

# Mechanical micronization of lipoaspirates for treatment of bleomycin-induced scleroderma in nude mice

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## Research

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# Abstract

## Background

Scleroderma is a chronic autoimmune disease that causes hardening of the skin. Adipose tissue is used as a regenerative treatment for scleroderma, and mechanical micronization of adipose tissue can concentrate stem cells, improving its therapeutic efficacy. Stromal vascular fraction gel (SVF gel) is produced by subjecting lipoaspirates to a series of mechanical processes. The present study aimed to assess the therapeutic effect of SVF gel on dermal scleroderma.

## Methods

Scleroderma was induced in nude mice by daily subcutaneous injections of bleomycin (BLM) for 4 weeks. SVF gel, cell-enriched lipoaspirates (CAL), or Coleman fat (CF) (0.1 mL) was injected subcutaneously into local sclerosis lesions. Histological analyses, TUNEL assay, and qRT-PCR were conducted 4 weeks post-transplantation.

## Results

SVF gel, CAL, and CV decreased dermal thickness in the scleroderma model ( $P < 0.05$ ), and sclerosis was significantly decreased in the SVF gel and CAL groups. SVF gel and CAL decreased macrophage infiltration, pro-inflammatory cytokine levels, and fibrosis in transferred fat and/or skin lesions ( $P < 0.05$ ). This revealed anti-inflammatory and anti-fibrotic effects of adipose-derived stem cells (ASCs) therapy in scleroderma ( $P < 0.05$ ). SVF gel exhibited the most potent anti-inflammatory and anti-fibrotic effects among treatment groups ( $P < 0.05$ ).

## Conclusion

SVF gel and CAL significantly reversed dermal sclerosis, and were more effective than CF. The SVF gel group exhibited the lowest inflammation both in transferred fat and in skin lesions, and also the lowest fibrosis among the three groups. This study suggested that SVF gel was a more suitable adipose-based therapy for topical treatment of scleroderma patients.

## Introduction

Scleroderma is a chronic autoimmune disease that results in hardening of the skin, vascular pathologies, and generation of autoantibodies [1]. Scleroderma causes various cutaneous manifestations, including morphea and en coup de sabre [2]. Although not life-threatening, its manifestations are difficult to conceal, and lead to disability and decreased quality of life. At present, there is no consensus treatment for scleroderma skin lesions.

Fat grafting has significant potential as a regenerative treatment in various diseases [3, 4]. The therapeutic effect of fat grafting was also tested in scleroderma patients, identifying robust beneficial effects [5]. Nicolas et al. injected various fat-derived products into the skin lesions of a mouse scleroderma model, demonstrating the regenerative potential of fat grafts, which improved vascular formation and suppressed fibrosis in scleroderma lesions [6]. However, the mechanisms for the therapeutic effects of transferred fat in scleroderma lesions are incompletely understood.

Several studies have demonstrated that the regenerative effect of transferred fat is associated with the abundance of ASCs in the tissue [7]. Several studies demonstrate that ASCs exert potent immunosuppressive effects via paraendocrine mechanisms [8]. The pathogenesis of scleroderma involves a complex interplay between vascular damage, inflammation, and fibrosis. ASCs upregulate IL-10 and VEGF, inhibiting the autoreactivity of T-cells, which helps to maintain self-tolerance, and inhibits the Th1 and Th17 responses, thereby decreasing the production of pro-inflammatory cytokines [9]. Macrophages are important cells for the induction of scleroderma. Factors released from innate immune inflammatory cells in the early stages of disease lead to the formation of myofibroblasts that produce collagens, resulting in fibrosis [10]. Thus, increasing the ASC level in fat grafts might contribute to improving its regenerative effect for scleroderma lesions.

Cell-assisted lipotransfer (CAL), which increased stem cell concentration in transferred fat by directly mixing additional stem cells, has been proved to have a better survival rate than traditional fat grafting [11]. Another way to concentrate the ASCs in lipoaspirates is mechanical process, including chopping, shredding, pureeing, and mincing [12, 13]. We previously reported a refined method for processing lipoaspirates. Stromal vascular fraction gel (SVF gel), an injectable mixture, was generated from lipoaspirates with a simple mechanical process. This process destroyed most of the adipocytes by intersyringe shifting, and removed oil by centrifugation [13]. The product is rich in ASCs, vascular endothelial cells (ECs), and adipose extracellular matrix (ECM), which improved wound healing and scars. It has been reported that the concentration of ASCs in SVF-gel reached  $2.7 \pm 0.3 \times 10^5$  cells/mL [13].

In the present study, we hypothesized that the immunosuppressive and therapeutic effects of transferred fat were dependent on the relative abundance of ASCs. Two different ways to increase ASC concentration in fat tissue were applied in this study: SVF gel (increased ASC concentration by a mechanical method), and CAL (increased ASC concentration by an enzymatic method). These products were used to compare with Coleman fat (CF) to evaluate their therapeutic, anti-inflammatory, and anti-fibrotic effects in BLM-induced scleroderma in nude mice.

## Materials And Methods

### Animals

All experiments were approved by the Nanfang Hospital Animal Ethics Committee and were conducted in accordance with the guidelines of the National Health and Medical Research Council of China. Eight-

week-old pathogen-free male nude mice were obtained from Southern Medical University, Guangzhou and Nanjing University, Nanjing, P. R. China. All mice were maintained on a 12 h day/night cycle under specific-pathogen-free conditions, and were fed with normal chow and water ad libitum.

## **Bleomycin and fat graft treatment**

Bleomycin (BLM; Sanofi-Aventis, Paris, France) was administered to the nude mice as previously [14]. Briefly, BLM was dissolved in 0.9% phosphate buffered saline (PBS) at a concentration of 300 µg/mL. Thirty mice (8 weeks old) were injected with BLM (100 µL, 30 µg) subcutaneously into both flanks using a 26 G cannula daily for 4 consecutive weeks.

## **Preparation of products from fat grafts**

Human lipoaspirates were obtained from three healthy women,  $33.4 \pm 6.3$  years of age (mean  $\pm$  standard deviation (SD)) and body mass index (BMI)  $23.2 \pm 1.9$  g/m<sup>2</sup>, with no systemic diseases. All volunteers underwent liposuction with a 3 mm multiport cannula, containing sharp side holes 1 mm in diameter at  $-0.75$  atm of suction pressure. The lipoaspirates were centrifuged at  $1200 \times g$  for 3 min to remove the oil and liquid fractions. The middle layer after centrifugation was considered CF.

For CAL, SVFs were obtained from lipoaspirates as described previously [11, 36]. Briefly, tissue was washed with PBS and digested with 0.075% type II collagenase (Sigma-Aldrich Co., St. Louis, MO, USA). Red blood cell lysis buffer (Leagene Biotechnology, Beijing, China) was used to remove erythrocytes. Supplemental cells (250,000 cells) were added in a small volume (20 µL) to a larger volume of fat (5 mL), which was considered CAL.

