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Bleomycin-induced Scleroderma in Nude Mice can be Reversed by Injection of Adipose Tissue: Evidence for a Novel Therapeutic Intervention in Systemic Sclerosis

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Abstract

Objective: Systemic sclerosis is an autoimmune disease characterised by uncontrolled fibrosis and vascular insufficiency of the skin and internal organs, without efficient treatments. Subcutaneous adipose transplants have been shown to exert trophic effects on surrounding tissue. We aimed to determine the effects of human adipose tissue in a murine model of sclerotic skin.

Methods: Scleroderma was induced in 48 nude mice by daily subcutaneous injection of bleomycin during four weeks. Immediately after the final bleomycin injection, human subcutaneous adipose tissue was implanted into the subcutaneous space of mice using either the Coleman method or the micro-injection method. Epidermal and dermal thicknesses were assessed on skin biopsy specimens six weeks after fat implantation. Capillary density was assessed using immunohistochemistry with an endothelial-specific marker (CD31).

Results: Bleomycin increased dermal thickness ($p<0.01$), collagen network, and density of elastic fibers. Adipose tissue implantation reduced dermal thickness by 10% ($p<0.01$) with the Coleman method and by 14% ($p<0.001$) with the micro-injection method. Delivery of adipose tissue by the micro-injection method led to significantly greater neovascularization than the Coleman method ($p<0.001$).

Conclusion: Implantation of human adipose tissue resulted in significant improvement of both fibrosis and peripheral vascular sufficiency in this mouse model of sclerotic skin. Use of a micro-injection method was associated with superior outcome. We postulate that this positive effect is likely due to the activity of the stromal vascular fraction found within adipose tissue. Thus, injection of autologous adipose tissue may be a promising novel therapy for patients with hand or perioral functional impairment.

Keywords: Systemic Sclerosis; Bleomycin-induced scleroderma; Mouse model; Sclerotic skin; Coleman method; Micro-injection method; Fat grafting; Adipose tissue

Introduction

Systemic sclerosis (SSc) is an auto immune disease characterised by fibrosis of the skin and internal organs leading to high morbidity and mortality. Although the pathogenesis of SSc is not yet fully understood, it is characterized by excessive accumulation of extracellular matrix (ECM) in the skin and various internal organs, micro vascular injury, and immune activation resulting in the production of auto-antibodies [1,2]. Briefly, vascular injury is an early event in scleroderma, with alterations including abnormal vascular tone, loss of integrity of the endothelial cells, progressive luminal obliteration and thrombosis, and rarefaction of capillaries leading to ischemia and its clinical consequences (digital ulcers for example). Extinction of small blood vessels is a characteristic finding in later stages of the disease. Perivascular infiltrates of mononuclear cells are observed in the early stage in the dermis and around the vessel wall. The immune recruited cells secrete fibrogenic cytokines, growth factors and chemokines which in turn increase inflammation and collagen synthesis in the surrounding fibroblasts. Fibroblasts harbour an activated phenotype in the affected areas and produce large amounts of collagen, proteoglycans, and elastic fibers. Dermal fibrosis and fibrosis surrounding the vessels increase the ischemic process. Thus, uncontrolled and extensive fibrosis combined with peripheral vascular insufficiency both represent the hallmark of this disease, and so far, no drugs in clinical practice specifically inhibit the fibrotic process or act on both components together.

Yamamoto et al. established a model for scleroderma by local injection of subcutaneous bleomycin in Balb/c mice [3-7]. From a histopathological point of view, Yamamoto et al. [3-7] demonstrated that bleomycin-induced sclerosis on mice was very similar to what is seen in the skin of patients with scleroderma, and that bleomycin-induced sclerotic changes persist for at least 6 weeks. Moreover, Yamamoto et al. [6] have also demonstrated that marked dermal sclerosis can be induced in the immunodeficient SCID mice, and the period needed for the development of dermal sclerosis was not longer than that seen in control Balb/c mice. Adipose tissue is used over a

