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Original Article

Adipose-derived mesenchymal stem cell-loaded β-chitin nanofiber hydrogel activates the AldoA/HIF-1α pathway to promote diabetic wound healing

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Abstract: Objectives: To identify the effect of adipose-derived mesenchymal stem cell-loaded β-chitin nanofiber (ADSC-loaded β-ChNF) hydrogel on diabetic wound healing and clarify its mechanism of action. Methods: We prepared the ADSC-loaded β-ChNF hydrogel to repair wounds of db/db diabetic mice. Wound healing rate, histopathology, enzyme-linked immunosorbent assay, and western blot were used to confirm its role and mechanism in promoting diabetic wound healing. Results: The ADSC-loaded β-ChNF hydrogel accelerated wound healing in db/db diabetic mice, as indicated by increased cell proliferation, epithelization, and tissue granulation in the skin. Moreover, expression of vascular endothelial growth factor (VEGF) and its receptor (VEGFR), matrix metalloproteinase 9 (MMP9), and TIMP metallopeptidase inhibitor 1 (TIMP1) were upregulated. These results demonstrate the beneficial effects of this ADSC-loaded β-ChNF hydrogel on diabetic wound healing. Furthermore, we show that the ADSC-loaded β-ChNF hydrogel activated aldolase A (AldoA)/hypoxia-inducible factor 1α (HIF-1α) signaling. An inhibitor of HIF-1α markedly decreased the promotive effects of the ADSC-loaded β-ChNF hydrogel on wound healing and reduced expression of VEGF, VEGFR, MMP9, and TIMP1. Conclusions: Our findings suggest that the ADSC-loaded β-ChNF hydrogel activated the HIF-1α/MMP9 axis through AldoA feedback to promote diabetic wound healing.

Keywords: Adipose-derived mesenchymal stem cell, β-chitin nanofiber hydrogel, HIF-1α, MMP9, AldoA

Introduction

Skin is the largest organ covering the surface of the human body. Trauma, surgery, ulcers, and many other causes can lead to the formation of skin wounds, thus damaging the body's defense barrier [1]. Wounds in patients with diabetes tend to develop into chronic wounds and heal more slowly than those in healthy people. The process of wound repair is complex, involving four stages: hemostasis, inflammation, proliferation, and remodeling. Each stage overlaps in time and progresses in an orderly manner through activation of different cell types and regulation of various functions. Processes of acute and chronic wound repair and reconstruction involve cell proliferation and migration, angiogenesis, collagen production, and extracellular matrix (ECM) remodeling, which require the involvement of many cytokines and key proteins. Although the healing process of chronic wounds is basically the same as that of acute wounds, abnormalities occur at some point during this process. Compared with acute wounds, hypoxia, dysfunction of epidermal cells and fibroblasts, angiogenesis disorders, changes in metalloproteinase levels, secondary damage of reactive oxygen species, increased advanced glycosylation end products, decreased host immune resistance, and neuropathy are observed in chronic wounds. Chronic wounds have no orderly and timely repair process or are

unable to complete wound healing after 1 month of treatment. In addition, chronic wounds show no healing tendency and involve more complex repair mechanisms than acute wounds. Accordingly, chronic wounds are difficult to heal without effective treatment, leading to infection, amputation, and even threats to patients' lives.

Current treatment methods for diabetic wounds mainly include autologous skin transplantation, biological dressing, negative pressure suction, tissue engineering, vascular revascularization, growth factor therapy, and stem cell therapy. However, most treatment methods for large-area and chronic wound repair are limited in use or effect. Cellular hypoxia is the main characteristic of diabetic wounds. In addition to cell proliferation, angiogenesis and ECM remodeling are essential for the healing of diabetic wounds. Vascular endothelial growth factor (VEGF) not only affects vascular remodeling and provides nutrients for new granulation tissue, it promotes collagen formation and deposition. Matrix metalloproteinases (MMPs) and tissue inhibitor of matrix metalloproteinases (TIMPs) are involved in ECM remodeling processes. Therefore, regulation of hypoxia, angiogenesis, and ECM remodeling is expected to accelerate diabetic wound healing. At present, the mechanisms and characteristics of diabetic wound injury are not completely clear, and the treatment strategy needs to be perfected [2, 3].

With the development of regenerative medicine, stem cells bring new possibilities for wound treatment. Adipose-derived mesenchymal stem cells (ADSCs) can improve the wound environment and contribute to vascularization, collagen generation, and ECM remodeling in damaged tissues. However, problems such as limited drug delivery, low colonization rates, and low cell survival rates continue to hinder clinical applications [4, 5]. Hydrogels can provide a carrier for local immobilization of stem cells and themselves exert certain effects to promote healing. Indeed, combinations of stem cells and hydrogels can exert greater biological activity [6, 7]. We previously observed beneficial effects of an ADSC-loaded β-chitin nanofiber (ADSC-loaded β-ChNF) hydrogel in healthy rats [8], but its therapeutic effect and underlying mechanism for diabetic wound injury remained unclear. Aldolase A (AldoA) reportedly has a beneficial role in cell proliferation. It can affect expression of downstream proteins through feedback activation of hypoxia-inducible factor 1α (HIF-1α) and plays an important role in promoting proliferation and angiogenesis in a variety of tissues [9, 10]. Based on omics analysis, we found that ADSC-exosome hydrogels could regulate AldoA protein expression in wounds [11]. Determining whether ADSC-loaded β-ChNF hydrogels can activate the AldoA/HIF-1α axis to promote diabetic wound healing could provide a new direction for wound treatment research.

