Research Article

Vitamin D ameliorates impaired wound healing in streptozotocin-induced diabetic mice by suppressing NF-κB-mediated inflammatory genes

YiFeng Yuan, Sushant K. Das and MaoQuan Li

Department of Intervention and Vascular Surgery, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai, China

Correspondence: MaoQuan Li (106013870@qq.com)

Diabetic wounds are characterized by delayed wound healing due to persistent inflammation and excessive production of reactive oxygen species. Vitamin D, which is well acknowledged to enhance intestinal calcium absorption and increase in plasma calcium level, has recently been shown to display beneficial effects in various vascular diseases by promoting angiogenesis and inhibiting inflammatory responses. However, the role of Vitamin D in diabetic wound healing is still unclear. In the present study, we investigated the role of Vitamin D in cutaneous wound healing in streptozotocin (STZ)-induced diabetic mice. Four weeks after injection of STZ, a full thickness excisional wound was created with a 6-mm diameter sterile biopsy punch on the dorsum of the mice. Vitamin D was given consecutively for 14 days by intraperitoneal injection. Vitamin D supplementation significantly accelerated wound healing in diabetic mice and improved the healing quality as assessed by measuring the wound closure rate and histomorphometric analyses. By monitoring the level of pro-inflammatory cytokines tumor necrosis factor-α (*TNF-α*), interleukin (IL) 6 (*IL-6*), *IL-1*β) in the wounds, reduced inflammatory response was found in VD treatment group. Furthermore, nuclear factor κB (NF-κB) pathway was found to be involved in the process of diabetic wound healing by assessing the relative proteins in diabetic wounds. Vitamin D supplementation obviously suppressed NF-κB pathway activation. These results demonstrated that Vitamin D improves impaired wound healing in STZ-induced diabetic mice through suppressing NF-κB-mediated inflammatory gene expression.

Introduction

Diabetic foot ulcer is a severe, persistent complication of diabetes mellitus (DM) [1]. It is estimated that 15–25% diabetic patients suffer from lower extremity ulcers during their lifetime, which seriously affects their quality of life [2]. Delayed diabetic wound healing may induce chronic infection, neuropathy, microvascular disorder, which may cause refractive diabetic foot ulcer. Although various treatments have been applied for diabetic foot ulcer, including surgical repair, endovascular treatment, infection control, moist dressings, and wound offloading, wounds are often notably slow to heal and 7–20% of patients will end up with an amputation [3]. Therefore, novel methods or adjuvant therapies that promote diabetic wound healing are continuously being investigated to reduce the morbidity and mortality.

Vitamin D is well acknowledged to enhance intestinal calcium absorption and increase plasma calcium level [4,5]. Mounting studies have reported that Vitamin D has anti-inflammation and cardiovascular protective properties [6-8]. Vitamin D is involved in various diseases, such as autoimmune disorders and cardiovascular diseases. Furthermore, epidemiological evidences show that low Vitamin D status is involved the development of diabetic vascular diseases [9-11]. However, it is still unclear whether Vitamin

D supplementation contributes to the improvement of delayed wound healing in DM through inflammation system. In the current study, we investigated whether Vitamin D accelerates cutaneous diabetic wound healing and explores underlying mechanisms.

Materials and methods
Animals and induction of DM
Male ICR mice (6 weeks) were purchased from SLAC Laboratory Animal Co., Ltd (Shanghai, China). All animals were lodged in individual cages in the Animal Facility of Tongji University. Diabetes were induced in the mice with streptozotocin (STZ) injected intraperitoneally once, at a dose of 100 mg/kg. Normal mice were injected with only a saline vehicle. After 1 week, mice with fasting blood glucose levels higher than 250 mg/dl were considered as diabetic. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Biological Research Ethics Committee of the Chinese Academy of Sciences.

Experimental design
Each *in vivo* experiment was conducted using at least 15 mice per group. (i) Normal group: normal mice who received a saline vehicle for 14 days. (ii) Diabetic group: diabetic mice were injected with a saline vehicle for 14 days. (iii) Vitamin D treatment group (VD treatment group): diabetic mice treated with Vitamin D (100 ng/ kg per day) for 14 days.

Wound biopsy and measurement of wound closure
Diabetic wound model was created on mice after 4 weeks of STZ: all animals were anesthetized with isoflurane, and dorsum were shaved and sterilized. With a disposable 6 mm skin biopsy punch and Westcott scissor, full thickness excisional wounds were made on the dorsum. Wounds in individual mice were photographed digitally every 3 days until the end (14 days). Pictures were taken by digital camera (EOS50; Cannon, Japan) and the ulcer area was analyzed by ImagePro Plus 4.5 software. The rate of wound closure was defined as the ratio of the wound size to the initial wound size. A smaller wound ratio indicated faster wound closure.

