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Propranolol improves cutaneous wound healing in streptozotocin-induced diabetic rats

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ABSTRACT

Sympathetic nerve failure has been proposed as a contributing factor in impaired cutaneous wound healing in diabetes mellitus. Nevertheless, no studies have shown whether β-adrenoceptor blockade through β-blocker (e.g., propranolol) administration may alter healing of diabetic cutaneous lesions. This study evaluated macro- and microscopically the effects of propranolol administration on cutaneous wound healing in streptozotocin-induced diabetic rats. Acute diabetes was induced by a single intraperitoneal injection of streptozotocin 14 days before wounding. Animals were treated with propranolol (50 mg/kg) dissolved in drinking water; controls received water only. Administration of β-receptor antagonist began 1 day before wounding and was continued daily until euthanasia. A full-thickness excisional lesion (1 cm^2) was created. The wound area was measured weekly and the animals were killed 14 days after wounding. Lesions and adjacent skin were formalin-fixed and paraffin-embedded. Sections were stained with hematoxylin-eosin, Sirius red, and toluidine blue, and immunostained for CD-68, alpha-smooth muscle actin and proliferating cell nuclear antigen. The wound area was significantly smaller in the propranolol-treated group than in the control group 7 and 14 days after wounding. Inflammatory cell numbers and metalloproteinase-9 levels were reduced in the propranolol-treated group compared to the control group 14 days after wounding. Cell proliferation, mast cell number, collagen deposition, blood vessel density, and nitric oxide levels were increased in the propranolol-treated group compared to the control group 14 days after wounding. Propranolol administration improves cutaneous wound healing of hyperglycemic diabetic rats by reducing the local inflammatory response and improving subsequent phases of the repair process.

1. Introduction

Diabetes mellitus affects approximately 170 million people worldwide, and by 2030 the number of diabetics is projected to double (Brem and Tomic-Canic, 2007; Wild et al., 2004). Diabetic status impairs cutaneous wound healing, resulting in chronic lesions and ulcers (such as foot ulcers) that lead to high morbidity and mortality and increased treatment costs. Furthermore, foot ulcers comprise more than 50% of the amputation cases among diabetic patients (Bild et al., 1989). However, the mechanism by which the disease impairs cutaneous wound healing is not completely understood. Previous work has suggested that decreased production of growth factors [such as transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF)], reduced collagen deposition, excessive protease activity, a delayed inflammatory response, and impaired nitric oxide (NO) synthesis all contribute to the impaired wound healing observed in diabetes mellitus (Bitar and Labbad, 1996; Lerman et al., 2003; Lobmann et al., 2002; Schaffer et al., 1997; Wetzler et al., 2000). In addition, some studies have proposed that absence of an intact cutaneous nervous system also contributes to the pathology (Gibran et al., 2002; Levy et al., 1989; Watkins and Edmonds, 1983).

The skin is equipped with a dense network of sensory and sympathetic nerve branches in all cutaneous layers. The former nerves pick up sensorial stimuli whereas the latter maintain skin homeostasis. Initially, it was demonstrated that a reduction in both neuropeptide secretion and numbers of cutaneous peripheral nerves is associated with impaired wound healing in both diabetic patients and genetically diabetic mice (Gibran et al., 2002; Levy et al., 1989). Moreover, diabetic patients may also present sympathetic nerve failure, which reduces skin blood flow and impairs wound healing (Watkins and Edmonds, 1983). Nonetheless, the role of β-adrenoceptors in diabetic wound healing is poorly understood. Previous studies have reported that β-receptor antagonists (such as propranolol and metoprolol) may reduce skin blood flow and hence be hazardous to hypertensive diabetic patients (Hyer et al., 1987). On the other hand, β_3-adrenoceptor activation increased the rate of cutaneous wound closure in animal models of type II diabetes (Schaeffer et al., 2006).

Considering that propranolol may activate β_3-adrenoceptors (Anesini and Borda, 2002), the aim of this study was to evaluate

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macro- and microscopically the effects of propranolol administration on cutaneous wound healing in streptozotocin-induced diabetic rats.

2. Material and methods

This study was approved by the Ethical Committee for Animal Use of the State University of Rio de Janeiro. Male Wistar rats weighing between 250–350 g were maintained one per cage with free access to food and water in a room with controlled humidity and temperature (22 °C) on a 12-h light/dark cycle.

2.1. Induction of acute diabetes mellitus

After a 12-h fast, animals were given a single intraperitoneal injection of streptozotocin (45 mg/kg body weight in 0.1 M citrate buffer, pH 4.5) (Sigma, Inc., St. Louis, MO) and received a solution of 6% sucrose in their drinking water. Seven days after the streptozotocin application, a blood glucose measurement was performed on tail-vein blood using an Accu-Chek® Advantage II (Roche Diagnostics, Mannheim, Germany). Diabetic status was defined as blood glucose levels higher than 300 mg/dl.

Seven days after induction of diabetes, daily water intake per animal was measured. Based on the daily water intake, drug was dissolved in 50 ml of drinking water to guarantee intake of the total dose per animal. This volume of drinking water was consumed by the rats all day long. Water or drug intake per animal was monitored daily to prevent dehydration.

2.2. Wounding model and propranolol administration

Animals were divided into two groups: a control group ($n=5$), which received water (in the same amount as the experimental group); and a propranolol-treated group ($n=5$), which received propranolol hydrochloride (a non-selective β_1- and β_2-adrenoceptor antagonist) (Farm Derm; Vitória, Brazil), 50 mg/kg, dissolved in water. Administration of propranolol began 1 day before wounding and was maintained daily until euthanasia. The study dose was obtained from a dose calculation based on body surface area, which permits conversion of drug doses from humans to rodents (Reagan-Shaw et al., 2008). Thus, the animal dose of propranolol used in this study was converted from a human dose of 8 mg/kg of propranolol (Reagan-Shaw et al., 2008; Souza et al., 2006). Fourteen days after induction of diabetes, rats were intraperitoneally anesthetized with ketamine (5 mg/kg) and xylazine (2 mg/kg). After shaving the dorsum, a full-thickness excisional wound (1 cm^2) was made to the level of the panniculus carnosus muscle. The wound was not sutured or covered and healed by second intention.

2.3. Macroscopic analyses

Body weight and blood glucose levels were measured on the day of wounding, and thereafter weekly until euthanasia. To evaluate wound contraction and re-epithelialization, a transparent plastic sheet was placed over the wound and its margins were traced (Amadeu et al., 2007; Nascimento and Costa, 2006). After digitization, the wound area was measured using KS400 image software (Zeiss-Vision; Oberkochen, Germany). The wound area was measured and photographed soon after wounding, and thereafter weekly until euthanasia without scab remotion. Re-epithelialization was measured 14 days after wounding only. Data are expressed as percentage of the initial wound area and as percentage of the re-epithelialized wound area.