Here, we studied the effect of an ADSC-loaded β-ChNF hydrogel on diabetic wounds and determined whether it could activate the AldoA/HIF-1α signaling axis to promote diabetic wound healing.

Methods

Preparation of ADSC-loaded β-ChNF hydrogel

Squid pens were used to prepare a β-chitin nanofiber dispersion by acid hydrolysis and alkali extraction. Briefly, squid pens were treated with 0.1 mol/L hydrochloric acid (Sinopharm Chemical Reagent, Shanghai, China) and 4 wt% sodium hydroxide (Sinopharm Chemical Reagent) until dissolved completely. An ultrasonic homogenizer (JY92-IIDN; Scientz, Zhejiang, China) was used to generate a β-ChNF suspension, which was subsequently autoclaved and reacted with culture medium to form a gel that was subsequently used for further culture of ADSCs. A total of $1*10^6$ ADSCs (MUBMD-01001; Cyagen Biosciences, China) were added to 500 μL of hydrogel and cultured for 3 d for wound intervention.

Animals

Diabetic mice (db/db, aged 12 w) were purchased from Liaoning Changsheng Biotechnology (Liaoning, China) and kept in cages maintained at 22 ± 3°C with a humidity of 45%-60%. Mice were allowed free access to food and water. After acclimation, mice were randomly divided into hydrogel, ADSCs, and ADSC-loaded β-ChNF hydrogel groups (n = 6 per group). The left wound of all mice was used as self control without any treatment, and the right wound was treated immediately after injury. Hydrogel group

mice were directly covered with 500 μL of β-ChNF hydrogel. ADSCs group mice were subcutaneously injected with $1*10^6$ ADSCs at multiple sites around the wound. ADSC-loaded β-ChNF hydrogel group mice were directly covered with 500 μL of ADSC-loaded β-ChNF hydrogel. Animal experiments were approved by the Ethics Committee of the General Hospital of the Northern Theater Command. At the end of the experiment, mice were intraperitoneally anesthetized with 2% sodium pentobarbital (30 mg/kg) and their skin was harvested for pathology and protein detection. After that, mice were performed the euthanasia by cervical dislocation.

Wound healing activity

A full-thickness defect wound model was established according to a procedure used in a previous report [8]. Circular full-thickness cutaneous wounds were created and carefully observed at 0, 3, 6, 9, 12, and 15 d post-surgery. Each wound was photographed and its area was measured using ImageJ (National Institutes of Health, Bethesda, MD, USA). The wound healing rate was calculated as follows: wound healing rate (%) = (A0 - At)/A0 × 100%.

Histological examination

Hematoxylin and eosin (HE) staining was used to observe histopathological changes in mice. Skin samples were fixed in 10% formalin solution, dehydrated, paraffin embedded, and cut into 5 μm-thick sections. Sections were stained with HE solutions for 10 min and 3 min, respectively. Immunohistochemistry was performed with a kit according to the manufacturer's instructions (Hangzhou Maixin Biotechnology Development, Hangzhou, China). The results were observed by light microscopy.

Enzyme-linked immunosorbent assay (ELISA)

After homogenizing tissue, the supernatant was taken for detection. Fifty microliters of standard, buffer, or sample diluted to various concentrations were added to standard, blank, or sample wells, respectively. The optical density value of each well was measured at a 450 nm wavelength. The linear regression curve of the standard was drawn and the concentration value for each sample was calculated according to the curve equation.

Western blot

Proteins for western blot analysis were extracted from skin tissues and evaluated using a BCA Protein Assay Kit (FD2001; Hangzhou Fude Biological Technology Company, Hangzhou, China) to determine concentrations. Antibodies included cytokeratin (1:1000; ab191208; Abcam, Cambridge, UK), Ki67 (1:1000; ab16667, Abcam), AldoA (1:1000; 11217-1-AP; Proteintech, Rosemont, IL, USA), HIF-1α (1:1000; ab179483, Abcam), GAPDH (1:4000, 2118, Cell Signaling Technology, Danvers, MA, USA), and an anti-rabbit IgG secondary (1:4000; ab6721, Abcam). Proteins were visualized using Clarity™ Western ECL Substrate (170-5061; Bio-Rad, Hercules, CA, USA) and a Tanon 5200 fully automatic chemiluminescence image analysis system (Tanon Science and Technology, Shanghai, China).

Statistical analysis

All data were analyzed using SPSS 22.0 (IBM, Armonk, NY, USA). Quantitative data are expressed as mean ± standard deviation. A two-tailed paired Student's t-test was used for comparisons of two groups. Comparisons between multiple groups were performed by one-way ANOVA. *P*-values less than 0.05 were considered statistically significant.

Results

ADSC-loaded β-ChNF hydrogel accelerated wound healing

After treatment with the ADSC-loaded β-ChNF hydrogel, skin wounds of mice were observed and photographed at 0, 3, 6, 9, 12, and 15 d. As shown in the representative images in **Figure 1A**, the wound areas of mice treated with ADSC-loaded β-ChNF hydrogel were notably smaller compared with the model group ($P < 0.05$). Indeed, wounds were basically healed at 15 d in mice treated with ADSC-loaded β-ChNF hydrogel but still large and had scabs in the model group. The wound area was measured and the relationship between wound healing rate and postoperative day was evaluated. As shown in **Figure 1B**, the wound healing rate of the ADSC-loaded β-ChNF hydrogel group increased from 18.7% to 80.5% from 3 d to 15 d, significantly faster than observed in the model group ($P < 0.05$). As shown in **Figure 1C**, there

