set of photographs of the patient by means of a calibrated digital camera. On the most representative images (typically frontal and sagittal planes), the numbers and initial position of entry points and tunnels, and peak angular values of feasible insertion pathways and eventual inaccessible or untreatable areas are manually defined and represent the boundaries of the optimization procedure. The iterative algorithm was based on a constrained objective function designed to minimize dimension and variability of the areas generated by the intersection of tissue deposition pathways associated with each set of entry points and tunnel directions. The optimization procedure takes a few seconds (depending on the number of entry points) to produce as output a composite representation of the optimized entry point position and direction of insertion pathways, superimposed onto the selected patient images. This is used for intraoperative guidance by the surgeon for the highest uniformity of adipose tissue deposition under the predefined set of boundaries.

Ultrastructural Study

For transmission electron microscopy, the specimens were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon-Araldite. Ultrathin sections were stained with lead citrate and observed under an EM10 electron microscope (Zeiss, Oberkochen, Germany). Ultrastructural analyses were performed on purified lipoaspirates after centrifugation as a way to describe the outcomes of the purification procedure and detail the cellular state of the transplanted medium, and on radiodamaged subcutaneous tissue of all patients before and after cell therapy. After treatment, ultrastructural examinations were carried out 1, 2, 4 to 6, and 12 months after the last surgical procedure; four patients had their last analysis after 18 months and one after 31 months.

RESULTS

Purified Lipoaspirate Ultrastructural Characterization

For all lipoaspirate undergoing ultrastructural analysis, structurally normal adipose tissue was found. However, adipocytes showed interruptions of the cytoplasmic membrane and exhibited various degrees of degeneration ranging up to cellular necrosis. Well-preserved adipocytes were so rare as to be virtually absent. In contrast, the stromal-vascular cell fraction (Fig. 1) appeared to be well preserved, with minimal loss.

Purified Lipoaspirate Cytologic Characterization

The immunophenotype of adipose tissue–derived and in vitro–expanded mesenchymal stem cells corresponded to that of bone marrow–derived mesenchymal stem cells,[16–18] as they were positive for surface CD105, CD73, CD29, CD44, and CD90, with the exception of CD106, which was not expressed; similarly, they were negative for hemopoietic (CD45, CD14, and CD34) and endothelial markers (CD31) (Fig. 2, *above* and *center*). They showed multilineage differentiation potential into adipocytes, osteocytes, and chondrocytes, as assessed by the specific staining (Fig. 2, *below*). CD105+ cells with mesenchymal stem cells physical properties (high fetal calf serum and saline sodium citrate values) in the stromal-vascular fraction cell suspension ($7.4 \pm 3.6 \times 10^5$ cells/lipoaspirate; range, 3.86 to 11.1×10^5) were 1.07 ± 0.5 percent ($n = 4$). However, CFU-F number at 10 days was $13.9 \pm 4.3/10^4$ cells (0.139 percent), thus suggesting that not all the CD105+ cells in the stromal-vascular fraction are clonogenic, and that at least 1.02×10^3 CFU-F could be obtained with a single lipoaspirate.

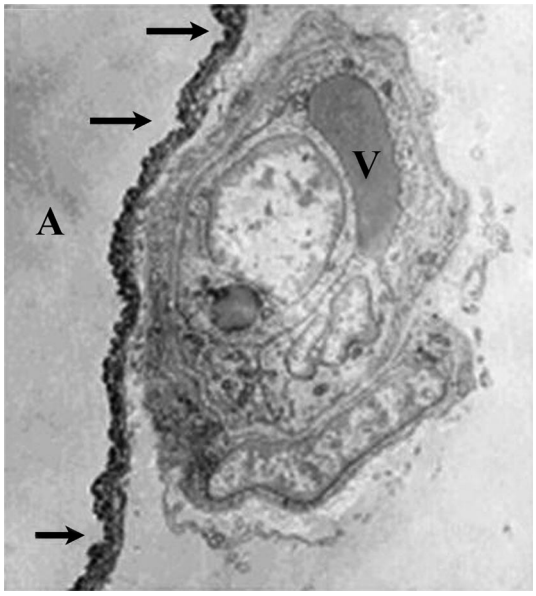

Fig. 1. Ultrastructural analysis revealed a well-preserved stromal-vascular component of purified lipoaspirate (original magnification, ×4000) (*V*, vessel; *A*, adipocyte). Adipocyte shows signs of cytoplasmic alterations (*arrows*).

Clinical Results

Generalized *dramatic* improvement of symptoms was observed in all patients (except one case) (Table 2), with a statistically significant decrease of LENT-SOMA scores before and after cell therapy ($Z(19) = 4.13$, $p < 10^{-5}$). The clinical follow-up varied between 18 and 33 months (mean follow-up, 30 months).

The 11 patients initially classified as LENT-SOMA grade 4 (irreversible functional damage) progressed to grade 0 (no symptoms), grade 1, and grade 2 in four, five, and one cases, respectively; in one case, no improvements were observed. In the four mastectomized patients carrying breast prostheses and exhibiting initial areas of skin necrosis, necrosis showed complete remission. Remaining wounds were sutured at the same time of lipoaspirate injection and healed, allowing prosthesis conservation in two cases. In one case, the wound healed only with two injections without any suture.

In the latest case, the severity of the initial clinical picture did not allow conservation of the implant. In the grade 4 patient without a breast implant who was suffering from a 10 × 15-cm ulceration in the chest region, osteoradionecrosis, exposure of the ribs, and grade 4 pain, the therapy supported the formation of an excellent granulation tissue, which was later covered by a skin graft (Fig. 3). The lack of recurrence of rib exposure, the stability of the skin graft with no complications, and the disappearance of pain after 2 years since the last lipoaspirate injection suggest that the osteoradionecrotic process is in remission.

