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Original Article

Mepenzolate bromide promotes diabetic wound healing by modulating inflammation and oxidative stress

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Abstract: Diabetic wounds are characterized by persistent inflammation and the excessive production of reactive oxygen species, thus resulting in impaired wound healing. Mepenzolate bromide, which was originally used to treat gastrointestinal disorders in clinical settings, has recently been shown to display beneficial effects in chronic obstructive pulmonary disease and pulmonary fibrosis of a mouse model by inhibiting inflammatory responses and reducing oxidative stress. However,the role of mepenzolate bromide in diabetic wound healing is still unclear. In this study, full-thickness excisional skin wounds were created on the backs of db/db mice, and mepenzolate bromide was topically applied to the wound bed. We found that mepenzolate bromide significantly promoted diabetic wound healing by measuring wound closure rate and histomorphometric analyses. Further studies showed that inflammation was inhibited by assessing the number of macrophages and levels of pro-inflammatory cytokines and pro-healing cytokines in the wounds. Furthermore, oxidative stress was reduced by monitoring the levels of MDA and H_2O_2 and the activities of glutathione peroxidase and catalase in the wounds. These results demonstrated the potential application of mepenzolate bromide for treating diabetic ulcers and other chronic wounds in clinics.

Keywords: Mepenzolate bromide, wound healing, diabetes, inflammation, oxidative stress

Introduction

Diabetic foot ulcer is one of the most serious complications of diabetes mellitus [1]. Approximately 25% of diabetic patients suffer fromdiabetic lower extremity ulceration throughout their lives [2]. These wounds tend to heal slowly and frequently reoccur. Moist dressings, debridement, infection control, and wound offloading are standard therapies for managing diabetic lower extremity ulcers. Even with the best conservative treatment, these wounds are often notably slow to healand 7%-20% of patients will subsequently need an amputation despite undergoingstandard care treatment [3]. Therefore, new treatments are neededfor diabetic wounds to prevent foot ulcers from leading to amputations.

Although the potential mechanisms of impaired diabetic healing are not yet well-understood, a common featureof a poorly healing wound is persistent inflammation with protracted accumulation of macrophages and elevated levels of pro-inflammatory cytokines [4-6]. Further anti-inflammation treatment improves diabetic wound healing [7]. Another important factor for impaired diabetic wound healing is excessive and prolonged reactive oxygen species (ROS) production [8]. Dhall [9] reports that an elevated level of oxidative stress leads to the formation of chronic wounds, which is then reversed by antioxidant treatment.

It has a great meaning to develop some new indications for medicines from a library of approved drugs. Considering that the safety aspects of approved drugs have already been well studied in humans, this method greatly decreases the risks for unexpected adverse effects in humans. Mepenzolate bromideis originally used for gastrointestinal disorders in a clinical setting (such as peptic ulcers and irritable bowel syndrome) [10, 11]. Recently, scientists from Japan have also reported that mepenzolate bromide displays beneficial effects in chronic obstructive pulmonary disease and pulmonary fibrosis by preventing inflammatory

Table 1. Primers for qPCR

Gene	Forward sequence	Reverse sequence
TNF-α	TGTCTCAGCCTCTTCTCATT	AGATGATCTGAGTGTGAGGG
IL-1β	GCAGGCAGTATCACTCATTG	CACACCAGCAGGTTATCATC
IL-6	ATGAAGTTCCTCTCTGCAAGAGACT	CACTAGGTTTGCCGAGTAGATCTC
TGF-β1	CCACCTGCAAGACCATCGAC	CTGGCGAGCCTTAGTTTGGAC
IGF-1	CACATCATGTCGTCTTCACACC	GGAAGCAACACTCATCCACAATG
VEGF	CTGCCGTCCGATTGAGACC	CCCCTCCTTGTACCACTGTC
GAPDH	AGAACATCATCCCTGCATCC	TCCACCACCCTGTTGCTGTA

responses and reducing oxidative stress [12-14]. However, whether mepenzolate bromide plays a role in inflammation and ROS-related diabetic wound healing is still unclear.

In this study, full-thickness excisional skin wounds were created on the backs of db/db mice, and mepenzolate bromide was topically applied to the wound bed. The efficacy of mepenzolate bromide was evaluated by measuring wound closure rate and histomorphometric analyses. To further study the related mechanisms, inflammation in the wound was evaluated by measuring the number of macrophages and levels of pro-inflammatory and pro-healing cytokines. Oxidative stress in the wound was assessed by monitoring levels of malondialdehyde (MDA) and H_2O_2 and activities of glutathione peroxidase (GSH-Px) and catalase (CAT).