Histological assessment of wound healing
The wounds, together with unwounded skin margins collected from each group, were fixed in paraffin, and sectioned at 5.0 μm. The sections were dehydrated with successive concentrations of ethanol and washed twice in distilled water. The sections were stained with Hematoxylin and Eosin (H&E) and Masson's trichrome, in accordance with the protocols of the manufacturer (Cyagen Biosciences Inc.), to detect the re-epithelialization/granulation tissue formation and collagen deposition, respectively. The percentage of re-epithelialization (distance traversed by epithelium over wound from wound edge/distance between wound edge) was calculated for two sections per wound and was averaged over sections to provide a representative value for each wound. The average granulation thickness was measured in the same sections by dividing the wound bed area by the wound length.

The sections were also incubated with the primary antibody against CD31 (1:50, Abcam, U.S.A.) to observe angiogenesis, ki67 (1:50, Abcam, U.S.A.) to observe proliferation in the ulcerative tissues, MPO (1:50, Abcam, U.S.A.), and CD68 (1:50, Abcam, U.S.A.) to observe infiltration of neutrophils and macrophages in the ulcerative tissues. For evaluation of staining, the histological sections were observed and analyzed under a microscope (Leica DMR 3000; Leica Microsystems) by three blinded, experienced investigators. The overview of the positive-signal density was scored semiquantitatively as 1 (absent), 2 (low), 3 (medium), 4 (strong), and 5 (very strong).

Measurement of tumor necrosis factor-α (**TNF-α**)**,** interleukin (IL) 6 (**IL-6**)**,** **IL-1**β in ulcerative tissues: mice wounds were harvested and homogenized in cold PBS supplemented with protease inhibitor cocktail (Sigma–Aldrich) by using a Dounce homogenizer, and then sonicated and centrifuged at 10000 rpm for 20 min at 4°C. Supernatants were used for the ELISA of IL-1β (R&D Systems), IL-6 (R&D Systems), and TNF-α (R&D Systems).

Western blot analysis
The ulcer samples were homogenized in a tissue protein extraction reagent (RIPA buffer: PBS supplemented with 135 mmol/l NaCl, 20 mmol/l Tris, 2 mmol/l EDTA, and 1 mmol/l PMSF, BI Yun Tian, China). Lysates were centrifuged at 12000 rpm for 20 min at 4°C, and then the supernatants were collected for Western blot analysis. The protein concentration of the supernatants was determined by the BCA protein assay kit (Beyotime Biotechnology, Shanghai,
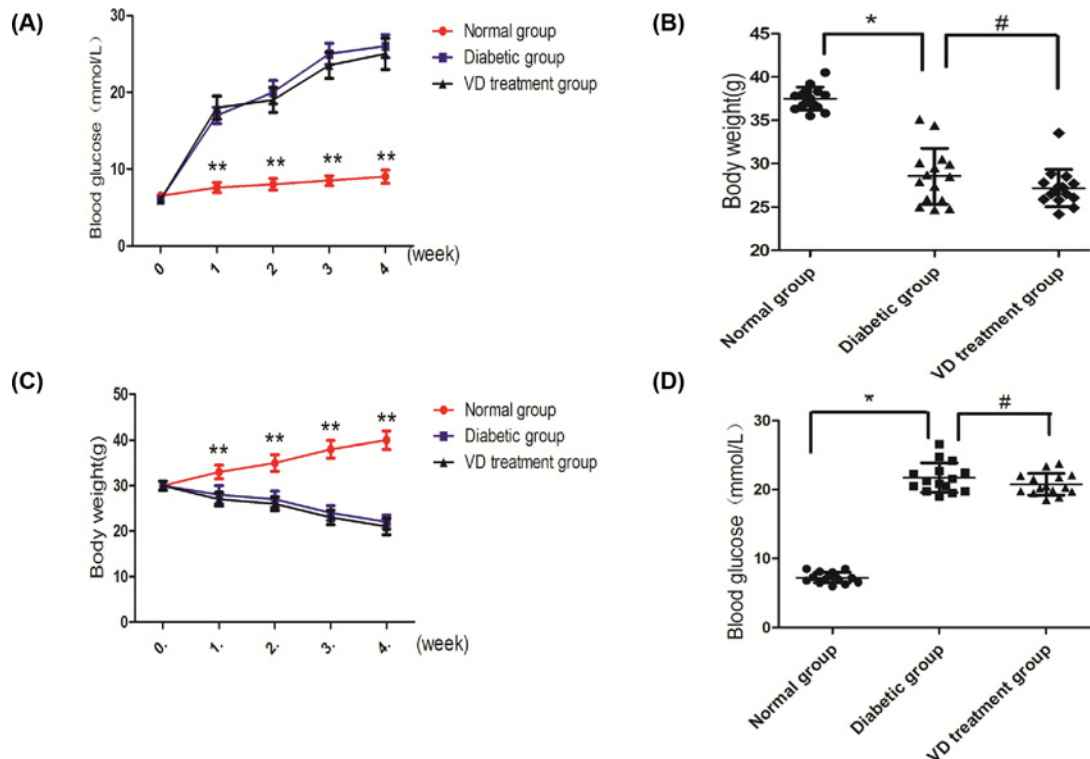
Figure 1. Establishment of STZ-induced diabetic mice