SVF gel was generated as described previously [13]. Briefly, CF was mechanically emulsified by shifting CF between two regular disposable syringes connected by a female-to-female Luer-Lok connector with an internal diameter of 1.4 mm at a rate of 10 mL/sec for 1 min. The emulsified CF was centrifuged at  $2000 \times g$  for 3 min. Finally, the substance under the oil layer was defined as SVF gel.

## **Fat product injections**

Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). Fifteen mice treated with BLM for 4 weeks as described above were injected subcutaneously with CF, CAL and SVF gel on both flanks of skin lesion (Fig. 1). Six BLM-treated mice received PBS injection in the lesions as a blank control. Animals were sacrificed 4 weeks after injection of fat graft preparations. Lesion skin and fat grafts were harvested.

## **Histologic analysis**

The lesion skin were fixed in paraformaldehyde for at least 48h and embedded in paraffin. The sections (5 µm) were dewaxed using graded xylene and ethanol. Masson trichrome (MT) staining was conducted according to the manufacturer's instructions. The dermal thickness and collagen area were calculated with Image J software (National Institutes of Health, Bethesda, Md.) to assess the relative degree of fibrosis.

Immunohistochemical analysis of lesion samples was performed following standard procedures. Briefly, endogenous peroxidase activity was blocked by immersing sections in 3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min. Antigen retrieval was performed by steaming slides in 0.01 M citrate buffer (pH 6.0) for 30 min. Slides were blocked in 1% bovine serum albumin for 30 min at room temperature, and subsequently incubated at 4°C overnight with antibodies against  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA). Slides were then incubated at 37°C for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody. Between each incubation, sections were washed three times with PBS. Sections were developed with 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide, and subsequently counterstained with hematoxylin. Sections were imaged using a Nikon Eclipse 80i microscope (Nikon, Badhoevedorp, Netherlands).

For immunofluorescence staining, the following primary antibodies were used: rat anti-mouse Mac2 (1:200; Cedarlane Corp., Burlington, ON, Canada) and rabbit anti-mouse CD206 (1:300; Abcam, Cambridge, MA, USA). The following secondary antibodies were used for double staining: donkey anti-rat-555 IgG (1:200; Abcam) and goat anti-chicken IgY-488 (1:200; ThermoFisher, Cambridge, MA, USA). Nuclei were stained with DAPI (1:200; Sigma-Aldrich). Images were captured and analyzed with a confocal laser scanning microscope (C1Si; Nikon, Tokyo, Japan).

## TUNEL assay

Paraffin sections were stained for apoptotic cells using a commercially available Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit (Apop-Tag Peroxidase In Situ Apoptosis Detection Kit S7100; Chemicon International Inc., Billerica, MA, USA). The TUNEL assay was performed according to the manufacturer protocol. Results were presented as the mean number of TUNEL-positive cells per power field.

## Quantitative reverse transcription polymerase chain reaction

Skin and fat tissue were quickly excised separately, immediately frozen in liquid nitrogen, and stored at -80°C. Total RNA was extracted from 50 mg frozen tissue using an RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany), according to manufacturer instructions. cDNA was synthesized using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) and amplified for 40 cycles using a Rotor-Gene 3000 Real-Time PCR Detection System (Corbett Research, Sydney, Australia). *Gapdh* was used as a reference gene. Expression levels were calculated using the 2- $\Delta\Delta$ Ct method. The following primers were used:

*Mcp-1*F:5'GCAAGATGATCCCAATGAGT, R:5'TAGCTTCAGATTTACGGGTC; *IL-6*F:5'CCTGTCTATAACCACTTCACA, R:5' TGCATCATCGTTGTTTCATAC;

*TGF-β1*F:5'GAGCAACATGTGGAAGTCTA, R:5'TGAATCGAAAGCCCTGTATT; and

*Gapdh*F:5'GGCCTCCAAGGAGTAAGAAA, R:5'GCCCCTCCTGTTATTATGG.

## Statistical analyses

Data were expressed as means ± standard error. The results were analyzed with repeated analysis of variance. Independent Student's t-test (two groups at a single time point) and one-way analysis of variance (more than two groups at a single time point) were performed. A value of  $p < 0.05$  was considered to indicate a statistically significant difference.

## Results

### Pathological changes of BLM-induced scleroderma

Daily subcutaneous BLM injections for 4 weeks induced stable skin sclerosis (evaluated at 4 and 8 weeks post-BLM treatment). MT staining revealed that all BLM-treated sites developed stable sclerosis (Fig. 2B) compared with untreated mice (Fig. 2A). A dense collagen network was observed, and elastic fibers were more abundant and denser in the dermis of BLM-treated skin than in that of control skin. The dermis was significantly thickened in BLM-treated skin (mean dermal thickness =  $391 \pm 33.73 \mu\text{m}$  in BLM-treated mice vs.  $152.0 \pm 15.23$  in untreated mice,  $P < 0.01$  (Fig. 2C)). The thicknesses of subcutaneous fat tissue were significantly decreased in BLM-treated mice ( $P < 0.01$ , (Fig. 2D)). These manifestations were indicative of typical scleroderma changes, and were similar to histopathological features reported previously for skin fibrosis [15]. Body weights of the BLM-treated mice (4th week) were significantly decreased compared with untreated mice ( $P < 0.01$ , (Fig. 2E)). The weight loss continued for 4 weeks after withdrawal of BLM treatment, but did not affect viability.

### Histopathological changes after treatment with fat preparations

After different fat preparation treatments for BLM-induced scleroderma, MT staining was used to evaluate the therapeutic effects of fat-derived products. PBS injection did not affect sclerosis, as PBS-injected lesions demonstrated a characteristically dense collagen network and thickened skin. However, all fat-derived cell therapy products significantly decreased sclerosis. In the SVF gel and CAL groups, the dermis exhibited decreased condensed collagen and increased follicles and vessels, which was more characteristic of normal skin. (Fig. 3A). Dermal thicknesses of samples from all four groups were measured (Fig. 3B). SVF gel exhibited the most robust therapeutic effect, with significantly reduced dermal thickness ( $197.8 \pm 31.81 \mu\text{m}$  vs. control  $433.3 \pm 68.91 \mu\text{m}$ ,  $P < 0.05$ ). CAL also decreased dermal thickness ( $271.0 \pm 39.57 \mu\text{m}$  vs. control,  $P < 0.05$ ), as did CF ( $339.0 \pm 48.15 \mu\text{m}$  vs. control,  $P < 0.05$ ).