2.4. Tissue harvesting and microscopic analyses

Rats were killed 14 days after wounding by CO_2 exposure. A fragment of lesion and adjacent normal skin was formalin-fixed (pH 7.2) and paraffin-embedded, and another fragment of lesion was frozen at −70 °C. Moreover, peripheral blood was collected from the tail vein and centrifuged; thereafter, the plasma was collected and frozen at −70 °C.

Sections (5 μm) were stained with hematoxylin-eosin to observe microscopically the wounded area, and were also stained with Sirius red and observed under polarization to evaluate the organization of collagen fibers. Furthermore, sections were stained with toluidine blue to quantify the number of mast cells. For this, ten random fields per animal (0.15 mm^2) were analyzed in the deep region of the granulation tissue using a ×40 objective lens (Zeiss Primo Star, Zeiss-Vision; Oberkochen, Germany). Results are expressed as the average of mast cells/mm^2. Quantification was done blindly and repeated without significant differences among them.

2.5. Immunohistochemistry and quantifications

For quantification of CD-68-positive macrophages, blood vessels, and cell proliferation, sections were respectively incubated with mouse anti-CD-68 (Serotec Inc.; Raleigh, NC; 1:300), mouse anti-alpha-smooth muscle actin (alpha-SM actin) (DAKO; Carpinteria, CA; 1:1000), or mouse anti-proliferating cell nuclear antigen (PCNA) (DAKO; 1:1000) antibodies. For antigen retrieval, sections were digested with 0.1% trypsin (Difco Laboratories; Detroit, MI), or incubated with citrate buffer (pH 6.0) before labeling. Subsequently, sections were incubated with 3% H_2O_2 in methanol to inhibit endogenous peroxidase. After washing, all primary antibodies were detected using the EnVision system (DAKO) and diaminobenzidine was used as chromogen. Sections were counterstained with hematoxylin. No labeling was observed on sections where primary antibody was omitted.

To quantify the number of CD-68-positive macrophages, ten random fields per animal (3636 μm^2) were analyzed in the superficial region of the granulation tissue using a ×40 objective lens. For this, a videomicroscopic system (Axiolab ZEISS microscope, a JVC video-camera, and Sony Trinitron monitor) (Sony, United Kingdom) was used (Souza et al., 2006). Data are presented as the average of CD-68-positive macrophages/mm^2.

Since stereological methods are precise tools for obtaining information about three-dimensional structures based mainly on observations made in two-dimensional sections, the quantitative distribution of blood vessels was evaluated using this method (vertical sections and cycloid arcs test-system) (Amadeu et al., 2007; Baddeley et al., 1986; Gundersen et al., 1988). Vessels were identified by the presence of blood cells in theirs lumens or positive staining for alpha-SM actin in the wall (to confirm the presence of smooth muscle cells or pericytes) (Souza et al., 2006). Five random fields per animal were analyzed in the superficial and deep regions of granulation tissue using a ×20 objective lens. For analysis, a videomicroscopic system (Axiolab ZEISS microscope, a JVC video-camera, and Sony Trinitron monitor) (Sony, United Kingdom) was used. Data are expressed as volume density of blood vessels ($Vv_{[blood\ vessels]}$%) in the superficial and deep regions.

Cell proliferation was evaluated in the neo-epidermis and granulation tissue (Souza et al., 2006). In neo-epidermis, the percentage of PCNA-positive epithelial cells was calculated by counting 240 cells (120 cells of each neo-epidermis side) from wound margin to neo-epidermis extremity. Results are presented as percentage of PCNA-positive basal epithelial cells. In the granulation tissue, the number of PCNA-positive connective tissue cells was analyzed by counting ten random fields per animal (3636 μm^2) in the granulation tissue using a ×40 objective lens. For this, a videomicroscopic system (Axiolab ZEISS microscope, a JVC video-camera, and Sony Trinitron monitor) (Sony, United Kingdom) was used (Souza et al., 2006). Data are presented as average of PCNA-positive connective tissue cells/mm^2.

All quantifications were done blindly and repeated without significant differences among them.

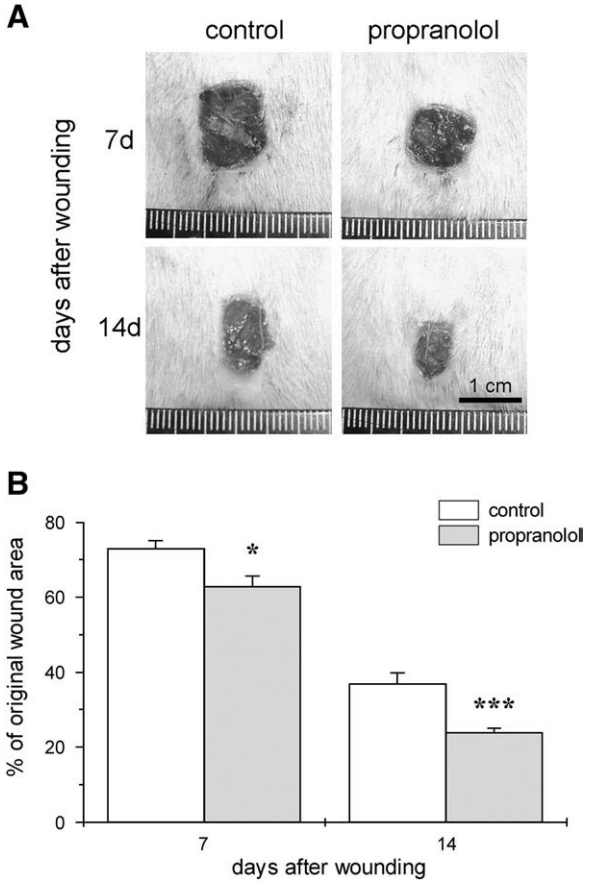

Fig. 1. Macroscopic evaluation of wounds in control and propranolol-treated animals. (A) Photographs of wounds in control and propranolol-treated animals 7 (7d) and 14 (14d) days after wounding. (B) Percentage of original wound area in control and propranolol-treated animals 7 and 14 days after wounding. Data are expressed as mean ± S.E.M. Bar: 1 cm. (*$P<0.05$ and ***$P<0.001$ vs. control group).

