The Effects of Adipose-Derived Stem Cell–Differentiated Adipocytes on Skin Burn Wound Healing in Rats

Yu-Wen Chen, PhD,* Tabita Timeea Scutaru, MD,*† Nicolae Ghetu, MD, PhD,*†‡ Eugen Carasevici, PhD,§‖ Cristian Dumitru Lupascu, MD, PhD,§¶ Dan Ferariu, MD, PhD,# Dragos Pieptu, MD, PhD,‡ Corneliu-George Coman,*† and Mihai Danciu, MD, PhD,§#

Both adipose-derived stem cells (ADSCs) and fat grafting promote burn wound healing, but whether adipogen-derived cells using various inducers such as 3-isobutyl-1-methylxanthine (IBMX) and insulin affect wound healing is unknown. Herein, ADSC-differentiated adipogenic lineages were used in rat burn wounds to evaluate wound healing potential. ADSCs were cultivated using six different adipogenic differentiation conditions (IBMX ± insulin, IBMX for 5 days, high and low Dulbecco's modified Eagle's medium) and in vitro morphological changes and cell proliferations during adipogenic differentiation were recorded. Intermediate burn wounds were inflicted in 15 Wistar male rats. Afterwards, the rats were divided into five groups for subcutaneous injections under the wounds: control; ADSCs; differentiated adipocytes (–IBMX+INSULIN and +IBMX[D1–5]+INSULIN) and fat prepared by Coleman technique. Macroscopic changes and histology were documented for 3 weeks. Repeated measures analysis of variance was performed to analyze cell growth and wound healing with a statistical level set of $P < .05$. Induction cocktails significantly reduced proliferation and induced lipid droplet accumulation. Conditioning without insulin induced the least lipid accumulation, while discontinuing IBMX generated larger adipocytes ($P < .001$). Adipogenic differentiated ADSCs had similar wound healing abilities with ADSC and fat injections, but differentiated adipocytes (+IBMX[D1–5]+INSULIN) and fat grafting accelerated the early healing process relative to ADSC ($P < .001$). Reduced fibrosis and mild inflammatory infiltration limited to superficial dermis were observed in +IBMX(D1–5)+INSULIN and fat injection groups, while those reactions were mild to moderate in ADSC group. Differentiated adipocytes achieve similar wound healing results compared with ADSC and fat injections, but differentiated adipocytes (+IBMX[D1–5]+INSULIN) and fat grafting accelerate early healing relative to ADSC. (J Burn Care Res 2017;38:1–10)

*From the *Center for Simulation and Training in Surgery, Grigore T. Popa University of Medicine and Pharmacy Iasi, Romania; †Faculty of Medicine, Grigore T. Popa University of Medicine and Pharmacy Iasi, Romania; ‡Department of Plastic and Reconstructive Surgery, Grigore T. Popa University of Medicine and Pharmacy Iasi, Romania; §Grigore T. Popa University of Medicine and Pharmacy Iasi, Romania; and ‖Department of Immunology, Faculty of Medicine, ¶Department of Surgery, and #Department of Pathology, Regional Oncology Institute Iasi, Romania.*

The research was funded by grants PNIIIDPCE201130906/ UEFISCDI 274/2011 and European Social Found, Human Resources Development Operational Program 2007–2013, project no. POSDRU/159/1.5/136893.

N.G. and Y.W.C. designed and conceptualized the study. T.T.S., Y.W.C., N.G., M.D., and C.G.C. performed literature review, data collection, and interpretation, and drafted the article. All authors edited and revised important intellectual contents critically, and approved the final version of the paper.

Address correspondence to Nicolae Ghetu, MD, PhD, Grigore T. Popa University of Medicine and Pharmacy, Department of Plastic and Reconstructive Surgery, Regional Oncology Institute, Plastic Surgery, 3rd Floor, 2-4, G-ral Berthelot St, 700483 Iasi, Romania. Email: ghetu.nicolae@umfiasi.ro, dr.ghetu@gmail.com

1559-047X/2016

DOI: 10.1097/BCR.0000000000000466

Burn injuries influence daily life greatly. In recent years, promising strategies such as mesenchymal cell-based (adipose-derived stem cells [ADSCs]) tissue-engineering substitutes[1–3] or paracrine-fashioned therapies[4–6] were applied extensively in laboratory or clinical settings. The contributions of ADSCs and adipose tissue in the complex wound repair processes, which comprise inflammation, granulation, and remodeling, have been documented.[7,8] In particular, adipocyte lineage cells or subcutaneous lipo-injections were found activated in acute wound healing and could enhance the appearance of scar tissues.[3,9] In the absence of adipocytes, fibroblast and extracellular matrix deposition were abrogated, and abnormal dermal remodeling/regeneration at the lesion site was noticed, suggesting that mature adipocytes may play an important role in skin wound healing.[7,8,10] Although the precise mechanisms of action or the specific role that adipocytes may play are not fully understood, evidence points out that fat

grafting accelerates wound healing surrounding the injection sites.[6,8,10] With the identified components from injected fat, such as adipocytes and ADSC as main factors enhancing the wound healing, ways to increase the content and wound healing properties of effector cells are currently under investigation. ADSCs differentiate in vitro into adipogenic lineages, thus mature adipocytes. The resulting adipocytes were not investigated for the possibility of retaining the wound healing abilities of their precursor ADSCs.

Various in vitro adipogenic differentiation protocols are available at present. Apart from maintenance medium recipe (Dulbecco's modified Eagle's medium [DMEM] plus 10% fetal bovine serum [FBS]), several cocktail inducers were commonly supplemented in adipogenic differentiation.[11] Dexamethasone is an anti-inflammatory steroid molecule that stimulates adipogenic differentiation at high concentration.[12] In addition, insulin (a peptide hormone regulates the metabolism of carbohydrates and fats) and 3-isobutyl-1-methylxanthine (IBMX, a competitive, nonselective phosphodiesterase [PDE] inhibitor) are the main regulators of peroxisome proliferator-activated receptor-γ (PPAR-γ) and/or CCAAT/enhancer-binding protein-α activation, which lead to adipogenic gene expression.[6,13–16] Despite an increased understanding of adipogenic inducers functions and working mechanisms, a standardized protocol regarding the selections of inducers and culture duration for adipogenic differentiation is still being disputed.[11] Variant adipogenic differentiation inducers that affect adipocytes maturation and proliferation[13] may indirectly influence the skin wound healing capacities of differentiated adipocytes.