The mice with blood glucose greater than 16.7 mmol/l were defined as diabetic. Blood glucose levels (**A,B**) monitored during the study period. The level of blood glucose was significantly higher in both Diabetic group and VD treatment group compared with Normal group (*$P<0.05$,**$P<0.01$). There was no significant difference between Diabetic group and VD treatment group (#$P>0.05$). Body weight (**C,D**) was significantly decreased in the Diabetic group and VD treatment group compared with Normal group (*$P<0.05$,**$P<0.01$). No significant difference between the Diabetic group and VD treatment group was seen (#$P>0.05$). The data are expressed as the means $\pm$ S.D. ($n=15$ per group, **$P<0.01$, compared with Normal group, #$P>0.05$, compared with Diabetic group). .

China). Protein samples were separated with SDS/PAGE and transferred on to PVDF membranes (Millipore, Marlborough, MA, U.S.A.). The proteins were separated by 10–12% SDS/PFGE and transferred on to a 0.45-µm PVDF membrane, which was then blocked in 5% skim milk for 1 h at room temperature, and incubated with primary antibodies against P-IκBα, IKKβ, IKKα, P-IKKα/β, nuclear factor κB (NF-κB) (p65), P-NF-κB (p65), and GAPDH (Abcam, U.S.A.) at 4°C overnight. The membranes were then washed three times with TBS-Tween 20 and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The expression of various proteins was subsequently visualizd by ECL. Housekeeping protein GAPDH was used as a reference control. The density of each band was quantitated using Quantity One software and normalized to their respective controls.

Statistical analysis

Data are expressed as mean $\pm$ S.D. Differences between experimental groups were assessed by Student's *t* test or two-way ANOVA. For all statistical analyses, $P<0.05$ was considered to be statistically significant.

Results
Effect of Vitamin D on blood glucose level and body weight in diabetic mice

During the treatment period, blood glucose levels (Figure 1A,B) of mice in Diabetic group and VD treatment group were significantly higher than those of mice in Normal group ($P<0.01$), while no significant difference was observed between Diabetic group and VD treatment group ($P>0.05$), indicating that Vitamin D had no effect on blood glucose.
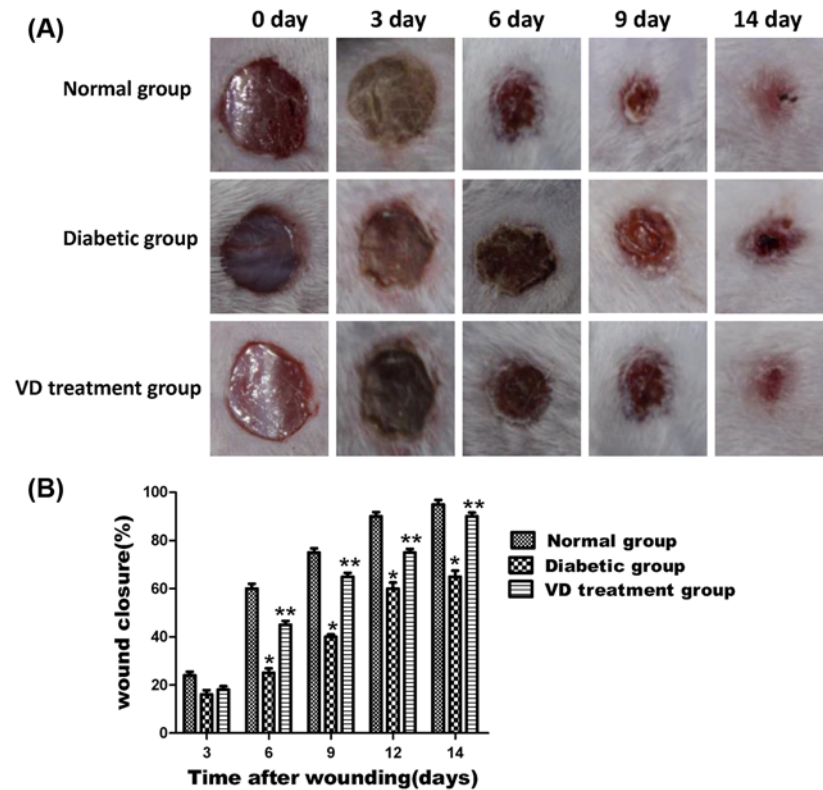
Figure 2. Vitamin D treatment accelerated diabetic wound healing

