—Original—

The characterization of a full-thickness excision open foot wound model in n5-streptozotocin (STZ)-induced type 2 diabetic rats that mimics diabetic foot ulcer in terms of reduced blood circulation, higher C-reactive protein, elevated inflammation, and reduced cell proliferation

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Abstract: Delayed foot wound healing is a major complication attributed to hyperglycemia in type 2 diabetes mellitus (DM) patients, and these wounds may develop into foot ulcers. There are at least two types of DM wound models used in rodents to study delayed wound healing. However, clinically relevant animal models are not common. Most models use type 1 DM rodents or wounds created on the back rather than on the foot. An open full-thickness excision wound on the footpad of type 2 DM rats is more clinically relevant, but such a model has not yet been characterized systematically. The objective of this study was to investigate and characterize how DM affected a full-thickness excision open foot wound in n5-streptozotocin (n5-STZ)-induced type 2 DM rats. We hypothesized that elevated inflammation, reduced blood circulation, and cell proliferation due to hyperglycemia could delay the wound healing of DM rats. The wounds of DM rats were compared with those of non-DM rats (Ctrl) at Days 1 and 8 post wounding. The wound healing process of the DM rats was significantly delayed compared with that of the Ctrl rats. The DM rats also had higher C-reactive protein (CRP) and lower blood circulation and proliferating cell nuclear antigen (PCNA) in DM wounds. This confirmed that elevated inflammation and reduced blood flow and cell proliferation delayed foot wound healing in the n5-STZ rats. Hence, this open foot wound animal model provides a good approach to study the process of delayed wound healing.
Key words: diabetes mellitus, foot wound, streptozotocin, wound healing

Introduction

Type 2 diabetes mellitus (DM) is a major medical problem and constitutes 90–95% of all diabetes in the population worldwide [18]. Delayed wound healing is one of complications of type 2 DM due to multiple factors such as poor blood circulation [4], prolonged inflammation, and hyperglycemia. It is a common cause of morbidity and mortality among DM patients [24]. Once a wound becomes chronic, it is prone to development of foot ulcers, including neuropathy, and foot deformities [4]. DM foot ulcers cause over 50% of all nontraumatic lower-leg amputations [44]. Evidence has shown that hyperglycemia is one of the major factors contributing to delayed wound healing [24] by increasing cell apoptosis and reducing cell survival in diabetic wounds. It has been shown to inhibit proliferation of endothelial cells and fibroblasts in humans [47], with up to 75% slower in an adult DM mouse compared with a control mouse [26].

Normal wound healing requires a well-orchestrated integration of the following overlapping events: hemostasis, appropriate inflammation, mesenchymal cell differentiation, proliferation, and migration to the wound site, angiogenesis, reepithelialization, granulation tissue formation, wound contraction, and tissue remodeling [4, 17]. The complex process requires proper circulation [1, 27] and involves the interaction and migration of various types of cells such as inflammatory cells, fibroblasts, keratinocytes, and endothelial cells with growth factors and enzymes [4, 23]. However, excessive or uncontrolled inflammation can promote tissue injury as observed in patients with type 2 DM [49], where DM disrupts each phase of healing progress by affecting various types of cells and molecular effectors [29]. DM wounds frequently enter a state of pathologic inflammation due to a postponed, incomplete, or uncoordinated healing process [17]. At the macroscopic level, delayed wound healing is associated with poor blood circulations [4]. Hence, this complex healing process requires a good animal model for research to understand its mechanism and explore new therapies.

Excision [34] and incision [31] wounds are two major types of wound models used for DM wound healing studies. An excision wound model is more suitable to study of open wound healing because it produces more wound and scar tissue for analysis [40] and does not require stitches after surgery [31]. In past DM foot ulcer/

wound studies, type 1 DM was induced in most rodents, such as rats and mice [43, 52, 54]. Furthermore, wounds were usually created on the back of the rat/mouse [3, 6, 15, 53]. There are a few papers in which wounds were created on the plantar skin of the paw [20] or at the foot dorsal [43], but none of these wound models used type 2 DM rats. Since 90% of DM patients worldwide have type 2 DM [41] and approximately 5–7% of type 2 patients will eventually have a foot ulcer [28], it is important to have a clinically relevant animal model for research, and it should be an open wound model on the foot in type 2 DM rats. There is a relevant animal model that induces an open full-thickness excision wound on the dorsal side of the footpad using type 2 DM rats [25]; however, the model has only been used to address the effect of oral medication without a systematic characterization of the model. The objective of this study was to investigate and characterize how DM affects an open full-thickness excision foot wound in n5-streptozotocin (n5-STZ)-induced type 2 DM rats. We hypothesized that elevated inflammation, reduced blood circulation, and reduced cell proliferation due to hyperglycemia could delay healing of the foot wound in n5-STZ-induced DM rats.