In the entire group of nine patients classified as LENT-SOMA grade 3, fibrosis, atrophy, and retraction progressed to grade 0 and grade 1 in five and four cases, respectively. In patients with telangiectasia and pain, complete healing and symptom remission was obtained (Table 2).

Ultrastructural Analyses

Before Treatment

An example of the fibrotic and microangiopathic initial aspect of radiodamaged tissue, which was systematically observed in all patients, is de-

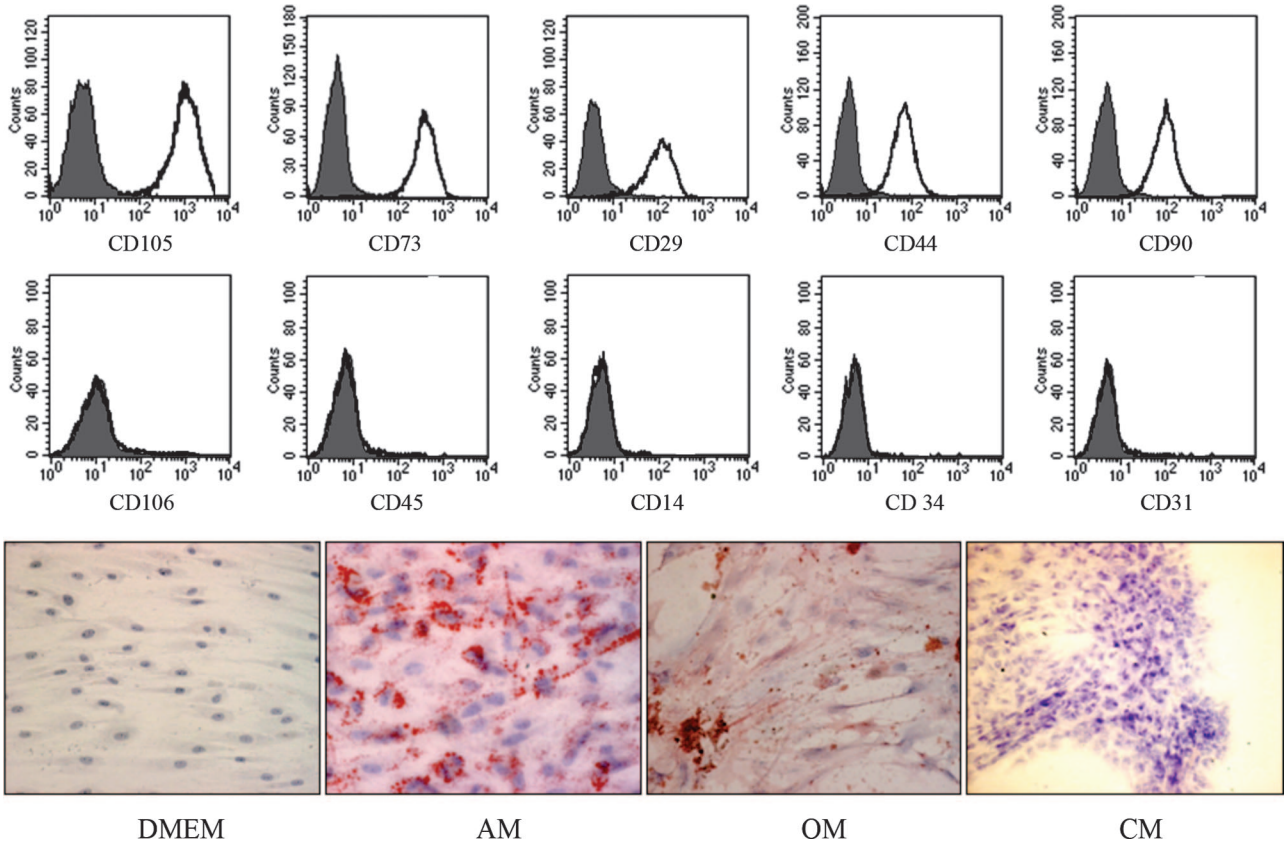

Fig. 2. (*Above* and *center*) Immunophenotype of adipose tissue–derived mesenchymal stem cells. In vitro–expanded mesenchymal stem cells were analyzed by flow cytometry for the expression of CD105, CD73, CD29, CD44, CD90, CD106, CD45, CD14, CD34, and CD31 biomarkers. The graphs report the entity of positivity for each specific biomarker versus the frequency of positive cells. The *shaded curves* represent the negative controls and the *open curves* represent the stem cell sample. When the curves are overlapped, the sample is negative. The shifting of the *open curve* with respect to the control curve indicated positivity of the sample for the specific biomarker under consideration. (*Below*) Multilineage differentiation potential of adipose tissue–derived mesenchymal stem cells. As detailed in the Materials and Methods section, mesenchymal stem cells were cultured for 2 weeks with simple medium (Dulbecco's Modified Eagle Medium) or with adipogenic, osteogenic, and chondrogenic medium, and then the cells were stained with Oil Red O, von Kossa, and toluidine blue dyes, respectively. In adipogenic medium, adipogenesis was indicated by the accumulation of neutral lipid vacuoles that stain with Oil Red O. In chondrogenic medium, chondrogenesis was indicated by the deposition of sulfated proteoglycan-rich matrix that stained with toluidine blue. In osteogenic medium, osteogenesis was indicated by von Kossa staining of extracellular matrix calcification.

picted in Figure 4. The capillary vessels showed duplication of the basal membrane; their lumen was usually ectatic and the endothelial cells showed abundant cytoplasm, micropinocytotic vesicles, and numerous Weibel-Palade bodies. A space was often visible between endothelial cells and pericytes. The adipocytes showed lysosomes or clusters of mitochondria. In the connective tissue, collagen accumulation and cell debris were visible, composed of membrane with fragments of external lamina, thus suggesting an origin from disrupted adipocytes. This suggests an ischemic cause of radiolesions, with similar patterns of scleroderma.