The aims of this study were to investigate the effects of ADSC-origin differentiated adipocytes on skin burn wound healing in rats relative to ADSC and fat injection control groups, and whether different adipogenic induction protocols affect their capacities in wound repair. To address the aforementioned questions, the morphological changes and cell proliferation abilities of differentiated adipocytes cocultured with alternative adipogenic cocktail inducers (IBMX and/or insulin) were analyzed. In addition, the effects of differentiated adipocytes (cocultured under different adipogenic protocols) on wound healing were compared with ADSC and fat injection (prepared by Coleman technique) groups. By implementing this study, we expect to establish an effective in vitro adipogenic differentiation protocol for burn wound healing in rats.

METHODS

Animals

Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals from the National Research Council (United States) and following the protocols approved by Grigore T. Popa University of Medicine and Pharmacy, Iasi, Romania.

Isolation and Culture of Adipose-Derived Stem Cells

The inguinal fat pads from male adult Wistar rats (procured from the animal farm of Grigore T. Popa University of Medicine and Pharmacy) were harvested in a sterile fashion and washed with sterilized phosphate-buffered saline (PBS) with 5% penicillin–streptomycin (500 UI/ml Penicillin, 500 mg/ml streptomycin). The protocols used for isolation and culture of ADSCs were the same as our previous study.[17] Briefly, the fat pads were minced and digested with 2 mg/ml of type IA collagenase (C9891, Sigma–Aldrich, St. Louis, MO, United States) for 30 to 45 minutes at 37°C. After centrifugation, the pelleted stromal vascular fraction containing ADSCs was resuspended, passed through a 70-μm cell strainer (#352350, Becton, Dickinson and Company, Franklin Lakes, NJ, United States), and incubated in stromal medium (low-glucose DMEM [D6046, Sigma–Aldrich] supplemented with 10% FBS and 1% penicillin–streptomycin) at 37°C in 5% CO_2 atmosphere. The medium was renewed every 2 to 3 days until the cells reached a confluence of 80 to 90%. Cells were then detached by the application of trypsin–EDTA for subculture.

Induction of Adipogenesis

In view of the existence of diverse protocols for adipogenic differentiation,[11] we established six separate conditions in order to observe the discrepancies of morphological changes, proliferation abilities, and the capacities in wound healing between different adipogenic inducers. Shortly, ADSCs (passage 2–3) were seeded at the density of 10,000 cells/cm^2 with the stromal medium in a six-well plate on day 0. After washing with PBS, basal differentiation medium (high-glucose DMEM [4500 mg/L glucose]+10% FBS + 1% P–S + 0.5 μM dexamethasone [H02AB02, Medochemie LTD., Limassol, Cyprus] + 50 μM diclofenac sodium [M01AB05, Salutas Pharma GMBH, Frankfurt am Main, Germany])[1,11,18,19] plus different adipogenic cocktail inducers were replenished starting from

day 1: +IBMX–INSULIN, 0.5 mM IBMX (I5879, Sigma–Aldrich) only; +IBMX+INSULIN, 0.5 mM IBMX+200 mUI/ml insulin (Insulin Humulin R, A10AB01, Lilly France SAS, Neuilly-sur-Seine Cedex, France); –IBMX+INSULIN, 200 mUI/ml insulin only; +IBMX(D1–5)+INSULIN, 0.5 mM IBMX + 200 mUI/ml insulin for 5 days and thereafter 200 mUI/ml insulin only for the rest of days[11]; LOW DMEM, low-glucose DMEM (1000 mg/L glucose); HIGH DMEM, high-glucose DMEM (4500 mg/L glucose). The differentiation media were renewed once every 3 days until adipocytes were developed (day 11).

On day 11, cells were fixed with 10% formalin solution and air dried for fat staining. A 2% of Sudan IV (S4261, Sigma–Aldrich) was applied for neutral lipid staining[19] followed by a counter staining the nuclei with Mayer's hematoxylin solution (MHS16, Sigma–Aldrich; Figure 1).

In vitro Morphological and Proliferation Changes During Adipogenic Differentiation

The photos of morphological changes in diverse adipogenic cocktails were taken sequentially on day 1, 3, 6, 9, and 11 by using a phase contrast microscope with a digital single-lens-reflex camera at 200 folds of magnification. Cell numbers between different adipogenic protocols were calculated by two investigators who were blind to group assignments.

Skin Burn Model and Wound Healing Assessment in Rats

Adult Wistar male rats weighing between 250 and 300 g were anesthetized using 3 to 4% isoflurane inhalation (N01AB06, Rompharm Company S.R.L., Otopeni, Romania) and the anesthesia was maintained with 1 to 2% isoflurane when performing burn infliction. The dorsum of the rat was shaved and a 3-cm-diameter burn wound was inflicted using hot steam for 2 seconds directed toward the skin through an electrovalve and a round-shaped rubber funnel.

Immediately after skin burn infliction, 12 rats were randomly divided into 4 groups. To allow equal distribution, each rat received a total 1 ml of liquid divided in four cardinal points (ie, 0.25 ml/point) in the subcutaneous tissue under the burn wound. Group 1 (control) received PBS injection only; group 2 (+ADSCs) received a total of 1 million of ADSC injection; group 3 (–IBMX+INSULIN) received a total of 1 million ADSC-origin differentiated adipocytes pretreated with 200 mUI/ml insulin without IBMX for 11 days; and group 4 (+IBMX[D1–5]+INSULIN) were injected with ADSC-origin differentiated adipocytes (1×10^6 cells) cocultured with 0.5 mM IBMX and 200 mUI/ml insulin for 5 days and 200 mUI/ml insulin only thereafter for the next 6 days.