Materials and methods

Animals, wound model, and treatment

All animal procedures were performed in accordance with the guidelines of the institutional animal care and use committee of the Second Military Medical University, Shanghai, China. The male db/db mouse (C57BL/KsJ, leptin receptor-deficient diabetes, 8-12 weeks, purchased from Slac Laboratory Animal Co. Ltd., Shanghai, China), was a well-established type 2 diabetic animal model with continuous hyperinsulinemia and high plasma glucose levels. One criterion for inclusion was a blood glucose level of more than 300 mg/dl. Mice were anesthetized with 1% sodium pentobarbital intraperitoneally. Two 8 mm full-thickness excisional splint wounds were inflicted on the dorsal skin of each mouse as previously described [15]. The mice were randomized equally into 2 groups: the mepenzolate group and saline group. A total of 600 μgkg^{-1} mepenzolate bromide (Sigma-Aldrich) in 30 μl saline or only saline was topically applied to the wound bed for 3 consecutive days. This treatment was applied 3 days after wounding to allow the initial inflammatory response to proceed normally. The mepenzolate bromide dose was determined in preliminary experiments: 200, 600, and 1000 μg doses were tested, and the 600 μg dose was the minimum dose of achieving the greatest acceleration of wound healing. Photographs of wounds were taken regularly from a certain distance at different time points. The wound healing rate was calculated using Image-Pro Plus Software.

Tissue harvesting in mice

The skin tissues were collected on day 10 and the wounds were excised with the wound margins. The tissues were fixed in 10% formalin for 24 hours and embedded in paraffin for further hematoxylin and eosin (HE) staining and immunohistochemistry staining.

Wound healing assays

Re-epithelialization and granulation tissue thickness were measured atday 10 after wounding by the histomorphometric analysis of sections stained with hematoxylin and eosin (HE). Digital images were obtained using a microscope (Leica, German) and analyzed using Image-Pro Plus Software. The percentage of re-epithelialization [(distance traversed by epithelium over wound from wound edge/distance between wound edges) ×100] was calculated for 2 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.

Angiogenesis at day 10 after wounding was observed using CD31 (1:200, Santa Cruz, USA) as the primary antibody and developed by DAB (Thermo, USA). The number of newly formed vessels was then calculated. For the infiltration of macrophages in the wounds, sections were incubated with primary antibody against F4/80 (1:50, ABcam, USA), and the number of F4/80-positive cells in day 10 wounds was calculated.

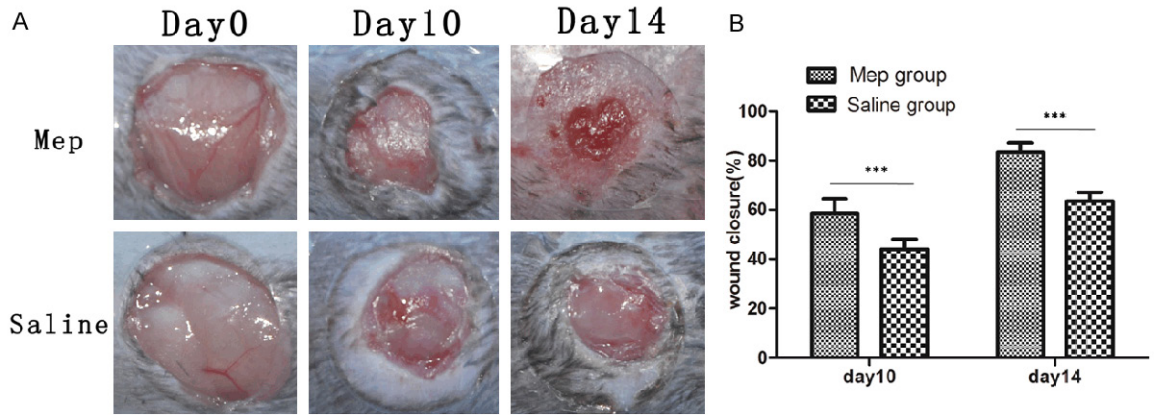

Figure 1. Topical administration of mepenzolate bromide accelerated wound healing in db/db mice. A. Full-thickness excisional skin wounds were created on the backs of db/db mice, treated with 600 μgkg^{-1} mepenzolate in 30 μl saline (Mep group) or saline alone for 3 consecutive days, and examined 0, 10, and 14 days after wounding. Wounds were digitally photographed. B. Quantitative analysis of wound healing rate showing faster wound healing rate in the Mep group. All data represent means ± SD; n=6; ***P<0.001.

Western blot analysis

The skin samples were homogenized in a tissue protein extraction reagent. Lysates were centrifuged at 12,000 rpm for 20 minutes at 4°C, and the supernatants were collected for Western blot analysis. The protein concentration of the supernatants was determined by the BCA protein assay kit. Western blot analysis was performed with primary antibody against F4/80, CD31, and GAPDH, as previously described [16].