Materials and Methods

Animal

A total of 60 female albino Wistar rats were used in this study, and they were equally divided into DM and non-DM (Ctrl) groups (30 rats/group). In the DM group, 5-day old Wistar rats were intraperitoneally injected with streptozotocin (STZ, Sigma-Aldrich, 70 mg/kg) that was freshly dissolved in 0.1 M citrate buffer (pH 4.5). The Ctrl group was injected with citrate buffer only. All rats were supplied by and kept in the Laboratory Animal Service Center of the Chinese University of Hong Kong. All rats resided in a room with a 12-h light-dark cycle and a constant temperature between 22–25°C. All rats were provided with a sufficient amount of a normal rodent diet *ad libitum* (Prolab 2500 rodent diet) and tap water daily. Body weights were measured weekly from Day 5 until the end point of the study (10–12 weeks old).

The animal experiments were conducted under a license issued under the Animals (control of experiments) Ordinance (Cap. 340) issued by the Department of Health of the Hong Kong government, and approval was obtained from the Animal Experimentation Ethics Com-

mittee (Ref: 13/085/GRF-5), the Chinese University of Hong Kong.

Foot wounding animal model

Induction of an open foot wound was based on an excisional model [25]. Nine weeks after STZ injection, a glucometer (Contour Plus, Bayer Healthcare, Germany) was used to determine if each rat's blood glucose level met the severe diabetes level of≥300 mg/dl by drawing blood at the tip of the tail. Adult DM rats with a blood glucose level greater than 300 mg/dl were used for open wound induction. On the day of open wound induction (Day 0), each rat was anesthetized with 75 mg/kg ketamine and 10 mg/kg xylazine by intraperitoneal injection. The surface skin of the right footpad was shaved and cleaned with an 70% ethanol wipe. A 2 mm × 5 mm rectangular full thickness wound was created in the skin of the footpad on the right hind leg of each rat using a scalpel [25]. This model was used to evaluate the epithelialization, contraction, and inflammation outcomes of the n5-STZ-induced DM rats.

Time point design

The wound area was measured with photo imaging software (SPOT 3.5.5 for Windows) at Day 1 post wounding as the initial wound size area and Day 8 post wounding as the wound at proliferation/angiogenesis stage. Blood glucose level and blood perfusion were measured at Day 1, Day 4, Day 8, and Day 13 post wounding with a Contour Plus glucometer (Bayer Healthcare, Germany) and laser Doppler imaging system (Moor Instruments Ltd., Devon, UK) [25].

Wound size measurements

Wound size (area) was evaluated by macro photography [25]. Rat wounds and a metric ruler with standardized 1 cm × 1 cm squares were photographed simultaneously. Digital photographs of the injury site were taken using a Canon SX50HS digital camera with white lighting. The area of the wound size was calculated using the photo imaging software (SPOT 3.5.5 for Windows) calibrated to reference squares. The metric ruler was used for calculating the wound area in the SPOT software.

Blood perfusion by laser doppler measurement

The wounded foot was placed flat on a platform and scanned by the laser Doppler imaging system (Moor Instruments Ltd., Devon, UK) using the repeat image measurement mode. The unwounded foot of each rat was also scanned as a control. Flux data were obtained from the process and used as the measurement data. All data were quantified and automatically calculated using the Moor FLPI measurement software (Version 2.1) [22].

ELISA of C-reactive protein (CRP)

Under general anesthesia, 1 ml of blood was extracted from each rat's heart through cardiac puncture before euthanasia. The blood sample was then centrifuged at 9,500 rpm for 10 mins. The serum was collected and diluted at 1:600,000. Fifty microliters of each sample was used to determine the CRP level according to the instructions of a Rat C Reactive Protein ELISA Kit (PTX1) (ab108827, abcam, Cambridge, UK).