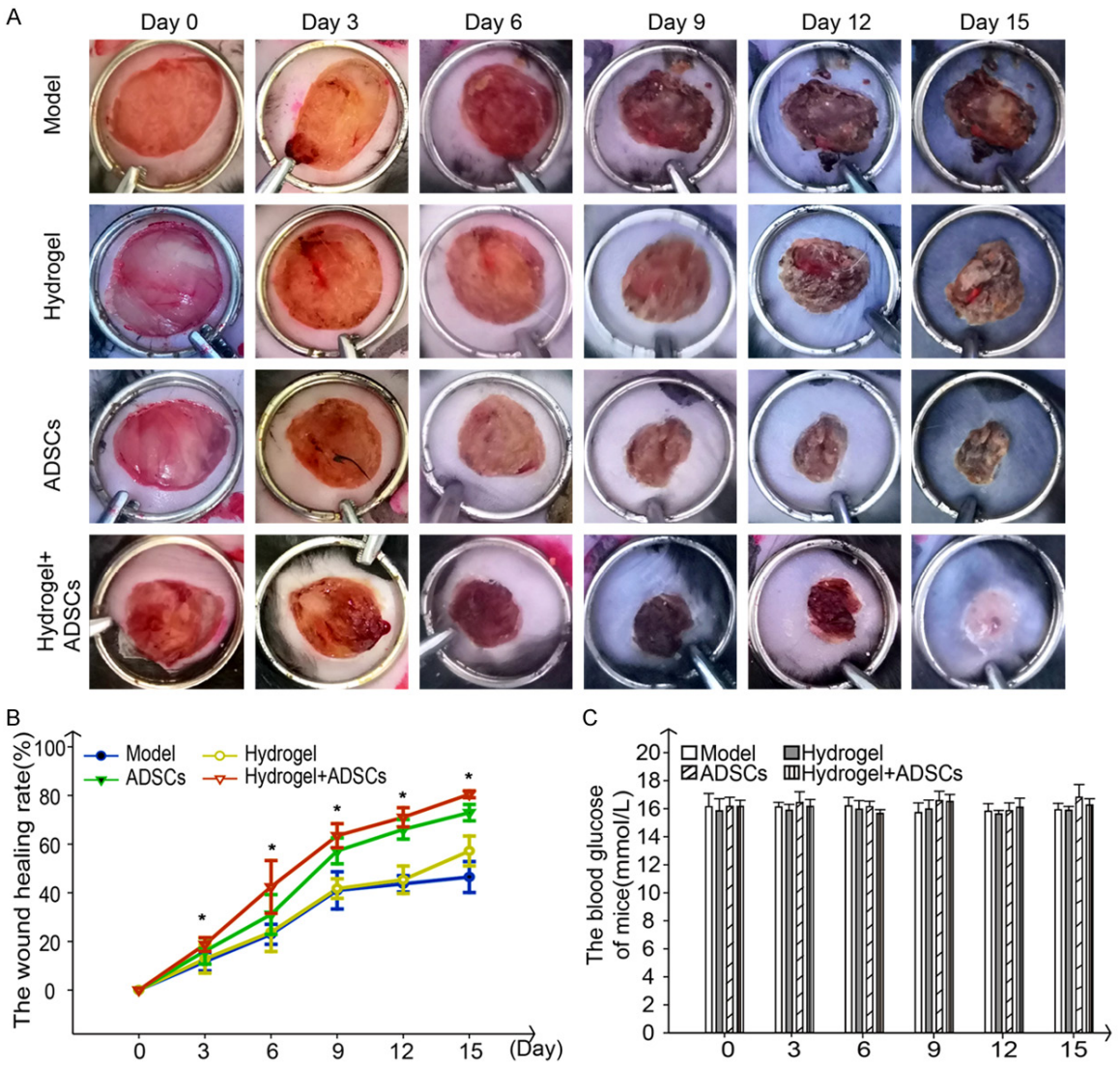

Figure 1. Effect of an adipose mesenchymal stem cell chitin nanofiber (ADSC-loaded β-ChNF) hydrogel on diabetic wound healing in mice. A. Representative images of wound healing. B. Wound healing rate. C. Blood sugar of mice. *$P < 0.05$ compared with model mice.

were no significant differences in blood glucose levels among the groups ($P > 0.05$). Collectively, these results indicate that the ADSC-loaded β-ChNF hydrogel significantly increased the rate of wound healing in diabetic mice.

HE staining indicated ADSC-loaded β-ChNF hydrogel promoted epithelialization

As shown in **Figure 2**, the internal structure of skin tissue in model-group mice was not fully developed and epidermal regeneration ability was poor. In hydrogel, ADSCs, and ADSC-loaded β-ChNF hydrogel groups, the internal tissue structure began to develop and an epithelium gradually formed. Compared with the other groups, hair follicles, blood vessels, and other skin appendages were more plentiful in the ADSC-loaded β-ChNF hydrogel group, which exhibited complete epithelialization.