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century in plastic surgery. Coleman described a technique of fat grafting in 1994 [8-10], which was became the reference in plastic surgery [11,12]. Initially used for its volumetric effect to correct volume and contour defects, a dynamic phenomenon of tissue regeneration occurs at the recipient site around the fat graft [13]. Therefore, fat grafting is commonly used for the regeneration and improvement of the skin at the grafted area for different indication, such as radiodermitis [14], instable wound healing [15] and localised forms of scleroderma such as "en coup de sabre scleroderma" [16,17]. To improve the fat grafted unit, we designed a new tool inspired from the Coleman technic to graft smaller fat units, what we called micro-injection [18]. Thus, using the mouse scleroderma model validated by Yamamoto et al., we aimed to determinate the beneficial effects of fat grafting on a sclerotic skin, testing the classical Coleman and the micro-injection method, as a requirement to treat human disease.

Materials and Methods

48 nude mice (Swiss nude nu/nu mice) were obtained from Charles River, L'arbresle France. Under general anaesthesia, each mouse was treated daily with 100 microliters of concentrated bleomycin (100 µg/ml) in 3 parts of the body: inter scapular region and in both flanks, according to the Yamamoto procedure [3-7]. An untreated area of skin in inter scapular region of each mouse served as a control to validate the development of bleomycin-induced sclerosis) (Figure 1).

Human adipose tissue was collected by a single operator from three healthy volunteer donors after informed consent; adipose tissue was removed from the flank area during an abdominal-dermolipectomy. For the Coleman's procedure [8-10], the aspiration of adipose tissue was performed with a manual and non-traumatic technique, using the S.R. Coleman's Blunt cannula (15 cm in length and 2.42 mm internal diameter that is 11 Gauge) and a 10cc syringe. For the micro-injection procedure, fat tissue is harvested in a 10 cc Luer Lock syringe, through a 15 Gauge cannula (15 cm in length and 1.5 mm internal diameter).

The adipose tissue was then centrifuged for 3 minutes at 1.200 g (microcentrifuge Medilite ref.448, Thermo Scientific) to remove oily and bloody residues. Adipose transplants were packaged in 1cc syringes for subsequent use. Under general anaesthesia a millimetre incision was made on the external side of each thigh. 0.2 cc of adipose tissue transfer was performed using the Coleman procedure with a 17 gauge blunt cannula (Figure 2, left) for half of the experiments, and using the micro-injection procedure with a 21 Gauge blunt cannula (Figure 2, right) for the other half. Compared to the Coleman procedure, micro-injection method has the advantage to deliver smaller transplants of adipose tissue in the subcutaneous area and should be easier to use for treatment of sclerotic skin.

For all nude mice, one flank was injected with adipose tissue, whereas the other side was injected with phosphate-buffered saline (PBS) and served as control. Half the mice received adipose tissue on the left, PBS on the right and half the mice received the opposite injections. All mice were sacrificed at day 42 (±1) after adipose tissue injection or PBS injection, according to the Yamamoto et al findings.

Histological evaluation

Four skin samples from each mouse were evaluated. First, skin biopsy samples were removed at week 0 (W0) in healthy skin and after the 4 weeks (W4 sample) of daily subcutaneous treatment with bleomycin, to validate the induction of skin sclerosis by bleomycin. Then, to evaluate the effect of subcutaneous injection of adipose tissue, we obtained skin biopsies of each flank 6 weeks after the end of bleomycin treatment (W10 sample) (Figure 3).

Skin biopsies were fixed in 10% formalin solution and embedded in paraffin, and then cut by microtome into 5 micrometer sections before microscopic analysis. Tissues were stained with Hematein Eosin Safran (HES), Masson Trichrome (for collagen detection), Orceine (to visualize elastin fibers), toluidine blue (to identify mast cells), and immunoperoxydase coupled anti-CD31 antibody (to identify the vasculature). For this last straining, a semi-quantitative evaluation was performed:

1: Capillaries present in normal number and normal aspect,

2: Increased number of congested capillaries,

3: Numerous congested capillaries

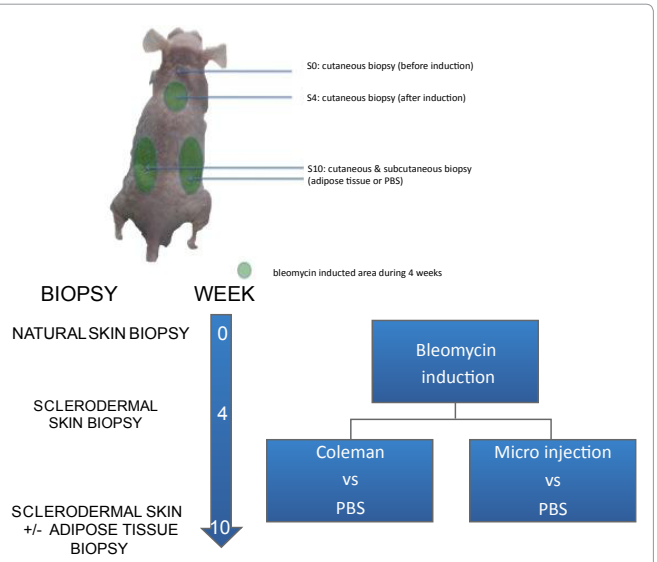


Figure 1: Up, the sites of the injection (bleomycin, PBS and adipose tissue) in each mouse, down, a schema illustrating the course including the different steps of the experiments.

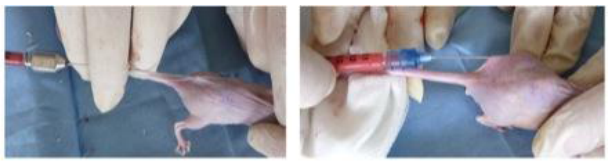


Figure 2: Adipose tissue transfer performed to the mouse using the Coleman procedure with a 17 gauge blunt cannula (left) and the microinjection procedure with a 21 Gauge blunt cannula (right).

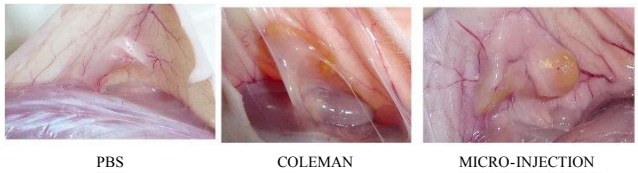


Figure 3: Photos of the mouse flank skin treated with PBS (the control) or with adipose tissue injection (Coleman or microinjection method).

Epidermal and dermal thickness was measured quantitatively using a Zeiss microscope (AXIOPHOT). Two measurements were made and the means were compared.

Statistical analysis

To avoid the bias of the cutaneous variation, by the hair cycle and the temporary effect of the bleomycin, each mouse was its own witness; we compared the effects of the adipose tissue injection to the PBS injection, at the same time and in the same mouse.

Statistical analysis was performed on Statview. Results are expressed as mean ± SD. Significance testing was done using the Mann ± Whitney U test. A p value <0.05 was considered to be significant.

Results

All mice are alive after the cutaneous bleomycin induction and the adipose graft. We report any local complication. Nine hundred and sixty micro slides were obtained to cover all the experimental conditions and were blindly analysed by the same pathologist (Lucile Andrac).

Validation of the Yamamoto model of bleomycin-induced dermal fibrosis in nude mice at W4

Macroscopically, skin sclerotic changes were localized to the skin around the bleomycin-injected site; the skin became thicker and indurated, revealing after skin incision a whitish and bright aspect compatible with a sclerosis.

Histopathological examination comparing normal skin to skin obtained after bleomycin treatment revealed change in the epidermis with loss of its normal papillomatous aspect and a decrease of its thickness (Figure 4). Mean epidermal thickness was significantly lower in treated than in untreated skin (0.018 mm ± 0.007 in treated compared to 0.019 mm ± 0.009 in normal nude mouse skin, $p<0.05$), which corresponded to a 5.3% decrease of epidermal thickness after bleomycin (Figure 5).