(**A**) Six millimeters diameter wounds were created by punch biopsy, and the closure of the wound area was measured by digital camera every 3 days until day 14. (**B**) Percentage of wound closure (means $\pm$ S.D.). Healing of diabetic wounds significantly delayed compared with normal wounds. Vitamin D began to improve diabetic wound closure on day 6. At the end of observation (14 days), VD treatment group exhibited improved wound healing, compared with Diabetic group. *$P<0.05$, compared with Normal group, **$P<0.05$, compared with Diabetic group.

Besides blood glucose level, body weight of mice (Figure 1C,D) was also detected. Body weight of mice in Diabetic group and VD treatment group were significantly lower than those of mice in Normal group ($P<0.05$), whereas no significant difference was observed between Diabetic group and VD treatment group ($P>0.05$), indicating that Vitamin D also had no effect on mice weight.

Supplementation of Vitamin D accelerated wound healing

The representative ulceration images for each group are presented (Figure 2A). Figure 2B shows the wound closure of ulceration at relevant time points. By the end of observation (D14 after wounding), normal wounds completely healed, while most of the diabetic wounds remained open with a low average closure rate of 60%. We provide the main-text citation for Figure 2A and Figure 3C and D. Vitamin D supplementation significantly improved diabetic wound closure rate and increased the healing rate of diabetic wound by 20.5%.

Supplementation of Vitamin D improved wound healing

Re-epithelialization was measured at D14 after wounding by the histomorphometric analysis of sections stained with H&E. As Figure 3A and 3C show: at the end of observation, wounds were not fully re-epithelialized in the Diabetic group, while wounds got close to fully re-epithelialized in the Normal group. With Vitamin D supplementation, the epithelia in VD treatment group were significantly longer compared with the Diabetic group. Figure 3B and 3D show that collagen formation in the ulcerative tissues at D14 was assessed by Masson's trichrome staining. Less amount of collagen deposition organized in aligned fibers in the Diabetic group, Vitamin D supplementation improved collagen deposition in ulcer tissues compared with Diabetic group.
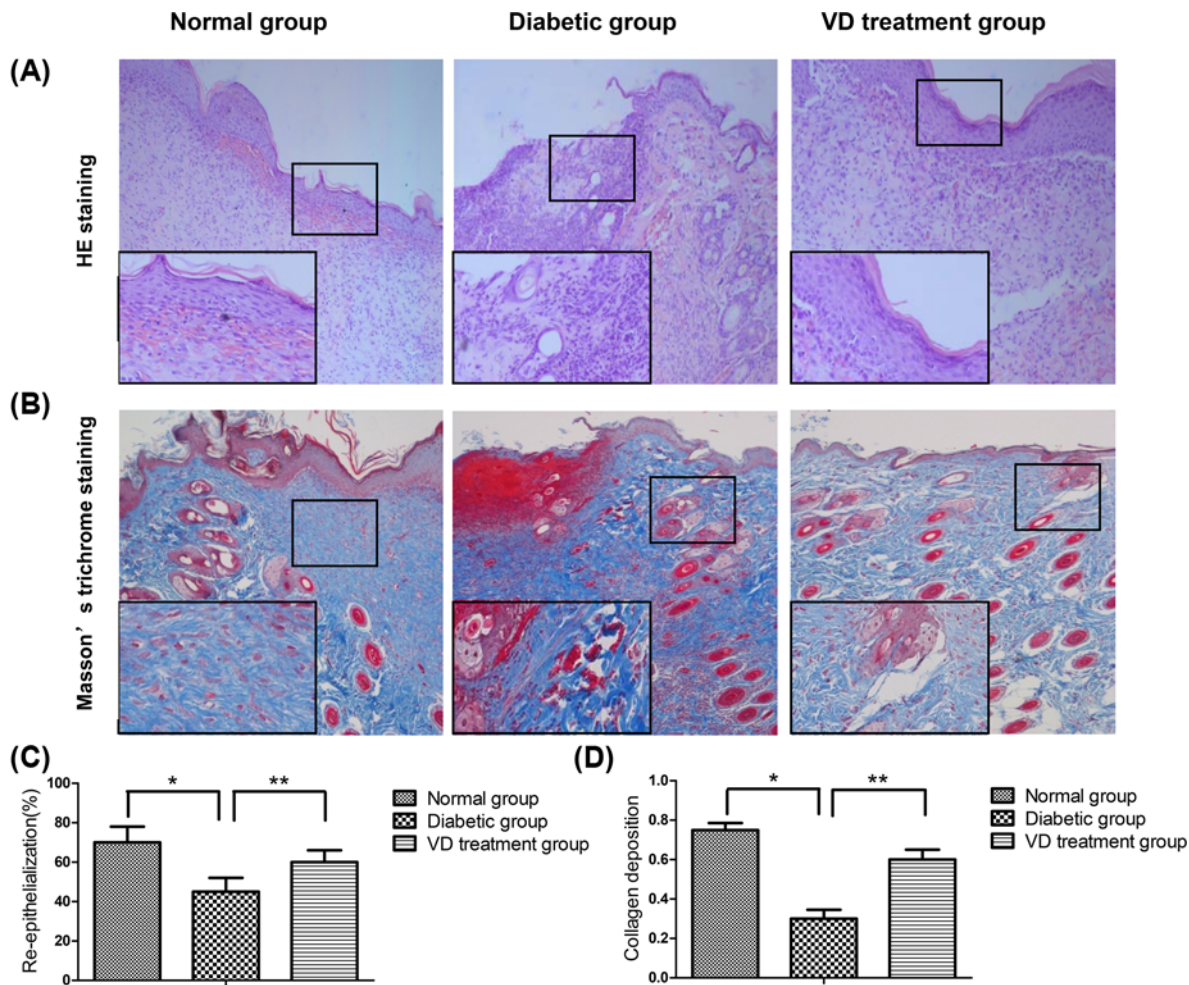
Figure 3. Effects of Vitamin D on the epithelialization and collagen deposition in ulcerative tissues at D14 after treatment
(**A**) H&E staining of sections showed better dermal re-epithelialization on the diabetic wounds in VD treatment group compared with Diabetic group.(B) Collagen deposition assessed by Masson's trichrome staining. Original magnification ×100 and inset magnification ×200. (**C and D**) Statistical re-epithelialization and thickness of collagen deposition of wounds by computer-assisted morphometric analysis. Data are represented as means $\pm$ S.D. *$P<0.05$, compared with Normal group, **$P<0.05$, compared with Diabetic group.