ELISA

Mouse wounds were harvested with a dermal biopsy punch, homogenized in cold PBS (10 μL of PBS per mg wound tissue) supplemented with protease inhibitor cocktail (Sigma-Aldrich) by usinga Dounce homogenizer, and then sonicated and centrifuged at 10,000 rpm for 20 minutes at 4°C. Supernatants were used for the ELISA of IL-1β (R&D Systems), IL-6 (R&D Systems), IL-10 (R&D Systems), TGF-β1 (R&D Systems), TNF-α (R&D Systems), and IGF-1 (R&D Systems).

Quantitative real-time PCR (SYBR Green method)

Total RNA was extracted from wound samples by using Trizol. Quantitative real-time PCR was performed using SYBR Green PCR Master Mix in a total volume of 10 μL with the Step One Plus Real-Time PCR System (Applied Biosystems). GAPDH was used as the reference gene. The sequence-specific primersare shown in **Table 1**.

Measurement of the levels of MDA and H_2O_2 and the activities of GSH-Px and catalase in wounds

The level of MDA and activities of GSH-Px and CAT in wounds were measured using commercial reagent kits (Jiancheng Bioengineering Institute, Jiangsu, China) according to the instruction manuals. Wound fluid was collected as previously described [17], and the level of H_2O_2 was measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Life Technologies) according to the manufacturer's instructions.

Statistical analysis

Data statistical analysis was performed by SPSS16.0, and the results were expressed as mean ± SD. Statistical analysis was conducted using unpaired two-tailed Student's t test. A *p*-value of <0.05 was considered significant.

Results

Topical administration of mepenzolate bromide accelerated wound healing in db/db mice

Full-thickness excisional skin wounds were created on the backs of db/db mice, treated with mepenzolate bromide or saline, and examined