ADSC-loaded β-ChNF hydrogel promoted wound cell proliferation

Expression of cytokeratin and Ki67 were detected by western blot. As shown in **Figure 3A** and **3B**, cytokeratin and Ki67 expression were increased following intervention with ADSC-loaded β-ChNF hydrogel compared with the model group ($P < 0.05$). Immunohistoche-

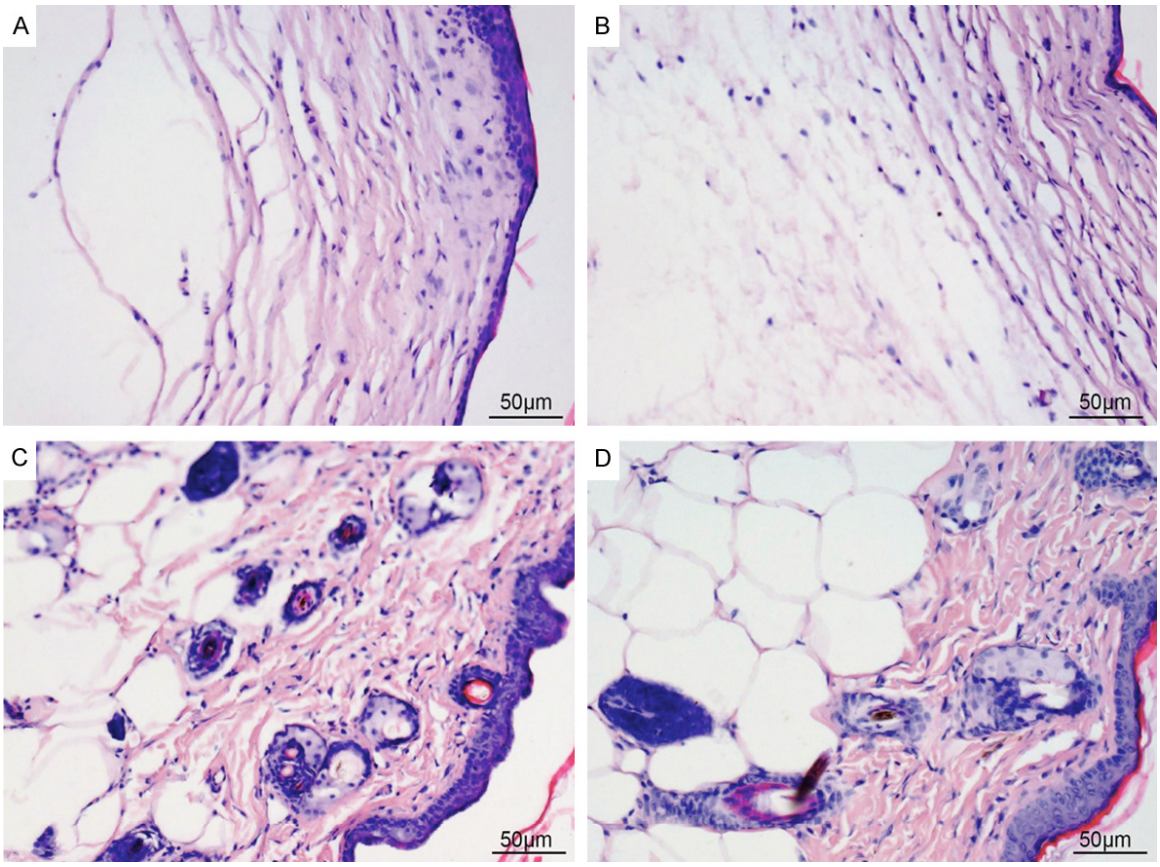

Figure 2. Effect of an adipose mesenchymal stem cell chitin nanofiber (ADSC-loaded β-ChNF) hydrogel on wound skin structures in diabetic mice. A. Control group. B. Chitin hydrogel group. C. ADSC group. D. ADSC-loaded β-ChNF group. 200× magnification.

mical staining further confirmed these results. Cytokeratin was mainly located in the cytoplasm. Compared with the model group, cytokeratin expression was increased in newly formed epithelial tissues after ADSC-loaded β-ChNF hydrogel intervention. Protein expression of the cell-proliferation marker Ki67 was mainly located in the nucleus. Compared with the model group, Ki67 expression was increased in newly formed epithelial tissues after ADSC-loaded β-ChNF hydrogel intervention (**Figure 3C**, **3D**).

ADSC-loaded β-ChNF hydrogel promoted wound angiogenesis and matrix remodeling

To further clarify the effect of the ADSC-loaded β-ChNF hydrogel on wound healing, we examined expression of angiogenesis-related proteins in newborn skin tissues of mice using ELISA. As shown in **Figure 4**, expression of angiogenesis-related proteins including VEGF and its receptor (VEGFR), MMP9, and TIMP1 were increased after ADSC-loaded β-ChNF hydrogel intervention compared with the model group ($P < 0.05$).

ADSC-loaded β-ChNF hydrogel promoted AldoA and HIF-1α protein expression

To further clarify the mechanism by which the ADSC-loaded β-ChNF hydrogel promoted wound healing in diabetic mice, we evaluated expression of AldoA and HIF-1α pathway proteins (**Figure 5**). Western blot results revealed increased expression of AldoA and HIF-1α proteins in diabetic mouse wound tissue after ADSC-loaded β-ChNF hydrogel intervention compared with the model group ($P < 0.05$).

HIF-1α inhibition decreased expression of angiogenesis and matrix remodeling proteins

Mice were administered the HIF-1α inhibitor BAY 87-2243 (9 mg/kg) by continuous local injection for 5 d at the same time as ADSC-loaded β-ChNF hydrogel treatment. On the