One Month after Treatment

The subcutaneous tissue was of normal morphology and the adipocytes generally appeared well conserved (Fig. 5, *left*). An evident advanced process of injected material removal was observed, and isolated lipid droplets were found in the fibrous connective tissue, where removal is probably slower. Macrophages or lymphatic cells were occasionally found and tissue appeared well hydrated. The spaces between adipocytes were large and had little collagen. There were elements with the characteristics of maturing preadipocytes (i.e., elongated or rounded, relatively poorly differentiated cells, with an abundance of polyribosomes

Table 2. Summary of Patient Clinical Assessment after Cell Therapy*

No. of Patients	Pretreatment LENT-SOMA Grade	Area Involved	Posttreatment LENT-SOMA Grade (No. of Patients)	Posttreatment Evaluation	Notes
7	4	Chest wall, breast (no breast prosthesis), supraclavicular	0 (1) 1 (5) 2 (1)	Ulcers healed, no pain	Osteoradionecrosis (1 case) in remission
4	4	Chest wall (with prosthetic implant)	0 (3) 4 (1)	Ulcers healed; prosthetic implants conserved (Fig. 3)	Breast prosthesis extruded (1 case)
9	3	Chest wall, breast	0 (5) 1 (4)	Total remission of fibrosis atrophy and retraction	Telangiectasia (1 case) and pain (1 case) remitted

*Evaluation was performed within the follow-up time frame varying between 18 and 33 months after the last procedure.

and lipid droplets), which were never observed in the radiodamaged tissue before cell therapy. The presence of a basal membrane proved that they belonged to the adipocyte line.

Capillaries were observed and exhibited a lack of basal membrane reduplication and their normal appearance, in contrast to what was observed in irradiated areas before treatment (Fig. 4). The

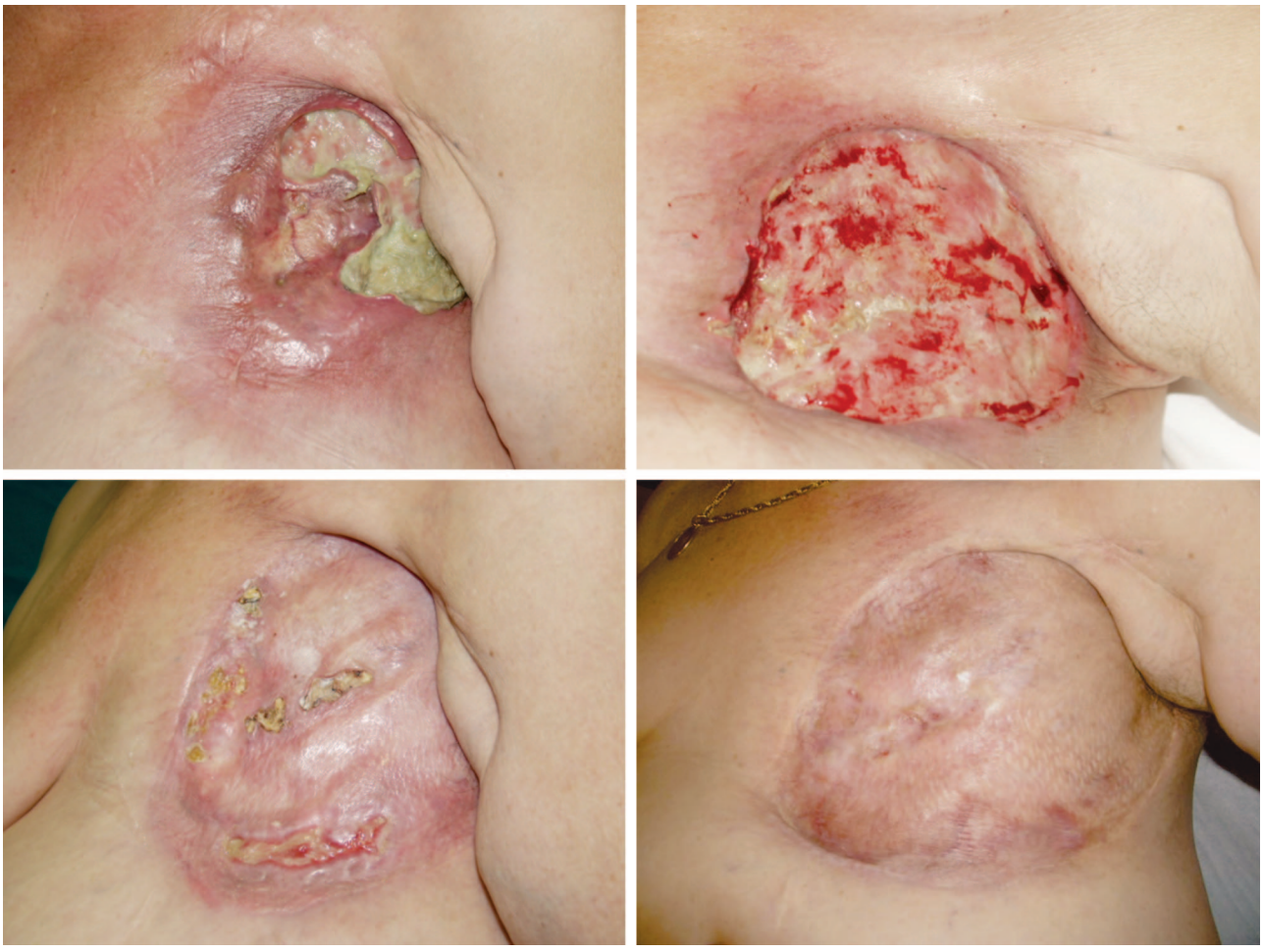

Fig. 3. (*Above*, *left*) Grade 4 patient 2: ulcerative phase with osteoradionecrosis of the ribs. (*Above*, *right*) First result after one treatment with adipose-derived adult stem cells showing good granulation tissue. (*Below*, *left*) Result after skin grafting with three residual ulcers and osteoradionecrosis. (*Below*, *right*) Note the healing of the residual ulcers and osteoradionecrosis after three more adipose-derived adult stem cell injections.