## Sclerosis lesion inflammation

Macrophages contribute to the onset of scleroderma and were associated with the progress of the disease [16]. M2 macrophages are the specific type of macrophage closely related to collagen deposition and fibrosis [17]. In the BLM-induced scleroderma model, macrophages are significantly elevated and tend to be M2-polarized. Thus, CD206 was used as an M2 macrophage marker to measure levels of M2 macrophages in skin lesions (Fig. 4A). In PBS-treated scleroderma lesions, high numbers of CD206<sup>+</sup> macrophages infiltrated. Injection of SVF gel and CAL significantly decreased CD206<sup>+</sup> macrophage levels in skin lesions (vs. PBS group,  $P < 0.05$ , Fig. 4B). SVF gel exhibited the most robust effects in relieving macrophage infiltration ( $P < 0.05$ , Fig. 4B). However, injection of CF did not significantly reduce skin lesion CD206<sup>+</sup> macrophage numbers (vs. PBS group,  $P > 0.05$ , Fig. 4B).

Skin lesion inflammation was further evaluated by qRT-PCR. Expression of *Mcp-1*, a chemokine related to macrophage recruitment, was significantly downregulated in the SVF gel and CAL groups relative to the PBS group, and was lowest in the SVF gel group ( $P < 0.05$ , Fig. 4D). *Mcp-1* expression was decreased in the CF group relative to the PBS group, but was still significantly higher than that in the SVF gel and CAL groups ( $P < 0.05$ , Fig. 4D). *Il-6* is a pro-inflammatory cytokine and exhibited a similar expression trend to that of *Mcp-1* (Fig. 4C).

## Inflammation of transferred fat tissue

Mac-2 (a pan-macrophage marker) and CD206 (an M2 macrophage marker) double immunofluorescence was used to identify the macrophage levels and polarization in the fat grafts. Few Mac-2<sup>+</sup>/CD206<sup>+</sup> M2 macrophages and Mac-2<sup>+</sup>/CD206<sup>-</sup> macrophages were present in the SVF gel group (Fig. 5A). CF exhibited the most severe macrophage infiltration (Fig. 5A). Inflammation level in transferred fat was further evaluated by qRT-PCR. Expression of *Mcp-1* was significantly decreased in the SVF gel group relative to other groups ( $P < 0.05$ , Fig. 5B). CAL exhibited lower expression of *Mcp-1* than did CF (Fig. 5B). *Il-6* exhibited a similar expression pattern among groups to that of *Mcp-1* (Fig. 5C).

## Apoptosis in transferred fat

Apoptotic cells in transferred fat were detected by TUNEL assay. Apoptotic nuclei were irregularly distributed in the stromal sites around mature adipocytes in the transferred fat (Fig. 6A). The number of apoptotic cells in the CF group was significantly higher than that of the SVF gel and CAL groups ( $P < 0.05$ , Fig. 6B). Apoptotic cell numbers did not differ between the SVF gel and CAL groups.

## Skin lesion myofibroblasts

In skin lesions,  $\alpha$ -SMA<sup>+</sup> spindle-shaped cells are defined as myofibroblasts, which play a key role in fibrosis during the scleroderma disease process [18]. In the CF group, significantly more myofibroblasts (red

arrows) appeared in the dermis within the fibrotic lesions (Fig. 7A). SVF gel exhibited the lowest level of skin lesion myofibroblast infiltration among the three groups (Fig. 7A). The relative abundance of myofibroblasts differed significantly between groups ( $P < 0.05$ , Fig. 7B). *Tgf- $\beta$*  expression [19] was measured in the lesions and transferred fat in the three groups (Fig. 7C). *Tgf- $\beta$*  expression was higher in transferred fat than in skin lesions, which was likely associated with active ECM remodeling after fat grafting. In the CF group, transferred fat *Tgf- $\beta$*  was higher than in the other groups. Skin lesion *Tgf- $\beta$*  levels exhibited a similar trend between groups (Fig. 7C).

## Discussion

Scleroderma is a clinically heterogeneous disease with a complex phenotype. Generally, scleroderma is characterized by autoimmunity, vascular dysfunction, tissue fibrosis, and in some cases, internal organ pathologies [20]. Skin lesions present on most patients and cause disfigurement [21]. Thus, treatment of skin lesions is a priority for clinical management of scleroderma. Topical corticosteroids are the standard topical treatment for localized scleroderma (LS) [2]. However, corticosteroids should be applied only during the active phase of disease, and total treatment time should be restricted to 3 months. This protocol has not been fully evaluated in well-controlled clinical trials. Other topical therapies such as methotrexate [22] and calcipotriol [23] target inflammation and fibrosis. Conventional treatments are generally well-tolerated but are not sufficiently effective [24].

LS is characterized by a markedly thickened and fibrotic acellular dermis in the form of plaques or macules, which are packed with dense collagen and extracellular matrix proteins, accompanied by inflammatory cell infiltration, loss of the microvasculature, and subcutaneous fat atrophy [25]. Lipotransfer fills in and repairs the subcutaneous tissue defects of LS [26]. Adipose tissue contains abundant ASCs, which have well-characterized angiogenic, anti-inflammatory, and anti-apoptotic properties [27]. Accordingly, we suggest lipotransfer as an optimal treatment for LS.

Lipotransfer has already been explored as a therapeutic approach for reversing fibrosis in a number of fibrotic conditions [28]. In 2012, Ould-ali et al. demonstrated that implantation of human adipose tissue significantly improved fibrosis in sclerotic skin lesions of the BLM-induced mouse model, suggesting autologous adipose tissue as a promising novel therapy for scleroderma [29]. Clinical studies have further demonstrated the promising therapeutic potential of adipose implantation for sclerosis [30]. Fat grafting can alleviate skin sclerosis, facial handicap, mouth opening limitations, sicca syndrome, and hand impairment by improving skin pliability and quality [5]. Thus, fat grafting is efficient and well-tolerated for the treatment of localized forms of scleroderma skin lesions.

Current exploration of the therapeutic benefits of fat grafting for scleroderma focused on the effects of ASCs. Fat-derived products vary between conventional CF, CAL, and mechanically processed fat grafts such as SVF gel. Because CF has been extensively applied, many fat-derived products have been developed to condense ASCs for not only improved volume retention, but also for improved therapeutic efficacy in wound healing and skin diseases [27, 31]. CAL attaches ASCs to the aspirated fat, converting

ASC-poor aspirated fat to ASC-rich fat [32]. As separating ASCs introduces exogenous collagenase and leaves them vulnerable to the attack of immune system in recipient sites, the isolation procedure is legislatively restricted for clinical use. However, SVF gel mechanically destroys and eliminates most mature adipocytes within the adipose tissue, getting rid of employing the collagenase and retaining the ECM scaffold. The whole process can be accomplished in 2 hours and ensures a high stromal vascular fraction cell density ( $>4.0 \times 10^5$  cells/mL) and ASCs ( $2.7 \pm 0.3 \times 10^5$  cells/mL) synchronously [13].

