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Research article

Dang-Gui-Bu-Xue decoction improves wound healing in diabetic rats by the activation of Notch signaling

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ABSTRACT

Diabetes serves as a severe chronic disease that severely affects the normal life of human beings. Diabetes causes the complication of diabetic wound dysfunction, which is characterized by sustained inflammation, altered angiogenesis, delayed epithelialization and abnormal secretion of protease. Dang-Gui-Bu-Xue decoction (DBD) is a Chinese traditional medicine that comprises Radix Astragali and Radix Angelicae sinensis and is widely applied in treatment of multiple diseases owing to its functions against inflammation, lipid peroxidation and oxidative stress. Nevertheless, the impact of DBD on diabetic wound healing remains elusive. In this study, we aimed to explore the function of DBD in the regulation of wound healing. We observed that the gavage administration of DBD reduced the wound area, inflammatory infiltration, inflammatory factor levesl, and enhanced granulation tissue formation, wound extracellular matrix (ECM) production, and CD31 accumulation in the diabetic rat wound model, and the co-treatment of gavage administration and the external administration of gauze containing DBD further improved the wound healing effect, while the combination of Notch signaling inhibitor DAPT ((N- [N- (3, 5-difluorophenacetyl)-*l*-alanyl]-*s*-phenylglycinet-butyl ester)) could attenuate the improvement. Regarding to the mechanism, the expression levels of Notch1, Delta-like canonical Notch ligand 4 (Dll4), Jagged1, and Hairy Enhancer of Split-1 (Hes1) were increased by DBD, while the treatment of DAPT impaired the effect in the rats. Furthermore, we found that the high glucose (HG)-inhibited viability and tube formation were induced by DBD in human umbilical vein endothelial cells (HUVECs), in which DAPT could reverse this effect. Therefore, we concluded that DBD contributed to wound healing by the activation of Notch signaling. Our finding provides new insight into the potential role of DBD in promoting diabetic wound healing.

1. Introduction

Diabetes is one of the most severe chronic diseases that severely affects the normal life of human beings [1,2]. Diabetes frequently causes various complication, among which the diabetic wound dysfunction happens in nearly 15% of patients with diabetes and is characterized by sustained inflammation, altered angiogenesis, delayed epithelialization and abnormal secretion of protease [3]. Diabetic wound dysfunction, including the ulcers in foot or other organs, has become a heavy burden to both patients and society due

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to the high morbidity and mortality [4–8]. Therefore, exploring the pathogenesis and effective therapeutic approaches for diabetic wound dysfunction have become an important focus in diabetes research. During the diabetic wound healing process, various cells, inflammatory factors, and extracellular matrix play critical roles during diabetic wound healing-related matrix remodeling and angiogenesis [9]. Studies has revealed that increased production and secretion of inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-a (TNF-a) prevent cell migration and proliferation required for wound healing [10]. High blood glucose level suppresses the function of endothelial cells and damaged neovascularization [11]. Besides, impaired neovascularization and the following decreased blood supply are involved in diabetic wound dysfunction of patients [12,13]. Therefore, recovering the local angiogenesis and controlling inflammatory response are promising approaches for diabetic wound dysfunction treatment.

Notch1 is an evolutionally conserved signaling that participates in multiple regulatory process including cell proliferation, apoptosis, and angiogenesis [14,15]. Notch receptors family contains four members that located on cell membrane, namely Notch1 to 4, which could be activated by binding with their ligands (such as Delta-like 1, 3, 4) on adjacent cells. Activated Notch receptor releases the intracellular domain which subsequently translocate into the nucleus and regulatory gene expression [14,16]. Abnormal Notch signaling plays critical role in multiple diseases, especially cardiovascular diseases and cancers [14]. Notch signaling was also found to be dysregulated in diabetes [17–19]. Besides, Notch signaling regulates cell migration, angiogenesis, and inflammation during wound healing process [20,21]. A recent study further revealed a Delta-like 4 (DLL4)-Notch1 loop in diabetic wound healing [22].

Dang-Gui-Bu-Xue decoction (DBD) is a Chinese traditional medicine that comprise of Radix Astragali and Radix Angelicae sinensis [23], and is widely applied in treatment of diseases owing to its functions against inflammation, lipid peroxidation and oxidative stress [24–27]. In addition, DBD treatment leads to decreased level of serum glucose and improves insulin resistance in rat diabetic model [28,29]. Administration with DBD also impaired inflammatory response and downregulated levels of inflammatory cytokines including IL-1β, IL-6, NF- κB, and TNF-α [30]. In present study, we tried to decipher the function of DBD in diabetic wound healing by using rat model. We found decreased inflammation and activated angiogenesis in diabetic rat model after DBD treatment, along with modulation of DLL4-Notch1 signaling. These data presented DBD as a potential effective therapy for diabetes-induced ulceration.

