



Anti-Aging Effect of Adipose-Derived Stem Cells in a Mouse Model of Skin Aging Induced by D-Galactose

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Abstract

Introduction: Glycation products accumulate during aging of slowly renewing tissue, including skin, and are suggested as an important mechanism underlying the skin aging process. Adipose-derived cells are widely used in the clinic to treat ischemic diseases and enhance wound healing. Interestingly, adipose-derived stem cells (ASCs) are also effective in anti-aging therapy, although the mechanism underlying their effects remains unknown. The purpose of the present study was to examine the anti-aging effect of ASCs in a D-galactose-induced aging animal model and to clarify the underlying mechanism.

Materials and Methods: Six-week-old nude mice were subcutaneously injected with D-gal daily for 8 weeks. Two weeks after completion of treatment, mice were randomized to receive subcutaneous injections of 10^6 green fluorescent protein (GFP)-expressing ASCs, aminoguanidine (AG) or phosphate-buffered saline (PBS). Control mice received no treatment. We examined tissue histology and determined the activity of senescence-associated molecular markers such as superoxide dismutase (SOD) and malondialdehyde (MDA).

Results: Transplanted ASCs were detectable for 14 days and their GFP signal disappeared at day 28 after injection. ASCs inhibited advanced glycation end product (AGE) levels in our animal model as well as increased the SOD level and decreased the MDA level, all of which act to reverse the aging phenotype in a similar way to AG, an inhibitor of AGE formation. Furthermore, ASCs released angiogenic factors *in vivo* such as vascular endothelial growth factor, suggesting a skin trophic effect.

Conclusions: These results demonstrate that ASCs may contribute to the regeneration of skin during aging. In addition, the data shows that ASCs provide a functional benefit by glycation suppression, antioxidation, and trophic effects in a mouse model of aging.

Citation: Zhang S, Dong Z, Peng Z, Lu F (2014) Anti-Aging Effect of Adipose-Derived Stem Cells in a Mouse Model of Skin Aging Induced by D-Galactose. PLoS ONE 9(5): e97573. doi:10.1371/journal.pone.0097573

Editor: Adam J. Engler, University of California, San Diego, United States of America

Received: February 26, 2014; **Accepted:** April 21, 2014; **Published:** May 15, 2014

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Funding: This work was financially supported by the National Nature Science Foundation of China (81171834, 81071589), Medical Scientific Research Foundation of Guangdong Province (A2013449, A2011453). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Aging is a biological process that induces changes to the structural integrity and physiological function of skin [1], such as the development of dyschromia, roughness, and fine rhytids followed by persistent deeper folds. Structural changes are a result of dermal atrophy, decreased collagen, the loss of subcutaneous fat, the loss of inherent elasticity, and increased melanogen [2]. Several theories have been proposed to explain this process, including the accumulation of genomic mutations, the accumulation of toxic metabolites, hormonal deprivation, the increased formation of free radicals (oxidative damage), and the cross-linking of macromolecules under glycation [3].

Glycation is a nonenzymatically driven reaction between free amine groups, such as amino acids in proteins, and reducing sugars like glucose. This reaction, also called the Maillard reaction, eventually leads to the formation of advanced glycation end

products (AGEs) such as carboxymethyl-L-Lysine and pentosidine, which may be responsible for cross-linking between macromolecules through covalent bonding. Glycation most commonly occurs in tissues in which macromolecular structures have a slow turnover rate and is therefore thought to play an important role in aging [4]. Accumulating evidence indicates that AGEs exacerbate and accelerate the aging process and contribute to the early phases of age-related diseases, including neurodegenerative disease, cataracts, renal failure, arthritis, and age-related macular degeneration [5,6]. Moreover, AGEs and their precursors usually contain reactive carbonyl groups generated by reactive oxygen species (ROS) [7,8]. ROS bind to polyunsaturated lipids, forming malondialdehyde (MDA), which is a reactive aldehyde and one of many reactive electrophile species that causes toxic stress in cells similarly to AGEs. Therefore, the level of MDA could be used as a marker of the aging process [9]. Superoxide dismutases (SOD) are enzymes that catalyze the dismutation of superoxide into oxygen

and hydrogen peroxide and play an important role in antioxidant defense in nearly all cells exposed to oxygen. For these reasons, the expression of SOD may be another marker related to the aging process.