Although adipose-tissue-based therapies are clinically effective in scleroderma, the relative efficacies of conventional CF and ASC-enriched preparations such as CAL and SVF gel had not yet been evaluated [33]. The present study was designed to compare the therapeutic efficacies of these preparations in the nude mouse BLM-induced scleroderma model.

The present study demonstrated the therapeutic efficacy of three different fat-derived products in induced scleroderma skin lesions. All three products significantly decreased dermal thickness and reversed dermal sclerosis. SVF gel and CAL decreased collagen and increased follicles and vessels in BLM-induced scleroderma, and the lesions were closer to the appearance of normal skin. All three experimental groups reduced dermal thickness. CAL significantly alleviated dermal sclerosis, although SVF gel had the most robust therapeutic effect. Both SVF gel and CAL successfully decreased inflammation in scleroderma lesions. SVF gel and CAL treatments significantly decreased *Mcp-1*, *Il-6*, and *Tgf- $\beta$*  in transferred fat, with parallel effects in skin lesions. Further CAL and SVF gel treatment significantly decreased macrophage infiltration into scleroderma lesions. SVF gel more significantly decreased local inflammation than other treatments. By fragmenting large pieces of adipose tissue and removing thick fibrous tissue, the SVF gel isolation process removes more lipid droplets, which could release inflammatory mediators, and leaves very few flat or fragmented adipocytes. Accordingly, the density of ASCs increases markedly after mechanical processing in SVF gel [12].

Though all treatment groups were fat-derived therapies, the prominent effect of SVF gel in the MCF-induced scleroderma model could be related to high level of ASCs in this preparation. ASCs possess anti-fibrotic and regenerative properties in many fibrotic conditions, including scars, radiation-induced fibrosis, and scleroderma [34]. ASCs downregulate expression of the pro-fibrotic markers *Tgf- $\beta$* ,  $\alpha$ -SMA, CTGF, NF- $\kappa$ B, IL-1, and IL-13, and upregulate expression of anti-fibrotic fibroblast growth factor and proangiogenic VEGF and HGF [35]. Topical application of ASCs to animal fibrosis models softens and thins fibrotic tissue, and decreases dermal collagen content with a looser and better-organized pattern of residual collagen fibers with lower expression of collagens 1-6 [36]. The anti-fibrotic properties of ASCs could be attributed to remodeling of the fibrotic matrix, tilting the balance between ECM deposition and ECM degradation in favor of degradation [37]. These results suggest that ASCs are a promising therapeutic agent for localized scleroderma. In a prior study using the BLM-induced scleroderma mouse model, ASC therapy significantly attenuated dermal fibrosis, and reduced skin thickness and hydroxyproline levels, accompanied by decreased *Tgf- $\beta$*  [38].

Macrophages are activated in patients with skin disease, including systemic sclerosis, and are potentially important sources of fibrosis-inducing cytokines [39]. The transferred adipose tissue was under severe ischemic environment and some fragile cells underwent apoptosis. This process recruited numerous macrophages to the injection sites. This study revealed that SVF-gel and CAL group had less cell apoptosis and macrophage infiltration. The local accumulation of macrophages in the transferred fat under the skin lesion may affect the inflammation level in skin lesion. The SVF-gel and CAL group, which had decreased cell death and restrained inflammation level, had lower macrophage infiltration level in the skin lesion.

ASCs were capable to inhibit activated macrophages and mitigate inflammation [40]. Dou et al. demonstrated that ASCs alleviate lung inflammation and skin fibrosis via immunomodulation in a bleomycin-induced murine model [41]. M2 macrophages are closely related to inhibition of collagen deposition and fibrosis. Several studies have demonstrated the capacity of ADSCs to promote phenotype switching in macrophages, inhibiting expression of M1 polarization genes. Consistent with previous reports [28], SVF gel decreased macrophage infiltration and pro-inflammatory cytokines, indicative of decreased inflammation. Further, apoptotic adipose cells and skin lesion fibrosis were decreased in mice treated with SVF gel. These parallel results in transferred fat and skin lesions may indicate a correlation between internal fat graft inflammation and host skin lesion inflammation and fibrosis in scleroderma patients.

## Conclusions

The present study assessed the efficacy of three different adipose-based therapies in scleroderma. SVF gel and CAL significantly reversed dermal sclerosis that developed after 4 weeks of BLM treatment. CF modestly decreased dermal sclerosis but did not significantly affect the other measured parameters. Macrophage infiltration and inflammatory cytokine levels in both transferred fat and skin lesions were lowest in mice injected with SVF gel. SVF gel also exhibited the most potent anti-fibrotic effects. The efficacy of adipose-derived therapies in the scleroderma animal model suggests the potential clinical application of this approach in scleroderma patients.

## List Of Abbreviations

CF, Coleman fat; SVF-gel, Stromal vascular fraction gel; CAL: cell-assisted lipotransplantation; ASC, adipose-derived stem cell; LS: localized scleroderma; H&E, hematoxylin and eosin; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; MT, Masson's trichrome; PBS, phosphate-buffered saline; TGF- $\beta$ 1, transforming growth factor  $\beta$ 1; VEGF, vascular endothelial growth factor;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin

## Declarations

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## **Authors' contributions**

Wenqing Jiang and Jing Wang: conception and design, manuscript writing, collection and assembly of data, data analysis and interpretation; Shenglu Jiang, Yuping Quan, Yunjun Liao: data analysis, interpretation, and collection data; Jianhua Gao and Junrong Cai: conception and design, financial support, and final approval of manuscript.

## **Availability of data and materials**

All data generated or analyzed in this study are included in this article.

## **Ethics approval**

All animal experiments were approved by Nanfang Hospital Institutional Animal Care and Use Committee and conducted according to the guidelines of the National Health and Medical Research Council (China).

## **Consent for publication**

Not applicable.