2. Materials and methods

2.1. Cell culture and treatment

The Human umbilical vein endothelial cell line (HUVEC) was obtained from the American Type Culture Collection (ATCC, USA), cultured in F12 medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA), and placed at a humidified 37 °C incubator filled with 5% CO_2. HUVECs were placed in culture medium that contains high level glucose (40 mM, 72 h) to induce diabetic environment, and named as HG-HUVECs. The DBD (10 μg/ml) was obtained following the reported article [27]. The working concentration of DBDin cellular experiments is 5 mg/ml. The inhibitor against Notch (N- [N- (3, 5-difluorophenacetyl)-*l*-alanyl]-*s*-phenylglycinet-butyl ester, DAPT) (St Louis, MO, Sigma, USA) was applied at concentration of 10 μM for *in vitro* study.

2.2. Diabetic skin wound model and treatment

All experiments were approved by the Animal Ethic Committee of Hebei Agricultural University. Male Sprague–Dawley rats weighted around 220 g were purchased from the Jackson Laboratory (USA). To establish the diabetic model, rats were treated with intraperitoneal injection of Streptozotocin (STZ, Sigma, USA) (60 mg/kg) once a day for two days. Seven days later, the serum glucose level (>300 mg/dL) was measured to determine the success of model establishment. Subsequently, the rats were anesthetized and full-thickness excision wounds of 2 cm × 2 cm were created on the backs of rats. The rats were randomly separated into six groups with 6 rats in each group and received corresponding treatment. The wounds were immediately covered with two layers of gauze that contains saline (control group), rb-bFGF (100 U/cm^2) (rb-bFGF group), gavage of DBD (5 g/kg) (oral DBD group), both gavage and external use of DBD (oral + external DBT group), or gavage and external use of DBD along with subcutaneous injection of DAPT (80 μM, 200 μl) (oral + external DBD + DAPT group). The drug was changed every day. Rats in blank group received no treatment and the control group received PBS treatment.

2.3. Histological analysis

Rats were killed at day 21 after surgery when the wounds were almost partially closed. The skin tissues were fixed with formalin, embedded with paraffined, and sliced into 5 μm tissue sections. After deparaffinization and rehydration, the tissues were stained with hematoxylin and eosin, and examined by two professional pathologists. Levels of IL-6 and TNF-α were examined by incubation with anti-IL-6 (1:200, Abcam, MA, USA) and anti- TNF-α (1:200, Abcam, USA) antibodies and the follwing DAB staining. The angiogenesis was examined by immunofluorescence staining with CD31 antibody (1:200, Abcam, USA). Masson's trichrome staining (Beyotime, Guangzhou, China) was applied to examine deposition of collagens following the manufacturer's instruction. At least five images were taken in each sample by a microscope (Leica, Germany).

2.4. Cell viability

To evaluate cell viability, 5000 HUVECs or HG-HUVECs were seeded in each well of 96-well plates, and treated with PBS, DBD (10