After bleomycin treatment, dermal sclerosis characterized by deposition of homogenous materials in the thickened dermis was observed (Figure 4). Masson trichrome stain showed a dense collagen network in the thickened dermis and elastic fibers appeared more abundant and dense in bleomycin-treated skin than in normal untreated skin (Figure 4). Mean dermal thickness was significantly higher in treated than in untreated skin (respectively 0.268 mm ± 0.05 compared to 0.244 mm ± 0.054, $p<0.01$), corresponding to a significant 9.0% increase of dermal thickness after bleomycin (Figure 5).

Toluidine blue stain revealed that there was no difference in mast cell number or morphology after treatment with bleomycin.

Mononuclear cells were rare or absent in all analysed skin, which was expected in nude mice; there was no evidence of graft versus host reaction.

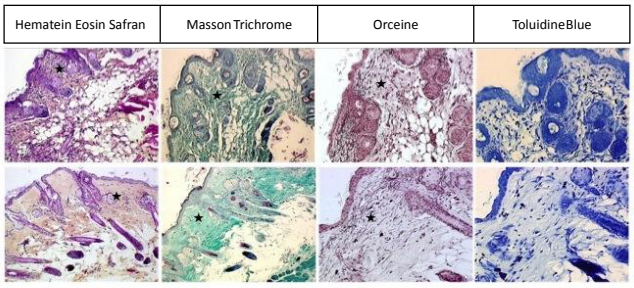
Adipose tissue injection reversed sclerosis and has a pro-angiogenic effect

Histopathological examination showed that adipose transplants (Coleman or micro-injection method) were located under the panniculus carnosus in analysed skin.

When adipose tissue was injected into areas which had been treated with bleomycin, a significant decrease of dermal thickness was observed without change in the epidermis. Mean dermal thickness was 0.30 mm ± 0.047 after Coleman treatment, 0.29 mm ± 0.007 after micro-injection of adipose tissue and 0.33 mm ± 0.057 after PBS treatment. Differences were significant between Coleman procedure and PBS ($p<0.01$) and between micro-injection of adipose tissue and PBS ($p<0.001$). This corresponded to a 10% decrease of dermal thickness with Coleman and a 14% decrease with micro-injection of adipose tissue, when compared to PBS treatment (Figure 6).

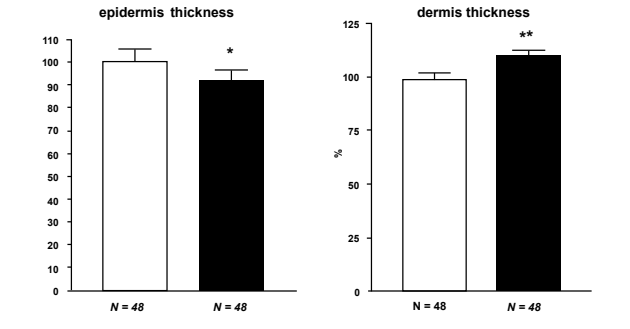
There were no significant changes in collagen architecture or density of elastic fibers in the adipose tissue-treated skin when compared to PBS-treated skin.

Finally, the number vessels in the deep dermis significantly increased after micro-injection of adipose tissue, in comparison to PBS treated skin ($p<0.001$, Figure 7). Qualitatively, vessels appeared larger in size and more visible. No significant difference was observed when adipose tissue injected using the Coleman method was compared to PBS treated skin (Figure 7).



*Visualization of dermal fibrosis (HES), collagen deposit (trichrome Masson) and the elastic fibers density (orceine)

Figure 4: Microscopic observation of stained histological sections evaluating the skin respectively before (first line) and after (second line) local injection of bleomycin.



Mean (mm)	*0,019*	*0,018*	*0,244*	*0,268*
SD	0,009	0,007	0,054	0,050

* p< 0.05, ** p<0.01

N: number of treated mice, % indicate the percent of change of epidermal and dermal thickness when normal skin was compared to bleomycin treated skin. Mean values (and standard deviation) are given below the figure.