## **Competing interests**

The authors declare that they have no competing interests

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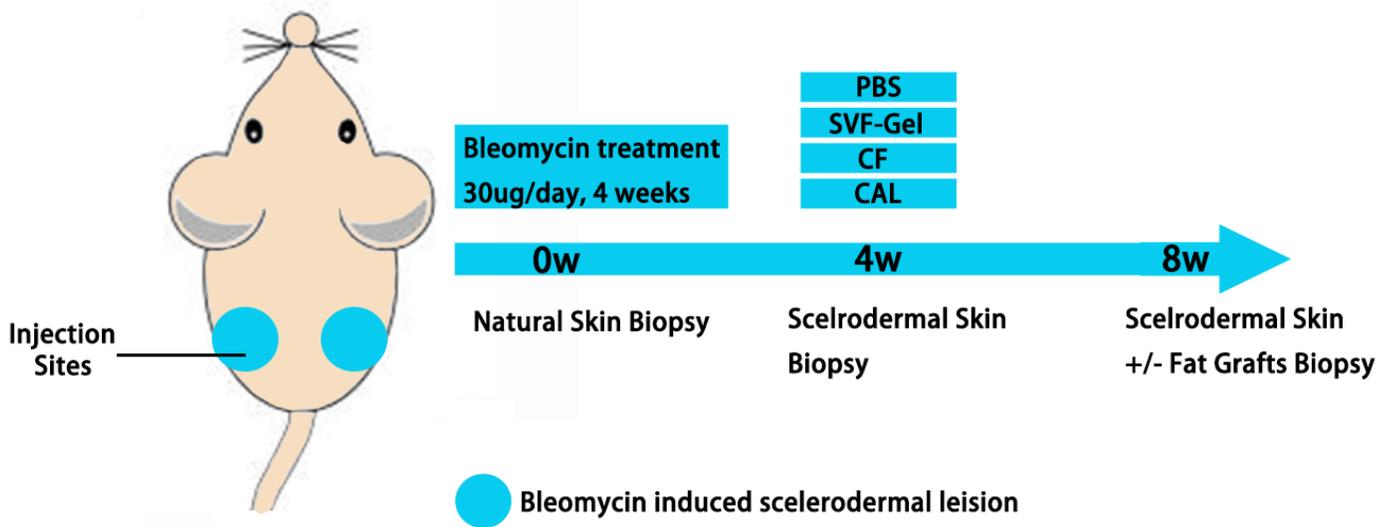
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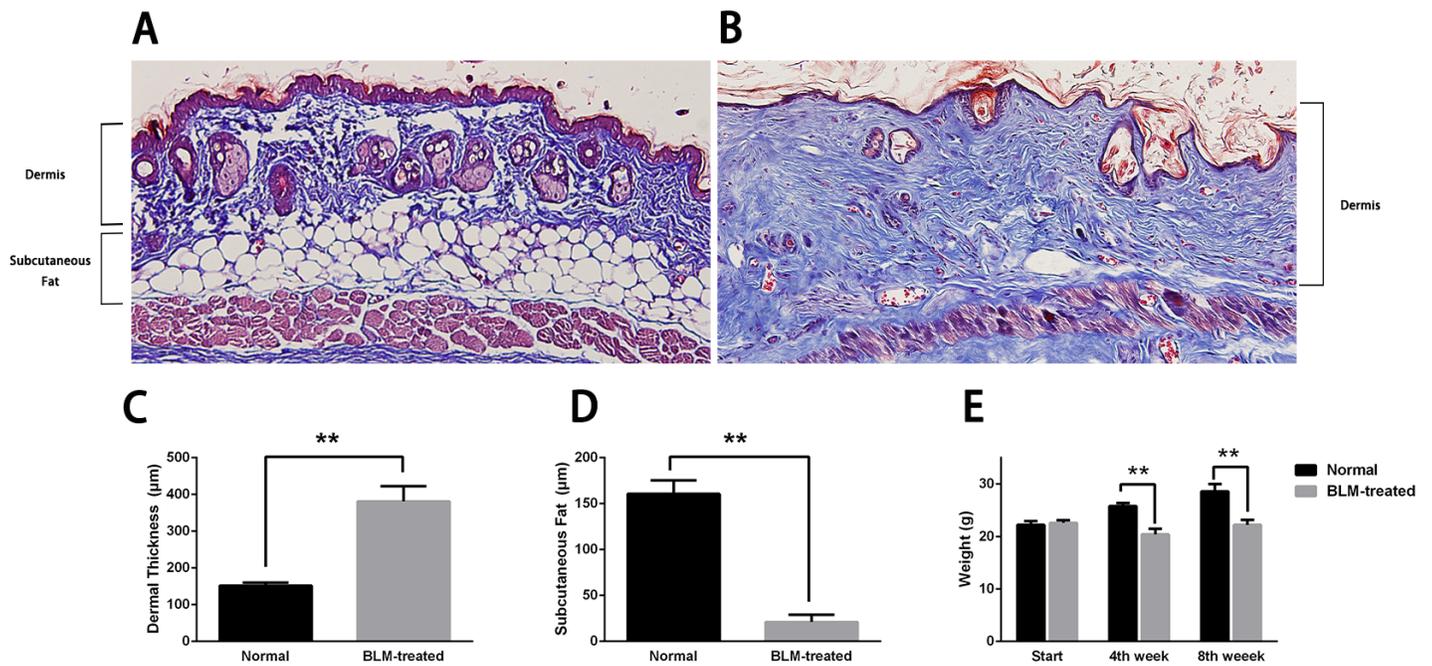
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## Figures