2.6. Biochemical analyses

To estimate the number of neutrophils in the wounded area, myeloperoxidase (MPO) activity in wound lysate was assayed according to Stark and collaborators with minor modifications (Stark et al., 1992). An aliquot of wound lysate was mixed in phosphate buffer (80 mM) containing 0.5% hexadecyltrimethyl ammonium bromide (Sigma-Aldrich; St. Louis, MO) at pH 5.5. The mixture was centrifuged at 12,000 rpm for 15 min to extract the MPO. Subsequently, the supernatant was mixed with 3,3′,5,5′-tetramethylbenzidine dihydrochloride (1.9 mg/ml) (Sigma-Aldrich; St. Louis, MO) and hydrogen peroxide (1 mM) (Vetec; Rio de Janeiro, Brazil). Thereafter, the mixture was incubated at 37 °C for 15 min and then mixed with acetate buffer pH 3.0 (1.64 M). The developed color was read spectrophotometrically at 630 nm. MPO concentrations in the samples were determined from a standard curve generated by different concentrations of MPO from human leukocytes (Sigma-Aldrich; St. Louis, MO). Data are expressed as mU MPO per mg total protein.

Collagen deposition was quantified by hydroxyproline assay in frozen lesions (Souza et al., 2006). Dry and defatted tissue (1.5–7.0 mg) was hydrolyzed in 6 M HCl for 18 h at 118 °C. Hydrolysate was then diluted with distilled water, neutralized with 6 M NaOH and centrifuged at 3000 rpm for 15 min. Hydroxyproline levels were measured in this hydrolysate as previously described (Woessner, 1961). Briefly, diluted aliquots of hydrolysate (80 µl) were mixed with 40 µl chloramin-T (0.05 M) (Merck & Co., Inc.; Whitehouse Station, NJ) and incubated for 20 min at 25 °C. Thereafter, 40 µl percloric acid (3.17 M) (Vetec; Rio de Janeiro, Brazil) and 40 µl 4-dimethylamino benzaldehyde (Merck & Co., Inc.; Whitehouse Station, NJ) were added, plates were again incubated for 20 min at 60 °C. The developed color was read spectrophotometrically at 550 nm. Hydroxyproline concentrations were determined from a standard curve generated by different concentrations of L-4-hydroxyproline (Sigma-Aldrich; St. Louis, MO). Data are expressed as ng hydroxyproline per mg tissue.

Levels of nitrite in wound lysates and plasma were determined by a spectrophotometric method based on the Griess reaction (Green et al., 1982). An aliquot of each sample (100 µl) was mixed and incubated with 100 µl of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylenediamide dihydrochloride in water) at room temperature for 10 min. Thereafter, the developed color was spectrophotometrically read at 557 nm. Nitrite concentrations in the samples were determined from a standard curve generated by different concentrations of sodium nitrite. Data are expressed as µmol nitrite per µg total protein.

All analyses were done in triplicate and repeated without significant differences among them.

2.7. Gelatin zymography

Gelatinase activities of matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9) were assessed by zymography as previously described (Varelias et al., 2006). Frozen wound fragments were placed into lysis buffer (20 mM Tris–HCl, pH 7.5, 138 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride), macerated and centrifuged at 4800 rpm for 30 min at 4 °C. Subsequently, total protein concentration of the obtained lysates was determined by using the Bradford assay. Twenty micrograms of each lysate were added to a volume of 5X sample buffer (0.125 M Tris–HCl, pH 7.4, 10% sodium dodecylsulfate, 50% glycerol, 1% bromophenol blue) and was electrophoresed on an 8% sodium dodecylsulfate (SDS)-polyacrylamide resolving gel containing 1 mg/ml gelatin (Sigma-

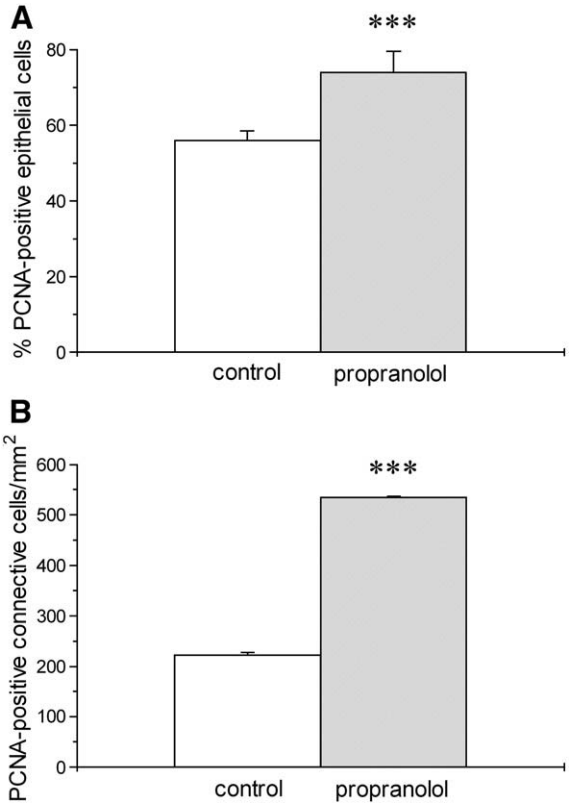

Fig. 2. Cell proliferation in neo-epidermis (A) and granulation tissue (B) in control and propranolol-treated groups 14 days after wounding. Data are expressed as mean ± S.E.M. (***$P<0.001$ vs. control group).

Aldrich; St. Louis, MO) layered with a 4% SDS-polyacrylamide stacking gel. Human placenta was used as a positive control (Niu et al., 2000). Protein molecular weight standard (Amersham Pharmacia Biotech; Buckinghamshire, UK) was included. Thereafter, the gels were washed in 2.5% Triton X-100 and incubated with development buffer (50 mM Tris–HCl, pH 8.4, 5 mM $CaCl_2$, and 2 μM $ZnCl_2$) for 12 h at 37 °C. Gels were stained with 0.25% Coomassie Blue and then destained to obtain contrast between the gelatinolytic bands and the gel background. Densitometry was performed using Adobe Photoshop version 7.01 (Adobe Systems Incorporated; San Jose, CA, USA) and data are expressed in arbitrary units (a.u.).

2.8. Statistical analyses

All data are reported as mean ± standard error of mean (S.E.M.). The results of wound contraction, re-epithelialization, epithelial proliferation and blood vessel density, and MMP activity were statistically analyzed by Mann–Whitney test using GraphPad InStat version 3.01 software (GraphPad Software Inc.; San Diego, CA). Data concerning body weight, blood glucose levels, number of PCNA-positive connective tissue cells, CD-68-positive macrophages and mast cells, MPO activity and levels of hydroxyproline and nitrite were statistically analyzed by Student's *t* test with Welch's correction using the same software. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. Macroscopic analyses

Hyperglycemic diabetic animals of both the control and propranolol-treated groups weighed significantly less at the end of the experiment compared to the day of wounding (control: 211 ± 18 g vs. 265 ± 22 g, $P<0.01$) (propranolol: 231 ± 11 g vs. 253 ± 9 g, $P<0.001$). Despite the weight loss, no difference in body weight was observed between the control and propranolol-treated groups over the course of the experiment. Blood glucose levels were higher than 300 mg/dl in both the control and propranolol-treated groups 7 days before wounding, on the day of wounding, and 7 and 14 days after wounding. Moreover, no differences in blood glucose levels were observed between the control and propranolol-treated groups.