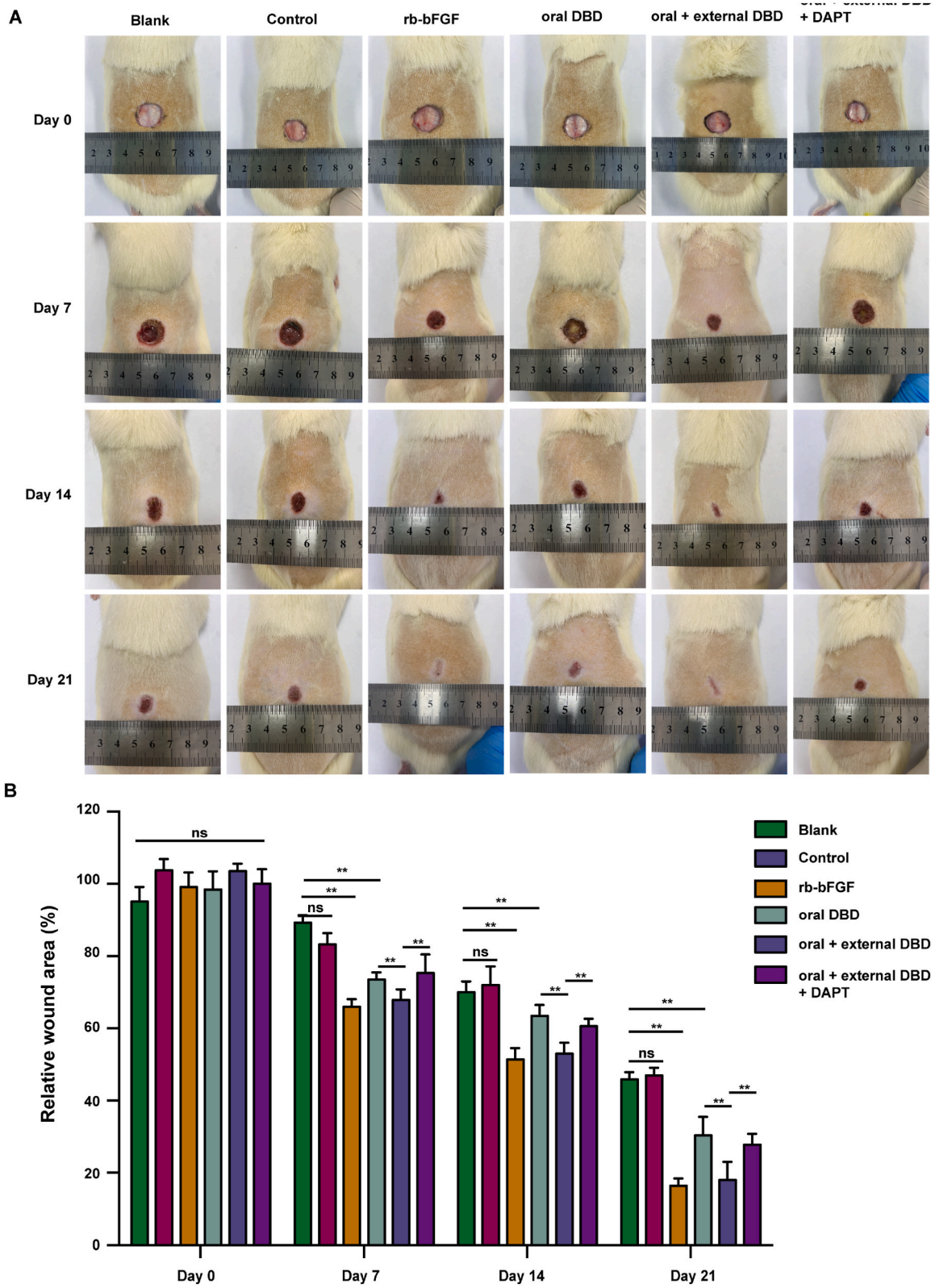

Fig. 1. DBD contributes to wound healing in diabetic rat wound model. (A and B) The representative images of full-thickness skin defects in diabetic rat wound model treated with PBS, rb-bFGF, gavage of DBD, DBD gavage and gauze containing DBD, or co-treated with DBD gavage, gauze containing DBD, and DAPT. The relative wound area was shown. Data are represented as the means ± SD (n = 6), ns no significance, **p < 0.01.

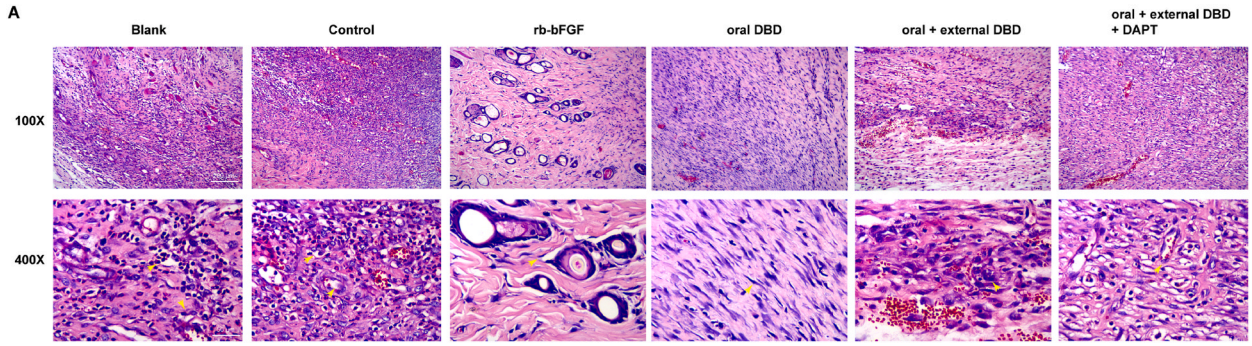

Fig. 2. DBD alleviates histopathological injury in diabetic rat wound model. (A) The representative images of H&E staining in diabetic rat wound model treated with PBS, rb-bFGF, gavage of DBD, DBD gavage and gauze containing DBD, or co-treated with DBD gavage, gauze containing DBD, and DAPT. Data are represented as the means ± SD (n = 3).

μg/ml), or DBD + DAPT (10 μM) for 48 h. After that, 10 μl cell counting kit-8 (CCK-8) reagent (Thermo, MA, USA) was added into each well and incubated at 37 °C for 1 h. The optical density at 450 nm was measured by a microplate reader (PerkinElmer, USA).

2.5. Tube formation assay

To analyze the *in vitro* angiogenic activity of HUVECs, we performed a tube formation assay in Matrigel (Corning, Corning, NY, USA). Briefly, cells (5×10^4 per well) were pretreated with high glucose, DBD and DAPT, followed by seeding in a 24-well plate pre-coated with Matrigel. Cells were culture in 37 °C incubator for 8 h, then the images of formed branches were taken by a microscope.

2.6. Western blotting

Cells derived from wound area were collected and lysed by chilled RIPA buffer containing protease and phosphatase inhibitors (Sigma). Total proteins were quantified by BCA kit (Thermo, USA), loaded, and separated by SDS-PAGE gels, transferred to NC membranes, incubated with anti-Notch1, anti-Dll4, anti-Jagged1, anti-Hes1 and anti-βactin antibodies overnight at 4 °C. Next day, the proteins were hatched with corresponding secondary anti-rabbit or anti-mouse antibody (1:5000, Abcam), and visualized by the enhanced chemiluminescence (ECL) reagents (Thermo) in a gel image system (BD Biosciences, NJ, USA). The gray values of the blots were quantified using Image J software and presented as bar charts. All antibodies were purchased from Abcam and diluted at ratio of 1:2000 in accordance with the manufacturer's protocols.