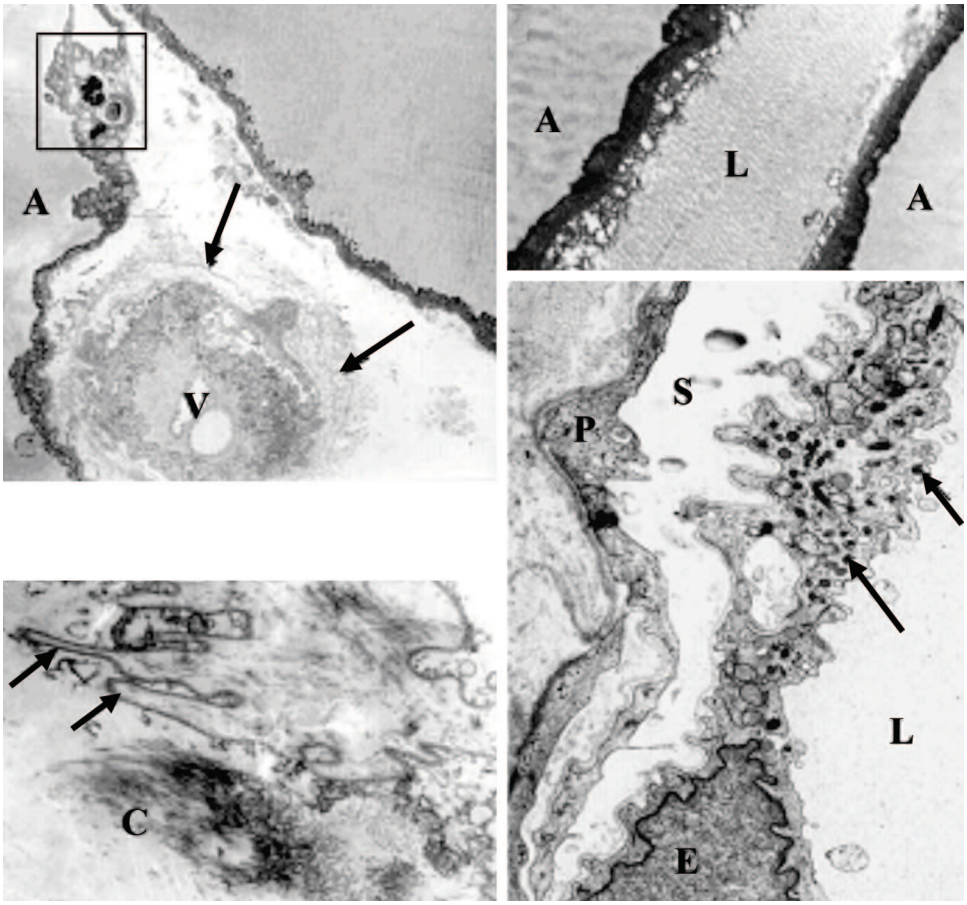

Fig. 4. Ultrastructure of subcutaneous tissue after irradiation. (*Above*, *left*) A capillary vessel shows duplication of the basal lamina (*arrows*). An adipocyte shows lysosomes (*inset*) (original magnification, ×2500) (*A*, adipocytes; *V*, vessels). (*Above*, *right*) A thick layer of collagen is visible between two adipocytes (original magnification, ×5000). (*Below*, *left*) In the connective tissue, collagen accumulation and cell debris were visible, composed of membrane with fragments of external lamina (*arrows*) (original magnification, ×7000). (*Below*, *right*) An endothelial cell (*E*) shows numerous Weibel-Palade bodies (*arrows*). A space (*S*) was often visible between the endothelial cell and a pericyte (*P*) (original magnification, ×8000).

overall picture was characterized by signs of removal of the injected material along with signs of regeneration. Phenomena indicating regeneration were the maturation of stem cells into both adipocytes and vascular cells. The preadipocytes seemed more mature 1 month after treatment than the preadipocytes found in the tissue ready for injection. The pattern was suggestive of an old microcirculation, recognizable from lesions caused by radiotherapy, coexisting in the same tissue with a newly formed microcirculation.

Two Months after Treatment

The process of injected material removal was found to be well advanced, with an almost complete absence of cell debris (Fig. 5, *above*, *right*). The tissue appeared hydrated, although areas of fibrosis were occasionally found. The spaces between adipocytes were large with little collagen. The adipocytes appeared well conserved. Blood vessels showed only occasional signs of hyperpermeability or reduplication of the basal membrane.

The overall picture showed that regenerative phenomena were at an advanced stage, as shown by the presence of almost mature multilocular adipocytes, which testifies to their progress toward complete maturity. The absence of reduplicated blood vessels can be interpreted as a sign of a newly formed microcirculation.