Figure 5: Effects of subcutaneous bleomycin treatment on epidermal and dermal thickness (white column: normal skin, black column: skin treated with bleomycin).

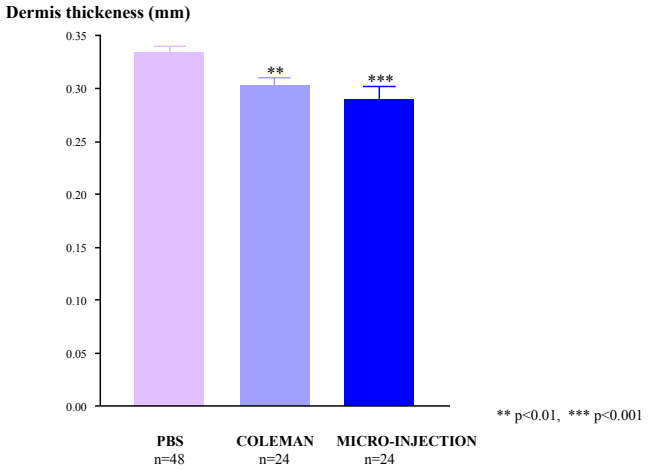


Figure 6: Effects of adipose tissue transfer on bleomycin treated skin: analysis of dermal thickness. All 48 mice were treated with bleomycin for 4 weeks and then received PBS and adipose tissue (Coleman or microinjection), n=number of treated mice.

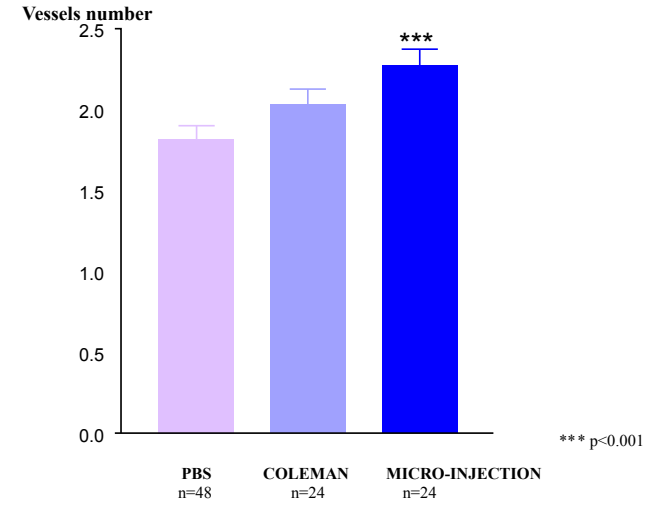


Figure 7: Effects of adipose tissue transfer on bleomycin treated skin: analysis of vessels in the deep dermis (vessels were stained with an immunoperoxydase coupled anti-CD31 antibody). All 48 mice were treated with bleomycin for 4 weeks and then received PBS and adipose tissue (Coleman or microinjection), n=number of treated mice.

Discussion

In this study, we showed that injection of adipose tissue significantly reduced dermal sclerosis in a scleroderma animal model and could constitute a novel therapeutic way in the treatment of one of the major and disabling manifestations of SSc.

First of all, we validated the animal model of sclerotic skin induced by bleomycin in the nude mouse. Indeed, using subcutaneous injection of bleomycin, dermal sclerosis was evidenced by increased dermal thickness as well as an increased of collagen network and density of elastic fibers. In contrast to prior reports, we also found a decrease in epidermal thickness after bleomycin-treatment; the epidermal atrophy could be related to the effect of bleomycin, which has been shown to stop epidermal cell cycle, and to induce epidermal cell apoptosis after multiple doses [19]. Moreover, our data are in accord to the classical histopathological findings observed in cutaneous and systemic form of scleroderma [20].

This model of sclerodermic skin appeared by odema and sclerosis, that means the early stage of scleroderma. It is a hope for patients who suffering from early limited cutaneous scleroderma, like morphea, but also the early stage, which is a inflammatory stage of systemic scleroris with local cutaneous functional impairments (such as perioral area).