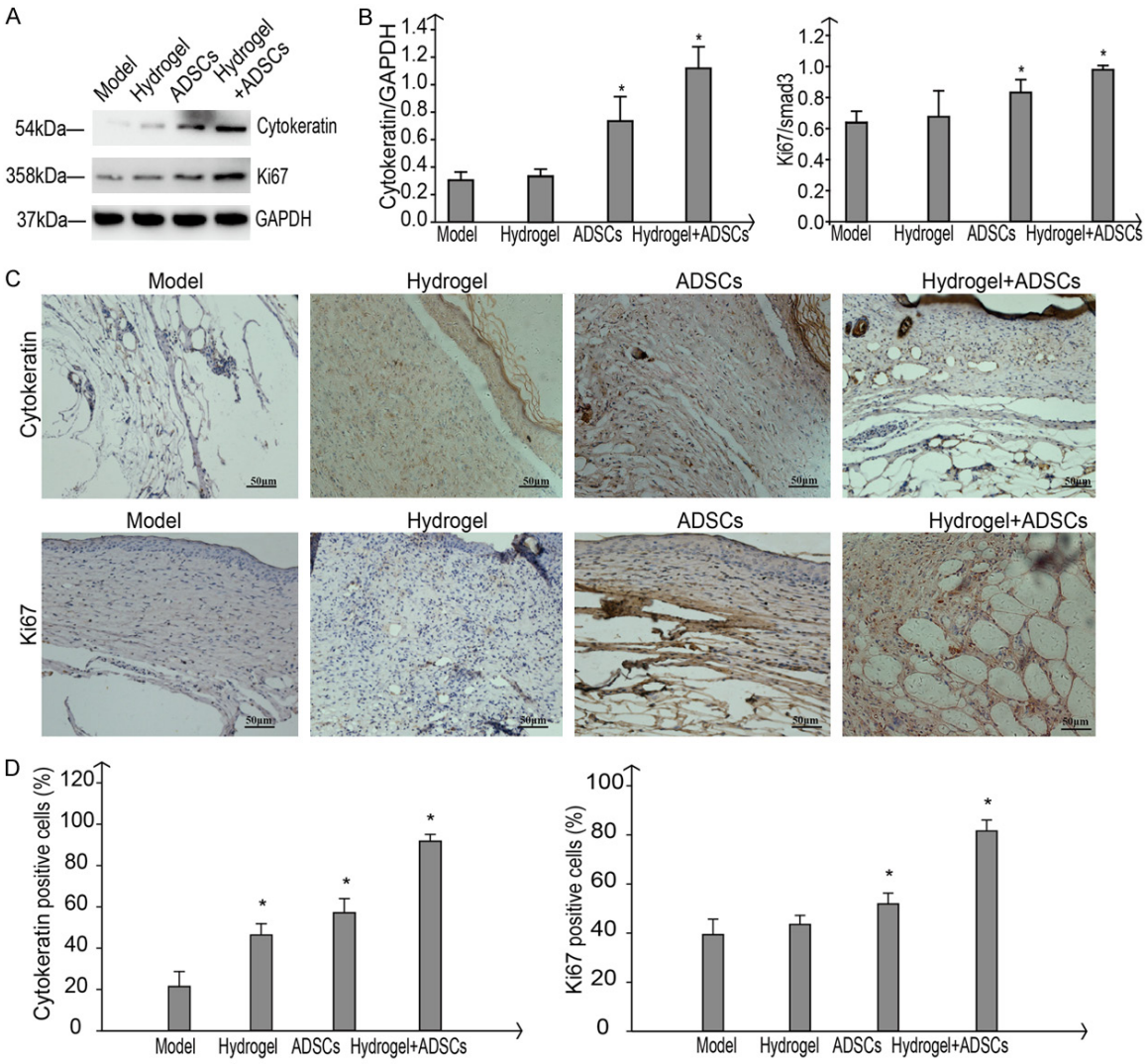

Figure 3. Effect of an adipose mesenchymal stem cell chitin nanofiber (ADSC-loaded β-ChNF) hydrogel on cytokeratin and Ki67 protein expression in diabetic mouse wound tissue. A. Representative images of western blot analysis. B. Relative gray values of western blot analysis. C. Immunohistochemistry analysis of cytokeratin and Ki67. D. Relative value of positive cells. 200× magnification. $^{*}P < 0.05$ compared with model mice.

ninth day, wound tissues were collected for western blot analysis. As shown in **Figure 6**, HIF-1α protein expression in wound tissues was lower compared with the uninhibited group ($P < 0.05$). Moreover, expression of VEGF, VEGFR, MMP9, and TIMP1 proteins in the HIF-1α group was significantly reduced compared with the uninhibited group ($P < 0.05$).

HIF-1α inhibition decreased wound healing

Mouse wounds were observed at 0, 3, 6, 9, 12 and 15 d, and the wound healing rate was calculated. As shown in **Figure 7**, a HIF-1α inhibitor reduced the promotive effect of the ADSC-loaded β-ChNF hydrogel on wound healing ($P < 0.05$), indicating an important role of HIF-1α during diabetic wound healing.

Discussion

In this study, an ADSC-loaded β-ChNF hydrogel promoted healing of a full-thickness defect diabetic wound model established in db/db mice [12, 13]. The results of our mechanistic study show that expression of AldoA, HIF-1α, VEGF, VEGFR, MMP9, and TIMP1 were upregulated in db/db mice after ADSC-loaded β-ChNF hydrogel treatment compared with model-group mice. Moreover, an inhibitor of HIF-1α markedly