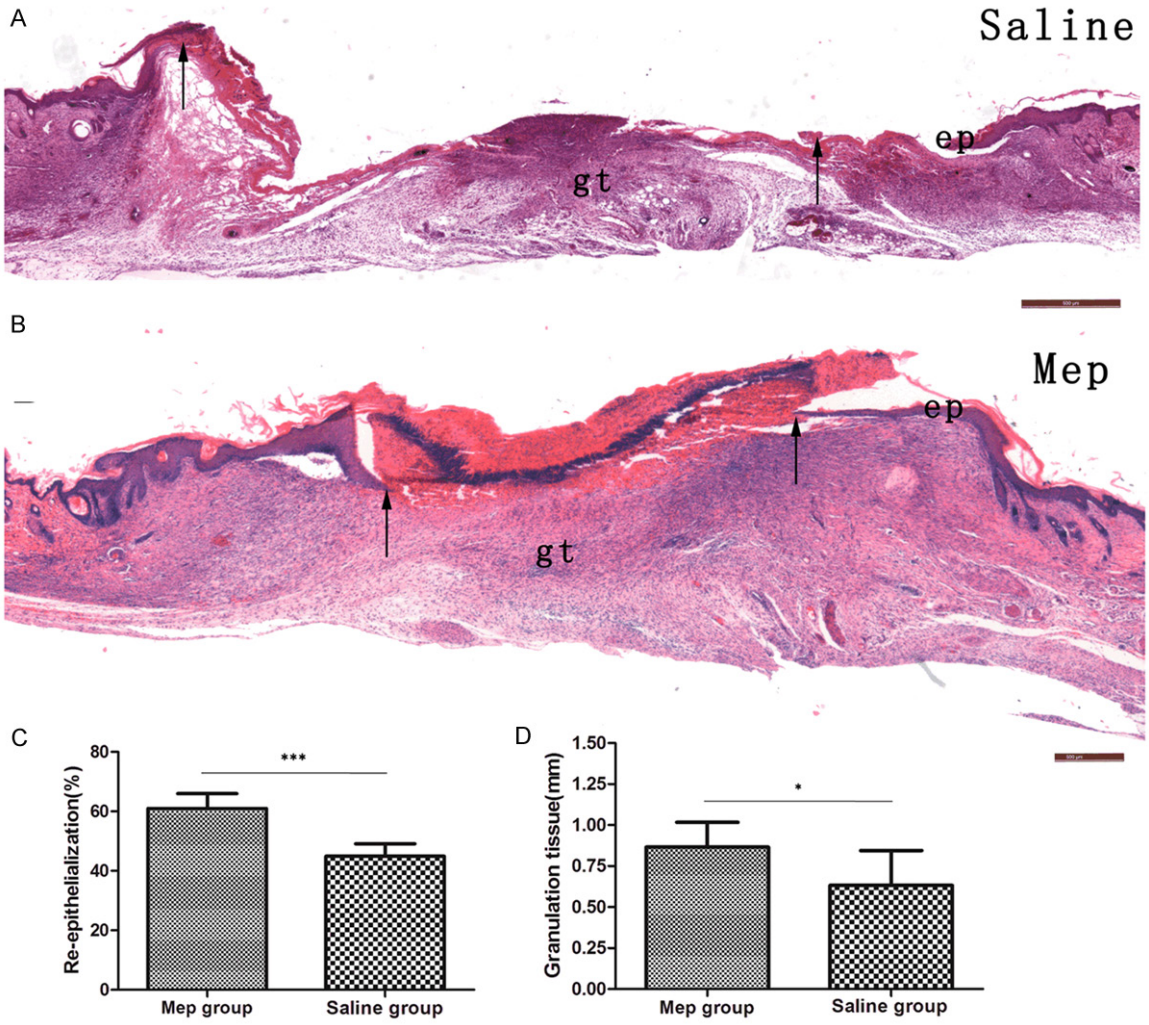

Figure 2. Histomorphometric analysis of diabetic wounds. A, B. Representative HE staining of wound tissues harvested atday 10 after wounding.ep, epithelium; gt, granulation tissue; arrows, ends of epithelial tongues. C, D. Quantitative analysis of re-epithelialization. C. Andgranulation tissue thickness D. Showing the increased re-epithelialization and granulation tissue formationin the mepenzolate group compared with the saline group. All data represent means ± SD; n=6; *P<0.05 and ***P<0.001; scale bar=500 μm.

at 0, 10, and 14 days after wounding (**Figure 1A**). Atday 10 after wounding, the percentage of wound closure was significantly greater in mice treated with mepenzolate bromide (58.33 ± 6.03%) than thattreated with saline (43.94 ± 4.07%, P<0.001, **Figure 1B**). At Day 14 after wounding, the gap of wound healing rate between these 2 groups gradually increased. To obtain further insights, we performed histomorphometric analyses on the wound biopsies (**Figure 2A**, **2B**). At day 10 after wounding, the epithelia were significantly longer and granulation tissues were significantly thicker in the mepenzolate group compared with thesaline group (**Figure 2C**, **2D**).

Mepenzolate bromide reduced macrophage infiltration and increased capillary density in diabetic wounds

Ten days after wounding, the cells stained positively for the macrophage-specific marker F4/80 were more prevalent in thesaline group than in the mepenzolate group (mepenzolate-group: 70.2 ± 11.5 cells per high-power field (HPF), saline group: 108.8 ± 9.5 cells per HPF; P<0.001; **Figure 3A**). Immunohistochemistry of CD31 showed that neovascularization in the mepenzolate group was more evident than that in the saline group at day 10. Capillary density in the mepenzolate group (20.5 ± 5.2/HPF) was