Other than glycation, alterations in skin collagen content and dermal vascularization also play key roles in aging. As the process of aging advances, collagen fibers become thinner, thereby changing the collagen proportion in tissues. In fact, with advanced age, collagen fibers in the deep dermis undergo lysis and become thinner. Moreover, a progressive reduction in dermis vasculature is also seen, resulting from a reduction in the number and size of vascular vessels, which is associated with alterations in vascular wall components and other changes that progress until the vessels are no longer functional [10,11].

Previous studies indicated that adipose tissue transplantation could improve skin quality at the recipient site in addition to increase skin volume [12,13]. This unexpected consequence of adipose tissue transplantation may be due to the effect of mesenchymal stem cells (MSCs) in the stromal-vascular fraction of subcutaneous adipose tissue, or adipose-derived stem cells (ASCs). ASCs exhibit multi-lineage developmental plasticity and are similar to bone-marrow-derived MSCs in terms of surface markers and gene profiling [14,15]. In addition, many clinical studies and animal experiments have confirmed that the injection of these cells has favorable effects on wound repairing, immunomodulation, and anti-apoptosis via a paracrine effect or differentiation [16,17]. Moreover, recent studies also revealed that ASCs improve wrinkles resulting from photo-aging and promote collagen synthesis and epidermal thickening of photo-aged fibroblasts *in vitro* [18].

However, the underlying mechanisms of the anti-aging effects of ASCs have not been extensively studied. Therefore, in an attempt to further understand these mechanisms, we designed an experimental animal study of skin aging induced by D-galactose (D-gal). The goal of this study was to use histologic and immunohistologic analyses to assess the anti-aging effects of ASCs, especially in the suppression of glycation and restoration of functional capacity.

Materials and Methods

Ethics statement

Animal experimental protocols were approved by the Southern Medical University Laboratory Animal Administration Committee, and experiments were performed according to the Southern Medical University Guidelines for Animal Experimentation. All efforts were made to minimize animal suffering.

Isolation and culture of ASCs

Mouse inguinal fat pad adipose tissue samples were acquired from 6-week-old green fluorescent protein (GFP)-expressing mice, which were provided by the Model Animal Research Center of Nanjing University (Nanjing, China). The obtained samples were cut into pieces and digested with 0.075% type I collagenase (Sigma-Aldrich, St. Louis, MO) under gentle agitation for 45 min at 37°C. Mature adipocytes and indigested connective tissue were separated from pellets by centrifugation (800 *g* for 10 min) and then discarded. The pellets were resuspended in phosphate-buffered saline (PBS) and filtered through a 200 μ m mesh followed by centrifugation (800 *g* for 10 min) to spin down stromal-vascular fraction cell pellets. The retrieved cell fraction was cultured overnight at 37°C with 5% CO₂ in a control medium (Dulbecco's modified Eagle media, 10% fetal bovine serum, 100 units/mL penicillin, 100 mg/mL streptomycin). The resulting cell popula-

tion was cultured for 3 to 5 days until confluent. ASCs were cultured and expanded in the control medium. Cells from P3 to P5 were used in the following experiments.

Differentiation of mouse ASCs

In vitro multi-lineage differentiation of ASCs was induced in the control medium supplemented with one of the three formulas described below, as previously described [19]. *In vitro*-cultured ASCs were detected using Oil-red O, Alizarin red, and Alcian blue staining, which identified fat, bone, and cartilage cells, respectively, differentiated from ASCs.

D-galactose (D-gal)-induced aging model and animal experiments

Chronic administration of a low dose of D-gal has been widely used as an animal model for aging in studies of skin aging or anti-aging pharmacology [20]. In this model, the AGE inhibitor aminoguanidine (AG) prevents aging phenotypes, suggesting AGEs as a pivotal player in the underlying mechanism of aging [21].

A total of 80 6-week-old nude mice (gender not considered) were provided by the Southern Medical University Experimental Animal Center (Guangzhou, China). Mice were randomly divided into four groups (*n* = 20 each). Three groups of animals received daily subcutaneous injections of D-gal (1,000 mg/kg, subcutaneously) for 8 weeks. Two weeks later, animals of these three groups received a subcutaneous injection of 10⁶ GFP-expressing ASCs, AG (100 mg/kg, intragastrically), or PBS at the midline of the dorsum and the injection sites were marked. After the injection, all four groups of mice were housed for another four weeks. All animals were allowed free access to water and a chow diet and were observed daily. Mice were sacrificed at the end of treatment, and skin tissue was immediately collected or stored at -80°C until further use.

Survival of ASCs

After injection of GFP-expressing ASCs, mice were anesthetized with isoflurane and underwent fluorescence live imaging using the Kodak *In-Vivo* Imaging System F (Carestream Health, Inc. Rochester, NY, US) at days 1, 3, 7, 14 and 28 after injection.