Histology

The skin tissue from the wound site and its surrounding unwounded area were collected from all the rats for histological analyses [35]. Samples were obtained after rats were euthanized with an overdose of sodium pentobarbital at Day 1, Day 4, Day 8, and Day 13 post wounding. Samples were fixed in 4% paraformaldehyde for 48 h, embedded in paraffin, and sectioned at 8 μm. The sections were deparaffinized in xylene and rehydrated before staining with hematoxylin and eosin (H&E). Section images were captured with a microscope (DM5000, Leica Microsystems GmbH, Wetzlar, Germany) to evaluate reepithelialization, granulation tissue, and inflammatory response.

Immunohistochemistry: proliferating cell nuclear antigen (PCNA)

Some histological sections were used for immunohistochemistry [7]. Sections were incubated with an anti-PCNA rabbit polyclonal antibody (ab18197, abcam, Cambridge, UK) diluted at 1/4,000 in PBS for 2 h, washed in PBS, and incubated with a Mouse and Rabbit Specific HRP/DAB Detection IHC kit (abcam, Cambridge, UK). Sections were counterstained with hematoxylin and then dehydrated with ethanol. Control specimens were also stained, but PBS was substituted for the primary antibody. All section images were captured with a microscope.

Statistical analysis

All data were expressed as the mean ± standard de-

Table 1. Comparision of the average blood glucose levels between DM and Ctrl rats at Days 1, 4, 8, and 13 post wounding

	Day 1	Day 4	Day 8	Day 13
Ctrl	109.00 ± 25.70 mg/dl	119.47 ± 28.76 mg/dl	115.65 ± 31.90 mg/dl	95.65 ± 20.45 mg/dl
DM	476.33 ± 93.11 mg/dl	476.95 ± 75.95 mg/dl	550.76 ± 64.93 mg/dl	491.26 ± 82.96 mg/dl

Significant differences ($P<0.001$ for all) were observed at all time points between DM and Ctrl rats.

viation. Two-way analysis of variance (ANOVA) was used to analyze the main effects between the two groups (Ctrl and DM), and time point differences were analyzed with post hoc Bonferroni tests. All tests were two-tailed. Student's *t*-test for two independent samples was used for comparisons between the Ctrl and DM groups. Statistical analyses were performed using IBM SPSS Statistics 20.0 (IBM, Armonk, NY, USA), and statistical significance was considered at $P<0.05$.

Sample size estimation

A sample size of 6 in each group is sufficient to detect a difference in treatment effect using an ANOVA (analysis of variance) test with 80% power and a 0.05 two-sided significance level (PASS 11.0, NCSS, LLC, Kaysville, UT, USA).

Results

Rat physical health

The weights of DM rats were significantly lower than those of the rats in the Ctrl group beginning in week 3 after birth (Fig. 1). The mortality rate was approximately 45%. DM rats also increased their food and water uptakes beginning at week 5 after birth. The glucose levels of the DM rats were significantly higher than those of the Ctrl rats from week 2 to week 12 ($P<0.05$ for all). The average blood glucose level of DM rats was 498 mg/dl (Table 1).

Wound morphology

Wounds created by scalpel and scissors (Day 0) were consistent in size and shape with minimal to no bleeding in both groups. All open wound inductions were conducted by the same researcher. At 24 h post wounding, the wound size was increased in both groups, as the edges of the wounds were stretched farther apart due to edema and swelling expanded to the whole foot (Fig. 2A). Swelling at the foot wound continued in DM rats at Day 13, but it subsided in Ctrl rats as early as Day 4
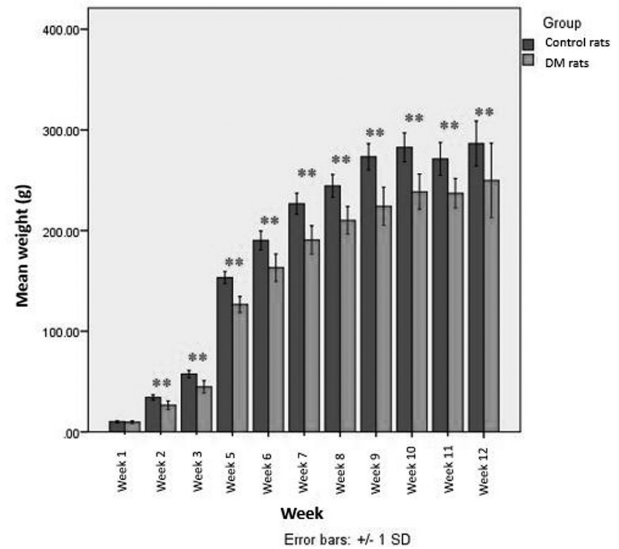