**Figure 1**

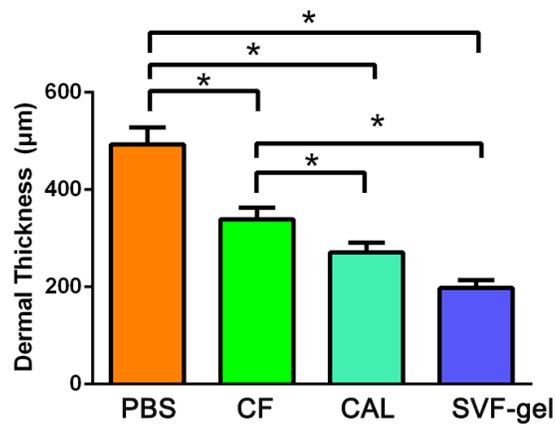
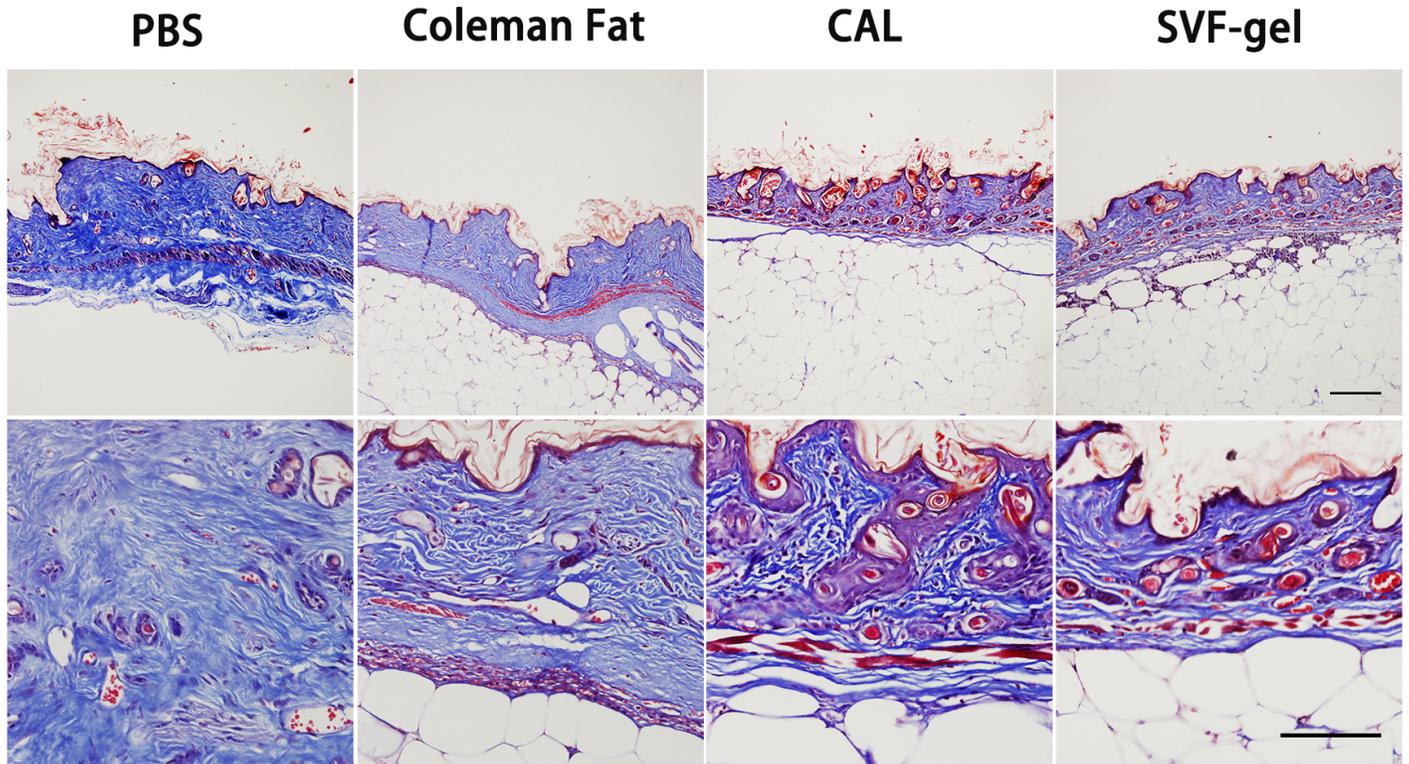
Injection sites (CF, CAL, SVF gel, or PBS) in each mouse, and a schema illustrating the time course, including the different steps of the experiments.



**Figure 2**

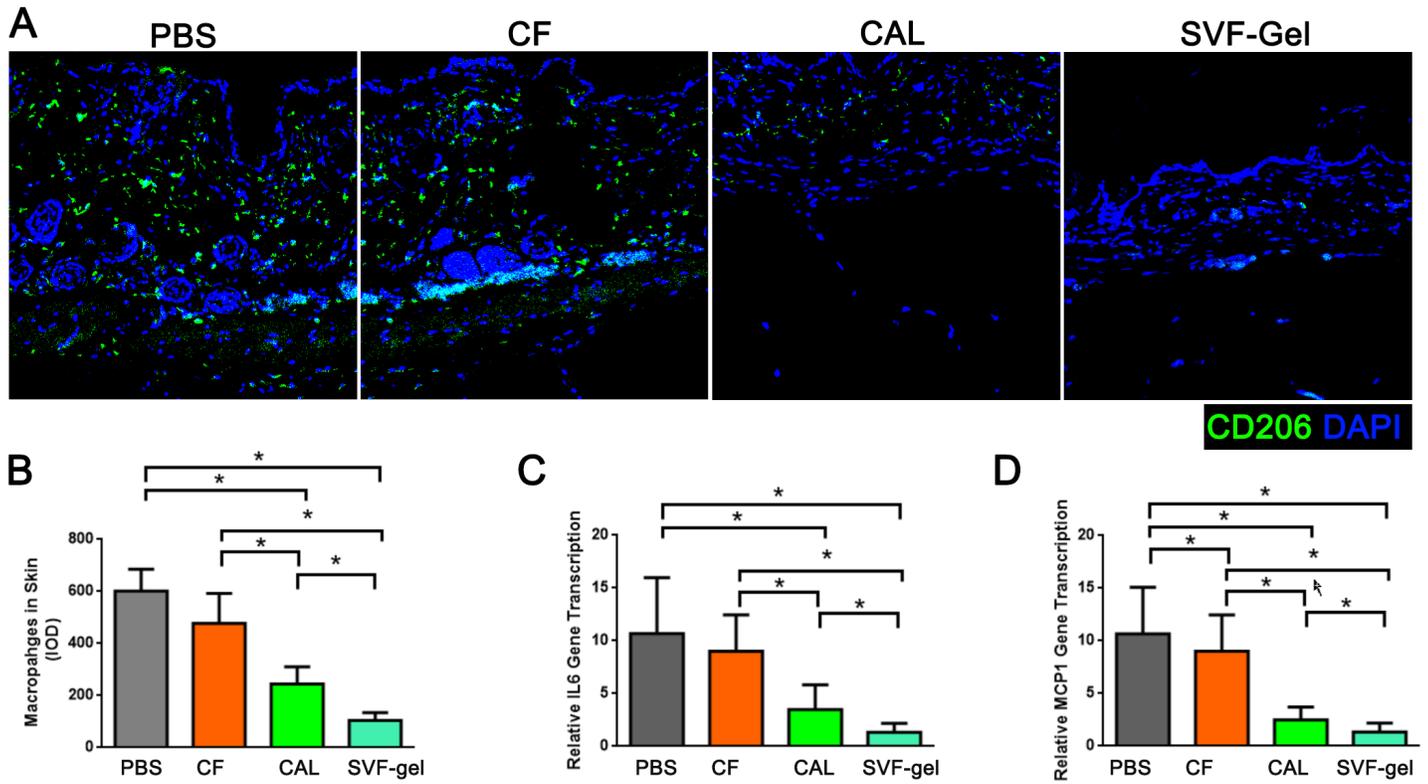
Pathological changes of BLM-induced scleroderma. (A-B) Microscopic observation of Masson Trichrome-stained histological sections evaluating the skin of (A) untreated mice and (B) BLM-treated mice. (C-D) After BLM injection, the (C) dermis was significantly thickened, and the (D) subcutaneous fat thickness

was significantly decreased in BLM-treated skin.  $**P < 0.01$ . (E) Effects of BLM treatment on body weights at initiation of the experiment, and in the 4th and 8th weeks.  $**P < 0.01$ .