Supplementation of Vitamin D reduced inflammation cells infiltration, stimulated cellular proliferation, and augmented neovascularization (CD31) on the wound bed.

MPO and CD68

Populations of neutrophil and macrophage at the wound site were assessed by determining the molecular markers MPO (neutrophil) and CD68 (macrophage) on day 14. The infiltration of neutrophils and macrophages in diabetic wounds was much stronger compared with normal wounds. Administration of Vitamin D led to a significant resolution of neutrophils and macrophages in wound beds on day 14. Ki67: cellular proliferation in the wound tissues may contribute to the ulcer healing, we investigated whether Vitamin D treatment promoted cellular proliferation by ki-67 staining. The ki-67 positive cells were distributed diffusely in the basal layer of epidermis of the VD treatment group but less in the Diabetic group. The mean density of ki-67 expression was significantly higher in the VD treatment group compared with Diabetic group. Neovascularization is an essential event in the healing of wounds. We evaluated the neovascularization by immunostaining of endothelial marker CD31. The vessel density of diabetic wounds was significantly decreased compared with normal wounds ($P<0.05$). Vitamin D supplementation significantly increased neovascularization of diabetic wounds, demonstrated by increased CD31 staining (Figure 4).
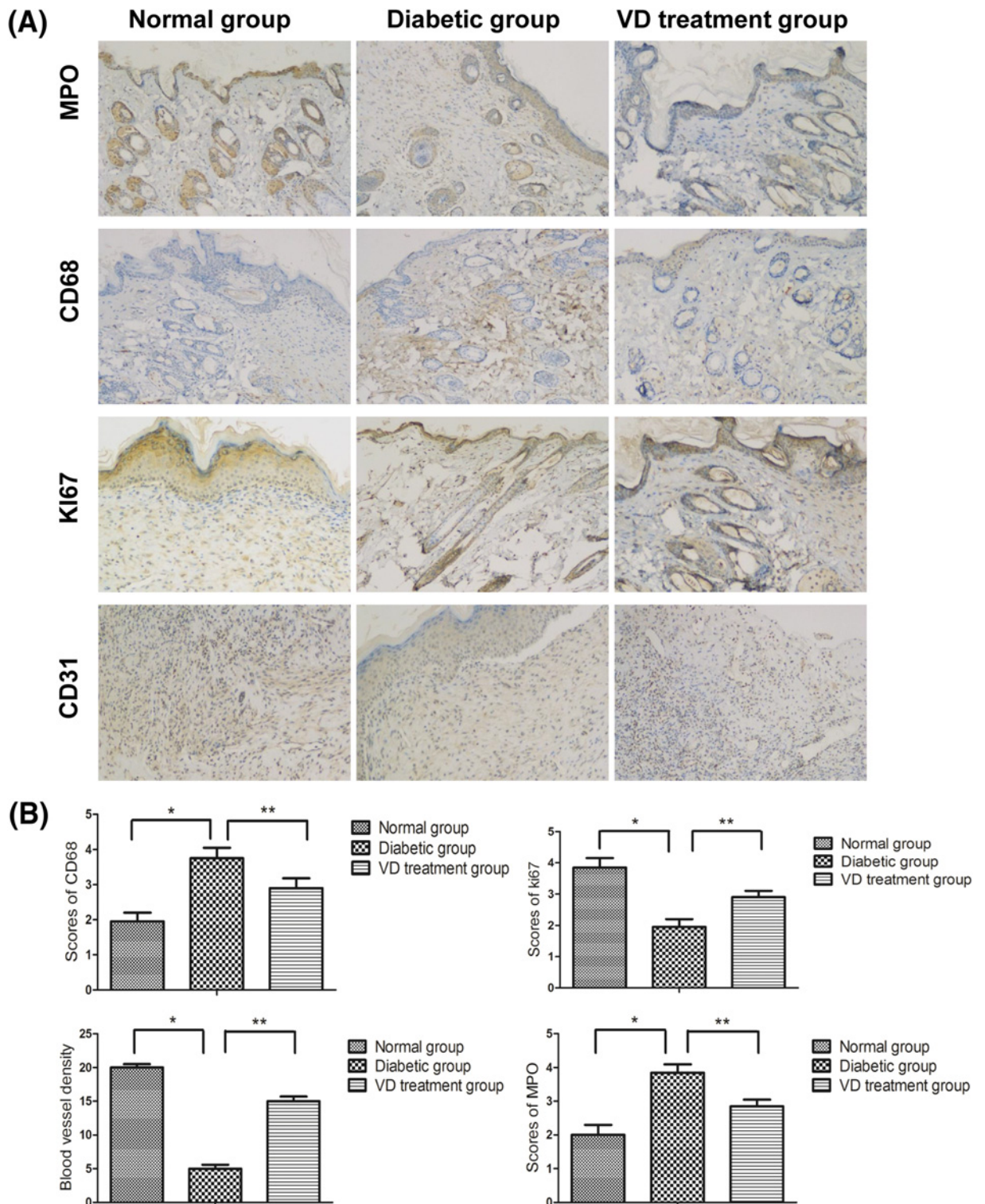
Figure 4. Vitamin D reduced inflammation cells infiltration, stimulated cellular proliferation, and augmented neovascularization on the wound bed