2.7. Statistical analysis

Data in this work were exhibited as mean ± SD and analyzed by Graphpad Prism 7.0 software. Statistical analysis was performed by using two-tailed Student's t-test or one-way ANOVA. The $p < 0\ 05$ was regarded as statistical significance.

3. Results

3.1. DBD contributes to wound healing in diabetic rat wound model

In order assess the impact of DBD on diabetic wound healing, we established the diabetic rat wound model and measured the wound area at day 0, day 7, day 14, and day 21. As expected, the treatment of rb-bFGF, as a positive control [31], inhibited the wound area from day 7 relative to the sham and control group in the rats (Fig. 1A and B). The gavage administration of DBD repressed the wound area compared with the sham and control group in the model from day 7 as well (Fig. 1A and B). Obviously, the co-treatment of gavage administration and the external administration of gauze containing DBD further improved the wound healing effect, while the combination of Notch signaling inhibitor DAPT was able to block the improvement (Fig. 1A and B).

3.2. DBD alleviates histopathological injury in diabetic rat wound model

Next, we evaluated the influence of DBD on histopathological alterations of wound in the rat model by H&E staining. We found that the inflammatory infiltration was decreased, and formation of granulation tissues were increased by the treatment of rb-bFGF or the gavage administration of DBD and co-treatment of gavage administration and the external administration of gauze containing DBD further improved the effect of DBD, while the combination of DAPT could reverse the effect in the model (Fig. 2A).

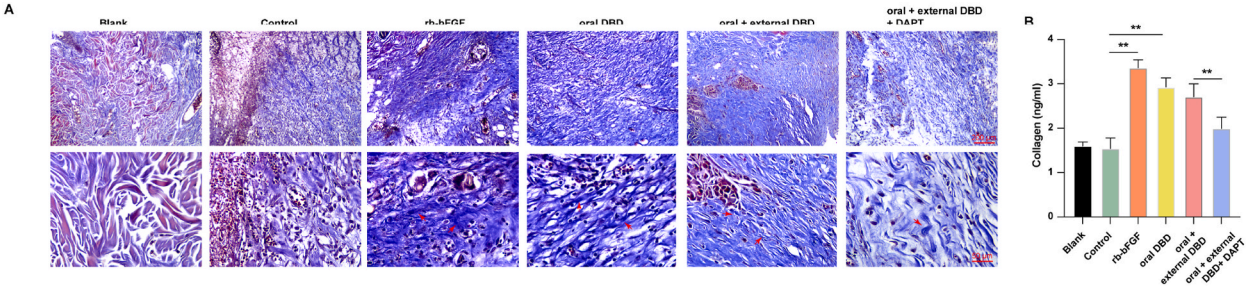

Fig. 3. DBD induces extracellular matrix (ECM) production in diabetic rat wound model. (A) The representative images of Masson's trichrome staining in diabetic rat wound model treated with PBS, rb-bFGF, gavage of DBD, DBD gavage and gauze containing DBD, or co-treated with DBD gavage, gauze containing DBD, and DAPT. (B) The collagen level in tissues was measured by ELISA. Data are represented as the means ± SD (n = 3). *p < 0.05, **p < 0.01.