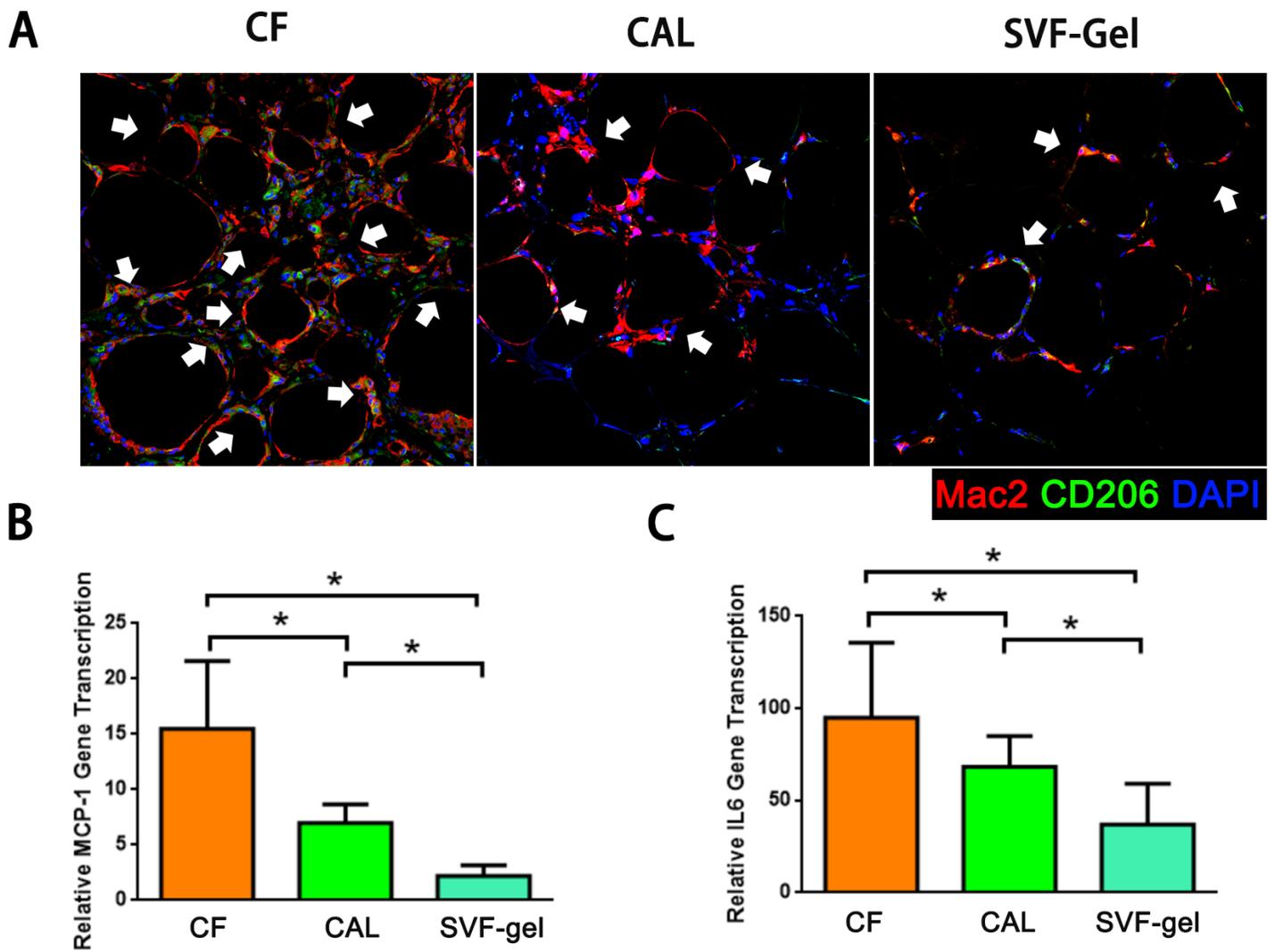
**Figure 3**

Histopathological changes after fat product treatment. (A) Microscopic observation of Masson Trichrome-stained histological sections of PBS, SVF, CAL, and SVF gel-treated sclerosis lesions. In the SVF gel and CAL groups, the dermis exhibited decreased condensed collagen and increased follicles and vessels, which was more characteristic of normal skin. (B) Dermal thickness was evaluated in each group. SVF gel exhibited the most robust therapeutic effect, with the largest reduction in dermal thickness.  $*P < 0.05$ .



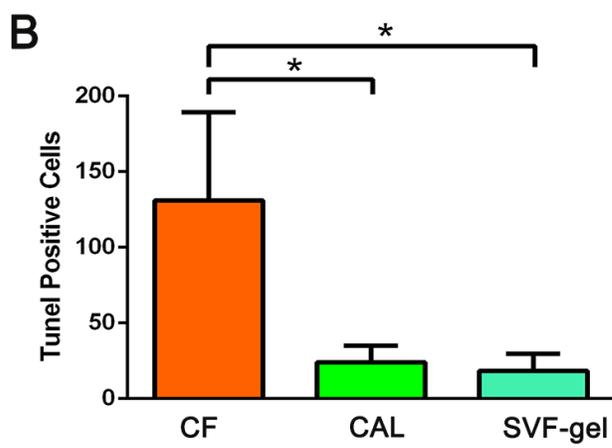
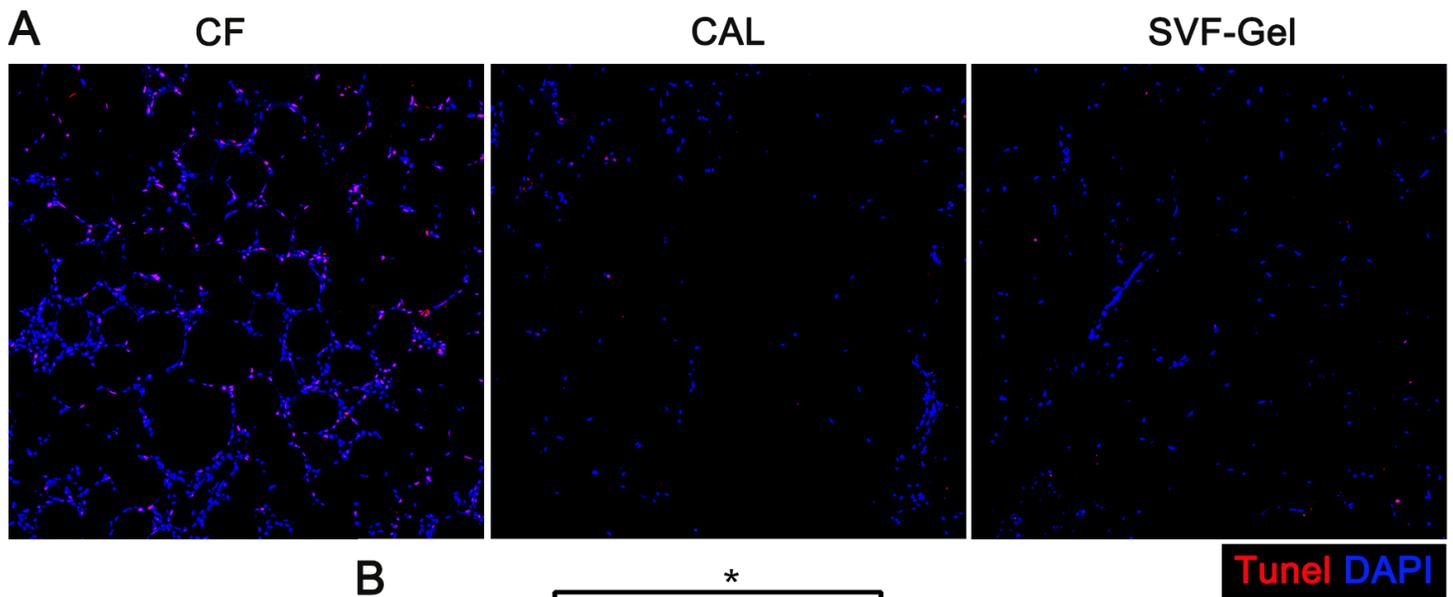
**Figure 4**