Visual macroscopic changes were documented using photographs taken with a single-lens-reflex

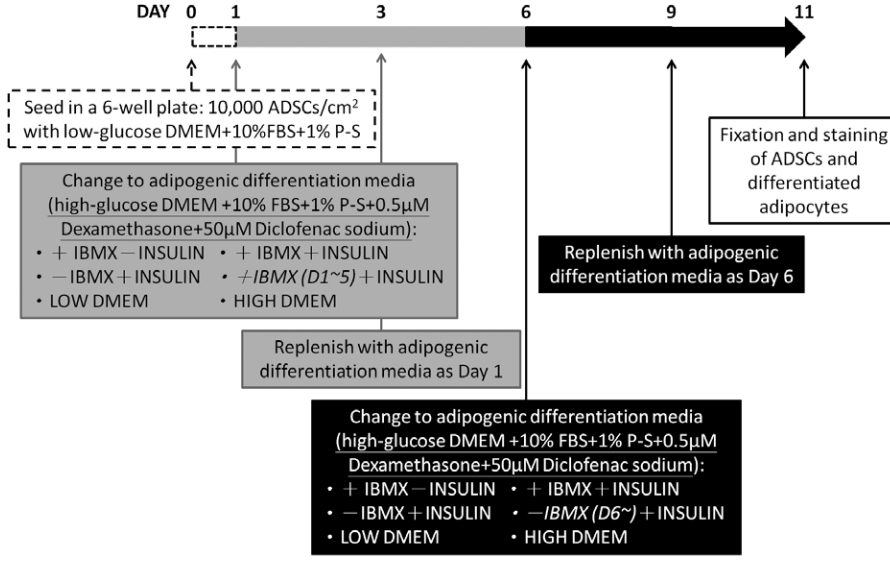

Figure 1. The flow chart of adipogenic differentiation. DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; P–S, penicillin–streptomycin; IBMX, 3-isobutyl-1-methylxanthine; +IBMX–INSULIN, 0.5 mM IBMX only; +IBMX+INSULIN, 0.5 mM IBMX + 200 mUI/ml insulin; –IBMX+INSULIN, 200 mUI/ml insulin only; +IBMX(D1–5)+INSULIN, 0.5 mM IBMX + 200 mUI/ml insulin for 5 days and then changed to 200 mUI/ml insulin only for the rest of days; LOW DMEM, low-glucose DMEM (1000 mg/L glucose); HIGH DMEM, high-glucose DMEM (4500 mg/L glucose).

camera at day 0, 7, 12, 14, 16, 18, and 21 after burn infliction. Actual wound size (cm^2) was calibrated and analyzed using Image J software in accordance with the scale in each image. Differences between the wound areas were measured by two investigators who were blind to group assignments.

In addition, 4-mm-diameter punch biopsies were taken from the cardinal points of the burn wounds and fixed with 10% of formalin solution for further histological examination. After fixation for 12 hours, biopsies were routinely processed by paraffin embedding. Four-micrometer hematoxylin and eosin stained sections were independently evaluated by two independent pathologists.

Fat Injection (Coleman Technique) in Skin Burn Wound Healing Model

In order to evaluate the effects of fat tissues on burn wound healing (as a comparative control to ADSCs or differentiated adipocytes), inguinal fat pads harvested from rats (n = 3) were washed with saline and minced using iris scissors until they became gel-like in appearance. Followed by suction through a 1-mm cannula into 10 ml syringes, the gel was centrifuged at 200 rpm for 2 minutes. The supernatant containing the fat was discarded, and then 10^6/ml of the cell suspension aliquot was injected 0.25 ml at each cardinal point under the burn area in the subdermal plane. The follow-up procedures for rats were similar to cell-injected groups.[20]

Statistical Analysis

The statistical software SPSS version 13.0 was used for data analysis. The results were expressed as mean ± SD. A 6 (differentiation protocols) × 5 (time sample points) repeated measures analysis of variance and Bonferonni post hoc comparisons were used to analyze the ability of cell proliferation in different adipogenic differentiations. In addition, a 5 (differentiation protocols) × 7 (time sample points) repeated measures analysis of variance and Bonferonni post hoc comparisons were used to evaluate the effects of ADSCs, differentiated adipocytes (with or without IBMX), and fat injection on skin burn wound healing. The significance level was set as $P < .05$.

RESULTS

Morphological Changes During Adipogenic Differentiation

Under phase contrast microscopy, cultured rat ADSCs showed a spindle-shaped, fibroblastic morphology. In cytochemistry observation, the nuclei of ADSCs were stained with dark blue by hematoxylin, but negative for Sudan IV (scarlet; Figure 2, E5 and F5). No significant morphological differences were found between low-glucose (Figure 2E) and high-glucose DMEM (Figure 2F) groups.

ADSCs treated with basal adipogenic medium (high-glucose DMEM + 10% FBS + 1% P–S + 0.5 μM dexamethasone + 50 μM diclofenac sodium)

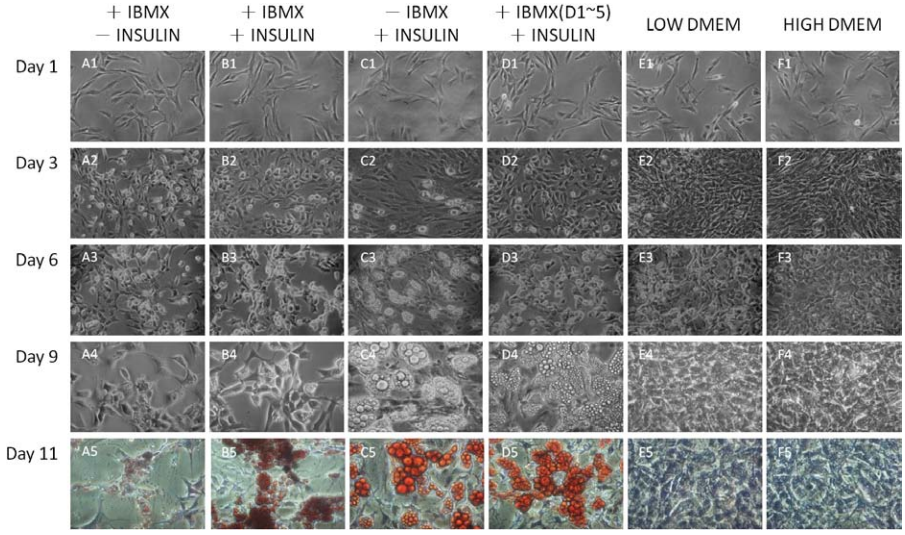

Figure 2. In vitro time course of morphological changes in adipogenesis. The photos of differentiated adipocytes were taken sequentially on day 1, 3, 6, 9, and 11 of adipogenic differentiation by using a microscope with a single-lens-reflex camera at 200 folds of magnification. Photos on day 9 and day 11 were locally magnified in proportion (400 folds) to exhibit comprehensive details of the cells.