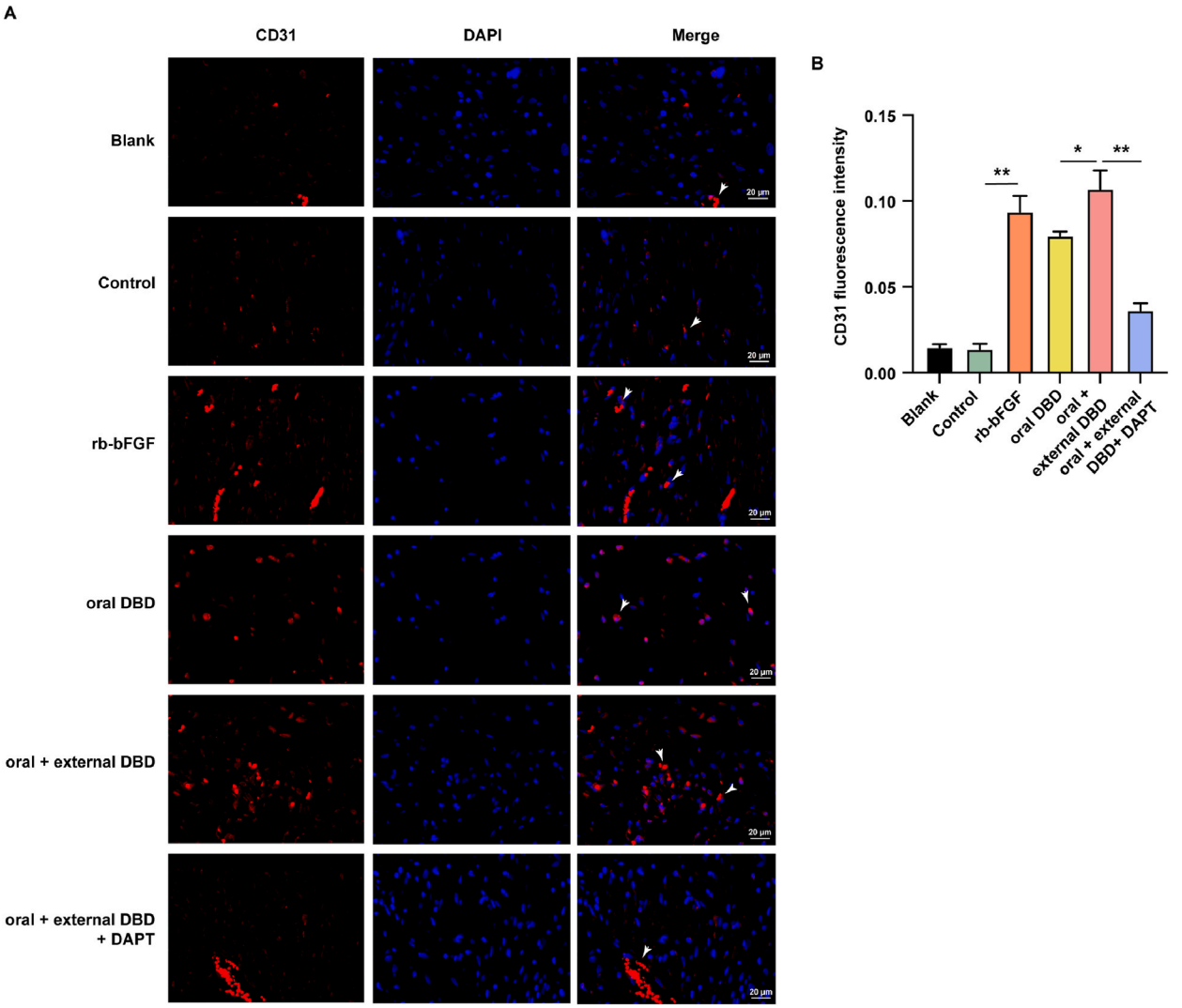

Fig. 4. DBD facilitates angiogenesis in diabetic rat wound model. The representative images of immunofluorescence staining of CD31 in diabetic rat wound model treated with PBS, rb-bFGF, gavage of DBD, DBD gavage and gauze containing DBD, or co-treated with DBD gavage, gauze containing DBD, and DAPT. (B) Relative expression of CD31 was presented as histogram. Data are represented as the means ± SD (n = 3). *p < 0.05, **p < 0.01.

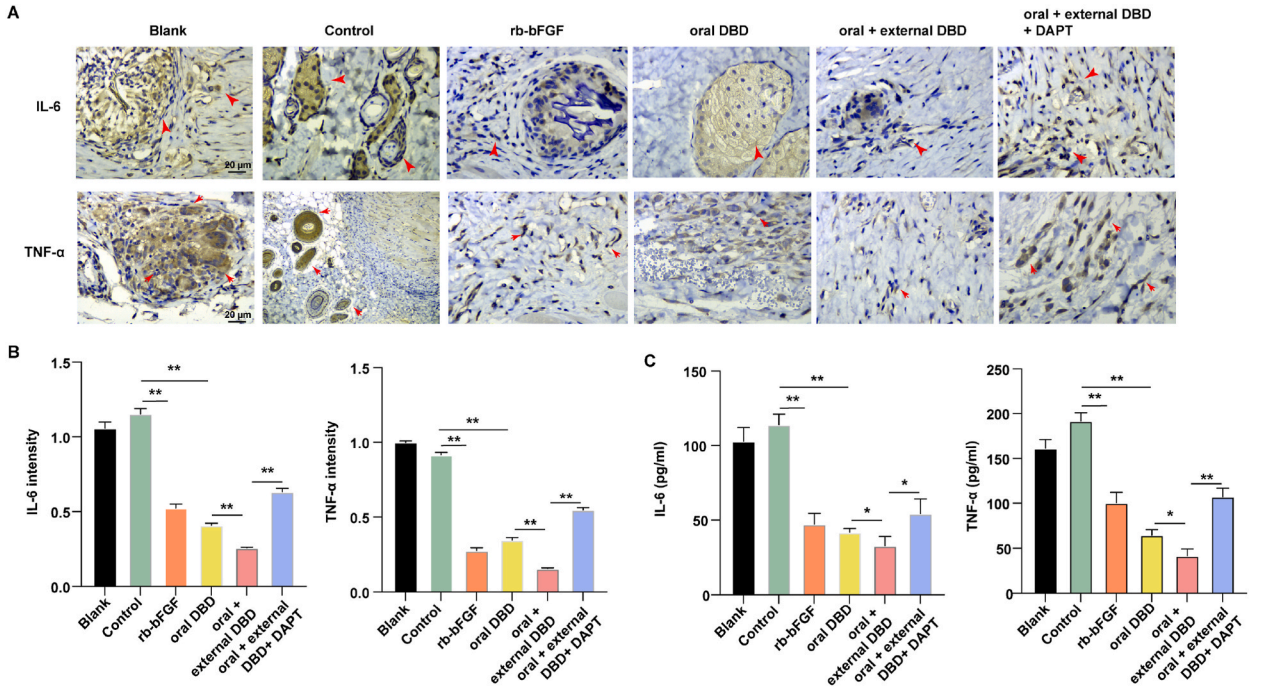

Fig. 5. DBD induces anti-inflammatory effect in diabetic rat wound model. (A) The levels IL-6 and TNF-α were analyzed by immunohistochemical in diabetic rat wound model treated with PBS, rb-bFGF, gavage of DBD, DBD gavage and gauze containing DBD, or co-treated with DBD gavage, gauze containing DBD, and DAPT. Data are represented as the means ± SD (n = 3). (B) Relative levels of IL-6 and TNF-α in IHC staining were calculated. (C) Production of IL-6 and TNF-α was measured by ELISA assay. *p < 0.05, **p < 0.01.