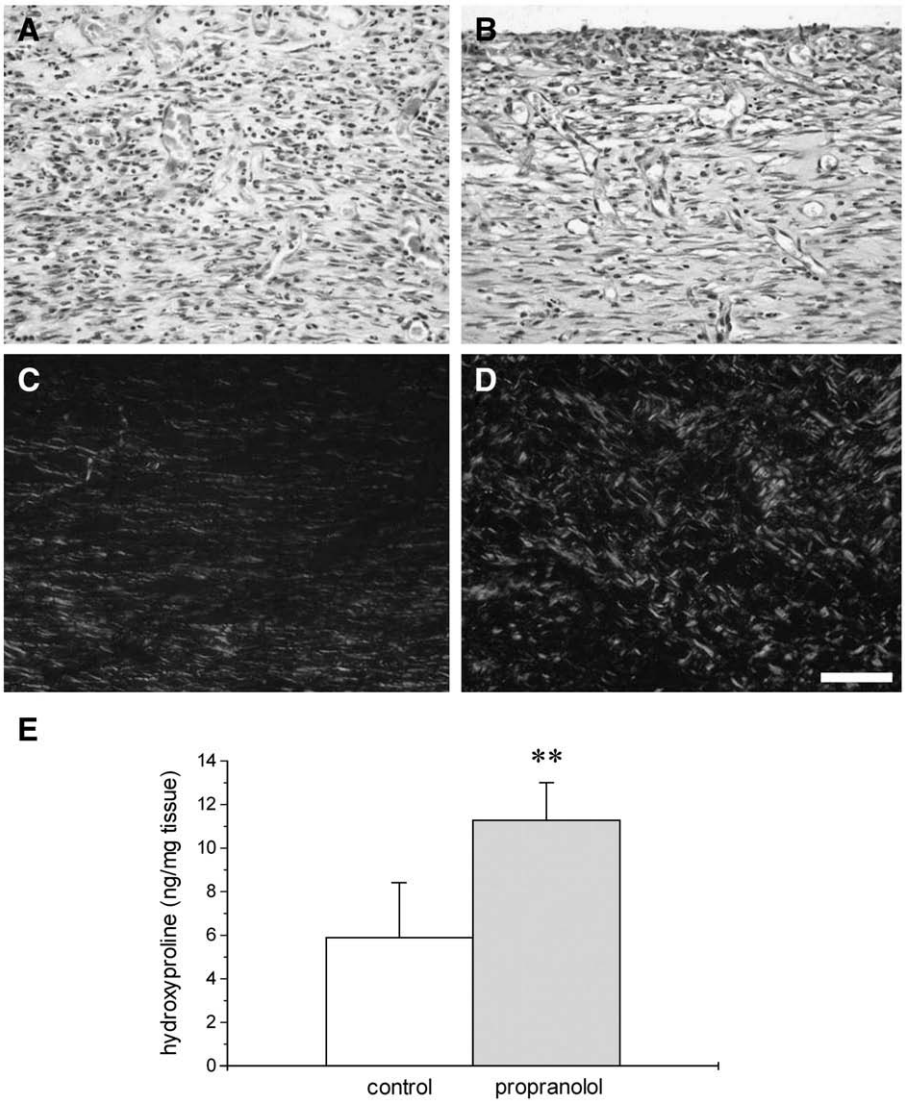

Fig. 3. Development of granulation tissue in control (A, C) and propranolol-treated groups (B, D) 14 days after wounding. In the hematoxylin-eosin stained sections (A, B), the control group presents a high number of inflammatory cells; in the propranolol-treated group, "fibroblast-like" cells predominate. In Sirius red-stained sections (C, D), collagen fibers are fragmented, thin and arranged parallel to the cell surface in the control group; in the propranolol-treated group the fibers are fragmented, thin and arranged similarly to those of normal skin. Bar: 50 μm. Hydroxyproline levels (E) in wound areas of the control and propranolol-treated groups 14 days after wounding. Data are expressed as mean ± S.E.M. (**$P<0.01$ vs. control group).

Table 1
Effects of propranolol administration on inflammatory cell migration in control and treated groups 14 days after wounding.

Measurements	Control ($n=5$)	Propranolol ($n=5$)
MPO (mU/mg total protein)	296.78 ± 16.31	231.67 ± 5.97[a]
CD-68-positive macrophages/mm^2	1199.1 ± 0.3	962.6 ± 0.3[b]
Mast cells/mm^2	11.0 ± 0.3	15.9 ± 0.3[b]

Note. All data are shown as mean ± S.E.M.
MPO = myeloperoxidase.
[a] Significantly different from the control group at $P<0.01$.
[b] Significantly different from the control group at $P<0.05$.

As illustrated in the Fig. 1A, the wound area was not completely re-epithelialized in either group 14 days after wounding. Moreover, the wound area seemed smaller in the propranolol-treated group than in the control group 7 and 14 days after wounding. In order to confirm this observation, the wound area was measured. The percentage of original wound area in the propranolol-treated group was 14% smaller at 7 days ($P<0.05$) and 35% smaller at 14 days ($P<0.001$) post-wounding compared to the controls (Fig. 1B). Nevertheless, the percentage of re-epithelialized wound area was similar in the control (26% ± 3) and propranolol-treated (28% ± 2) groups 14 days after wounding.

3.2. Quantification of cell proliferation

Epithelial proliferation is one of the essential processes of re-epithelialization, so the percentage of PCNA-positive epithelial cells was estimated 14 days after wounding. The PCNA-positive basal keratinocytes were concentrated in the neo-epidermis margin in both groups. However, the percentage of PCNA-positive epithelial cells was 40% higher in the propranolol-treated group than in the control group ($P<0.001$) (Fig. 2A).

Cell proliferation is an early event in the development of granulation tissue, so the number of PCNA-positive connective tissue cells was counted 14 days after wounding. The PCNA-positive connective tissue cells were homogeneously distributed in the granulation tissue in both groups. However, the number of PCNA-positive connective tissue cells was 140% greater in the propranolol-treated group than in the control group ($P<0.001$) (Fig. 2B).

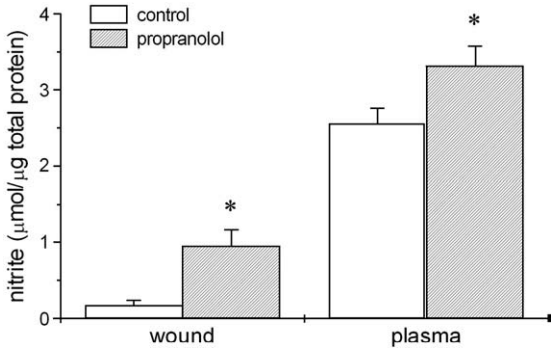

Fig. 5. Nitrite levels in wounds and plasma of control and propranolol-treated groups 14 days after wounding. Data are expressed as mean ± S.E.M. (*$P<0.05$ vs. control group).