Thus, we validated the animal model of sclerotic skin induced by bleomycin in the nude mouse, confirming a T-cell independent component to fibrosis. The major result of our study is the anti fibrotic effect obtained after adipose tissue injection, with a significant decrease in dermal thickness. Furthermore a pro-angiogenic effect was also obtained with the micro-injection method as evidenced by the increase in vessels number.

The mouse nude model provided us an interesting tool to study the effect of adipose tissue injection. Although adipose tissue was obtained from human fat, it was well tolerated without any signs of graft versus host reaction (absence of any mononuclear cell infiltration), testifying to the purity of the injected adipose tissue. We report any complications, such as infectious process or cytosteatonecrosis. Adipose graft tissue is a safe well-tolerated therapy in plastic surgery practice since over a century [8-12]. We observed an anti fibrotic effect of adipose tissue injection with a significant decrease in dermal thickness ($p<0.01$ with the Coleman method and $p<0.001$ with the micro-injection method). Furthermore a pro-angiogenic effect was obtained with the micro-injection method as evidenced by the increase in vessels number and size, in comparison to PBS-treated skin. The superiority of the micro-injection vascular effect is probably due to the fact that the micro-injection transfers smaller adipose tissue units with a better vascular exchange area with the host site, compared to the Coleman method. The consequence of this better exchange area is a better functionality of the micro-injection of adipose tissue and a better trophic effect.

These data are only preliminary results, as no previous study to our knowledge, has been published on the histopathological effect of adipose tissue injection on sclerosis skin. We acknowledge some limits to the short term results of our study. Although we detected a decrease in dermal thickness after treatment with adipose tissue, there was no difference in collagen architecture or density of elastin fibers.

As immunodeficient mice were required to avoid host versus graft reaction, our results are difficult to extrapolate to human beings. However, in human beings autologous adipose tissue will be used, bypassing the problem of histocompatibility.

Finally, we observed mild epidermal atrophy after bleomycin treatment which did not revert after adipose tissue injection. The meaning of this epidermal atrophy induced by bleomycin is still unclear and is contrary to prior reports of experiments in immunocompetent mice where epidermal thickness was increased [5].

Importantly, mature adipocytes account for only 40-60% of adipose tissue cells. This tissue also contains a stroma vascular fraction (SVF) which contains mesenchymal stem cells. Many studies have shown that adipose tissue-derived SVF contains multipotent cells able to differentiate in vitro into multiple cell types including muscle, bone, chondrocyte, neuron, epithelium, macrophage, hepatocyte and fibroblast [21,22]. Finally, several animal studies demonstrate the potential value of the SVF for cell therapy, particularly for mesodermal

tissue repair as well as for revascularization [23,24]. Thus we speculate that the anti-fibrotic and pro-angiogenic effects that we observed in our experiments could be based on the SVF of the fat graft but further study will be required to confirm this fact.

Therefore we believe that the subcutaneous injection of adipose tissue improves the local balance of pro and anti-fibrogenic factors and increases the microvascular network, reducing significantly the clinical consequences of scleroderma. Although preliminary, these data offer a new hope for sclerotic skin therapy in human SSc disease.