3.3. DBD induces extracellular matrix (ECM) production in diabetic rat wound model

Then, the function of DBD in the regulation of wound extracellular matrix (ECM) production was analzyed by Masson's trichrome staining in the rat model. We observed that organized and dense collagen were deposited and a large number of sparse and scattered collagen fibers in the wounds were identified in rb-bFGF group and gavage administration of DBD group. Consistently, co-treatment of gavage administration and the external administration of gauze containing DBD demonstrated a more efficient impact on the production of ECM, but the combination of DAPT repressed the effectiveness in the model (Fig. 3A and B).

3.4. DBD facilitates angiogenesis in diabetic rat wound model

As angiogenesis serves as an essential process in wound healing, we further determined the effect of DBD on newly formed blood vessels by analyzing the levels of CD31, a critical indicator of neovascularization, using immunofluorescence staining. We found that the treatment of rb-bFGF induced the expression of CD31 in wound area compared with the sham and control group in the rats (Fig. 4). Similarly, the gavage administration of DBD enhanced the expression of CD31 in wound area compared with the sham and control group in the model (Fig. 4). Obviously, the co-treatment of gavage administration and the external administration of gauze containing DBD further promoted the expression of CD31, in which the combination of DAPT was able to reverse the promotion in the model (Fig. 4).

3.5. DBD induces anti-inflammatory effect in diabetic rat wound model

We then investigation the anti-inflammatory effect of DBD in the rat model by analyzing the levels of inflammatory factors, including IL-6 and TNF-α. The immunohistochemical analysis revealed that the expression and production of IL-6 and TNF-α were suppressed by rb-bFGF and the gavage administration of DBD (Fig. 5A–C). Moreover, the co-treatment of gavage administration and the external administration of gauze containing DBD showed a more obvious anti-inflammatory impact, while the combination of DAPT could impair the effect of DBD in the model (Fig. 5A–C).

3.6. DBD activates Notch signaling in diabetic rat wound model

Next, we verified the association of DBD with Notch signaling in the regulation of diabetic wound healing. Interestingly, expression levels of Notch1, DII4, Jagged1, and Hes1 were enhanced by the treatment of rb-bFGF and the gavage administration of DBD in the rats