plus 200 mUI/ml insulin without IBMX (Figure 2C) showed a spindle-shaped morphology and developed significant lipid accumulation in cytoplasm starting from day 6 after the adipogenic induction (Figure 2, C3). However, ADSCs cocultured with adipogenic medium with IBMX but without insulin (Figure 2A) exhibited the least lipid accumulation (Figure 2, A4 and A5). By contrast, spherical-shaped changes were noticed in IBMX-positive groups (+IBMX+INSULIN, +IBMX–INSULIN, or +IBMX[D1–5] +INSULIN, Figure 2A, B, and D), and marked lipid accumulation appeared only after IBMX treatment was discontinued (Figure 2, D4 and D5). Interestingly, larger lipid droplet formation was observed in IBMX-negative group (Figure 2, C4 and C5) than in the IBMX-positive (for 5 days) group (Figure 2, D4 and D5). However, continuous IBMX treatment (for 11 days, Figure 2A, B) not only confined lipid droplet accumulation (Figure 2, A5 and B5), but also accelerated cell detachment (Figure 2, A4–A5 and B4–B5, respectively).

Proliferation Abilities of Differentiated Adipocytes

Cell numbers in all groups were significantly increased from day 0 to day 11 (**P* < .001, day 1 vs day 3, 6, 9, and 11), but only cells cultured with stromal media (low and high DMEM) continued to proliferate until day 9. Cells cocultured with adipogenic media plus cocktail inducers did not show significant mitotic expansion after day 3 of differentiation (+*P* < .001, day 3 vs day 6, 9, and 11; Figure 3). In addition, there were no significant differences of cell proliferation between low- and high-glucose DMEM groups (*P* = .517). However, in the group treated with IBMX for 5 days (+IBMX[D1–5]+INSULIN), not only lipid droplets started to accumulated significantly (Figure 2, D4–D5), but also the ability of cell proliferation resumed after discontinuing IBMX treatment on day 6 [‡*P* < .001, LOW or HIGH DMEM or +IBMX(D1–5)+INSULIN vs +IBMX+INSULIN and +IBMX–INSULIN] (Figure 3).

Skin Burn Wound Healing

ADSCs, differentiated adipocytes, and fat tissues promoted skin burn wound healing in rats; differentiated adipocytes and fat grafting accelerated early healing relative to ADSCs.

Three-centimeter-diameter dorsum skin burn inflictions were performed on 15 Wistar male rats, and then the rats were randomly assigned to five groups of different cell injections for wound healing assessments: control (PBS-injected), +ADSCs

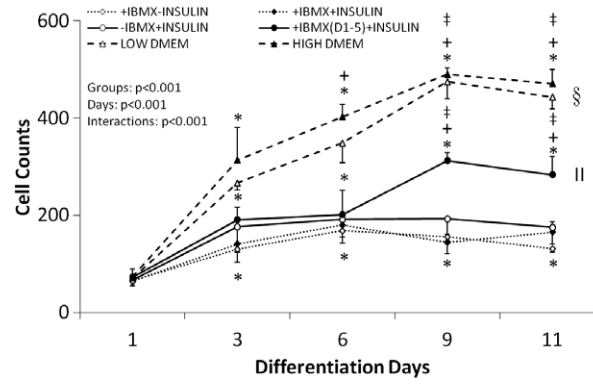

Figure 3. Proliferation abilities of ADSC-origin differentiated adipocytes. Data were expressed as mean ± SD (n = 3). Cell numbers were counted on day 1, 3, 6, 9, and 11 by using a microscope at 200 folds of magnification. **P* < .001, day 1 vs day 3, 6, 9, and 11 in all six groups; †*P* < .001, day 3 vs day 6, 9, and 11 in LOW and HIGH DMEM; ‡*P* < .001, day 6 vs day 9, 11 in +IBMX(D1–5)+INSULIN, LOW and HIGH DMEM; §*P* < .001, LOW or HIGH DMEM vs other groups; ‖*P* < .001, +IBMX(D1–5)+INSULIN vs +IBMX+INSULIN and +IBMX–INSULIN).

(ADSC-injected), –IBMX+INSULIN (adipocytes pretreated with differentiation cocktails without IBMX), +IBMX(D1–5)+INSULIN (adipocytes pretreated with differentiation cocktails with IBMX for only 5 days), and +fat injection (prepared by Coleman technique). Wound size was calibrated and analyzed by using Image J software in accordance with the scale attached in each photo on day 0, 7, 12, 14, 16, 18, and 21 after burn infliction (Figure 4A).

Overall, the wound healing process was observed early at day 7 after the burn infliction and continued for 3 weeks (day 0 vs day 12, 14, 16, 18, and 21 in all groups, *P* < .001). However, ADSC-origin differentiated adipocytes cocultured with IBMX for 5 days (+IBMX[D1–5]+INSULIN) as well as the injected fat initiated wound healing process earlier than all other groups in the first week after burn infliction (day 7), and had achieved significant wound size reduction (*P* < .001, Figure 4B).

Compared with the natural healing process (control group), all other groups exhibited accelerated wound healing on day 12, 14, 16, 18, and 21 after burn infliction (*P* < .001, Figure 4B). Although burn wounds injected with ADSCs, differentiated adipocytes, or fat tissues (prepared by Coleman technique) expedited wound healing, the changes of wound sizes in these groups did not reach significant differences on day 16 and thereafter (*P* > .05, day 16 vs day 18 and 21). On the contrary, wound healing process in control group continued markedly to