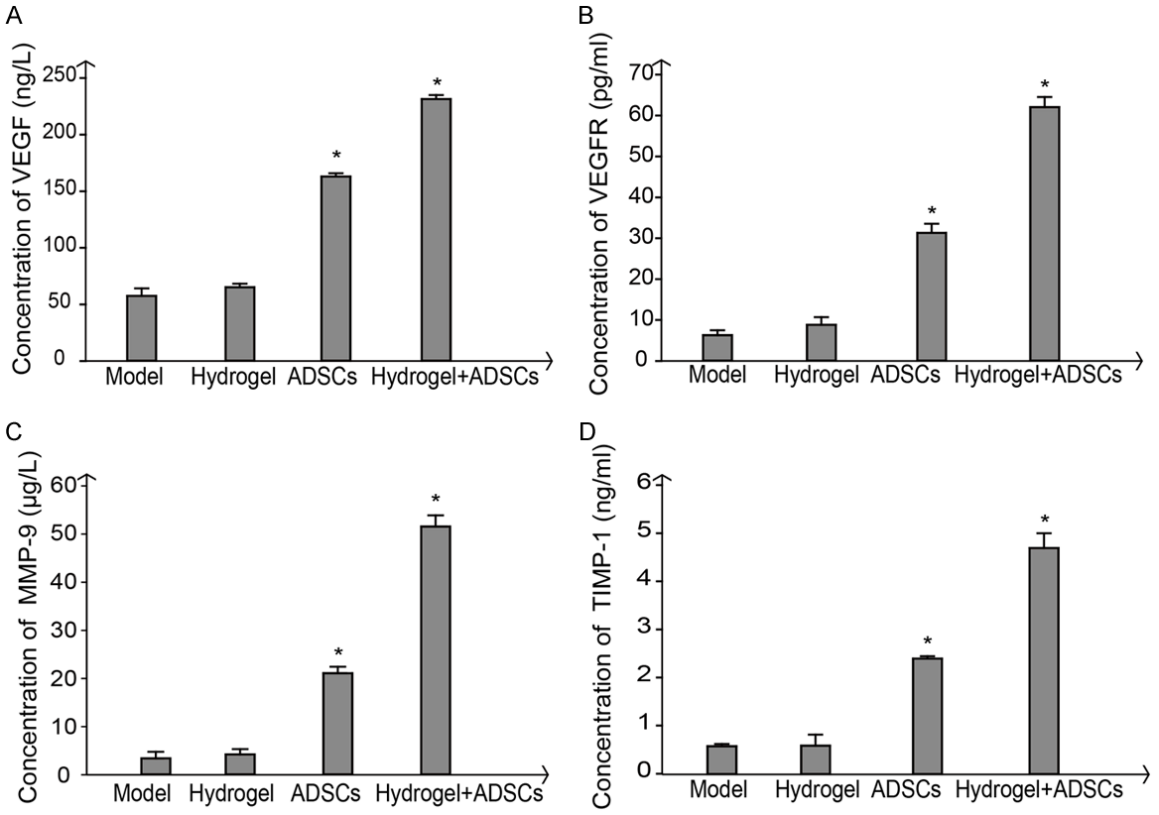

Figure 4. Effect of an adipose mesenchymal stem cell chitin nanofiber (ADSC-loaded β-ChNF) hydrogel on expression of angiogenesis- and neovascularization-related proteins in diabetic mouse wound tissue. A. Vascular endothelial growth factor (VEGF) expression. B. VEGFR-receptor (VEGFR) expression. C. Matrix metalloproteinase 9 (MMP-9) expression. D. TIMP metallopeptidase inhibitor 1 (TIMP-1) expression. $^{*}P < 0.05$ compared with the model group.

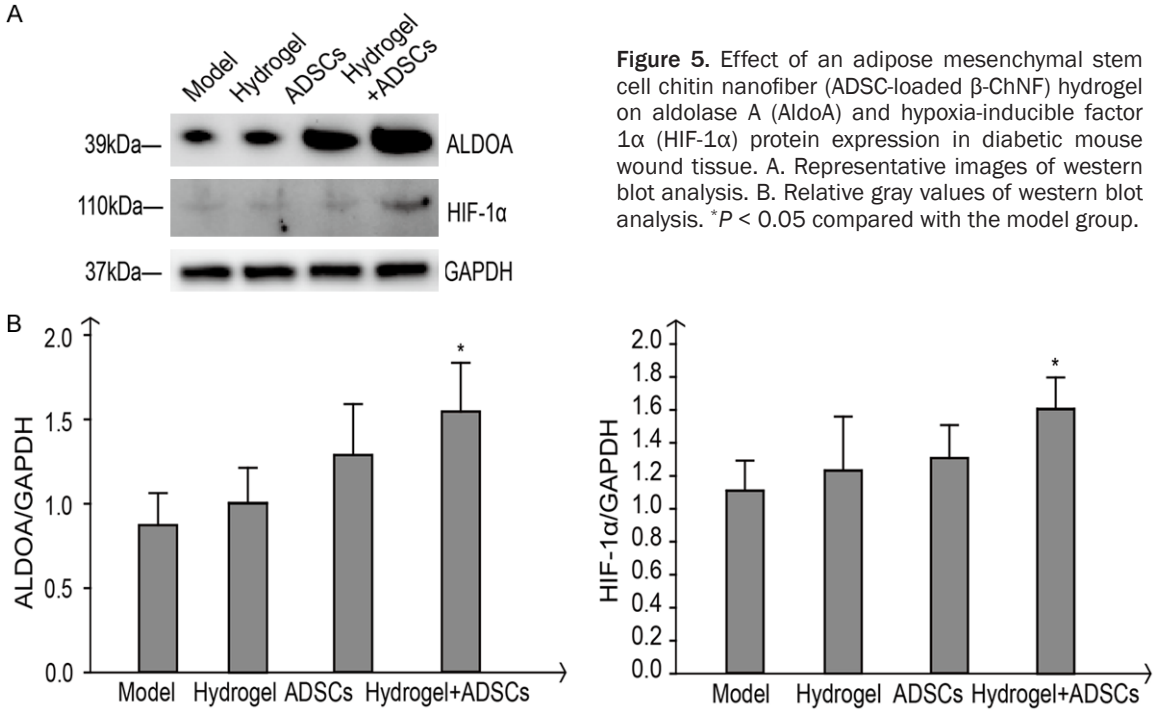

Figure 5. Effect of an adipose mesenchymal stem cell chitin nanofiber (ADSC-loaded β-ChNF) hydrogel on aldolase A (AldoA) and hypoxia-inducible factor 1α (HIF-1α) protein expression in diabetic mouse wound tissue. A. Representative images of western blot analysis. B. Relative gray values of western blot analysis. $^{*}P < 0.05$ compared with the model group.