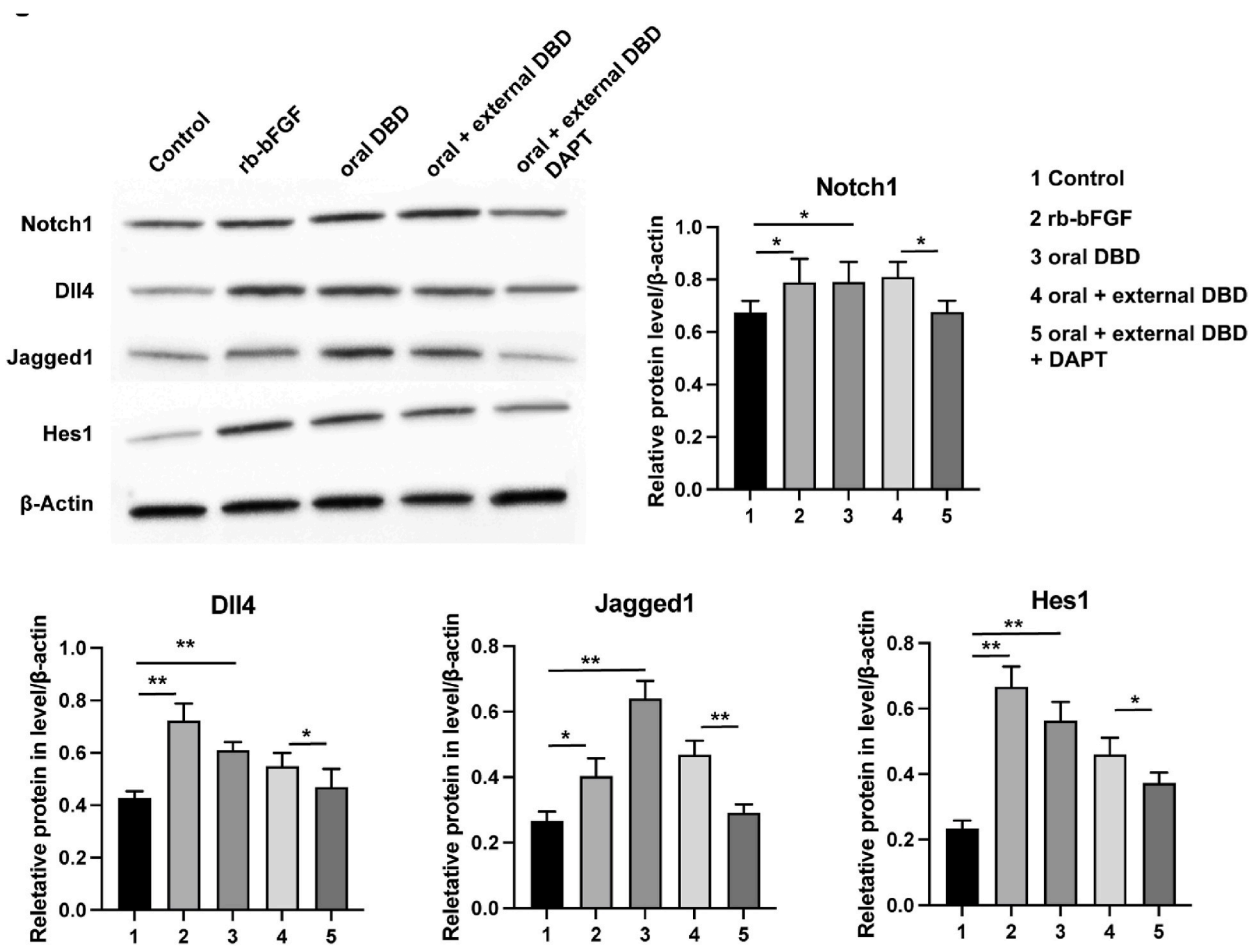

Fig. 6. DBD activates Notch signaling in diabetic rat wound model. The expression levels of Notch1, DII4, Jagged1, and Hes1 were analyzed by Western blot analysis in diabetic rat wound model treated with PBS, rb-bFGF, gavage of DBD, DBD gavage and gauze containing DBD, or co-treated with DBD gavage, gauze containing DBD, and DAPT. Data are represented as the means ± SD (n = 3). Relative levels of Notch1, DII4, Jagged1, and Hes1 were calculated. *p < 0.05, **p < 0.01.

and the co-treatment of gavage administration and the external administration of gauze containing DBD further promoted the levels compared with the single gavage administration of DBD group, while the combination of DAPT was able to reverse the promotion in the system (Fig. 6).

3.7. DBD promotes angiogenesis and proliferation by activating Notch signaling in HG-HUVECs

Next, we determined the effect of DBD by analyzing the proliferation and angiogenesis in the HG-induced HUVECs model *in vitro*. We found that the treatment of HG inhibited the cell viability of HUVECs and DBD restored the cell viability in the HG-HUVECs, in which the co-treatment of DBD and DAPT could reverse the effect of DBD (Fig. 7A). Furthermore, the numbers of tube formation were reduced in HG-HUVECs and the treatment of DBD increased the tube formation in the HG-HUVECs, while the combination of DAPT was able to impair the enhancement in the model (Fig. 7B).

4. Discussion

Diabetes is a severe chronic disease and causes the complication of diabetic wound dysfunction, which is characterized by sustained inflammation, altered angiogenesis, delayed epithelialization and abnormal secretion of protease. Various studies have been introduced for the treatment of diabetic wounds. For example, the platelet-rich plasma (PRP) and platelet gel products has been reported to accelerate the healing of chronic wounds [32]. The US Food and Drug Administration has approved only one topical-growth-factor (GF)-based therapeutic, Becaplermin (0.01% Regranex® gel), with efficacy to promote healing of diabetic foot ulcer [32]. The embryonic stem cell extracts facilitate wound closure, contraction and re-epithelialization in diabetic db/db mice [33]. DBD is a Chinese traditional medicine and widely applied in treatment of multiple diseases owing to its functions against inflammation, lipid