Histological examination

Skin tissue from animals of all four groups was fixed in 4% paraformaldehyde, dehydrated, and paraffin-embedded for haematoxylin and eosin (H&E) staining. All the skin samples used for histology were taken from the cell injection site of the mice at the midline of the dorsum. Tissue blocks were serially sectioned (6 μ m sections), mounted onto a 3-Aminopropyl-Triethoxysilane (APES)-treated glass slide, assessed under an Olympus BX51 microscope, and photographed using an Olympus DP71 digital camera. The dermal thickness of the skin samples was measured.

Collagen quantification

To determine the amount of total collagen, samples obtained from all four groups were stained with Masson's trichrome. Sections were deparaffinized in xylene, rehydrated in graded ethanol, and post-fixed in Bouin's fixative for 1 h at 55°C. The nuclei were stained with an equal volume of ferric chloride solution, and then collagen was stained with an alcoholic hematoxylin and trichrome solution. Total collagen content was reported as a percentage of the aniline blue staining divided by the total tissue area of the section using the Image J software (National Institute of Mental Health, Maryland, USA).

Immunohistochemistry of CD31 and vascular endothelial growth factor (VEGF)

Immunohistochemistry was used to detect angiogenesis in the samples. Sections obtained from each group were stained with an anti-CD31 antibody (Abcam, Cambridge, UK) and an anti-VEGF antibody (Abcam, Cambridge, UK). Paraffin sections were dewaxed and hydrated before immunohistochemical staining. Slides were washed with PBS, incubated in 3% H₂O₂ for 10 min, washed once more in PBS, and then incubated in a protein block solution for 30 min. Then, sections were incubated with the primary antibody at 4°C overnight. The next day, the sections were washed three times and then incubated with a biotinylated secondary antibody. After a 30-min incubation with a complex of avidin and biotinylated horseradish peroxidase, the enzyme activity was visualized using 3,3'-diaminobenzidine. Slides were scored by two independent observers using an Olympus BX51 microscope and photographed with the use of an Olympus DP71 digital camera. The number of CD31-positive vessels was counted and the VEGF-positive area was quantified using the Sigma Scan software on five nonconsecutive tissue sections for each image.

Measurement of superoxide dismutase activity and lipid peroxidation

Skin tissue samples were weighed and homogenized in normal saline to generate 5% homogenates. Homogenates were sonicated twice at 30 s intervals. Homogenization and sonication were performed at 4°C. After sonication, homogenates were sequentially centrifuged at 3,000 rpm for 10 min and 12,000 rpm for 15 min. Aliquots of supernatants were used for further experiments. The protein content of the aliquots was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co.).

SOD activity of the skin was examined using the xanthine oxidase method with a commercial kit (Nanjing Jiancheng Bioengineering Institute, China), as previously described [21]. This assay involves a xanthine-xanthine oxidase system that reacts with 2-(4-iodophenyl)-3-(4-nitrophenol-5-phenyl)tetrazolium chloride to form a red formazan dye at an absorbance at 550 nm and produces superoxide ions. The protein concentration was determined using a BCA protein assay kit (Pierce Chemical Co.), with one unit of SOD defined as the amount of SOD inhibiting the rate of reaction by 50% at 25°C.

Lipid peroxidation was evaluated by assessing the MDA content using a thiobarbituric acid (TBA) method as recommended (Nanjing Jiancheng Bioengineering Institute, China). This method is based on the spectrophotometric measurement of color produced during the MDA reaction with TBA. MDA concentrations were calculated through the absorbance of TBA reactive substances (TBARS) at 532 nm.

Inhibition of AGEs formation *in vitro*

AGE-modified bovine serum albumin (BSA) was prepared, as previously described [22]. Briefly, BSA (100 mg/mL) was incubated under sterile conditions with 0.5 M D-gal in 0.2 M PBS (pH 7.4) at 37°C for 8 weeks. For ASC treatment or AG inhibition, AGE-modified BSA samples were incubated with ASCs (1×10^6) or AG (100 nm), respectively, under identical conditions. A control BSA sample was incubated under identical conditions but without D-gal. Samples were dialyzed (10 kDa cut-off) against PBS, and the BSA-AGEs content was determined using a commercial enzyme linked immunosorbent assay (ELISA) kit, as previously described [23].

Statistical analysis

The results of the quantitative and morphometric analyses were calculated as the means \pm SEM. Statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL). Results were compared using ANOVA, with post-hoc least significant difference (LSD) test as appropriate. A P-value of <0.05 was considered statistically significant.