Four to 6 Months after Treatment

The process of removal of injected material was almost finished. Very few cells were found in

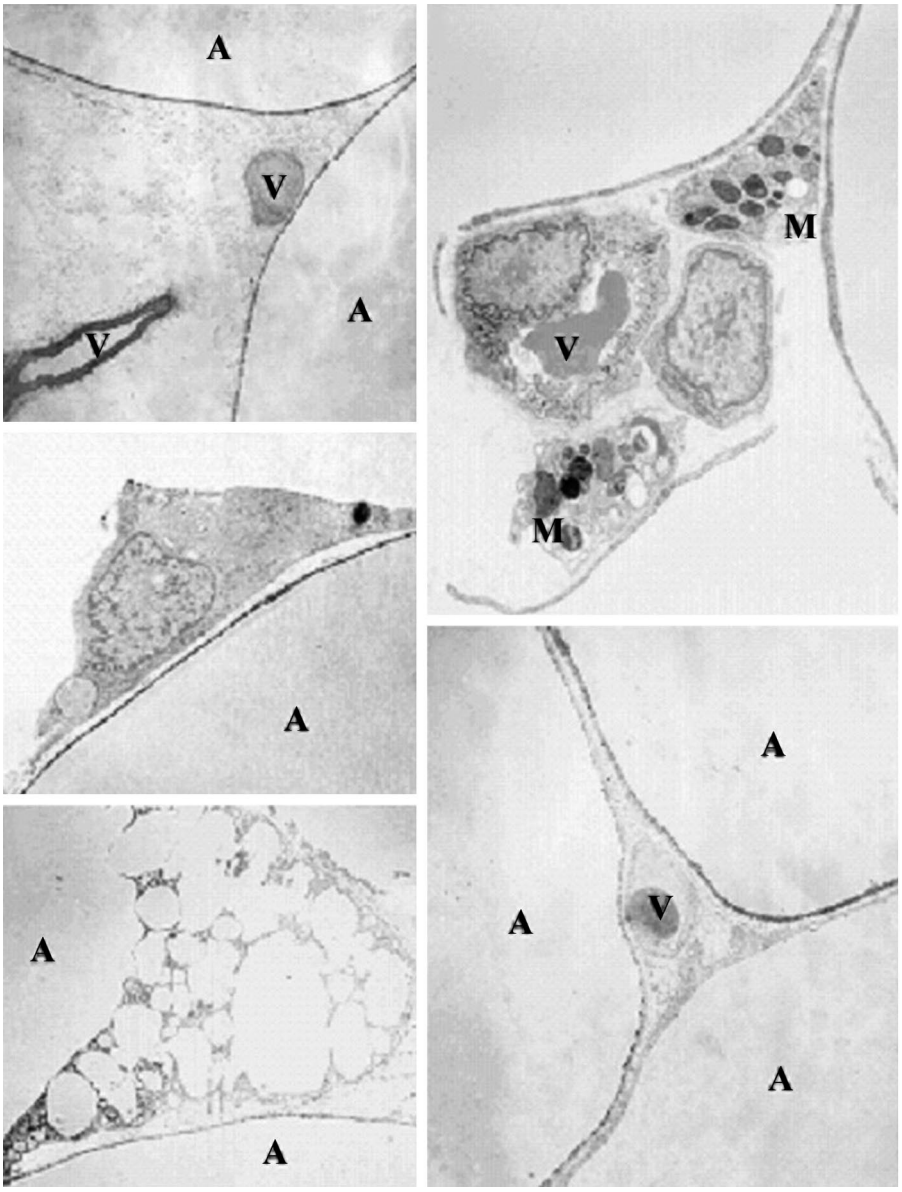

Fig. 5. Ultrastructure of subcutaneous tissue after cell therapy (*A*, adipocytes; *V*, vessels; *M*, macrophages). (*Left*) Photomicrographs obtained at 1 month. (*Above*, *left*) The adipocytes are well conserved and separated by large spaces with little collagen. Vessels do not show reduplication of the basal lamina (original magnification, ×2500). (*Center*, *left*) Macrophages (original magnification, ×4000). (*Below*, *left*) Maturing preadipocyte (original magnification, ×2500). (*Right*) Photomicrographs obtained at 2 months. (*Above*, *right*) Multilocular adipocyte (original magnification, ×2500). (*Below*, *right*) Mature adipocytes with thin extracellular spaces (original magnification, ×2500).

the connective tissue, which appeared well hydrated, with very little collagen. The adipocytes were normal. Maturing adipocytes were no longer evident. The microvessels exhibited a normal ultrastructure, with a very low percentage of vessels showing reduplication of the basal membrane. Areas of fibrosis were found in a single case. The tissue was well hydrated and the newly formed microcirculation showed no lesions.

One Year or More after Treatment

The picture was substantially unchanged, apart from a tendency toward shrinkage of the extracellular spaces. The adipocytes were large and the overall appearance was of mature adipose

tissue with a well-formed microcirculation (Fig. 5, *below, right*).

DISCUSSION

We have shown here that the transplant of lipoaspirates containing adipose-derived adult stem cells is a highly effective therapeutic approach for the treatment of degenerative, chronic lesions induced as late effects by oncologic radiation treatments (Figs. 6 through 8). We have also provided evidence supporting the hypothesis that the reported clinical results could be associated with the observed signs of neovascularization of the targeted tissue, which initially exhibited microvascular alterations similar to several chronic ischemic diseases. The reported regenerative potential of autologous adipose tissue was related to the observed presence of multipotent mesenchymal stem cells, which have been recently reported to secrete multiple potentially synergistic proangiogenic growth factors[8] and, despite the loss of CD34 after long-term culture maintain the capability of differentiating into endothelial cells in vitro and improving postnatal neovascularization in vivo.[9]

The results of the ultrastructural analysis before transplant revealed that radiodamaged tissue significantly featured reduction of the capillary bed. The considerable presence of damaged adipocytes and the duplication of vessel basal membranes (Fig. 4) are put forward to represent signs of suboptimal perfusion, chronic damage, and subsequent repair. In subcutaneous fibrosis, the perfusion was supported mainly by a small number of vessels with morphology suggesting increased transendothelial transport. One possible interpretation of the provided ultrastructural evidence is that the capillary leakage was not caused by interruptions of the endothelial layer but by an increase in transendothelial transport. This aspect