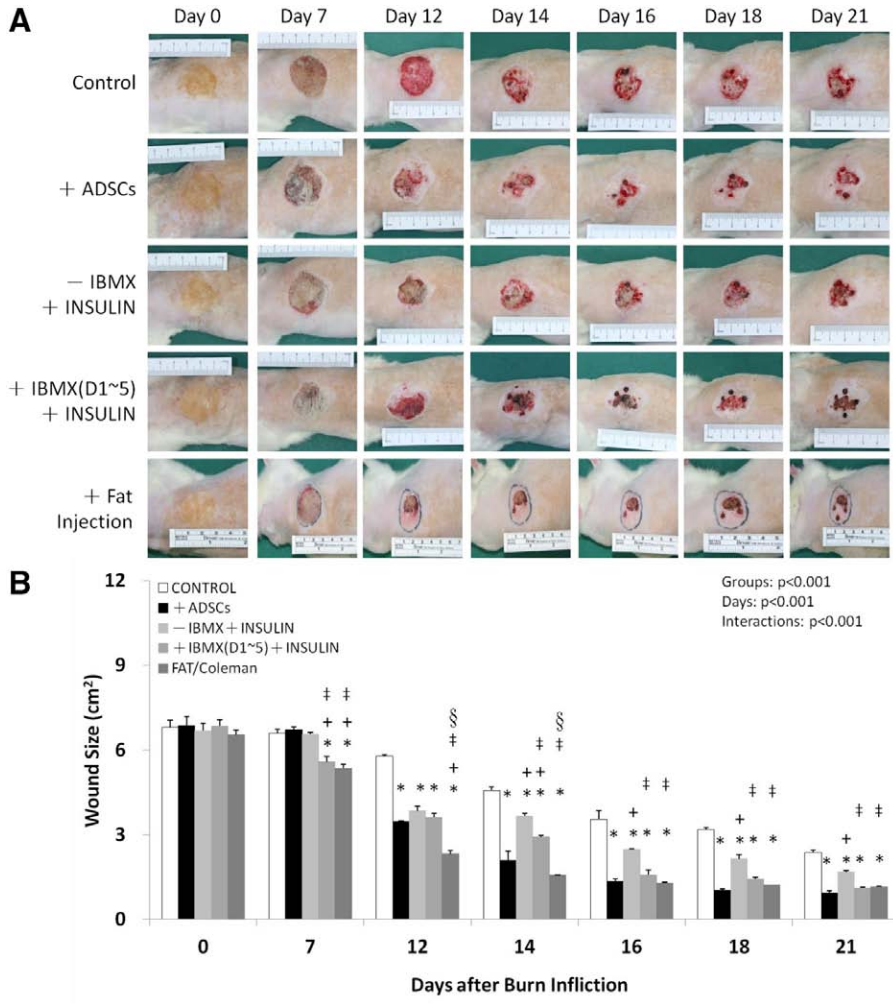

Figure 4. ADSCs, differentiated adipocytes, and fat tissues improved skin burn wound healing in rats, but differentiated adipocytes and fat grafting accelerated early healing process relative to ADSC. Rat skin burns were inflicted as indicated in methodology and then injected with ADSCs, adipocytes pretreated with different cocktail inducers, or fat tissues prepared by Coleman technique. Photos were taken sequentially on day 0, 7, 12, 14, 16, 18, and 21 after burn infliction. A. Representative images of wound healing on day 0, 7, 12, 14, 16, 18, and 21 after burn infliction. B. The unhealed wound sizes (cm^2) of the control (PBS-injected, blank bar), ADSC-injected (filled bar), adipocyte-injected (without IBMX [light-gray bar] and with IBMX treatment for 5 days [medium-gray bar]), and fat-injected groups (dark-gray bar). Data were expressed as mean ± SD (n = 3). *$P < .001$, control vs all groups; †$P < .001$, ADSC-injected group vs adipocyte- and fat-injected groups; ‡$P < .001$, adipocytes pretreated without IBMX group vs adipocytes pretreated with IBMX and fat injection groups; §$P < .001$, adipocytes pretreated with IBMX group vs fat injection group.

the end of the third week (day 21). Furthermore, for the first 2 weeks, fat-injected group showed the most prominent wound healing capacity compared with ADSCs or ADSC-origin differentiated adipocyte-injected groups ($P < .001$, Figure 4B). Even though burn wounds injected with adipocytes pretreated with differentiation cocktail inducers without IBMX (–IBMX+INSULIN) demonstrated more potent wound healing capacity than control group (*$P < .001$), this group exhibited minor wound healing capacity compared with ADSC-injected, adipocyte (+IBMX(D1–5)+INSULIN)-injected, and fat-injected groups ($P < .001$, Figure 4B).

Histological Evaluation of Skin Burn Wound Healing

Four-millimeter punch biopsies were performed from the wounds for all groups on day 12, 14, 16, 18, and 21. Microscopic evaluation revealed that epithelial regeneration was complete in +ADSC and +IBMX(D1–5)+INSULIN samples starting with day 12. In fat-injected rats, the epithelium was

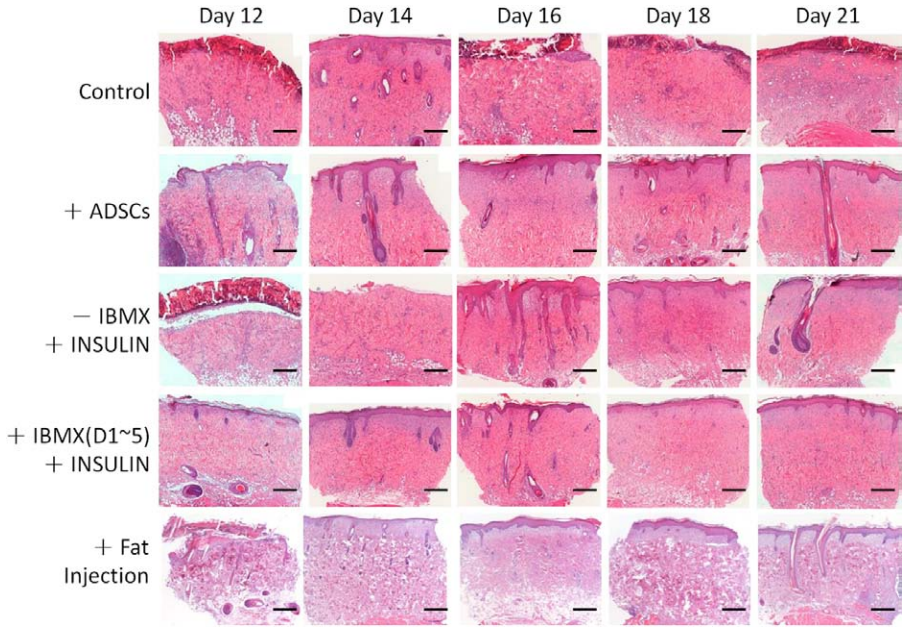

Figure 5. Microscopic evaluation of burn wound healing. Reduced fibrosis and mild lymphocytic inflammatory infiltration limited to the superficial dermis were observed in +IBMX(D1–5)+INSULIN and +Fat Injection groups, while the fibrosis and the inflammatory infiltration were mild to moderate in +ADSC group. In –IBMX+INSULIN group, the healing process was uneven due to delayed reepithelization, and moderate chronic inflammation with dermis and hypodermis infiltration was also noticed. In CONTROL group, large areas with no reepithelization and abundant chronic inflammation were observed. Bar: 500 μm.