Results

Characterization of ASCs

ASCs expanded easily *in vitro* and showed fibroblast-like morphologic features (Fig. 1A). To verify their multipotent differentiation, ASCs were incubated in media known to induce an adipogenic, osteogenic, or chondrogenic lineage. Adipogenic differentiation was determined by Oil Red O staining of intracellular lipid droplets (Fig. 1B), osteogenic differentiation through Alizarin red S staining of matrix mineralization (Fig. 1C), and chondrogenic differentiation through Alcian blue staining of cartilage-specific proteoglycans (Fig. 1D).

Retention rate of transplanted ASCs in a mouse aging model

GFP signals were detected in fluorescence live imaging of mice throughout the experiment (Fig. 2A). Signals were limited to the dorsum at day 1, with the injection site showing the strongest signal. At day 3, the intensity of signals at the injection area decreased but remained strong. From days 7 to 14, the intensity of signals in the dorsum area decreased and was weak by day 28 (Fig. 2B).

Effect of ASCs on the formation of AGEs in mice

After ASC treatment, visual inspection revealed no major abnormalities in mice. Mice of all groups gained weight normally throughout the study (Fig. 3A). As expected, mice treated with D-gal showed a remarkably increased level of skin AGEs compared to the control group ($P < 0.05$) (Fig. 3B), and AG treatment significantly reversed the increased level of AGEs in D-gal-treated mice ($P < 0.05$) (Fig. 3B). Similar to the effect of AG, ASC treatment was effective in significantly blocking the increase in the

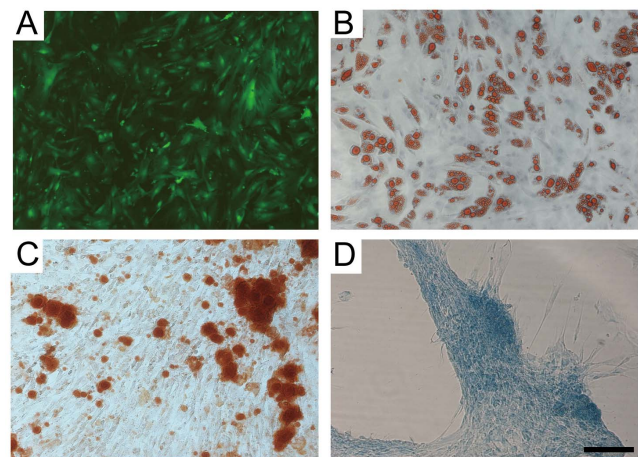


Figure 1. *In vitro* multi-lineage differentiation potential of adipose-derived stem cells (ASCs). A. Green fluorescent protein-expressing mouse ASCs. B. Oil Red O staining. C. Alizarin red staining. D. Alcian blue staining. Scale bars = 50 μ m. doi:10.1371/journal.pone.0097573.g001