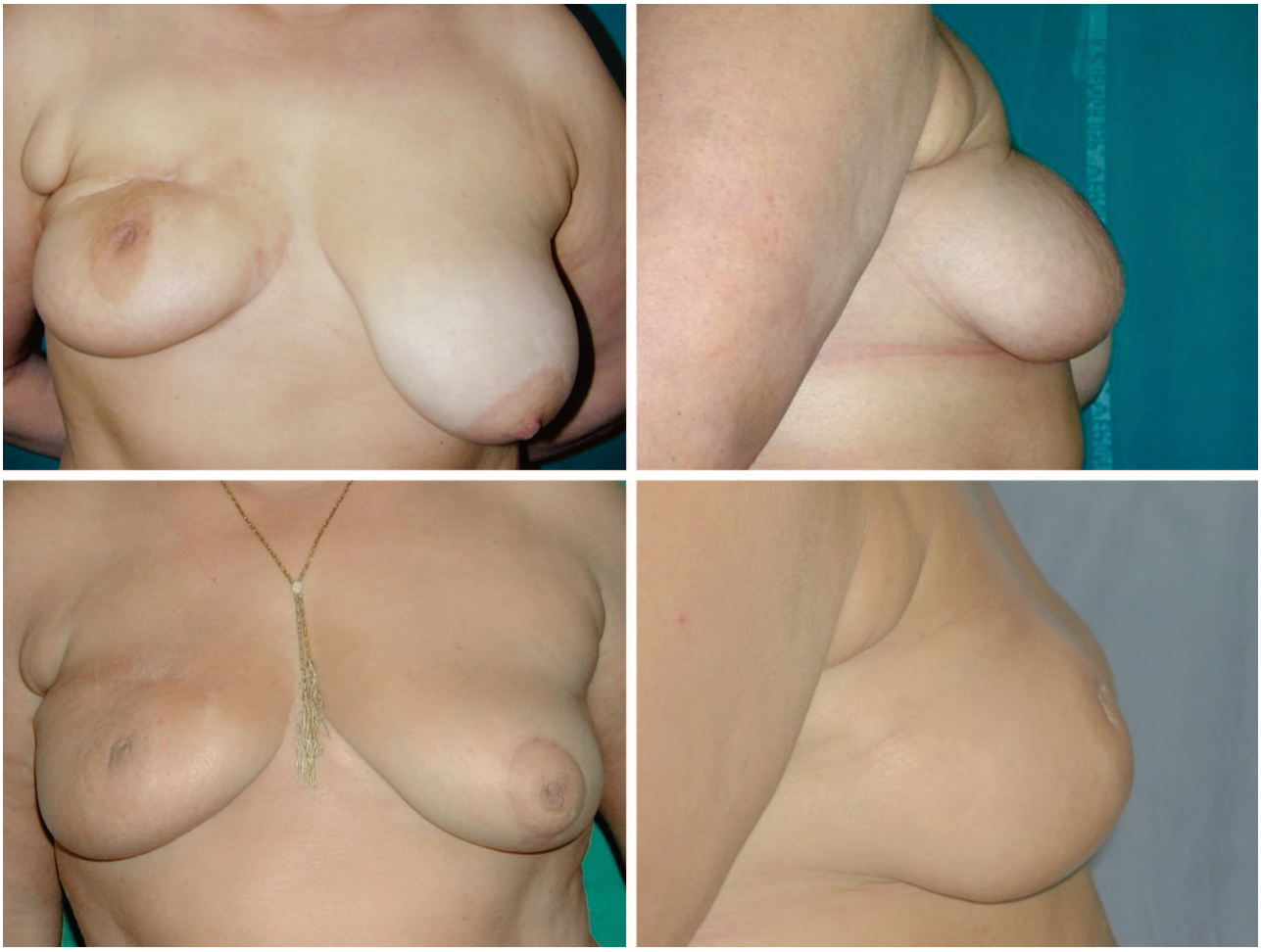

Fig. 6. One case following stem cell therapy after a severe outcome of quadrantectomy irradiation. Stiffness and scarring were improved enormously. This follow-up is 1 year after the last treatment.