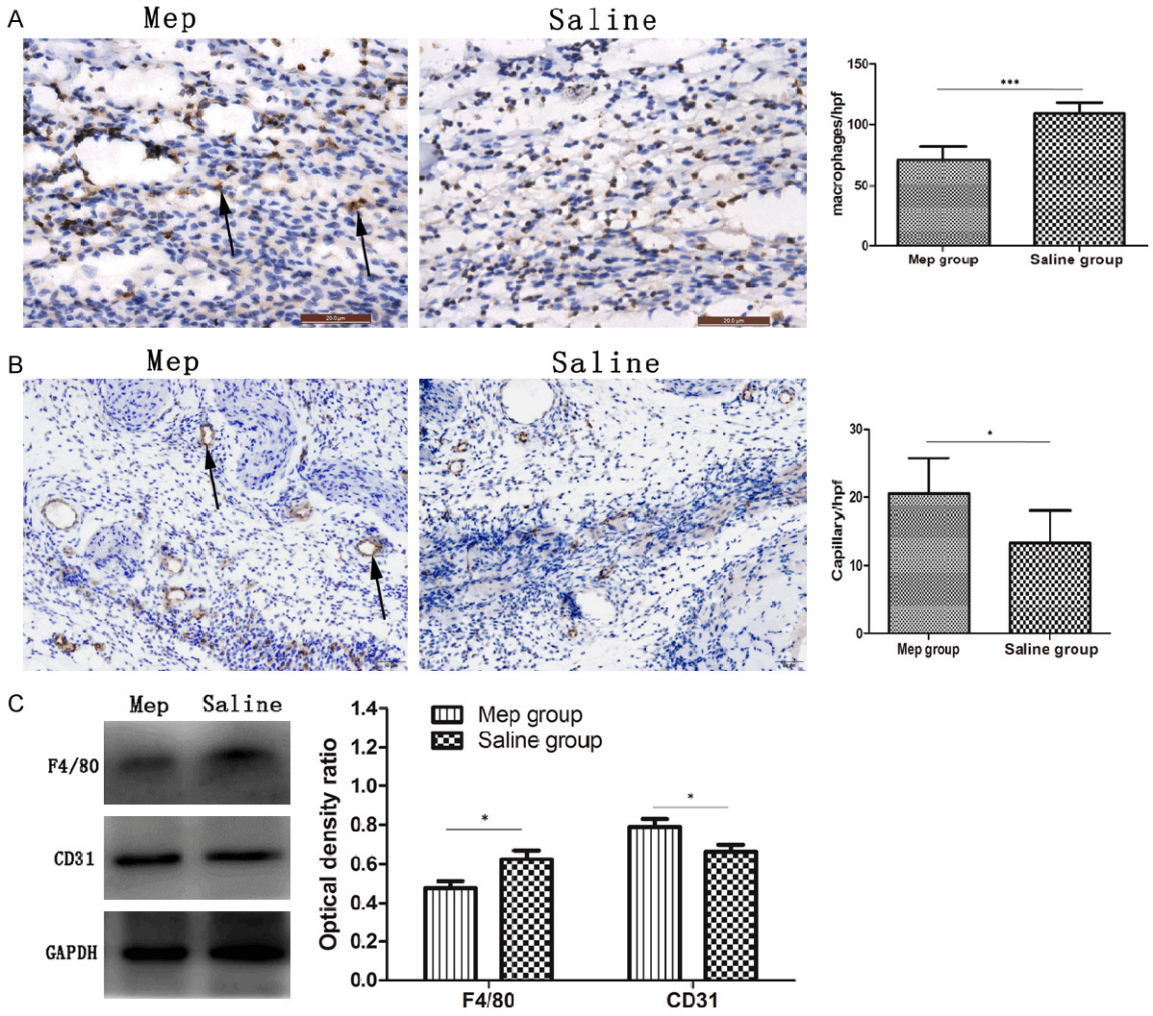

Figure 3. Mepenzolate bromide reduced macrophage infiltration and increased capillary density in diabetic wounds. (A) Wound tissues were harvested atday 10 after wounding and were stained with macrophage-specific antibody F4/80. The results showed reduced macrophage infiltration in the mepenzolate group compared with the saline group. Arrows pointed to F4/80 positive macrophages. (B) Specimens from day 10 wounds were stained with CD31, a blood vessel endothelium cell marker. Quantitative analysis of neovascularization showed that the number of the newly formed vessels in mepenzolate-treated wounds was significantly higher than that in saline-treated wounds. Arrows pointed to the $CD31^+$ blood vessel and HPFindicatedthehigh-power field. (C) The expression of F4/80 and CD31 proteins in the day 10 wounds of the mepenzolate group and saline group was assessed by Western blotting. All data represent means ± SD; n=6; *P<0.05 and ***P<0.001; Scale bars: 20 μm (A); 50 μm (B).

significantly higher than that in the saline group (13.3 ± 4.6/HPF; P<0.05; **Figure 3B**). This finding is consistent with the Western blotting results in **Figure 3C**.

Mepenzolate bromide promoted switch from pro-inflammatory status to pro-healing status ofdiabetic wounds

Next, we determined whether mepenzolate bromide down-regulated the inflammatory cytokines and promotedthe expression of pro-healing cytokines in wounds. Pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 and pro-healing cytokines VEGF, IGF-1, and TGF-β1 were measured using ELISA (**Figure 4A**, **4B**). The expression levels of IL-1β, TNF-α, and IL-6 significantly decreased in the mepenzolate bromide-treated wounds compared with the saline-treated wounds (**Figure 4A**). By contrast, the levels of TGF-β1 and VEGF significantly increased in mepenzolate bromide-treated wounds. However, the level of IGF-1 in wounds had aninsignificant difference between these two groups