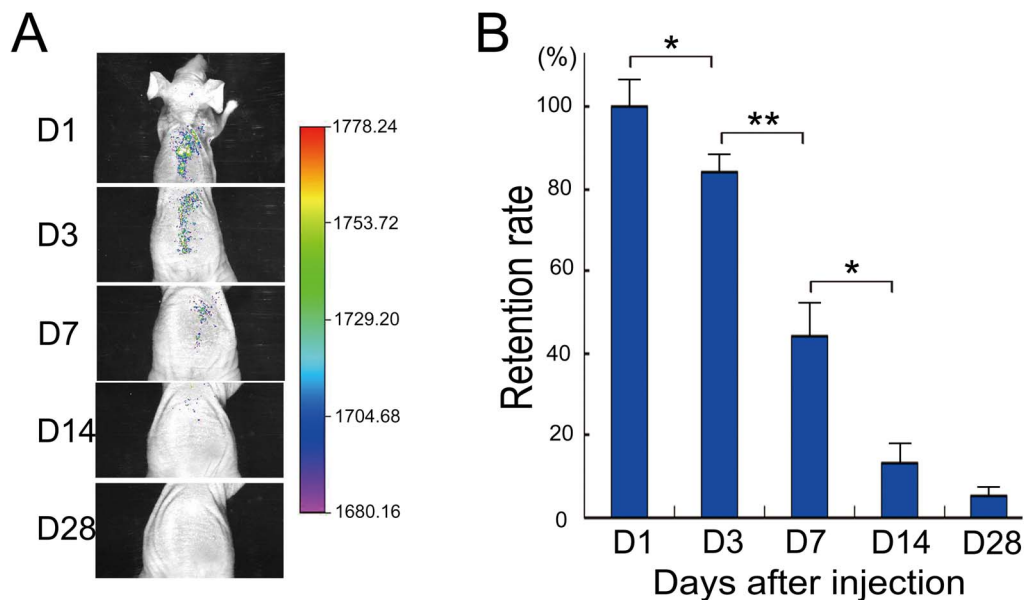


Figure 2. The retention rate of transplanted adipose-derived stem cells (ASCs). **A.** Fluorescence live imaging of ASCs trafficking *in vivo* in aging mice. **B.** The retention rate of ASCs at days 1, 3, 7, 14, and 28 after injection. doi:10.1371/journal.pone.0097573.g002

AGEs level ($P < 0.05$) (Fig. 3B), suggesting that ASCs have an inhibitory effect on BSA-AGE formation.

Effect of ASCs on antioxidant enzyme activity and lipid peroxidation in mice

To further confirm that ASCs have a protective effect on the skin by antioxidant action, we measured the levels of SOD and MDA, an indicator of lipid peroxidation, in mouse skin tissue. As expected, SOD levels decreased, while MDA levels significantly increased in D-gal treated only group; however, treatment with ASCs increased the SOD and decreased the MDA expression levels in D-gal-treated mouse skin similar to the effect of AG (Figs. 3C and D).

Histological observation

H&E staining showed significant changes in skin appendages in samples from D-gal-treated mice compared to control mice (Fig. 4A). Moreover, dermal thickness was significantly lower in D-gal-treated mice and significantly higher in the ASC-treated group compared to that of the control group (Fig. 4B). Quantification of collagen content showed that the ASC-treated group also had higher amount of total collagen than the D-gal-treated group (Fig. 4C).

VEGF levels and skin tissue angiogenesis

To further confirm that ASCs induce skin angiogenesis, we measured CD31-positive microvessels and VEGF expression in skin tissue (Fig. 5A). As expected, the ASC-treated group had higher microvessel density and VEGF expression levels than the D-gal-treated group (Figs. 5B and C).

Discussion

Stem cells have various potential uses in most medical areas due to their differentiation and paracrine effects. In particular, ASCs have several advantages in clinical applications because they are easy to harvest and abundant in the human body, meaning that

there are no ethical problems in harvesting these cells. In this study, we examined the anti-aging effects of ASCs, particularly focusing on the suppression of the glycation reaction and restoration of the functional capacity of skin in a mouse model of accelerated aging induced by D-gal. Our findings can be summarized as follows: (1) ASCs can survive up to almost 28 days after being injected into dermal tissue; (2) ASCs can decrease the AGE level, therefore reversing the aging phenotype, which is a similar effect to that of AG, and inhibitors of AGEs and ASCs can decrease the expression of senescence-associated markers such as SOD and MDA; (3) ASCs can significantly increase dermal thickness and collagen content of the skin; and (4) ASCs can increase the expression level of VEGF and increase the vessel density of the skin, indicating a possible skin trophic effect of ASCs.

Skin aging occurs through intrinsic and extrinsic pathways. Intrinsic aging, so-called normal aging, is confirmed by changes in the levels of senescence-associated molecular markers. A previous study demonstrated that D-gal injection leads to an accelerated aging phenotype, as well as changes in AGE level and in the expression levels of senescence markers such as SOD and MDA [24,25]. In our study, nude mice treated with D-gal showed significant changes resembling normal aging. In addition, the AGE inhibitor, AG, prevented the accelerated aging process. These results strongly suggest that AGEs are a crucial mediator in our D-gal-induced aging model.

An important barrier in cell therapy remains the low engraftment rate of transplanted cells, which diminishes the efficiency of cell therapy [26]. Previous studies suggested that transplanted ASCs have very low retention in the later stages of the transplant [27,28,29]. Remarkably, in our study, the GFP signal of injected ASCs was undetectable after day 28. The low survival rate of transplanted ASCs may be a result of phagocytosis by local immune cells. However, transplanted cells cannot be entirely responsible for the beneficial effect on aging; the paracrine effect is more likely to be the mechanism explaining the functional results [30]. The viability of cells post-injection is critical to the success of injectable cell-based therapies. Injection of functional