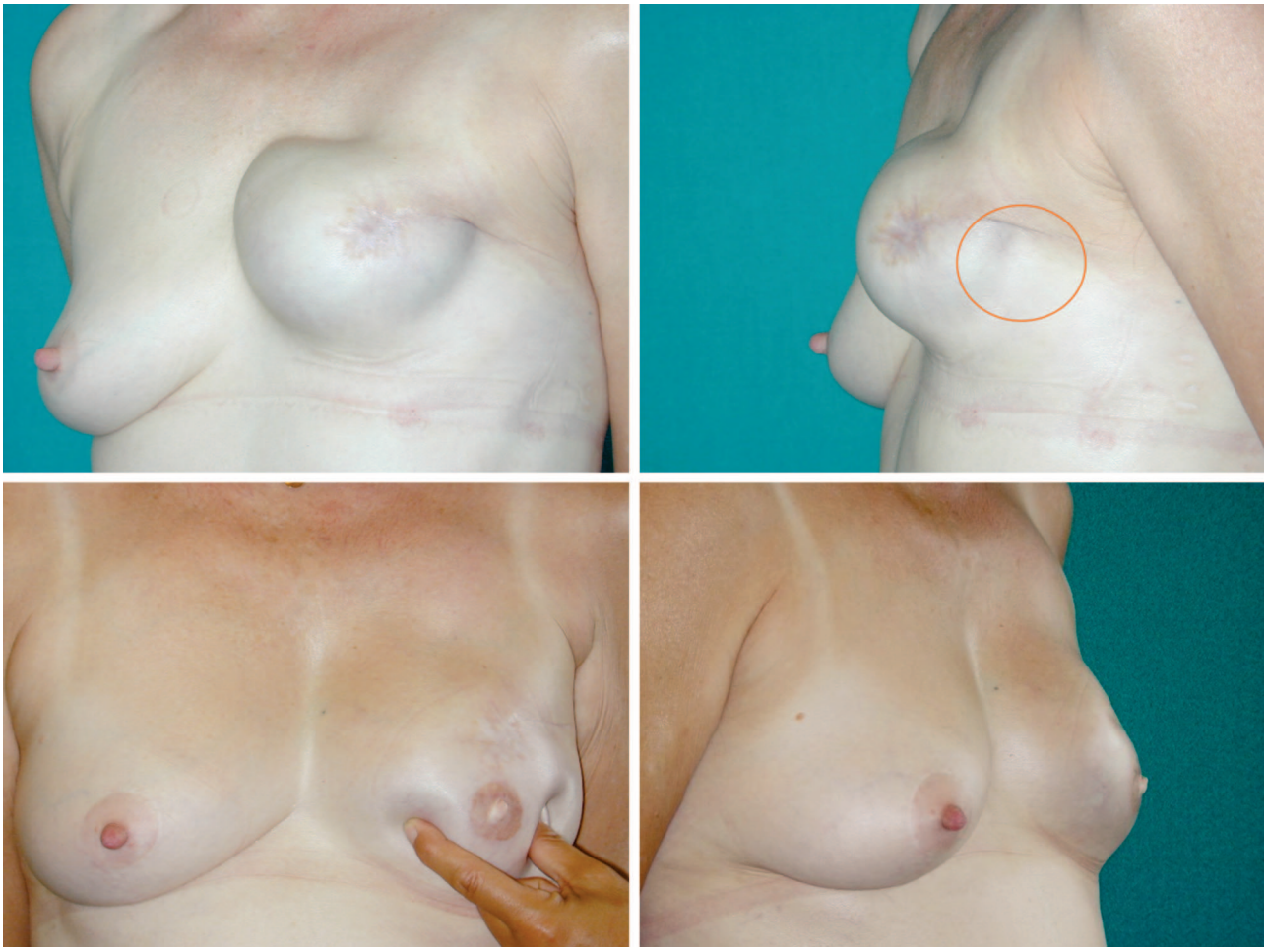

Fig. 7. Severe irradiation performed after expander insertion with dramatic capsular contracture (*above*, *left*) with a high risk of exposure laterally (*above*, *right*). (*Below*) At 18 months after three treatments followed by expander substitution with a prosthesis, no capsular contracture is visible.

can still be found years after radiotherapy. The concentric layers of the basal membrane are generated during phases of repair and are visible in several chronic microvascular diseases. As a whole, the clinical picture was quite similar to the one described in some forms of connectivitis, particularly in systemic sclerosis.[20] Megavoltage irradiation might therefore share similar patterns of microvascular lesions with systemic sclerosis. This is supported by the fact that in patients suffering from scleroderma and other connective diseases, postirradiation complications are frequent and severe, as if irradiation would act as a triggering factor in patients exhibiting a predisposition to microangiopathies.

The objective clinical evaluation of the patient population[13] led to the conclusion that severe radiation-induced lesions do not improve spontaneously but potentially evolve toward severe fibrosis and ultimately to ulceration.[21] For this reason, the reported pilot study was organized without including a control group of patients not receiving a manifestly effective therapy for their chronic and progressive injuries. According to the proposed interpretation of the reported clinical results and ultrastructural analysis, the therapeutic approach has to be designed aiming at breaking the vicious circle (i.e., vascular lesion, ischemia, hyperpermeability, fibrosis, and increased ischemia) and at favoring the growth of a microvascular bed with a correct ratio of adipocytes to capillaries. Our interpretation is that this might be obtained by exploiting the recently described proangiogenic capabilities of adipose-derived mesenchymal stem cells.[8]

Previous studies have already demonstrated that adipose tissue contains a clonogenic pool of stromal cells with the same immunophenotypic and functional properties of bone marrow–derived mesenchymal stem cells.[16–18] On the basis of