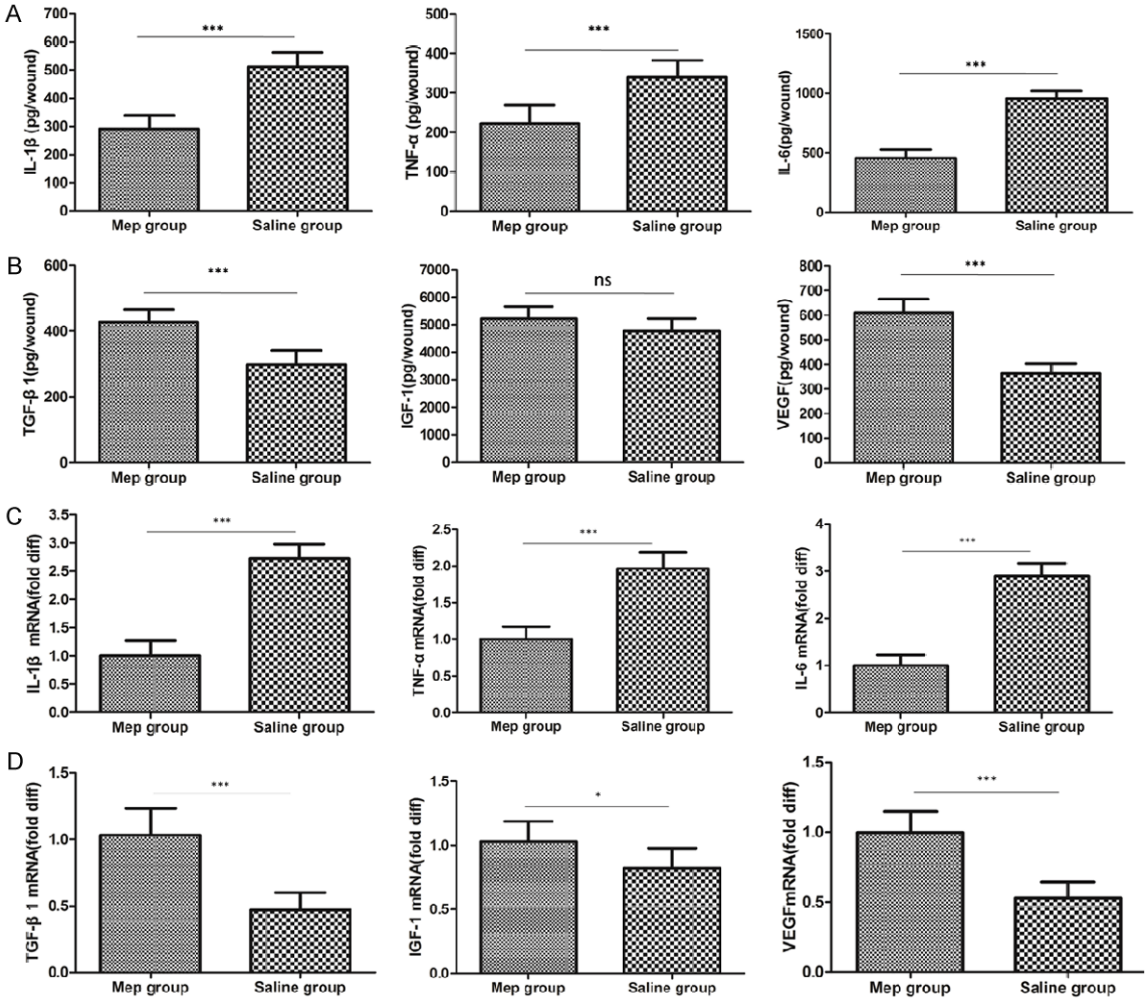

Figure 4. Mepenzolate bromide down-regulated pro-inflammatory cytokines and up-regulated healing-associated cytokines in diabetic wounds. A. The expression levels of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α inthe day 10 wounds of the mepenzolate group and saline group were measured using ELISA. B. The healing-associated cytokines TGF-β1, IGF-1, and VEGF at day 10 in the wounds of the 2 groups were measured using ELISA. C. Relative levels of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α inthe day 10 wounds of the 2 groups were assayed by real-time PCR. D. The healing-associated cytokines TGF-β1, IGF-1, and VEGF inthe day 10 wounds of the 2 groups were assayed by real-time PCR. All data represent means ± SD; n=6; *P<0.05 and ***P<0.001; n.s., no significance.

(**Figure 4B**). This finding is consistent with the real-time PCR results in **Figure 4C**, **4D**.

Mepenzolate bromide reducedoxidative stress in diabetic wounds

To evaluate the effects of mepenzolate bromide treatment on oxidative stress in diabetic wounds, we monitored thelevels of MDA and H_2O_2 and theactivities of GSH-Px and CATin wounds. As shown in **Figure 5A**, **5B**, the levels of H_2O_2 and MDA in mepenzolate-treated wounds significantly decreased compared with saline-treated wounds. To understand the underlying mechanism, we tested the activities of GSH-Px and CAT, both of which are related to H_2O_2 decomposition. As shown in **Figure 5C**, **5D**, both of them significantly increased in mepenzolate-treated wounds compared with saline-treated wounds.