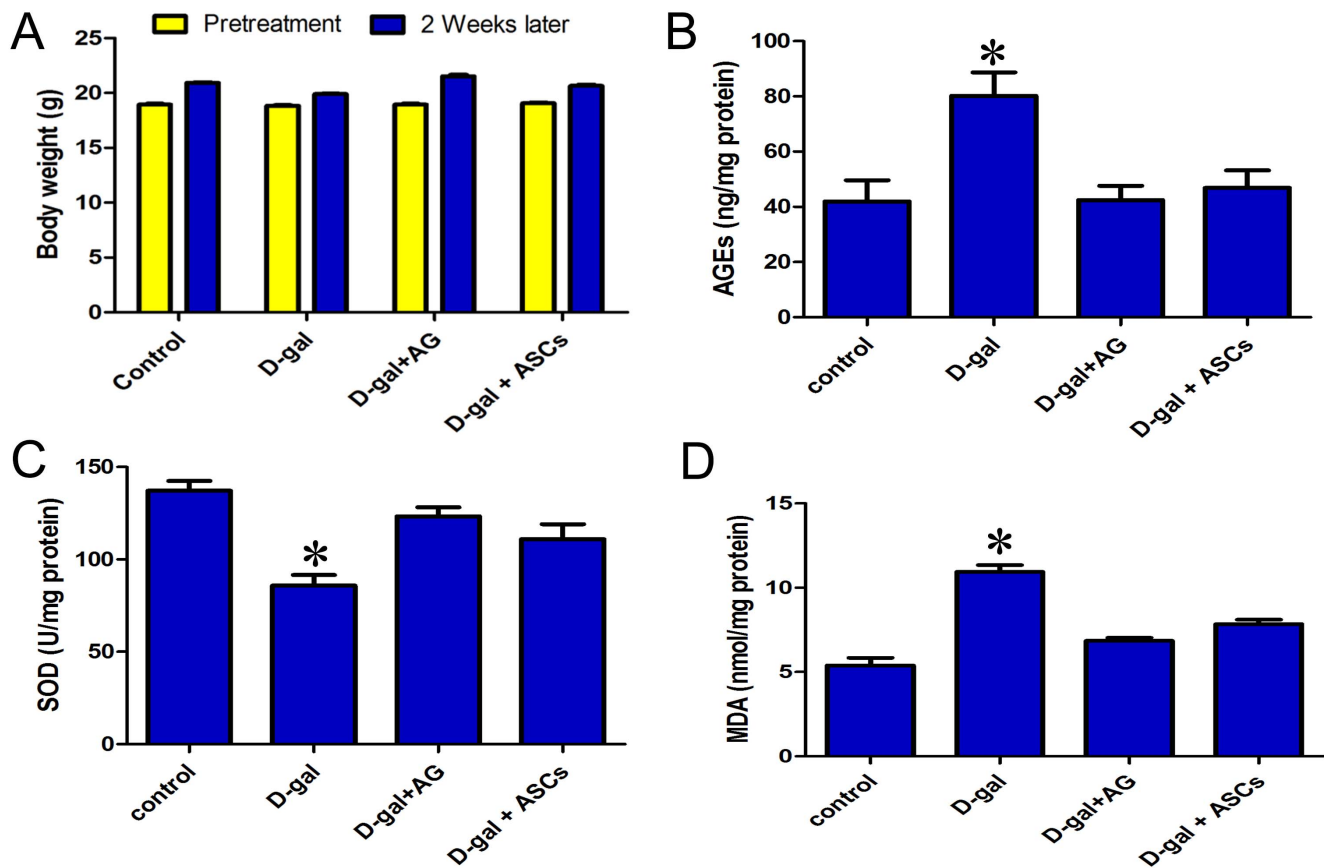


Figure 3. The dynamic changes of aging-associated markers. **A.** The changes in body weight, advanced glycation end product (AGE), and level of superoxidase dismutase (SOD) and malondialdehyde (MDA) in control, D-gal-treated, D-gal plus adipose-derived stem cells-treated, and D-gal plus aminoguanidine-treated mice. **B.** The content of bovine serum albumin-AGEs. **C and D.** SOD and MDA levels in skin. Statistically significant difference, * $P < 0.05$ versus control. doi:10.1371/journal.pone.0097573.g003