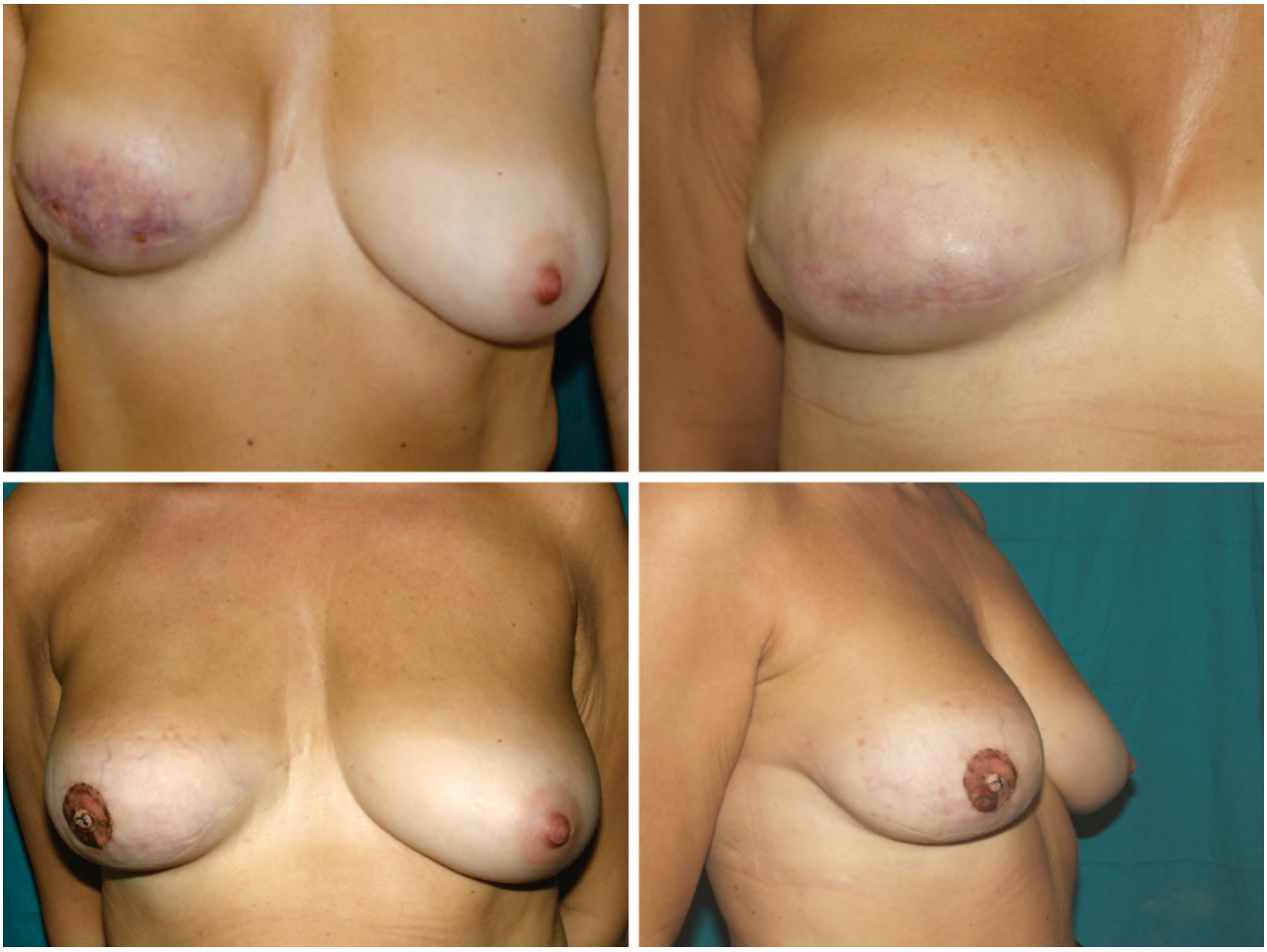

Fig. 8. An implant covered only by undamaged periprosthetic capsula and skin with initial radionecrosis (*above*). Lipoaspirate was injected between the two layers four times. Notice the newly formed adipose tissue exactly in the treated area that allowed nipple reconstruction with local flaps and reduction of the capsular contracture (*below*). No additional surgery was performed.

this evidence, we characterized and quantified the mesenchymal stem cell potential of small samples of adipose tissue collected by lipoaspiration as a way to support the use of adipose tissue to regenerate damaged subcutaneous tissues. We have found that the lipoaspirate-derived stromal-vascular fraction contains a mesenchymal stem cell pool with multilineage differentiation potential that may be responsible for the clinical improvement observed in the treated patients.

According to the ultrastructural analysis results, in the early stages after adipose-derived stem cell transplant, signs of tissue "mesenchymalization" were found to occur. Tissue appeared well hydrated and with large extracellular spaces, resembling fetal connective tissue. Later on, tissue matured and showed aspects similar to those of normal mature adipose tissue. According to the outcomes of recent studies,[8,9] the administration of the stromal-vascular component of normal adipose issue, which has been documented to be rich in stem cells, would elicit the excretion of angiogenic factors. This would lead to the production of new microvessels, which ultimately would ameliorate the circulation. In light of the reported results, we advanced the idea that the chain of events leading to mesemchymalization of the tissue would be the following: (1) targeting of damaged areas by stem cells (favored by their direct injection into the damaged areas), (2) release of angiogenic factors, (3) formation of new vessels, and (4) oxygenation. This process would favor the development of stem cells in mature adipocytes and in a newly formed microcirculation replacing the existing, seriously damaged one. Damaged vessels could still be found in persisting areas of fibrosis late after treatment (Fig. 5). This emphasizes the importance of repeated computer-assisted injections to obtain homogeneous improvement throughout the entire radiodamaged area. In-

deed, we noticed a linear relationship between clinical improvements and the number of transplants. The reason is probably because the healing of tissue microangiopathic status increased as a function of the total number of stem cells introduced. We are aware of the fact that, in light of the data provided, alternative interpretation of the reported results are feasible. Concerning the microangiopathic cause of radiation ulcers, Rudolph et al.[22] proposed an alternative hypothesis not dependent on decreased blood supply. According to their study, the dominant radiation effect would act intrinsically on fibroblasts or cause a selective ablation of a faster growing fibroblast subpopulation. In addition, a potential role of the inflammatory response inherent in any surgical trauma should also be considered, even if the timing of the response to the lipoaspirate injection procedures and the ultrastructural data provided suggest a marginal role of inflammatory processes.

From the clinical point of view, a radical change in the therapeutic approach of radiodamaged tissues might be taking place. Tissue damage is usually considered a matter for surgical removal followed by replacement with distant flaps. In our approach, damaged tissues were conserved rather than eliminated: stem cell therapy led to their radical structural change, transforming them into normal tissue. The beneficial effects turned out to be particularly evident in patients with cutaneous ulcers and even more complicated by osteoradionecrosis.

In light of the reported outcomes of this pilot study, the current approach to autologous adipose tissue transplants for tissue regeneration seems to sway. Although most authors focus on lipoaspirate transplantation procedures aiming at preserving mature adipocytes as a way to maximize the survival rate within the treated area,[23,24] we showed that mature adipocytes were already seriously damaged during the adipose tissue sampling procedure and therefore could not survive in the host tissue. Only the adipose stem cell fraction seems to be the regenerative active component in the transplanted tissue. From the technical point of view, the reported surgical approach does not differ from traditional fat grafting for aesthetic/filling purposes; the innovative aspect consists of focusing the technique on the treatment of late radiotherapy injuries. Regarding clinical complications of the described procedure, no case of infection, postinjection necrosis, or any other complication forcing suspension of therapy was experienced.

The significant presence of stem cells in the adipose tissue with multilineage capacity and therapeutic neovascularization potential, and the ease and minimal invasiveness of procurement, make autologous adipose tissue transplantation a recommended therapy for the treatment of radiation-induced injuries.

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DISCLOSURE

None of the authors has a financial interest in any of the products, devices, or drugs mentioned in this article.

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