(**A**) Populations of neutrophil and macrophage at the wound site by determining the molecular markers MPO (neutrophil) and CD68 (macrophage) at day 14. Cellular proliferation in the wound tissues was detected with immunostaining of Ki67. The neovascularization of wounds was detected by immunostaining of endothelial marker CD31. (**B**) Scores of MPO , CD68, ki67, and CD31 staining; n=5 for each group. Data are represented as means $\pm$ S.D. *P<0.05, compared with Normal group, **P<0.05, compared with DM group.
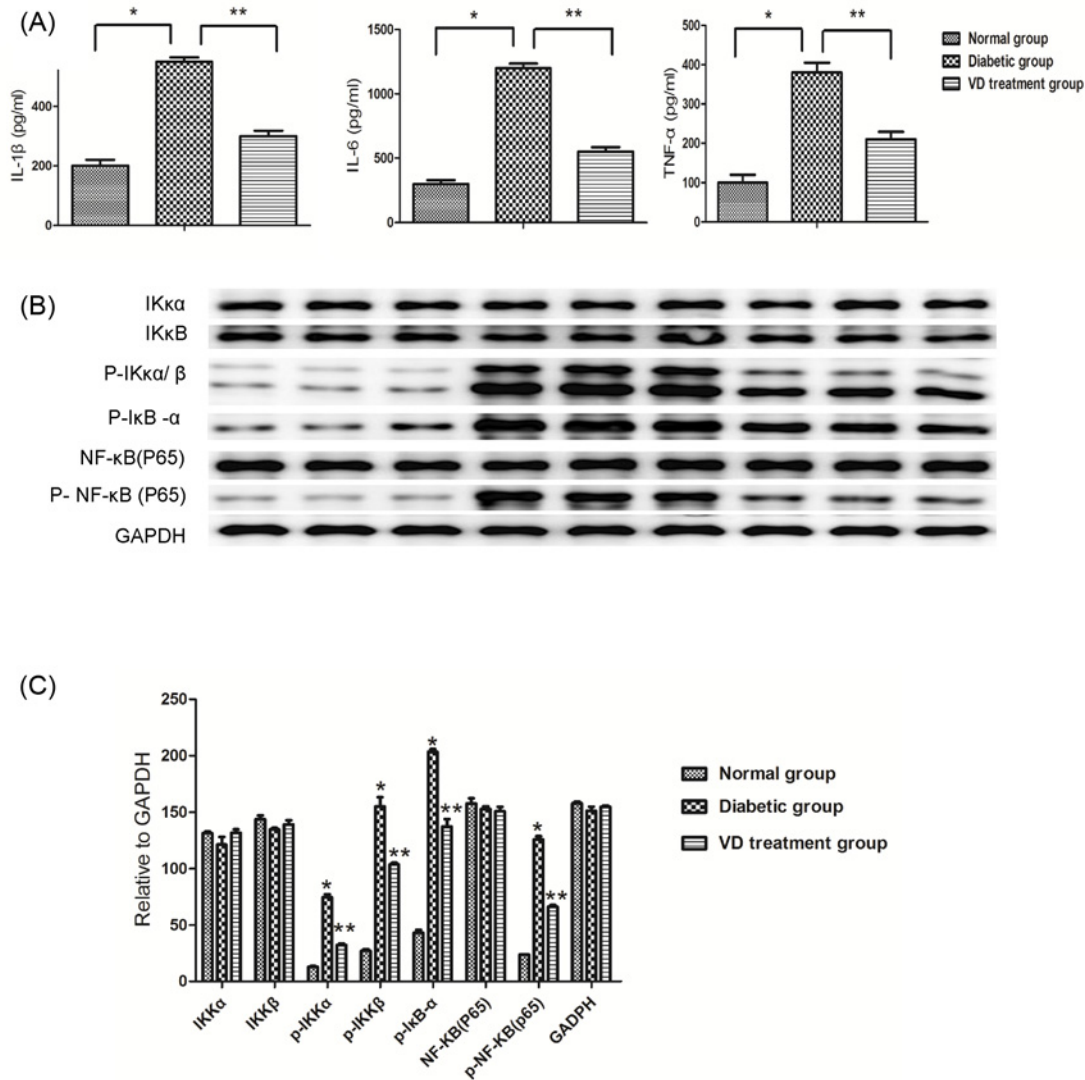
Figure 5. Vitamin D significantly down regulated pro-inflammatory cytokines and effect on NF-κB pathway in diabetic wounds

(**A**) The expression of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in the day 14 wounds of different groups were measured with ELISA. The expression level of pro-inflammatory cytokines were significantly down-regulated in VD treatment group, compared with diabetic group ($P<0.05$). (**B**) Expressions of p-IKKα/β, IKKα, IKKβ, P-NF-κB (p65), NF-κB (p65), P-IKBα in ulcerative tissues (D14) were assessed by Western blot. (**C**) Quantitation of p-IKKα/β, IKKα, IKKβ, P-NF-κB(p65), NF-κB(p65), P-IKBα expression. All data present means ± S.D. *$P<0.05$, compared with Normal group, **$P<0.05$, compared with Diabetic group.

Vitamin D decreased pro-inflammatory factors and effected NF-κB pathway in wounds