uniformly regenerated at day 14. In +IBMX(D1–5)+INSULIN group and in fat-injected group, we observed reduced fibrosis and mild lymphocytic inflammatory infiltration limited to the superficial dermis (maturation and remodeling phase) from day 12 to day 21 (Figure 5), while the fibrosis and the inflammatory infiltration was mild to moderate in +ADSC group (fibroplasia and granulation phase; Figure 5). In –IBMX+INSULIN group, the healing process was uneven, areas with complete reepithelization alternating with areas with no epithelial buds, covered only by fibrinoleukocytic exudates. In addition, the fibrotic changes affected the upper reticular dermis and were accompanied by moderate chronic inflammatory infiltration in dermis and hypodermis (day 12 to day 20; Figure 5). The microscopic results also showed that the morphology in control group was similar to that observed in –IBMX+INSULIN group, except the fact that areas with no reepithelization were more extensive in the control group (Figure 5).

DISCUSSION

Summary of the Results

To our knowledge, this is the first study to compare the effects of various adipogenic cocktail inducers on the morphological changes, proliferation abilities, and wound healing capacities of adipocytes between different protocols. Our results indicated that among well-known adipogenic inducers (eg, dexamethasone, insulin, and IBMX), a prolonged treatment of IBMX (with or without insulin for 11 days) resulted in cell deterioration (rounding-up and detachment of cells) and mitotic inhibition. However, a short-term treatment of IBMX (with insulin for 5 days) or a cocktail medium lack of IBMX led to successful adipogenesis. Although ADSCs, adipogenic differentiated cells, and processed fat injection groups achieved similar wound healing results, differentiated adipocytes (+IBMX[D1–5]+INSULIN) and fat injections showed early skin wound healing compared with ADSC injection groups only. Furthermore, ADSC-injected, adipocyte-injected (briefly cocultured with IBMX for 5 days), and fat-injected groups exhibited better wound healing capacities than adipocytes treated without IBMX (–IBMX+INSULIN) group.

Morphological Changes and Cell Proliferation

In view of the existence of diverse protocols for mesenchymal cell–originated adipogenic differentiation, the uses of adipogenic cocktail inducers such as dexamethasone, insulin, and IBMX were mentioned

repeatedly.[11] Among them, inducers such as insulin and IBMX that are involved in the master regulator of adipogenesis, PPAR-γ, are of interest to disparate specialties of medicine.[14,15] In our study, we used in vitro culture system to test the necessities of these two inducers for primary ADSC-origin adipogenic differentiation. The cytochemistry staining results showed that ADSCs cocultured with adipogenic medium (high-glucose DMEM + 10% FBS + 1% P–S + 0.5 μM dexamethasone + 50 μM diclofenac sodium) plus 200 mUI/ml insulin without IBMX (Figure 2C) displayed a spindle-shaped morphology and developed significant lipid accumulation in cytoplasm starting from day 6 after adipogenic induction (Figure 2, C3). However, ADSCs cocultured with adipogenic medium with IBMX but without insulin (Figure 2A) exhibited the least lipid accumulation (Figure 2, A4 and A5), implying that insulin played an important role in lipid accumulation. Our findings were consistent with the results published by Rieusset et al,[16] which concluded that insulin regulated the mRNA expression of PPAR-γ. Moreover, under the condition supplemented with insulin, the addition of IBMX and the coculture duration with IBMX also affected adipogenesis. Marked lipid accumulation appeared only after IBMX treatment was discontinued on day 6 (Figure 2, D4–D5). When the ADSC-originated cells were cocultured with IBMX for longer period (11 days), signs of cell deterioration such as detachment of the cells from the culture surface were observed (Figure 2, B4-B5). The results indicated that with the existence of insulin, IBMX was also a potent inducer for adipogenesis, but only short-term treatment of IBMX (for 5 days) preserved successful adipogenic differentiation. IBMX is a competitive, nonselective PDE inhibitor, which raises intracellular cyclic AMP (cAMP) and thus activates protein kinase A. Gabrielli et al[14] proved that protein kinase A signaling pathway is required for transcriptional activation of PPAR-γ and the expression of adipogenic genes. Although we have found that either insulin itself or insulin combined with IBMX could regulate adipogenesis, Gabrielli et al[14] and Kim et al[15] both mentioned that insulin and IBMX were prerequisite inducers for adipogenic differentiation of 3T3-L1 fibroblasts. Unfortunately their results were inconsistent with our findings. However, in the review of Scott et al, IBMX was not the mandatory component for murine ADSC-originated adipogenesis. On the contrary, insulin was widely used to induce proliferation and differentiation of pre-adipocytes.[11] The discrepancies of the results between ours and Gabrielli et al or Kim et al may be resulted from the cell types used for adipogenic differentiation. At least for primary ADSCs, a precise identity and heterogeneity are still under investigation. In our study, we discovered that primary ADSCs could be differentiated into adipocytes only by coculturing with insulin and dexamethasone except IBMX, which exhibited similar results to those of Rieusset et al.[16]