3.3. Quantification of inflammatory infiltrate

Fourteen days after wounding, inflammatory infiltrate was reduced and the quantity of "fibroblast-like" cells was increased in superficial regions of the lesions in the propranolol-treated group (Fig. 3B) compared to the control group (Fig. 3A). To evaluate the inflammatory infiltrate, MPO activity and the number of CD-68-positive macrophages and mast cells were measured 14 days after wounding. MPO activity was 22% lower in the propranolol-treated group compared to the control group ($P<0.01$) (Table 1). In addition, the number of CD-68-positive macrophages was 20% smaller in the propranolol-treated

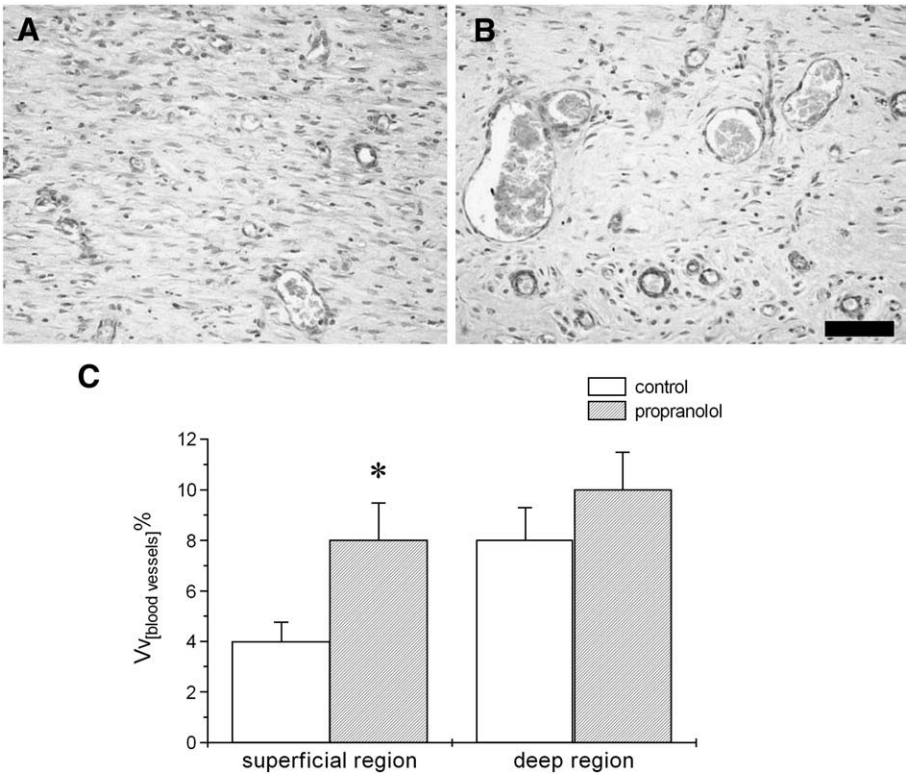

Fig. 4. Distribution of blood vessels in wound areas of (A) control and (B) propranolol-treated groups 14 days after wounding. In the alpha-SM actin immunostained sections (A, B), blood vessels are more numerous and dilated in the propranolol-treated group than in the control group on granulation tissue. Bar: 50 μm. Stereological analysis showing volume density for blood vessels ($Vv_{[blood\ vessels]}$%) (C) in granulation tissue of control and propranolol-treated groups. Data are expressed as mean ± S.E.M. (*$P<0.05$ vs. control group).

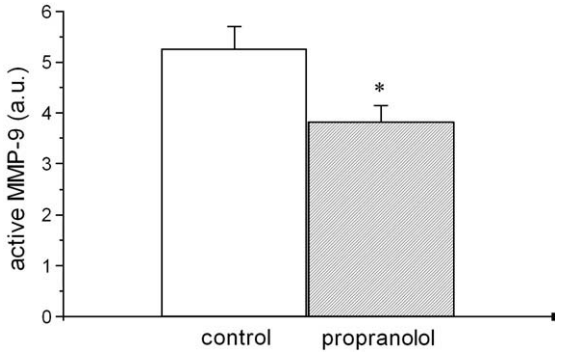

Fig. 6. Gelatinolytic activity of active metalloprotease-9 (MMP-9) in the control and propranolol-treated groups 14 days after wounding. Densitometry is expressed as arbitrary units (a.u.). Data are expressed as mean ± S.E.M. (*$P<0.05$ vs. control group).

group than in the control group ($P<0.05$) (Table 1). Finally, the number of mast cells was 45% greater in the propranolol-treated group than in the control group ($P<0.05$) (Table 1).

3.4. Collagen deposition

Collagen deposition is another important event in the development of granulation tissue, so both the organization of collagen fibers and the levels of hydroxyproline were evaluated 14 days after wounding. Collagen fibers were mainly greenish, fragmented and thin, and arranged parallel to the cell surface in the control group (Fig. 3C). In the propranolol-treated group the fibers were yellow-reddish, fragmented and thin, and arranged similarly to those of normal skin (Fig. 3D). Thus, collagen fibers were more organized and dense in the propranolol-treated group than in the control group (Fig. 3C and D). To confirm these histological observations, hydroxyproline levels were measured in the lesions 14 days after wounding. Hydroxyproline levels were 103% greater in the propranolol-treated group than in the control group ($P<0.01$) (Fig. 3E).

3.5. Angiogenesis

Angiogenesis also is an initial event in the development of granulation tissue, so the density of blood vessels was estimated by a stereological method 14 days after wounding. Blood vessels were concentrated in the superficial region of the granulation tissue in the control group, whereas in the propranolol-treated group they were homogeneously distributed in the granulation tissue (Fig. 4A and B). The volume density was used to determine the volume occupied by vessels in the lesions of each group. The volume occupied by the blood vessels was 98% greater in the propranolol-treated group than in the control group in the superficial region ($P<0.05$) (Fig. 4C). In the deep region, the volume occupied by the blood vessels was increased in the propranolol-treated group compared to the control group, but the difference was not significant (Fig. 4C).

3.6. Measurement of nitrite, MMP-2 and MMP-9 levels

Nitrite dosage was used as an index of NO synthesis, because nitrite is a stable molecule and accounts for more than 90% of total measurable nitrite and nitrate (Schaffer et al., 1997). Fourteen days after wounding, the nitrite levels were respectively 6-fold and 1.3-fold greater in the propranolol-treated group than in the control group in both the wound and plasma ($P<0.05$ for both) (Fig. 5).