Discussion

Mepenzolate bromide isa safe and cheapdrug used in clinics for gastrointestinal disorders andis known to provide therapeutic effects in a mouse model of chronic obstructive pulmonary disease and pulmonary fibrosis via reducing

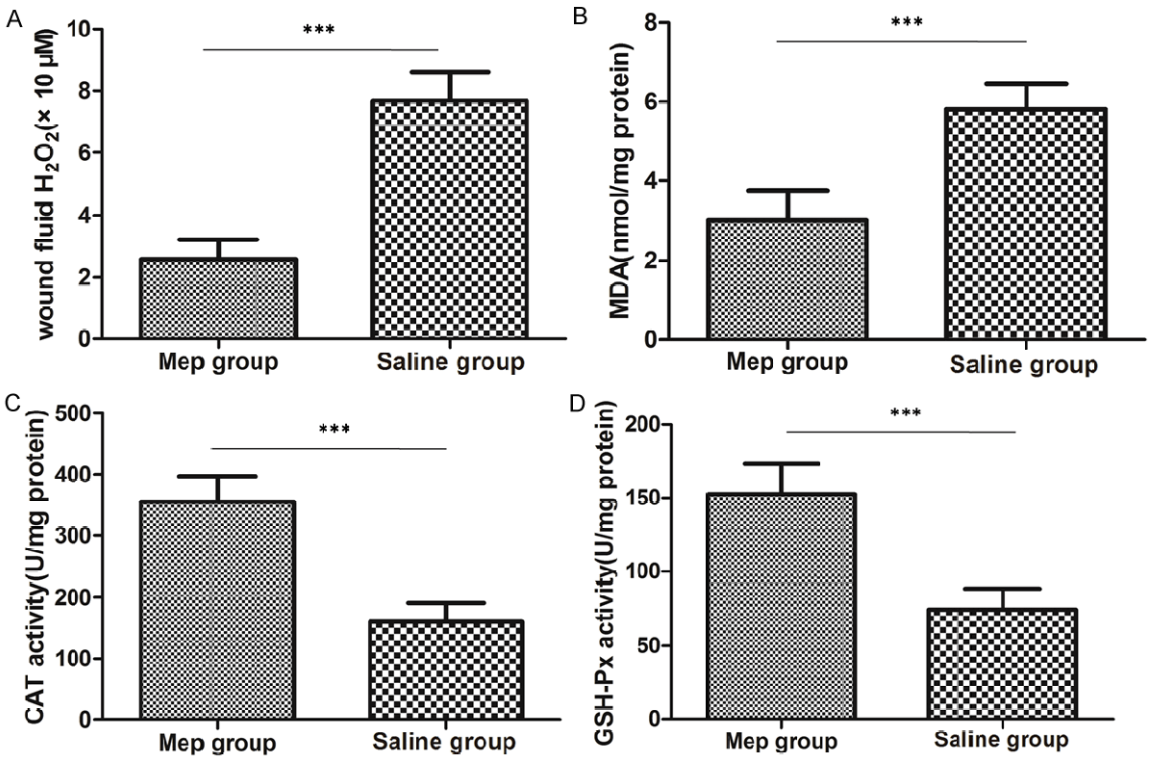

Figure 5. Mepenzolate bromide reduced oxidative stress in diabetic wounds. (A) H_2O_2 levels were measured by Amplex Red inday 10 wound fluids. The result showedthe decreased levels of H_2O_2 in mepenzolate-treated wounds compared with saline-treated wounds. (B) The levels of MDA in the wounds of themepenzolate group and saline group were measured using commercial reagent kits according to the instruction manuals,showing decreased levels of MDA in mepenzolate-treated wounds. (C, D) The activities of CAT (C) and GSH-Px (D) in day 10 wounds were measured using commercial reagent kits. The result showed the increased activities of CAT and GSH-Px in mepenzolate-treated wounds compared with saline-treated wounds. All data represent means ± SD; n=6; ***P<0.001.

inflammation and ROS [12-14]. This finding prompted us to investigate whether mepenzolate bromide plays a role in inflammation and ROS-related diabetic wound healing. We found that mepenzolate bromide significantly accelerated the diabetic wound healing rate, granulation tissue formation, re-epithelialization, and increased capillary density. Further studies showed that mepenzolate bromide could inhibit inflammatory responses and reduce oxidative stress in diabetic wounds. For the first time, these data demonstrate the potential application of mepenzolate bromide for treating diabetic ulcers.

The full-thickness excisional skin wound of db/db mouse is well-accepted for studying diabetic wound healing. In this study, we showed that thetopical application of mepenzolate bromide accelerated wound healing in db/db mice. Wound closure was significantly faster atDays 10 and 14 after treatment with mepenzolate bromide compared with the saline group. This result was consistent withthick granulation tissues formation, improved re-epithelialization, and increased capillary density in mepenzolate group.