Besides morphological changes, the abilities of cell proliferation were also varied between different adipogenic inducers. In order to compare the effects of adipogenic inducers on mitotic expansions of differentiated adipocytes, we have calculated cell numbers following the time courses of adipogenic differentiation (Figure 3). Our results demonstrated that cells cocultured with stromal media (low- and high-glucose DMEM supplemented with 10% FBS + 1% P–S) continued to proliferate from day 0 to day 9, but cells cocultured with adipogenic cocktail inducers exhibited growth arrest since day 3 of differentiation ($+P < .001$, day 3 vs day 6, 9, and 11; Figure 3). However, cell growth resumed when IBMX was discontinued (Figure 3). Similarly, Wang et al[21] discovered that by day 2 of the differentiation course, CCAAT/enhancer-binding protein-α protein initiated to accumulate, and after it was phosphorylated by the cyclin D3, a proliferation inhibition effect was induced.[13] Furthermore, a biphasic effect of IBMX incorporation with insulin depended on the intracellular level of cAMP was noted in Takahashi et al's study. In the beginning, the IBMX-potentiated intracellular cAMP accumulation markedly elevated DNA synthesis; once cAMP exceeded a certain level, an inhibition of DNA synthesis was observed,[22] suggesting that cAMP was equipped with both stimulatory and inhibitory effects on the regulation of mitosis. This also explains the abrogation of cell growth in long-term IBMX treatment groups in our study (Figures 2A, B and 3). Nevertheless, the cell growth arrest was reversible upon removal of the PDE inhibitor.[22] Therefore, when IBMX treatment was discontinued starting on day 6 in our study, the ability of cell proliferation of differentiated adipocytes returned (Figure 3), indicating that the inhibition of cell growth was IBMX-dependent.

Wound Healing Capacities

Due to their adipogenic and angiogenic properties, ADSCs have been widely used in clinical settings for cosmetic or reconstructive surgeries,[23] including as an adjunct to wound healing therapies following thermal injuries.[2,4] By contrast, intradermal adipocytes were considered harmful for wound healing because of its poor circulation and easy liquefaction,

which offered bacteria an excellent environment to propagate and resulted in local and systemic infections.[24] Nonetheless, these traditional concepts have been challenged by the novel discoveries of adipose tissue, including its secretion functions (eg, cytokines, growth factors, eicosanoids, and more) and potential for skin wound healing.[4,7,8,10] So far most of the tissue-engineered substitutes for clinical soft-tissue reconstruction involved a concomitant incorporation of ADSCs and adipocytes,[8,10] an independent functional examination of differentiated adipocytes on wound healing has been missing.

Our study compared the effects of ADSCs and differentiated adipocytes on wound size reduction after skin burn infliction, respectively. The results showed that compared with the control group (injection of PBS), subcutaneous injections of ADSCs, differentiated adipocytes (–IBMX+INSULIN and +IBMX[D1–5]+INSULIN), or processed fat under the wound accelerated wound healing after the first week (day 7) of burn infliction and continued for 3 weeks (day 21; $P < .001$ for day 0 vs day 12, 14, 16, 18, and 21 in all groups). Previous studies have proved that the wound healing process was promoted through either direct ADSCs or adipocytes and/or paracrine effects.[5,8,25,26] In Martin et al's[8] study, the levels of pro-proliferative and angiogenic factors such as vascular endothelial growth factor, hepatocyte growth factor, and leptin were significantly elevated in adipose dressing-containing in vitro culture system, so a paracrine-fashioned mechanism was suggested in skin wound healing. Besides the well-known role in satiety and weight control, leptin also possessed angiogenic activity.[8,27] In our case, although we did not investigate the hormone levels in burn models, significant weight reduction was observed in adipocyte-injected groups (data not shown), suggesting that adipocyte-secreted leptin (paracrine-fashioned) might be involved in wound healing.

According to the results published by Schmidt and Horsley,[10] adipocytes were also responsible for fibroblast (precursor) proliferation, repopulation, and dermal reconstruction following inflammation during acute skin wound healing, which suggested that mature adipocyte-released adipokines might be the key chemotactic agents for fibroblast recruitment. In the absence of adipocytes, fibroblast and extracellular matrix depositions were abrogated, leading to abnormal dermal remodeling/regeneration at lesion site.[7,8,10] Our results were consistent with the findings of Schmidt and Horsley.[10] In our study, ADSCs, differentiated adipocytes, and fat injection accelerated wound healing (*$P < .001$, control vs all other groups; Figures 4B and 5), but differentiated adipocyte injection group (+IBMX[D1–5]+INSULIN) as well as fat injection group initiated wound healing process earlier than any of the other groups ($P < .001$, Figure 4B). Similarly, according to our hematoxylin and eosin observations, we found reduced fibrosis and mild lymphocytic inflammatory infiltration limited to the superficial dermis (maturation and remodeling phase) from day 12 to day 21 in +IBMX(D1–5)+INSULIN and fat-injected groups, while the fibrosis and the inflammatory infiltration was mild to moderate (fibroplasias and granulation phase) in +ADSC group (Figure 5), pointing out that mature adipocytes, not mesenchymal stem cells (ADSCs), might expedite acute wound healing (at day 7) by promoting fibroblast recruitment.[10] ADSC-promoted wound healing may be initiated through intercellular and/or paracrine mechanisms as previously mentioned,[5,8,25,26] and thus was slightly slower than adipocyte-injected group at the beginning of wound healing. Adipocyte-injected group without coculturing with IBMX (–IBMX+INSULIN) exhibited the least wound healing capacity compared with ADSC-injected and adipocyte-injected groups (+IBMX[D1–5]+INSULIN; ‡$P < .001$; Figures 4B and 5). Short-course IBMX (+IBMX[D1–5]+INSULIN) have more potent wound healing abilities than IBMX-free differentiated adipocytes (–IBMX+INSULIN). Further research is needed in order to identify the favorable conditioning agent responsible for the increased wound healing ability.

Study Limitations

This study examined morphological changes, cell growth, and wound healing capacities of ADSCs and ADSC-origin differentiated adipocytes. The results were mainly based on macroscopic and microscopic observations. To obtain a better knowledge in the progress/quality of wound healing, associated mechanisms and the fate of implanted cells, the use of traceable fluorescence-labeled ADSCs and adipocytes as well as corresponding adipokines' measurements or specific fibroblast staining are suggested. Adipocytes cocultured with continuous IBMX (for 11 days) were missing in skin wound healing groups due to their cell deterioration phenomenon during in vitro cell culture. We have excluded the cell toxicity effects of the solvent (DMSO) by coculturing ADSCs with adipogenic cocktail inducers plus DMSO (data not shown). Moreover, ADSCs-plus-adipocytes injection group in wound healing model was lacking. We only replaced such group with fat injection prepared by Coleman technique. Whether ADSCs-plus-adipocytes therapy

has synergistic effects in wound repair will be added in future studies.