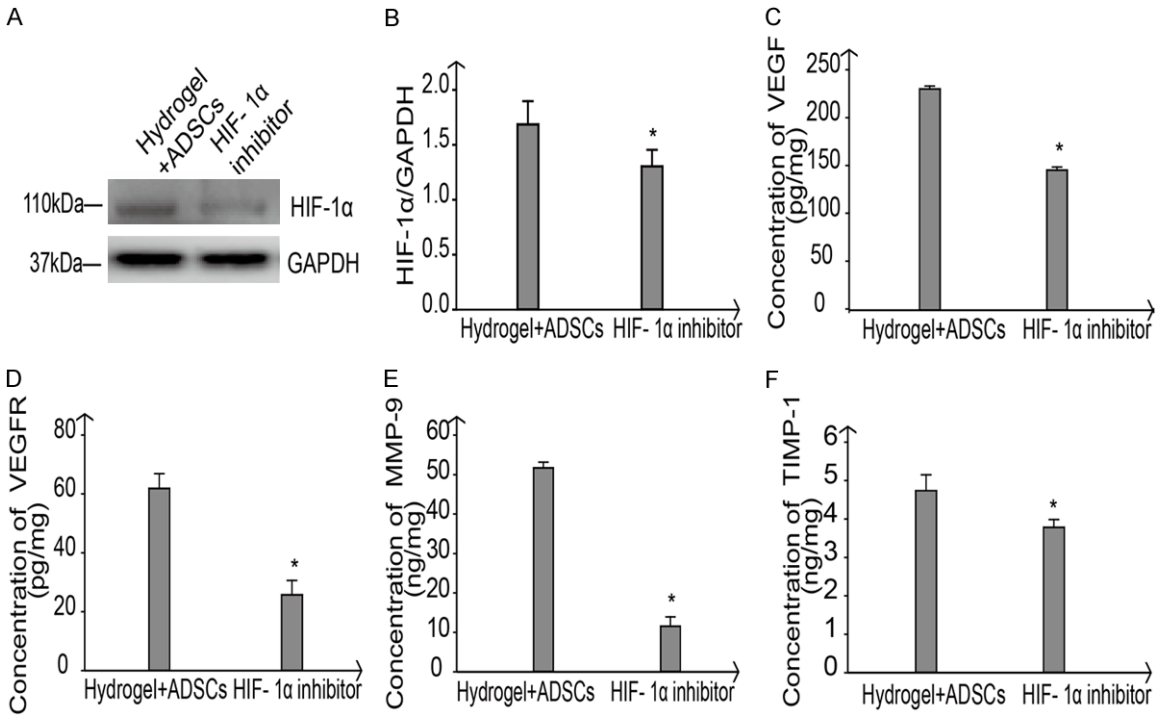

Figure 6. Effect of a hypoxia-inducible factor 1α (HIF-1α) inhibitor on skin protein expression in diabetic mice. A, B. Western blot to detect expression of HIF-1α. C-F. Enzyme-linked immunosorbent assay of angiogenesis-related protein expression. $^{*}P < 0.05$ compared with the adipose-derived stem cells chitin nanofiber (ADSC-loaded β-ChNF) hydrogel group.