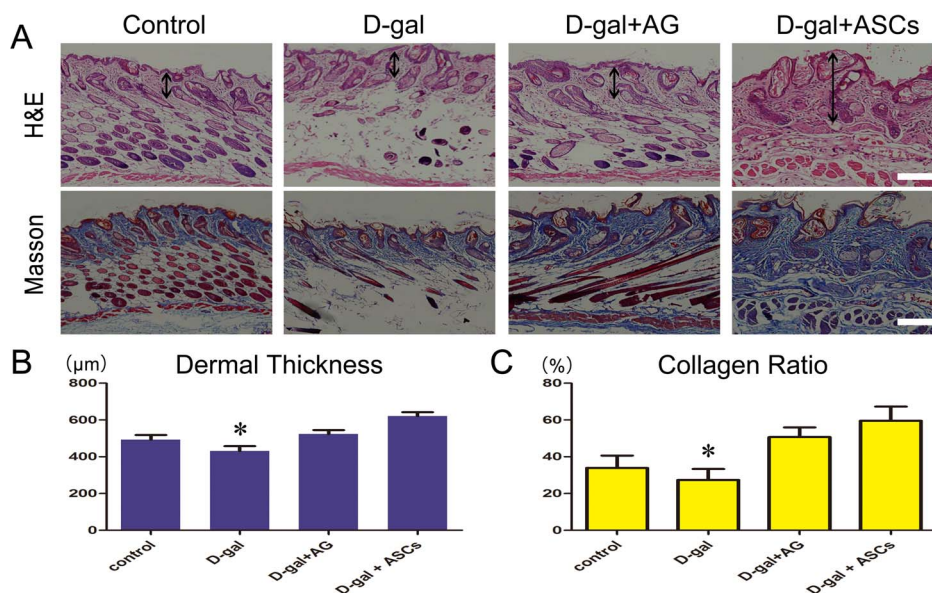


Figure 4. Haematoxylin and eosin staining and Masson's trichrome staining. **A.** Adipose-derived stem cells treatment increased dermal thickness (the double head arrows) and collagen ratio of mice skin. **B.** The thickness of the dermal portion of skin. **C.** Collagen ratio (collagen fibers stained blue) was measured with an image analysis program. $n = 4$. * $P < 0.05$, Scale bars = 100 μm . doi:10.1371/journal.pone.0097573.g004

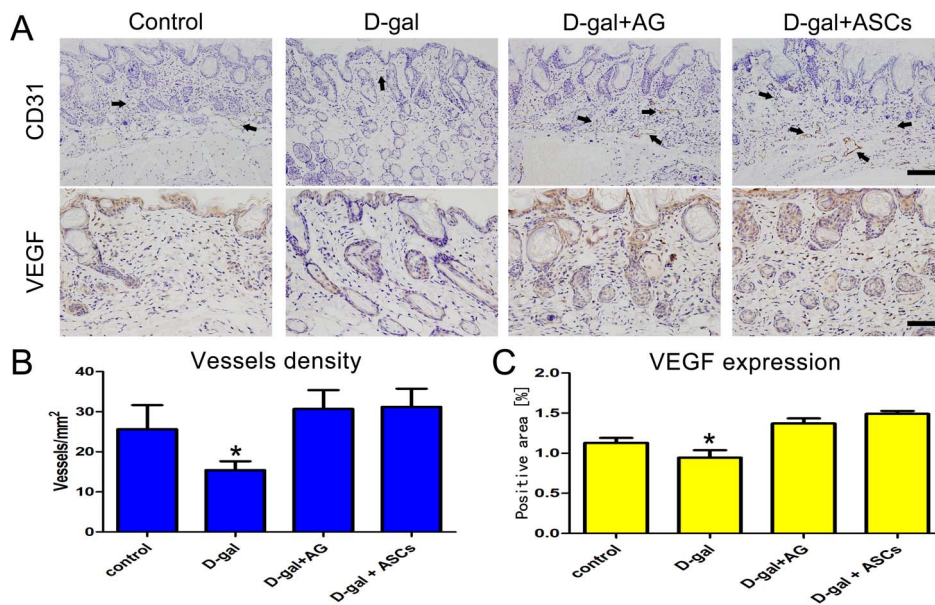


Figure 5. The changes in angiogenesis in skin tissue. **A.** Immunohistochemical detection of CD31-positive microvessels (arrows) and vascular endothelial growth factor (VEGF) expression in skin tissue in control, D-gal-treated, D-gal plus adipose-derived stem cells (ASCs)-treated, and D-gal plus aminoguanidine (AG)-treated animals. CD31: Scale bar = 200 μ m, VEGF: Scale bar = 100 μ m. **B.** The changes in vascular density/mm² in control, D-gal-treated, D-gal plus ASCs-treated, and D-gal plus AG-treated groups determined by counting the number of CD31-positive vessels within visual fields (n = 8). *P < 0.05. **C.** The D-gal plus ASCs-treated showed the highest expression of VEGF among the four groups. doi:10.1371/journal.pone.0097573.g005

cells is known to result in low viability ranging from 1% to 32% [31]. Recently, Shirae K. Leslie et al. developed a degradable and injectable hydrogel to deliver target cells for tissue regeneration [32]. Once the cells were injected, this material can provide further protection while retaining them at the injection site and slow released. Therefore, an injectable, degradable biomaterial is needed is necessary in our further research and clinical application.