CONCLUSIONS

Evidence reported in this study suggests that differentiated adipocytes achieve similar wound healing results as ADSC and fat injection, but differentiated adipocytes (+IBMX[D1–5]+INSULIN) as well as fat grafting (prepared by Coleman technique) accelerate early healing process relative to ADSC. Our study provides an alternative option (adipogenic differentiated cells vs fat prepared by Coleman technique—the mixture of stem cells, mediators, and cytokines) for future researchers who may be interested in elaborating on the single-lineage effects and underlying mechanisms of adipocytes in skin burn wound healing. However, further research is needed in order to explore the conditioning that would enhance differentiated adipocytes' abilities for wound healing.

ACKNOWLEDGMENTS

The authors would like to thank colleagues from Immunology laboratory of Grigore T. Popa University of Medicine and Pharmacy, Iasi, Romania, and to Dr. Daniel Murariu for their invaluable technical assistance.

REFERENCES

1. Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. Circ Res 2007;100:1249–60.
2. Huang SP, Huang CH, Shyu JF, et al. Promotion of wound healing using adipose-derived stem cells in radiation ulcer of a rat model. J Biomed Sci 2013;20:51.
3. Klinger M, Marazzi M, Vigo D, Torre M. Fat injection for cases of severe burn outcomes: a new perspective of scar remodeling and reduction. Aesthetic Plast Surg 2008;32:465–9.
4. Fu X, Fang L, Li H, Li X, Cheng B, Sheng Z. Adipose tissue extract enhances skin wound healing. Wound Repair Regen 2007;15:540–8.
5. Hu L, Zhao J, Liu J, Gong N, Chen L. Effects of adipose stem cell-conditioned medium on the migration of vascular endothelial cells, fibroblasts and keratinocytes. Exp Ther Med 2013;5:701–6.
6. Kim S, Moustaid-Moussa N. Secretory, endocrine and autocrine/paracrine function of the adipocyte. J Nutr 2000;130:3110S–5S.
7. Broughton G 2nd, Janis JE, Attinger CE. The basic science of wound healing. Plast Reconstr Surg 2006;117:12S–34S.
8. Morissette Martin P, Maux A, Laterreur V, et al. Enhancing repair of full-thickness excisional wounds in a murine model: Impact of tissue-engineered biological dressings featuring human differentiated adipocytes. Acta Biomater 2015;22:39–49.
9. Patel N. Fat injection in severe burn outcomes: a new perspective of scar remodeling and reduction. Aesthetic Plast Surg 2008;32:470–2.
10. Schmidt BA, Horsley V. Intradermal adipocytes mediate fibroblast recruitment during skin wound healing. Development 2013;140:1517–27.
11. Scott MA, Nguyen VT, Levi B, James AW. Current methods of adipogenic differentiation of mesenchymal stem cells. Stem Cells Dev 2011;20:1793–804.
12. Yin L, Li YB, Wang YS. Dexamethasone-induced adipogenesis in primary marrow stromal cell cultures: mechanism of steroid-induced osteonecrosis. Chin Med J (Engl) 2006;119:581–8.
13. Moreno-Navarrete JM, Fernández-Real JM. Adipocyte differentiation. In: Symonds ME, editor. Adipose tissue biology. 1st ed. New York: Springer-Verlag; 2012. p. 17–38.
14. Gabrielli M, Martini CN, Brandani JN, Iustman LJ, Romero DG, del C Vila M. Exchange protein activated by cyclic AMP is involved in the regulation of adipogenic genes during 3T3-L1 fibroblasts differentiation. Dev Growth Differ 2014;56:143–51.
15. Kim SP, Ha JM, Yun SJ, et al. Transcriptional activation of peroxisome proliferator-activated receptor-gamma requires activation of both protein kinase A and Akt during adipocyte differentiation. Biochem Biophys Res Commun 2010;399:55–9.
16. Rieusset J, Andreelli F, Auboeuf D, et al. Insulin acutely regulates the expression of the peroxisome proliferator-activated receptor-gamma in human adipocytes. Diabetes 1999;48:699–705.
17. Cheng HY, Ghetu N, Huang WC, et al. Syngeneic adipose-derived stem cells with short-term immunosuppression induce vascularized composite allotransplantation tolerance in rats. Cytotherapy 2014;16:369–80.
18. Adamson DJ, Frew D, Tatoud R, Wolf CR, Palmer CN. Diclofenac antagonizes peroxisome proliferator-activated receptor-gamma signaling. Mol Pharmacol 2002;61:7–12.
19. Bunnell BA, Flaat M, Gagliardi C, Patel B, Ripoll C. Adipose-derived stem cells: isolation, expansion and differentiation. Methods 2008;45:115–20.
20. Gir P, Brown SA, Oni G, Kashefi N, Mojallal A, Rohrich RJ. Fat grafting: evidence-based review on autologous fat harvesting, processing, reinjection, and storage. Plast Reconstr Surg 2012;130:249–58.
21. Wang GL, Shi X, Salisbury E, et al. Cyclin D3 maintains growth-inhibitory activity of C/EBPalpha by stabilizing C/EBPalpha-cdk2 and C/EBPalpha-Brm complexes. Mol Cell Biol 2006;26:2570–82.
22. Takahashi SI, Nedachi T, Fukushima T, et al. Long-term hormonal regulation of the cAMP-specific phosphodiesterases in cultured FRTL-5 thyroid cells. Biochim Biophys Acta 2001;1540:68–81.
23. Naderi N, Wilde C, Haque T, et al. Adipogenic differentiation of adipose-derived stem cells in 3-dimensional spheroid cultures (microtissue): implications for the reconstructive surgeon. J Plast Reconstr Aesthet Surg 2014;67:1726–34.
24. Nascimento AP, Costa AM. Overweight induced by high-fat diet delays rat cutaneous wound healing. Br J Nutr 2006;96:1069–77.
25. Rehman J, Traktuev D, Li J, et al. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation 2004;109:1292–8.
26. Nie C, Yang D, Xu J, Si Z, Jin X, Zhang J. Locally administered adipose-derived stem cells accelerate wound healing through differentiation and vasculogenesis. Cell Transplant 2011;20:205–16.
27. Murad A, Nath AK, Cha ST, Demir E, Flores-Riveros J, Sierra-Honigmann MR. Leptin is an autocrine/paracrine regulator of wound healing. FASEB J 2003;17:1895–7.