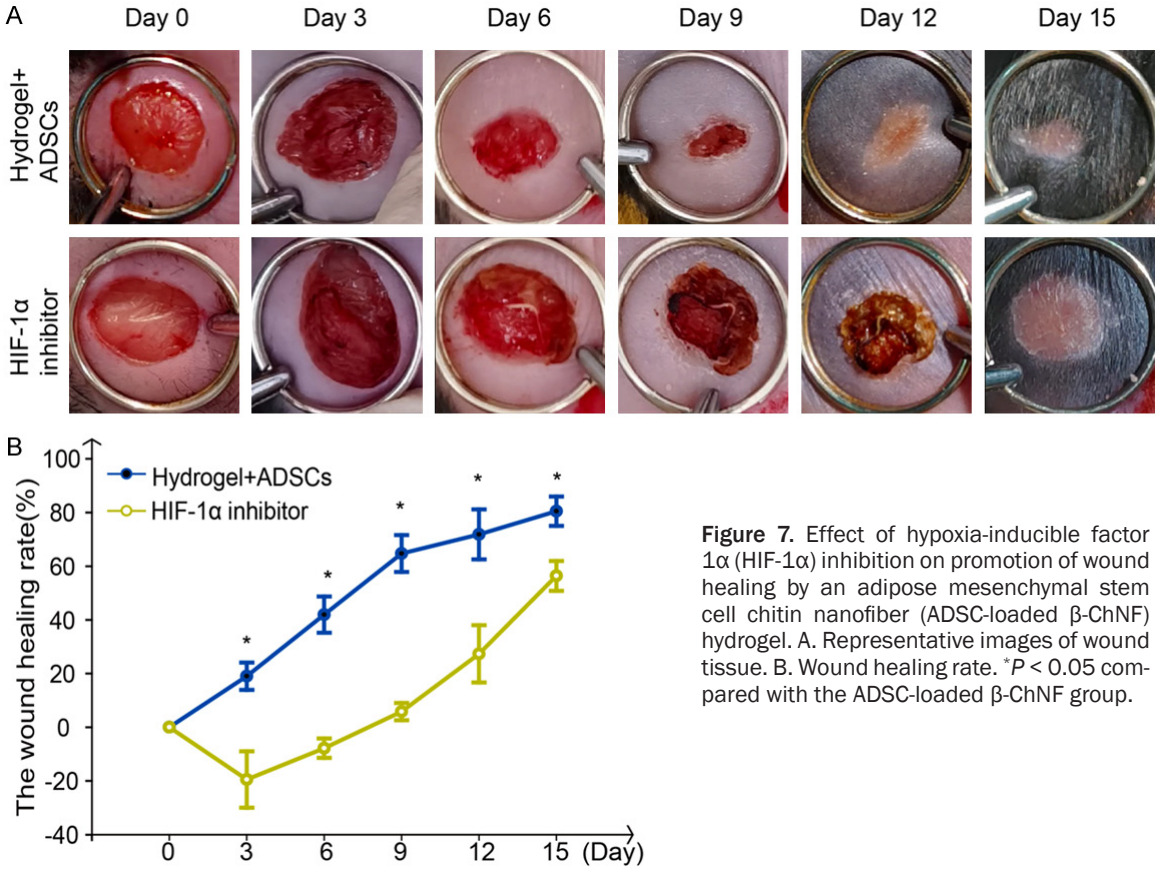

Figure 7. Effect of hypoxia-inducible factor 1α (HIF-1α) inhibition on promotion of wound healing by an adipose mesenchymal stem cell chitin nanofiber (ADSC-loaded β-ChNF) hydrogel. A. Representative images of wound tissue. B. Wound healing rate. $^{*}P < 0.05$ compared with the ADSC-loaded β-ChNF group.

decreased the beneficial effects of the ADSC-loaded β-ChNF hydrogel on wound healing and reduced expression of VEGF, VEGFR, MMP9, and TIMP1.

As a global health problem, the prevalence and incidence of diabetes are increasing. Diabetes is a type of metabolic disease and diabetic wounds are characterized by tissue hypoxia caused by a high-glucose environment and insufficient local vascular supply; moreover, the healing ability of wound tissue cells is decreased [14-16]. The ADSC-loaded β-ChNF hydrogel promoted wound healing, providing new possible strategies for chronic wound treatment. AldoA is involved in a variety of cell functions and may have key roles in cell cycle regulation and cell migration. AldoA, a multifunctional enzyme in the glycolysis pathway, also promotes HIF-1α expression and its overexpression can improve HIF-1α stability by inhibiting its hydroxylation [17, 18]. HIF-1α is a major regulatory factor involved in glucose uptake, glycolysis, angiogenesis, and stress resistance. By increasing HIF-1α expression, cells can induce expression of glucose transporter 1 and some glycolysis enzymes, and further stimulate the glycolysis pathway including AldoA [19]. Therefore, AldoA protein may have an important role during diabetic wound healing.

HIF-1α can also regulate numerous genes involved in cell proliferation, motility, metabolism, and angiogenesis by inducing expression of its downstream target genes [20, 21]. Hypoxia reportedly inhibited the proliferation of mouse embryonic fibroblasts, and HIF-1α inactivation enhanced this effect. Indeed, HIF-1α is a master regulator involved in glucose uptake, glycolysis, angiogenesis, and stress resistance [22, 23]. Hypoxia increased VEGF expression through HIF-1α in alveolar epithelial cells, which stimulated angiogenesis and increased oxygen delivery [24-26]. Furthermore, AldoA was found to substantially inhibit the hydroxylation status of HIF-1α by inhibiting PHD, thereby stabilizing HIF-1α at the protein level and activating its downstream signaling targets, including MMP9. Moreover, MMP9 was substantially upregulated following AldoA overexpression and an MMP9 inhibitor inhibited cell invasion and migration in AldoA/HIF-1α axis-induced lung cancer [27, 28]. Consistent with these results, we found that expression of AldoA, HIF-1α, and angiogenic proteins in neonatal skin tissues of diabetic mice was increased following intervention with ADSC-loaded β-ChNF hydrogel compared with the model group; by contrast, there was no significant difference in hydrogel or ADSCs groups compared with the model group. These results further implicate regulation of AldoA in the reparative mechanism elicited by the ADSC-loaded β-ChNF hydrogel. Notably, the effect of the ADSC-loaded β-ChNF hydrogel on wound healing likely combined the effects of ADSCs and the β-ChNF hydrogel. Furthermore, HIF-1α is one of the downstream pathways of AldoA changed by the ADSC-loaded β-ChNF hydrogel. In this study, a HIF-1α inhibitor partially inhibited HIF-1α expression and decreased the effect of the ADSC-loaded β-ChNF hydrogel. In future studies, the dose of the HIF-1α inhibitor could potentially be increased to completely inhibit HIF-1α expression.

In conclusion, the ADSC-loaded β-ChNF hydrogel proved to be an effective treatment for skin wounds in diabetic mice. Mechanistically, our findings reveal that the ADSC-loaded β-ChNF hydrogel could substantially promote cell proliferation and angiogenesis by activating the HIF-1α/MMP9 axis through AldoA feedback. Our study demonstrates the promise of ADSC-loaded β-ChNF hydrogels as a new method for healing chronic wounds.

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Disclosure of conflict of interest

None.

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