Vitamin D stimulated inflammation resolution in diabetic wounds. Therefore, the effect of Vitamin D on inflammatory cytokines expression on gene levels was examined (Figure 5A). On day 14 after wounding, expression of inflammatory cytokines, such as IL-1β, IL-6, and TNF-α was significantly higher in diabetic wounds than in normal wounds ($P<0.05$), and Vitamin D supplementation significantly decreased these inflammatory cytokines expression.

TNF-α, IL-6, IL-1β is well known as downstream inflammatory genes of NF-κB pathway. It is reasonable to postulate that NF-κB pathway is involved in the process of wound healing. In the current study, NF-κB pathway related proteins were detected in ulcerative tissues by Western blot analysis (Figure 5B,C). The expression of P-NF-κB(p65), p-IKKα/β, and P-IKBα were significantly increased in DM group, compared with Normal group ($P<0.05$). That

means NF-κB pathway is activated in the process of diabetic wound healing. The imbalanced inflammatory response may lead to delayed diabetic wound healing. According to our previous assumption, Vitamin D may suppress NF-κB activation to improve diabetic wound healing. The results supported the assumption as the level of p-IKKα/β, P-IKBα, P-NF-κB (p65) was significantly decreased in VD treatment group, compared with Diabetic group ($P<0.05$).

Discussion

Delayed wound healing is a hallmark of diabetes, leading to prolonged hospitalization and lower extremity amputation. Even with the best conservative treatment, diabetic wounds are often notably slow to heal and 7–20% of patients will subsequently need an amputation despite undergoing standard care treatment [3]. Therefore, new treatments or adjuvant therapy is an urgent clinical demand.