To evaluate protease levels in the wound area, the gelatinolytic activity of MMP-2 and MMP-9 was measured 14 days after wounding. The level of active MMP-9 (82 kDa) was 27% lower in the propranolol-treated group than in the control group ($P<0.05$) (Fig. 6). The levels of pro- and active MMP-2 (72 kDa and 62 kDa, respectively) were similar between the control (3.22 a.u. ± 0.25 and 1.03 a.u. ± 0.14, respectively) and propranolol-treated groups (4.13 a.u. ± 0.61 and 0.47 a.u. ± 0.23, respectively).

4. Discussion

In this study, propranolol-treated hyperglycemic animals presented reduced wound areas, lower inflammatory infiltrate and MMP-9 levels, but greater cell proliferation, collagen deposition, blood-vessel density, and NO synthesis when compared to control hyperglycemic animals.

Diabetes mellitus induces poor wound healing of cutaneous lesions by a mechanism which is still unclear. Therefore, several studies have used diabetic animal models to better understand the healing process of those lesions. In this study, we used the model of acute streptozotocin-induced diabetes which does not have the metabolic or pathologic features of long-standing human diabetes, but provides a good and analogous model to study acute wound healing events (Andriessen et al., 1995; Shi et al., 2003). In the present study, the diabetic rats developed typical physiological characteristics observed in diabetic humans such as hyperglycemia, weight loss and poor wound healing (Andriessen et al., 1995). Hyperglycemia impairs wound healing by decreasing cell proliferation and affecting collagen synthesis (Hehenberger et al., 1998). Furthermore, weight loss is associated with poor nutrient intake and contributes partially to the wound-repair defect observed in diabetic animals (Schaffer et al., 1997).

In acute wound healing, the inflammatory response should occur rapidly to permit the development of subsequent phases of wound healing. This requires that inflammatory cells (such as neutrophils and macrophages) migrate to the wound area and phagocytize necrotic tissue and microorganisms, and subsequently secrete several cytokines and growth factors [such as interleukin-1β (IL-1β) and TGF-β] which stimulate the development of granulation tissue (Li et al., 2007). Diabetic wounds show stronger expression of pro-inflammatory cytokines [such as macrophage inflammatory protein-2 (MIP-2), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-alpha (TNF-alpha) and IL-1β] during early and later phases of tissue repair, which stimulates the prolonged persistence of neutrophils and macrophages in the wound area, leading to delay of the subsequent phases of tissue repair (Galkowska et al., 2006; Hubner et al., 1996; Wetzler et al., 2000). In addition, reduced expression of anti-inflammatory cytokines (as IL-10) and growth factors (as TGF-β, VEGF and, IGF-1) as well as impaired activation of macrophages and leukocytes contribute to the chronic inflammatory response of those lesions (Bitar and Labbad, 1996; Delamaire et al., 1997; Galkowska et al., 2006; Maruyama et al., 2007). In the present study, propranolol administration significantly reduced the number of neutrophils and macrophages in the wound area of hyperglycemic diabetic rats when compared to the controls. However, in normoglycemic rats, propranolol administration increased the inflammatory infiltrate in excisional cutaneous lesions (Souza et al., 2006). The positive effect of propranolol on the inflammatory response of diabetic lesions may be explained by propranolol-stimulated NO synthesis. Previous work has demonstrated that NO may directly stimulate the production of chemoattractant cytokines of inflammatory cells (such as IL-8), thus improving the migration of those cells to wound areas (Schwentker et al., 2002). In addition, topical application of NO donors has been shown to reduce the inflammatory infiltrate in excisional cutaneous lesions of normoglycemic rats (Amadeu et al., 2007). Thus, we propose that propranolol administration may reduce the inflammatory response to diabetic wounds through stimulation of NO synthesis and consequently accelerate the development of the subsequent phases of wound healing.

Re-epithelialization is a process of restoring the epidermis and involves proliferation and migration of keratinocytes (Li et al., 2007).

Cell proliferation is an essential event during re-epithelialization, so proliferating keratinocytes ensure an adequate supply of cells to migrate into and cover the wound (Li et al., 2007). Keratinocyte proliferation may occur independently of keratinocyte migration during re-epithelialization (Andriessen et al., 1995). In diabetic foot ulcers, keratinocyte proliferation occurs normally at the wound margin, whereas keratinocyte migration is inhibited (Galkowska et al., 2006). In the present study, propranolol administration enhanced epithelial proliferation, but it did not alter re-epithelialization in hyperglycemic diabetic rats compared to controls. However, in normoglycemic animals, propranolol administration impaired both epithelial proliferation and re-epithelialization in excisional cutaneous lesions (Souza et al., 2006). The positive effect of propranolol on epithelial proliferation of diabetic lesions may be explained by the propranolol-stimulated NO synthesis. It has been showed that NO increases the secretion of VEGF and TGF-β1, which stimulate the proliferation of keratinocytes (Frank et al., 2000; Schwentker et al., 2002). Thus, we propose that propranolol administration may increase the epithelial proliferation on diabetic wounds through NO synthesis.

Dermal reconstruction is characterized by the formation of granulation tissue, which includes cell proliferation, extracellular matrix (ECM) deposition, wound contraction, and angiogenesis (Li et al., 2007). Initially, fibroblasts proliferate and subsequently migrate to a wound area and then deposit and organize the ECM (Li et al., 2007). In diabetic wounds, the formation of granulation tissue is seriously impaired due to reduced fibroblast proliferation and lower levels of collagen deposition and wound contraction (Darby et al., 1997; Galkowska et al., 2006; Lerman et al., 2003). Previous work demonstrated that diabetes significantly reduces NO synthesis, resulting in impaired fibroblast proliferation and collagen deposition (Schaffer et al., 1997; Schwentker et al., 2002). Furthermore, high concentrations of inflammatory cytokines stimulate the persistence of increased levels of proteases (such as MMP-9) which destroy both growth factors (such as TGF-β1) and ECM, leading to reduced cell proliferation and wound contraction (Bitar and Labbad, 1996; Darby et al., 1997; Lobmann et al., 2002). In the present study, propranolol administration enhanced cell proliferation, collagen deposition, and wound contraction in hyperglycemic diabetic rats compared to controls. However, it was demonstrated that propranolol administration impaired cell proliferation, collagen deposition and wound contraction in normoglycemic rats (Souza et al., 2006). The beneficial effect of propranolol on granulation tissue formation in cutaneous diabetic lesions may be explained by the propranolol-stimulated NO synthesis. Several studies have demonstrated that the supplementation with L-arginine or administration of NO donors improves fibroblasts proliferation, collagen deposition and the closure rate of cutaneous lesions in diabetic animals (Masters et al., 2002; Schaffer et al., 2007; Shi et al., 2003; Weller and Finnen, 2006; Witte et al., 2002). Furthermore, NO donors also reduce the expression of MMP-9 by human diabetic skin fibroblasts, which may reduce chronic inflammation and promote fibroblastic proliferation and ECM synthesis (Burrow et al., 2007). It was also shown that activation of β_3-adrenoceptors stimulates the cyclic guanosine monophosphate/NO pathway in human skin fibroblasts via endothelial NO synthase activity (Furlan et al., 2005). Furthermore, administration of selective β_3-receptor agonist was shown to enhance proliferation of neonatal mouse skin fibroblasts and the rate of wound closure in animal models of type II diabetes (Anesini and Borda, 2002; Schaeffer et al., 2006). Nevertheless, administration of propranolol does not block those effects (Anesini and Borda, 2002). Thus, we speculate that propranolol treatment may have enhanced NO synthesis via activation of β_3-adrenoceptors, leading to a reduction of MMP-9 levels and subsequently increased cell proliferation, deposition of collagen fibers, and wound contraction.