Macrophages play an important part in the repair of various tissues by clearing damages tissues, killing pathogens, and producing a variety of growth factors that induce collagen deposition, angiogenesis, and wound closure [18-20]. However, during the process ofdiabetic wound healing, wounds exhibit prolonged accumulation of macrophages associated with increased levels of pro-inflammatory cytokines and proteases and reduced levels of various growth factors [21, 22], which contribute to impaired diabetic wound healing. Furthermore, pro-inflammatory cytokines are part of a pro-inflammatory positive feedback loop that sustains a persistent pro-inflammatory macrophage phenotype in wounds. Mirza [7] reports

that macrophages isolated from wounds in diabetic mice and humans exhibit a pro-inflammatory phenotype and secrete increased levels of IL-1β, thus inducing a persistent pro-inflammatory macrophage phenotype. Further blocking of IL-1β induces the macrophages to switch from pro-inflammatory to healing-associated phenotypes, which improve the healing of diabetic wounds. We showed in this study that mepenzolate bromide dramatically decreased inflammation in diabetic wounds. We found that mepenzolate bromide reduced macrophage infiltration in day 10 wounds. Furthermore, levels of pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 were significantly decreased in mepenzolate bromide-treated wounds compared with saline-treated wounds. The expressions of pro-healing cytokines, including TGF-β1 and VEGF, were significantly increased in wounds after treatment with mepenzolate bromide. This result may partially explain the thick granulation tissues and increased capillary density in mepenzolate-treated wounds.

Excessive and prolonged ROS production is another important feature for impaired wound healing in diabetes [8]. In moderate concentrations for a short time periods, ROS ismainly produced by inflammatory cells during the early phase of wound healing; ROS is importantfor protecting wounded skin against invading pathogens [23]. However, excessive ROS accumulation can cause cell senescence and death and impair keratinocyte migration in vitro, thus potentially inhibiting re-epithelialization [24, 25]. Furthermore, high levels of ROS increase the expression of matrix metalloproteinases and causethe destruction of the extracellular matrix, thus further resulting in tissue damage and perpetuation of inflammation [26]. Exogenous antioxidants should be included to treat chronic wounds bycontrolling excessive ROS production. Given the short half-life of ROS, determining their concentrations in vivo is difficult. In the current study, ROS levels at the wound site have been determined indirectly by analyzing H_2O_2 and MDA expression. We found that mepenzolate bromide significantly reduced levels of H_2O_2 and MDA in diabetic wounds. Further experiments showed that activities of GSH-Px and CAT, which would facilitate the conversion of H_2O_2 to H_2O, increased in the mepenzolate group compared with the saline group. This result may partially explain the reduced oxidative stress in mepenzolate-bromide-treated wounds.

Wound healing is divided into three phases with limited overlap: coagulation-inflammation, proliferation, and remodeling [22]. Inflammation mainly occurs in the beginning phase of the wound healing process. In this study, we used mepenzolate bromide for three consecutive days rather than every day until complete wound healing. We intend to regulate prolonged and excessive inflammation in diabetic wounds without influencing the proliferation and remodeling phases of wound healing.

One limitation of thisstudy is that we onlyapplied mepenzolate bromide to the wound bed topically without trying systemic administration routes (oral and intravenous). Tanaka [13] found that the topical pulmonary route of mepenzolate administration wassuperior to other routes (oral, intravenous, or intrarectal) in protective effects against elastase-induced pulmonary damage and bronchodilation, with a lower dose than other routes and decreased risk of adverse effects. Althoughthe above findings suggest topical administration may besuperior to other routes for diabetic wound healing, further studies are still necessary to determine the appropriate route of mepenzolate administration toevaluate the effect of different administration routes on this drug's beneficialand adverse effects for diabetic wound healing.

Another limitation of this study is that we did not provide direct evidence to show if mepenzolate bromide could modulate the macrophage phenotype switch from pro-inflammatory to healing-associated phenotype. Increased oxidative stress makes macrophages adopt aproinflammatory phenotype [27] and blocking IL-1β induces an M2 phenotype in diabetic wounds [7]. In the present study, we found that mepenzolate bromide reduced oxidative stress, down-regulated inflammatory cytokines, and promoted the expression of pro-healing cytokines in diabetic wounds, thus indicating thatmepenzolate bromide may lead to a macrophage polarization toward the M2 phenotype. Further studies will be required to explore the role of mepenzolate bromide in macrophage polarization.

Our results showed that mepenzolate bromide significantly promoted diabetic wound healing-by inhibiting inflammation and oxidative stress in wounds.This might provide a potential drug for thetreatment of diabetic ulcers and other chronic wounds in clinics.

Acknowledgements

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Disclosure of conflict of interest

None.

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