Various factors may lead to delayed diabetic wound healing, such as uncontroled hyperglycemia, imbalanced inflammation, vascular diseases, and neuropathies [12-15]. One pathogenic abnormality can lead to another, developing vicious cycles of pathogenicity in the diabetic ulcers. Diabetes impairs wound healing through magnifying the inflammatory response, inhibiting angiogenesis, and decreasing extracellular matrix deposition [16]. Inflammation is the first phase and plays a vital role in the recovery mechanism. Usually, inflammation response gradually subsides less than 5 days after wounding [17]. However, abnormal microenvironment, such as hyperglycemia and oxidative stress, induce excessive inflammatory response, and may lead to prolonged inflammation. As shown in the present study, by 14 days after wounding, neutrophils and macrophages were still abundant in diabetic wounds, while non-diabetic wounds already moved to the tissue remodeling phase. Pro-inflammatory cytokines (***IL-1***β, *IL-6, TNF-α*) were detected in different groups by 14 days after wounding. The outcomes showed excessive pro-inflammatory cytokines production in DM group, compared with Normal group. All those outcomes revealed that persistent inflammatory response in diabetic wounds contribute to the delayed wound healing.

Vitamin D is well known as a regulator of epidermal and hair follicle differentiation. Tian et al. [18], observed that topical 1,25(OH)$_2$D enhanced wound healing. Luderer et al. [19], observed that in the global Vitamin D receptor (VDR) knockout mouse, there was a reduction in TGF-β signaling in the dermis. Oda et al. [20] observed that re-epithelialization is impaired when the deletion of VDR is accompanied by a low calcium diet. Besides that, several studies documented an anti-inflammatory effect of Vitamin D in variety of cell types, including endothelial cells [21,22], dentritic cells [23,24], T cells [25], and macrophages [26], which was in part linked to an inhibition of NF-κB activation and signaling [27]. Mounting researches observed that Vitamin D supplementation has a positive effect on diabetic wound healing. But the true mechanism is still uncertain, especially the relationship between Vitamin D and NF-κB pathway.

In the present study, inflammatory response in diabetic ulcer tissues is more obvious than tissues in Normal group. It supported the inflammation impairment mechanism. Pro-inflammatory cytokines were significantly decreased in VD treatment group, compared with Diabetic group. IL-1β, IL-6, and TNF-α are well known as downstream inflammatory genes of NF-κB pathway. It is reasonable to presume that NF-κB pathway is involved in diabetic wound healing. NF-κB pathway related proteins were detected in ulcer tissues by Western blot analysis. The level of p-IKKα/β, P-IKBα, P-NF-κB (p65) was significantly increased in Diabetic group, which imply that NF-κB pathway is activated in the process of diabetic wound healing. In VD treatment group, the expression of p-IKKα/β, P-IKBα, P-NF-κB (p65) was significantly down-regulated, compared with Diabetic group. These outcomes supported our presumption: Vitamin D supplementation significantly suppressed NF-κB pathway activation and its downstream inflammatory genes expression, which improve diabetic wound healing.

In summary, the presents study showed that Vitamin D has a positive effect on diabetes-impaired wounds. The improved wound healing is associated with reduced inflammation in diabetic wounds. Therefore, the supplementation of Vitamin D could provide an alternative and effective approach for refractive diabetic ulcer.

Limitation

In our study, the animal model is established with full thickness excisional wounds of STZ mice, which is characterized of acute inflammation (within 2 weeks). It does not completely coincide with natural clinical course of diabetic ulcer.

Author contribution

YiFeng Yuan contributed to study concepts and design; manuscript editing; manuscript preparation. Sushant K. Das contributed to statistical analysis; experimental studies/data analysis; MaoQuan Li was responsible for the conception and design of the study, and gave final approval for the article to be published. All authors read and approved the final version of the manuscript.

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Competing interests
The authors declare that there are no competing interests associated with the manuscript.

Abbreviations
DM, diabetes mellitus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, Hematoxylin and Eosin; HRP, Horseradish Peroxidase; IkB, Inhibitor of nuclear factor factor kappa-B; ICR, Institute of Cancer Research; IKK, Inhibitor of nuclear factor factor kappa-B kinase; IL, interleukin; MPO, myeloperoxidase; NF-κB, nuclear factor κB; PFGE, Pulsed Field Gel Electrophoresis; RIPA, Radio-Immunoprecipitation Assay; STZ, streptozotocin; TGF-β, Transforming growth factor beta; TNF-α, tumor necrosis factor-α; VDR, Vitamin D receptor; VD treatment group, Vitamin D treatment group.

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