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References

1. Abraham DJ, Varga J (2005) Scleroderma: from cell and molecular mechanisms to disease models. Trends Immunol 26: 587-595.
2. Gabrielli A, Avvedimento EV, Krieg T (2009) Scleroderma. N Engl J Med 360: 1989-2003.
3. Yamamoto T, Takahashi Y, Takagawa S, Katayama I, Nishioka K (1999) Animal model of sclerotic skin. II. Bleomycin induced scleroderma in genetically mast cell deficient WBB6F1-W/W(V) mice. J Rheumatol 26: 2628-2634.
4. Yamamoto T, Takagawa S, Katayama I, Yamazaki K, Hamazaki Y, et al. (1999) Animal model of sclerotic skin. I: Local injections of bleomycin induce sclerotic skin mimicking scleroderma. J Invest Dermatol 112: 456-462.
5. Yamamoto T, Kuroda M, Nishioka K (2000) Animal model of sclerotic skin. III: Histopathological comparison of bleomycin-induced scleroderma in various mice strains. Arch Dermatol Res 292: 535-541.
6. Yamamoto T, Nishioka K (2001) Animal model of sclerotic skin. IV: induction of dermal sclerosis by bleomycin is T cell independent. J Invest Dermatol 117: 999-1001.
7. Yamamoto T (2002) Animal model of sclerotic skin induced by bleomycin: a clue to the pathogenesis of and therapy for scleroderma? Clin Immunol 102: 209-216.
8. Coleman SR (2002) Hand rejuvenation with structural fat grafting. Plast Reconstr Surg 110: 1731-1744.
9. Coleman SR (2006) Structural fat grafting: more than a permanent filler. Plast Reconstr Surg 118: 108S-120S.
10. Coleman SR (2008) Fat injection. From filling to regeneration. Saint louis: Quality medical publishing: 406.
11. Kaufman MR, Bradley JP, Dickinson B, Heller JB, Wasson K, et al. (2007) Autologous fat transfer national consensus survey: trends in techniques for harvest, preparation, and application, and perception of short- and long-term results. Plast Reconstr Surg 119: 323-331.
12. Coleman SR (2006) Structural fat grafting: more than a permanent filler. Plast Reconstr Surg 118: 108S-120S.
13. Meruane MA, Rojas M, Marcelain K (2012) The use of adipose tissue-derived stem cells within a dermal substitute improves skin regeneration by increasing neoangiogenesis and collagen synthesis. Plast Reconstr Surg 130: 53-63.
14. Sultan SM, Stern CS, Allen RJ Jr, Thanik VD, Chang CC, et al. (2011) Human fat grafting alleviates radiation skin damage in a murine model. Plast Reconstr Surg 128: 363-372.
15. Klinger M, Caviggioli F, Vinci V, Salval A, Villani F (2010) Treatment of chronic posttraumatic ulcers using autologous fat graft. Plast Reconstr Surg 126: 154e-5e.
16. Oh CK, Lee J, Jang BS, Kang YS, Bae YC, et al. (2003) Treatment of atrophies secondary to trilinear scleroderma en coup de sabre by autologous tissue cocktail injection. Dermatol Surg 29: 1073-1075.
17. Palmero ML, Uziel Y, Laxer RM, Forrest CR, Pope E (2010) En coup de sabre scleroderma and Parry-Romberg syndrome in adolescents: surgical options and patient-related outcomes. J Rheumatol 37: 2174-2179.
18. Nguyen PS, Desouches C, Gay AM, Hautier A, Magalon G (2012) Development of micro-injection as an innovative autologous fat graft technique: The use of adipose tissue as dermal filler. J Plast Reconstr Aesthet Surg.
19. Thorud E, Clausen OP (1980) Epidermal cell kinetics in hairless mice after bleomycin. II. Perturbations after multiple doses. Cancel Treat Rep 64: 1039-1045.
20. Puzenat E, Aubin F (2002) Sclérodermies. Encycl Méd Chir (Edns) Scientifiques et Médicales, Elsevier SAS, Paris, tous droits reserves: 98-505.
21. Bacou F, el Andalousi RB, Daussin PA, Micallef JP, Levin JM, et al. (2004) Transplantation of adipose tissue-derived stromal cells increases mass and functional capacity of damaged skeletal muscle. Cell Transplant 13: 103-111.
22. Vermette M, Trottier V, Ménard V, Saint-Pierre L, Roy A, et al. (2007) Production of a new tissue-engineered adipose substitute from human adipose-derived stromal cells. Biomaterials 28: 2850-2860.
23. Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, et al. (2006) Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nat Med 12: 459-465.
24. Valina C, Pinkernell K, Song YH, Bai X, Sadat S, Campeau RJ, et al. (2007) Intracoronary administration of autologous adipose tissue-derived stem cells improves left ventricular function, perfusion, and remodelling after acute myocardial infarction. Eur Heart J 28: 2667-2677.