Angiogenesis refers to new vessel growth by the sprouting of pre-existing vessels adjacent to the wound (Li et al., 2007). New blood vessels are important to sustain the development of granulation tissue (Amadeu et al., 2003). Angiogenesis is severely impaired in diabetic wounds, probably due to reduced production of VEGF and IGF-1 (Bitar and Labbad, 1996; Galkowska et al., 2006). A previous study reported that β-receptor antagonists (such as propranolol and metoprolol) might reduce skin blood flow and therefore be hazardous to hypertensive diabetic patients (Hyer et al., 1987). Moreover, propranolol administration impairs both angiogenesis and mobilization of mast cells in normoglycemic diabetic rats (Souza et al., 2006). However, in the present study, propranolol administration enhanced blood vessel density and mast cell number in excisional lesions of hyperglycemic diabetic rats when compared to controls. The beneficial effect of propranolol on angiogenesis in diabetic lesions may be explained by the increased NO synthesis. It has been shown that NO increases VEGF expression, which is the most potent angiogenic factor during wound healing (Schwentker et al., 2002). Furthermore, topical application of NO donors increases the number of mast cells, which may stimulate the formation of new blood vessels (Amadeu et al., 2007; Azizkhan et al., 1980; Meininger and Zetter, 1992). Thus, we suggest that propranolol treatment may result in enhanced angiogenesis and mast cell mobilization through its stimulatory effects on NO synthesis leading to improved formation of granulation tissue.

In conclusion, propranolol administration improves cutaneous wound healing of hyperglycemic diabetic rats by reducing both the local inflammatory response and MMP-9 levels, by improving wound contraction, development of granulation tissue, collagen deposition and angiogenesis, and by stimulating NO synthesis.

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References

Amadeu, T.P., Braune, A., Mandarim-de-Lacerda, C., Porto, L.C., Desmouliere, A., Costa, A.M.A., 2003. Vascularization pattern in hypertrophic scars and keloids: a stereological analysis. Pathol. Res. Pract. 199, 469–473.

Amadeu, T.P., Seabra, A.B., de Oliveira, M.G., Costa, A.M.A., 2007. S-nitrosoglutathione-containing hydrogel accelerates rat cutaneous wound repair. J. Eur. Acad. Dermatol. Venereol. 21, 629–637.

Andriessen, M.P., van Bergen, B.H., Spruijt, K.I., Go, I.H., Schalkwijk, J., van de Kerkhof, P.C., 1995. Epidermal proliferation is not impaired in chronic venous ulcers. Acta Dermatol. Venereol. 75, 459–462.

Anesini, C., Borda, E., 2002. Modulatory effect of the adrenergic system upon fibroblast proliferation: participation of beta 3-adrenoceptors. Auton. Autacoid. Pharmacol. 22, 177–186.

Azizkhan, R.G., Azizkhan, J.C., Zetter, B.R., Folkman, J., 1980. Mast cell heparin stimulates migration of capillary endothelial cells in vitro. J. Exp. Med. 152, 931–944.

Baddeley, A.J., Gundersen, H.J., Cruz-Orive, L.M., 1986. Estimation of surface area from vertical sections. J. Microsc. 142, 259–276.

Bild, D.E., Selby, J.V., Sinnock, P., Browner, W.S., Braveman, P., Showstack, J.A., 1989. Lower-extremity amputation in people with diabetes. Epidemiology and prevention. Diabetes Care 12, 24–31.

Bitar, M.S., Labbad, Z.N., 1996. Transforming growth factor-beta and insulin-like growth factor-I in relation to diabetes-induced impairment of wound healing. J. Surg. Res. 61, 113–119.

Brem, H., Tomic-Canic, M., 2007. Cellular and molecular basis of wound healing in diabetes. J. Clin. Invest. 117, 1219–1222.

Burrow, J.W., Koch, J.A., Chuang, H.H., Zhong, W., Dean, D.D., Sylvia, V.L., 2007. Nitric oxide donors selectively reduce the expression of matrix metalloproteinases-8 and -9 by human diabetic skin fibroblasts. J. Surg. Res. 140, 90–98.

Darby, I.A., Bisucci, T., Hewitson, T.D., MacLellan, D.G., 1997. Apoptosis is increased in a model of diabetes-impaired wound healing in genetically diabetic mice. Int. J. Biochem. Cell Biol. 29, 191–200.

Delamaire, M., Maugendre, D., Moreno, M., Le Goff, M.C., Allannic, H., Genetet, B., 1997. Impaired leucocyte functions in diabetic patients. Diabet. Med. 14, 29–34.

Frank, S., Kampfer, H., Podda, M., Kaufmann, R., Pfeilschifter, J., 2000. Identification of copper/zinc superoxide dismutase as a nitric oxide-regulated gene in human (HaCaT) keratinocytes: implications for keratinocyte proliferation. Biochem. J. 346, 719–728.

Furlan, C., Sterin-Borda, L., Borda, E., 2005. Activation of beta3 adrenergic receptor decreases DNA synthesis in human skin fibroblasts via cyclic GMP/nitric oxide pathway. Cell. Physiol. Biochem. 16, 175–182.