Previous studies have suggested that excess AGE intake and chronic accumulation of AGE-related glycosylated proteins in tissue may further potentiate the aging process, resulting in impaired mitochondrial function and decreased life span in *Caenorhabditis elegans* and mice [33,34,35]. Our results showed that ASC treatment inhibits AGE formation and that ASCs had less of an inhibitory effect on the formation of AGEs than AG, suggesting that ASC treatment inhibits BSA-AGE formation. Cells in the body possess a wide range of inter-linked antioxidant defense mechanisms to protect themselves against damage caused by ROS. Among these mechanisms, antioxidant enzymes, including SODs, are important in scavenging remaining ROS in cells. SODs are metalloenzymes that catalyze the dismutation of superoxide anion to molecular O₂ and H₂O₂ and are, therefore, a crucial part of the cellular antioxidant defense mechanism. SODs exist in cells and tissues in three forms, namely, cytosolic Cu/Zn-SOD (SOD1), mitochondrial Mn-SOD (SOD2), and extracellular SOD (SOD3). Cu/Zn-SOD and Mn-SOD are thought to be important in defense against oxygen toxicity [36]. Proteomic analysis revealed 112 proteins, including SODs, which are upregulated by ASC treatment. Of these proteins, many showed antioxidant effects on epithelial cells in previous studies [37,38,39]. An important finding of the present study is that ASCs reversed the effects of D-gal-induced oxidative stress in mouse skin, as shown by the expression levels of senescence-associated molecular markers such as SOD and MDA. Secretory proteins from ASCs such as SOD and several cytokines may mediate the protective effects and play key

roles *in vivo*. Stem cells may possess potent antioxidant effects as suggested by the decrease in expression level of senescence-associated molecular markers following ASC treatment.

Kim et al. suggested that ASC-conditioned medium enhanced type 1 collagen secretion and fibroblast migration of human dermal fibroblasts in an *in vitro* wound-healing model [40]. In another study, wrinkles induced by UVB irradiation were significantly improved by subcutaneous injection of ASC in hairless mice. In addition, dermal thickness and collagen content were higher in animals of ASC-injection groups than of control groups [18]. Our histological observations showed lower expression levels of collagen and dermal thickness in D-gal-induced mice, which was increased by ASC injection. While some studies suggested that ASCs secrete collagen [41,42], the low retention rate of ASCs indicate that the increasing collagen expression level was more likely due to the upregulation of collagen expression in local fibroblasts caused by paracrine ASCs.

Another potential role of ASC treatment in skin anti-aging is angiogenesis. Substantial evidence indicates that ASCs may increase angiogenesis through secretion of angiogenic factors such as VEGF and hepatocyte growth factor [43,44,45]. In this study, our results support the notion that ASCs transplantation strongly induces the revascularization of skin tissue along with the secretion of VEGF, which was clearly detected in transplanted cells in our model, thereby lending support to the trophic hypothesis. CD31 staining studies further confirmed this hypothesis. Although previous studies indicated that ASCs can differentiate into vascular endothelial cells [46,47], we found that GFP-positive ASCs were undetectable after 28 days, indicating that angiogenesis was mainly a result of a paracrine effect of ASCs.

Conclusions

In summary, we examined the glycation suppression of ASCs in a mouse aging skin model induced by D-gal. ASCs may have the

potential to contribute to the regeneration of skin and provide a functional benefit. ASC injection prevented the expression of senescence-associated molecular markers. Similarly to AG, the inhibitor of AGE formation, ASCs inhibited D-gal-increased AGE levels, therefore reversing the aging phenotype in our mouse model. The expression level of SOD in skin was increased and MDA was decreased with ASC injection, suggesting that ASCs may suppress glycation in skin. ASCs may be a good candidate for the control and prevention of skin damage caused by glycation in various skin conditions, including wounding and aging.

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Supporting Information

File S1 Ethics statement.
(DOCX)

File S2 Certificate for proofreading by Bioedit.
(DOCX)

Author Contributions

Conceived and designed the experiments: SZ FL. Performed the experiments: SZ ZD ZP. Analyzed the data: SZ ZD. Contributed reagents/materials/analysis tools: SZ. Wrote the paper: SZ ZD.