Inflammation in sclerosis skin lesions. (A-B) Macrophage infiltration into skin lesions was determined by (A) CD266 immunofluorescence staining of PBS, CF, CAL, and SVF gel-treated sclerosis lesions, and (B) was quantified as number of CD266+ cells per field. \*P<0.05. (C-D) Inflammation in skin lesions from each group was determined by qRT-PCR for (C) IL-6 and (D) MCP-1. \*P<0.05.



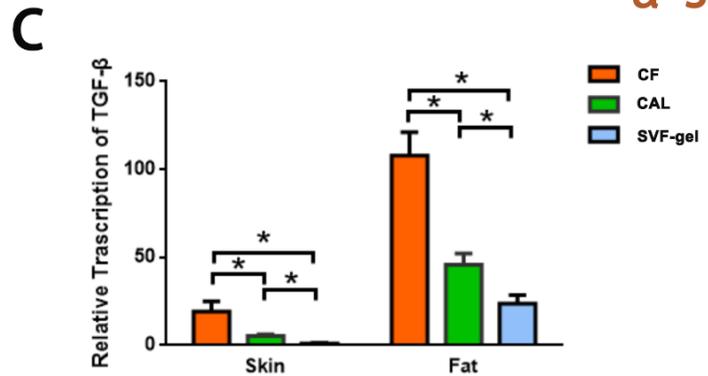
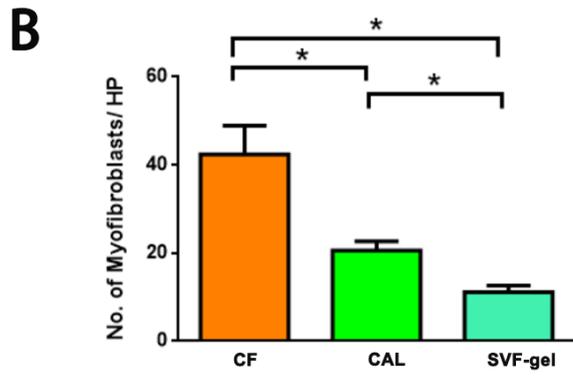
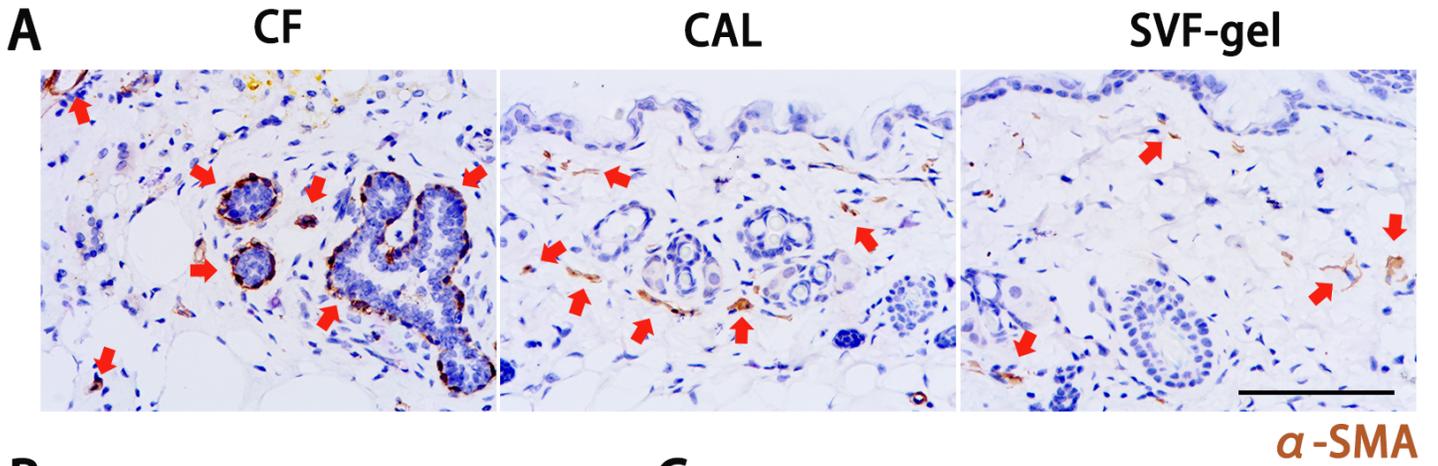
**Figure 5**

Inflammation in transferred fat. (A) Macrophage infiltration and polarization were determined by Mac-2 and CD206 double immunofluorescence in transferred fat from CF, CAL, and SVF gel groups in BLM-treated mice. (B-C) Inflammation in transferred fat was determined by qRT-PCR for (B) Mcp-1 and (C) Il-6. \*P<0.05.



**Figure 6**

Apoptosis in transferred fat. (A-B) Apoptotic cells were (A) labeled by TUNEL assay and (B) quantified in transferred fat from CF, CAL, and SVF gel groups in BLM-treated mice. \*P<0.05.



**Figure 7**

Skin lesion myofibroblast numbers and skin lesion/transferred fat Tgf- $\beta$  expression. (A) Histologic fibrosis determined by  $\alpha$ -SMA labeling of myofibroblasts in skin lesions from the PBS, SVF, CAL, and SVF gel groups. (B)  $\alpha$ -SMA+ myofibroblast density was examined by immunohistochemistry. \* $P$ <0.05. (C) mRNA levels of the fibrosis-related cytokine Tgf- $\beta$  were measured with qRT-PCR. \* $P$ <0.05.