Galkowska, H., Wojewodzka, U., Olszewski, W.L., 2006. Chemokines, cytokines, and growth factors in keratinocytes and dermal endothelial cells in the margin of chronic diabetic foot ulcers. Wound Repair Regen. 14, 558–565.
Gibran, N.S., Jang, Y.C., Isik, F.F., Greenhalgh, D.G., Muffley, L.A., Underwood, R.A., Usui, M.L., Larsen, J., Smith, D.G., Bunnett, N., Ansel, J.C., Olerud, J.E., 2002. Diminished neuropeptide levels contribute to the impaired cutaneous healing response associated with diabetes mellitus. J. Surg. Res. 108, 122–128.
Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal. Biochem. 126, 131–138.
Gundersen, H.J., Bagger, P., Bendtsen, T.F., Evans, S.M., Korbo, L., Marcussen, N., Moller, A., Nielsen, K., Nyengaard, J.R., Pakkenberg, B., et al., 1988. The new stereological tools: dissector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS 96, 857–881.
Hehenberger, K., Heilborn, J.D., Brismar, K., Hansson, A., 1998. Inhibited proliferation of fibroblasts derived from chronic diabetic wounds and normal dermal fibroblasts treated with high glucose is associated with increased formation of l-lactate. Wound Repair Regen. 6, 135–141.
Hubner, G., Brauchle, M., Smola, H., Madlener, M., Fassler, R., Werner, S., 1996. Differential regulation of pro-inflammatory cytokines during wound healing in normal and glucocorticoid-treated mice. Cytokine 8, 548–556.
Hyer, S.L., Taylor, D., Barham, J., Wilson, J., Shaw, J., Vince, F.P., 1987. The effects of propranolol and metoprolol on skin blood flow in diabetic patients. Br. J. Clin. Pharmacol. 23, 769–771.
Lerman, O.Z., Galiano, R.D., Armour, M., Levine, J.P., Gurtner, G.C., 2003. Cellular dysfunction in the diabetic fibroblast: impairment in migration, vascular endothelial growth factor production, and response to hypoxia. Am. J. Pathol. 162, 303–312.
Levy, D.M., Karanth, S.S., Springall, D.R., Polak, J.M., 1989. Depletion of cutaneous nerves and neuropeptides in diabetes mellitus: an immunocytochemical study. Diabetologia 32, 427–433.
Li, J., Chen, J., Kirsner, R., 2007. Pathophysiology of acute wound healing. Clin. Dermatol. 25, 9–18.
Lobmann, R., Ambrosch, A., Schultz, G., Waldmann, K., Schiweck, S., Lehnert, H., 2002. Expression of matrix-metalloproteinases and their inhibitors in the wounds of diabetic and non-diabetic patients. Diabetologia 45, 1011–1016.
Maruyama, K., Asai, J., Ii, M., Thorne, T., Losordo, D.W., D Amore, P.A., 2007. Decreased macrophage number and activation lead to reduced lymphatic vessel formation and contribute to impaired diabetic wound healing. Am. J. Pathol. 170, 1178–1191.
Masters, K.S., Leibovich, S.J., Belem, P., West, J.L., Poole-Warren, L.A., 2002. Effects of nitric oxide releasing poly(vinyl alcohol) hydrogel dressings on dermal wound healing in diabetic mice. Wound Repair Regen. 10, 286–294.
Meininger, C.J., Zetter, B.R., 1992. Mast cells and angiogenesis. Semin. Cancer Biol. 3, 73–79.
Nascimento, A.P., Costa, A.M.A., 2006. Overweight induced by high-fat diet delays rat cutaneous wound healing. Br. J. Nutr. 96, 1069–1077.
Niu, R., Okamoto, T., Iwase, K., Nomura, S., Mizutani, S., 2000. Quantitative analysis of matrix metalloproteinases-2 and -9, and their tissue inhibitors-1 and -2 in human placenta throughout gestation. Life Sci. 66, 1127–1137.
Reagan-Shaw, S., Nihal, M., Ahmad, N., 2008. Dose translation from animal to human studies revisited. FASEB J. 22, 659–661.
Schaeffer, P., Bernat, A., Arnone, M., Manara, L., Gallas, J.F., Dol-Gleizes, F., Millet, L., Grosset, A., Herbert, J.M., 2006. Effect of SR58611A, a potent beta-3 adrenoceptor agonist, on cutaneous wound healing in diabetic and obese mice. Eur. J. Pharmacol. 529, 172–178.
Schaffer, M.R., Tantry, U., Efron, P.A., Ahrendt, G.M., Thornton, F.J., Barbul, A., 1997. Diabetes-impaired healing and reduced wound nitric oxide synthesis: a possible pathophysiologic correlation. Surgery 121, 513–519.
Schaffer, M., Bongartz, M., Fischer, S., Proksch, B., Viebahn, R., 2007. Nitric oxide restores impaired healing in normoglycaemic diabetic rats. J. Wound Care 16, 311–316.
Schwentker, A., Vodovotz, Y., Weller, R., Billiar, T.R., 2002. Nitric oxide and wound repair: role of cytokines? Nitric Oxide 7, 1–10.
Shi, H.P., Most, D., Efron, D.T., Witte, M.B., Barbul, A., 2003. Supplemental L-arginine enhances wound healing in diabetic rats. Wound Repair Regen. 11, 198–203.
Souza, B.R., Santos, J.S., Costa, A.M.A., 2006. Blockade of beta1- and beta2-adrenoceptors delays wound contraction and re-epithelialization in rats. Clin. Exp. Pharmacol. Physiol. 33, 421–430.
Stark, J.M., van Egmond, A.W., Zimmerman, J.J., Carabell, S.K., Tosi, M.F., 1992. Detection of enhanced neutrophil adhesion to parainfluenza-infected airway epithelial cells using a modified myeloperoxidase assay in a microtiter format. J. Virol. Methods 40, 225–242.
Varelias, A., Cowin, A.J., Adams, D., Harries, R.H., Cooter, R.D., Belford, D.A., Fitridge, R.A., Rayner, T.E., 2006. Mitogenic bovine whey extract modulates matrix metalloproteinase-2, -9, and tissue inhibitor of matrix metalloproteinase-2 levels in chronic leg ulcers. Wound Repair Regen. 14, 28–37.
Watkins, P.J., Edmonds, M.E., 1983. Sympathetic nerve failure in diabetes. Diabetologia 25, 73–77.
Weller, R., Finnen, M.J., 2006. The effects of topical treatment with acidified nitrite on wound healing in normal and diabetic mice. Nitric Oxide 15, 395–399.
Wetzler, C., Kampfer, H., Stallmeyer, B., Pfeilschifter, J., Frank, S., 2000. Large and sustained induction of chemokines during impaired wound healing in the genetically diabetic mouse: prolonged persistence of neutrophils and macrophages during the late phase of repair. J. Invest. Dermatol. 115, 245–253.
Wild, S., Roglic, G., Green, A., Sicree, R., King, H., 2004. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 27, 1047–1053.
Witte, M.B., Thornton, F.J., Tantry, U., Barbul, A., 2002. L-Arginine supplementation enhances diabetic wound healing: involvement of the nitric oxide synthase and arginase pathways. Metabolism 51, 1269–1273.
Woessner, J.F., 1961. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch. Biochem. Biophys. 93, 440–447.