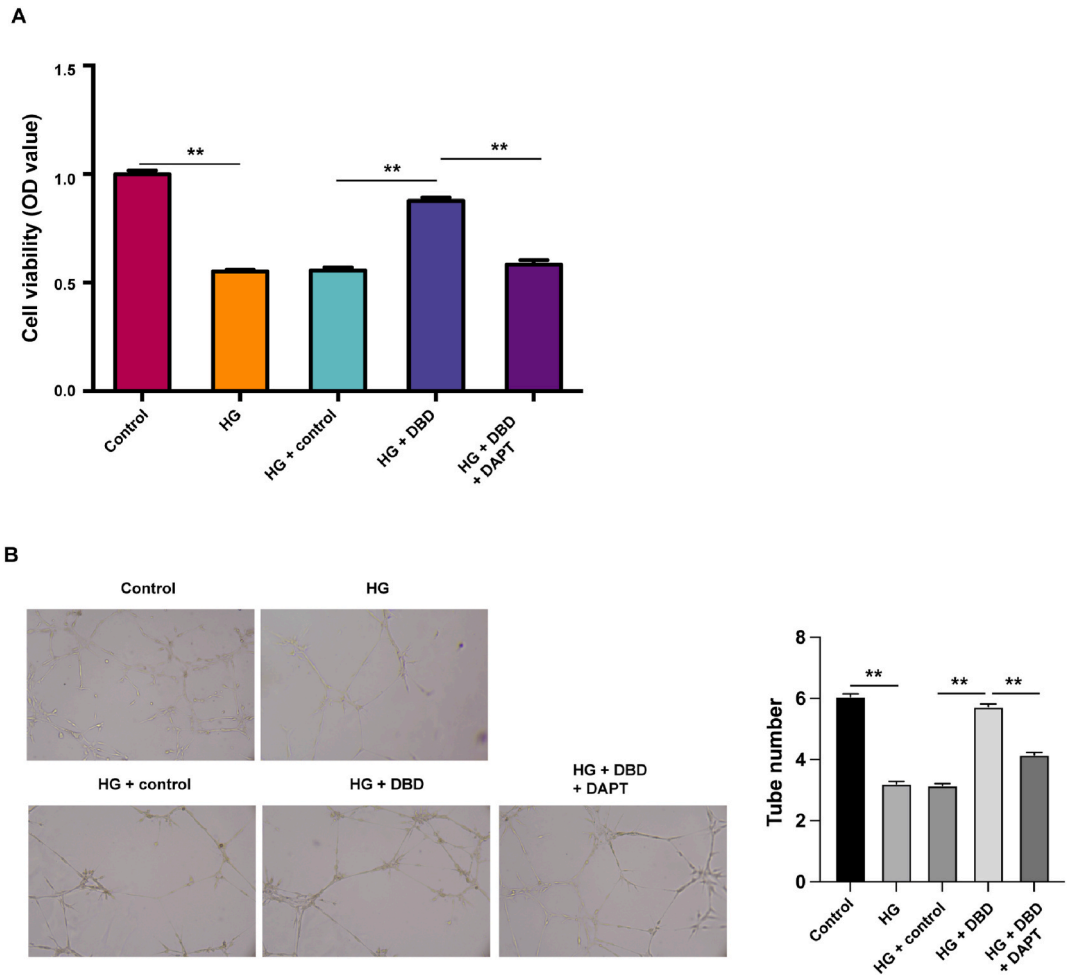

Fig. 7. DBD promotes angiogenesis and proliferation by activating Notch signaling in HG-HUVECs. (A and B) The HUVECs with or without HG were treated with DBD or co-treated with DBD and DAPT. (A) The cell viability was detected by CCK-8 assays. (B) The angiogenesis was analyzed by tube formation assay and quantified. Data are represented as the means ± SD (n = 3), **p < 0.01.

peroxidation and oxidative stress. However, the impact of DBD on diabetic wound healing remains obscure. In the present study, we identified the function of DBD in the regulation of wound healing.

The biomedical properties of DBD have widely reported in multiple investigations. It has been reported that DBD attenuates oxidant injury of H9c2 cells via modulating cellular glutathione synthesis and regeneration [34]. DBD inhibits lipid metabolic defects in diabetic atherosclerosis [35]. DBD improves the gemcitabine sensitivity of non-small-cell lung cancer cell by targeting p-glycoprotein and deoxycytidine kinase [36]. DBD protects β-amyloid-induced cell death of neurons [37]. In the present work, we found that DBD inhibited the wound area, inflammatory infiltration, and enhanced granulation tissue formation, wound (ECM production, and CD31 accumulation in the diabetic rat wound model. Furthermore, the HG-repressed viability and tube formation were promoted by DBD in HUVECs. These data suggest that DBD can improve wound healing *in vitro* and *in vivo*. Our finding provides new evidence of the crucial biomedical function of DBD in diabetic wound healing. The related clinical significance of DBD should be evaluated by more investigation.

Notch signaling contributes to the modulation of diabetic wound healing. It has been reported that platelet-rich plasma (PRP) treatment contributes to diabetic wound healing through enhancing Notch signaling in rats [39]. The (−)-epigallocatechin gallate promotes diabetic wound healing by Notch signaling [38]. Notch signaling enhances wound healing by regulating macrophage-regulated inflammation [21].The combined treatment by PRP and adipose-derived mesenchymal stem cells (ADSCs) enhance the wound closure, increased collagen production and promoted angiogenesis via modulating the Notch pathway [39]. Increasing number of studies have indicated the involvement of Notch signaling in treatment of traditional medicine for diabetic wound [40]. Our mechanism investigations demonstrated that the expression levels of Notch1, DII4, Jagged1, and Hes1 were increased by DBD, while the treatment of DAPT impaired the effect in the rats and in HUVECs. DAPT could reverse the effect of DBD on diabetic wound healing. These data imply that DBD exerted its properties by activating Notch signaling. Our finding provides new insight into the mechanism by which DBD contributes to diabetic wound healing by targeting Notch signaling. Nevertheless, further

studies such as the silencing experiments and transgenic mice would be required to better understand the mechanistic action of DBD through the Notch pathway. Besides, the Notch signaling may be just one of the multiple downstream signaling in the modulation of diabetic wound healing. Further investigation on critical pathways correlated with wound healing is necessary in future studies.

Thus, we concluded that DBD contributed to wound healing by the activation of Notch signaling. Our finding provides new insight into the potential role of DBD in promoting diabetic wound healing.

Data availability statement

Data in this study are available when emailing to the corresponding author for requisition. Data associated with your study has not been deposited into a publicly available repositor.

CRediT authorship contribution statement

Xian Zhang: Writing – original draft, Validation, Methodology, Investigation. **Song Zhao:** Validation, Investigation. **Xiaogui Zhao:** Validation, Investigation. **Zhiwei Yang:** Validation, Investigation. **Xiaodan Wang:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e26711.

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