Fig. 1. Weight comparison between the DM and Ctrl groups (n=20/group). Weight differences between DM and Ctrl rats from weeks 1 to 12. Week 4 was excluded because it was the end of the weaning period and the rats were adjusting to a new diet. Other than in week 1, the weights of the DM rats were significantly lower than those of the Ctrl rats. Data are presented as the mean ± SD. ** Significant difference.

post wounding. At Day 8 post wounding, the area of the wound in the DM rats (9.29 ± 0.88 mm^2) was significantly larger ($P=0.048$) than that in the Ctrl rats (7.91 ± 1.80 mm^2) at Day 8 (Fig. 2B).

Blood perfusion by laser doppler measurement

Laser Doppler imaging system was used to scan the surface of the wounded foot to evaluate the blood flow around the wound in terms of flux (n=6/ time point) (Fig. 3A), where flux was expressed in terms of arbitrary perfusion units rather than absolute values for the blood flow speed. The flux values of DM rats (Day 1, 167.91 ± 89.32; Day 4, 252.83 ± 100.24; Day 13, 49.89 ± 16.46) were significantly lower than those of Ctrl rats (Day 1, 287.48 ± 174.73; Day 4, 341.79 ± 142.207; Day 13, 163.63 ± 79.83) ($P=0.002$, 0.017, and 0.000, respectively). The data indicated that the blood flow around
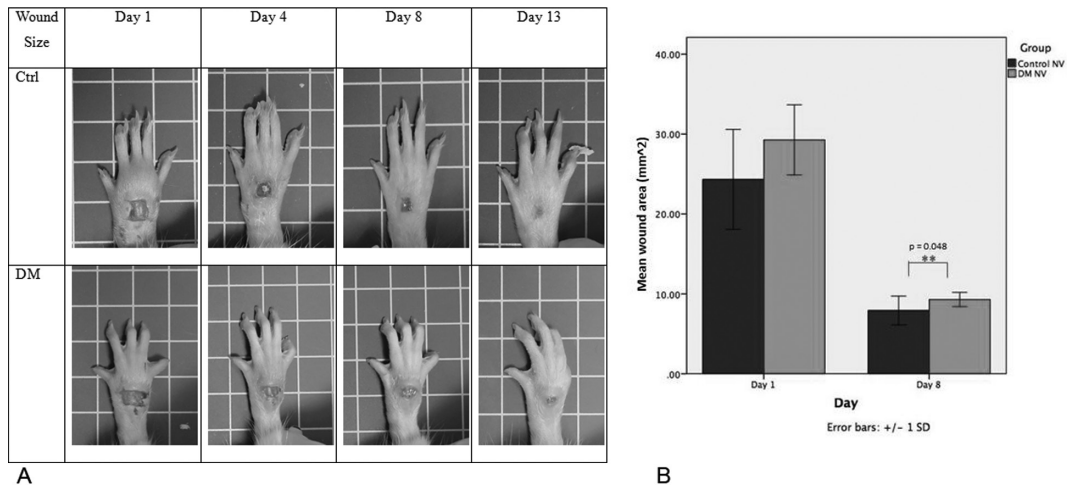
Fig. 2. Images of the foot wounds at Days 1, 4, 8, and 13 post wounding and comparison of wound size (area) between the DM and Ctrl groups (n=6/time point). A) At Day 1, the foot wounds of both the DM (a) and Ctrl (e) rats expanded from their original wound sizes within 24 h after open wound induction. At Day 4, the foot wound of the DM rats (f) remained swollen, and the hypodermis layer can still be observed from the outside. The foot wound edges of the Ctrl rats (b) were no longer stretched apart due to edema. Secondary wound healing was observed in the wound of the Ctrl rats, with the wound edges pulling into the center of the wound, forming an eclipse, and a scab was observed on the surface of the wound. At Day 8, the wound size of the Ctrl rats (c) had shrunken into half of its original size (a). Swelling can still be observed at the wound edges of the DM rats (g), and the hypodermis layer can still be seen from the outside. At Day 13, the wound of the Ctrl rats (d) was fully closed, while that of the DM rats remained open. B) The area of the wounds are shown for the DM and Ctrl rats at Days 1 and 8 post wounding. The area of the wound in the DM rats was significantly bigger than that in the Ctrl rats at Day 8 post wounding (*P*=0.048). ** Significant difference.
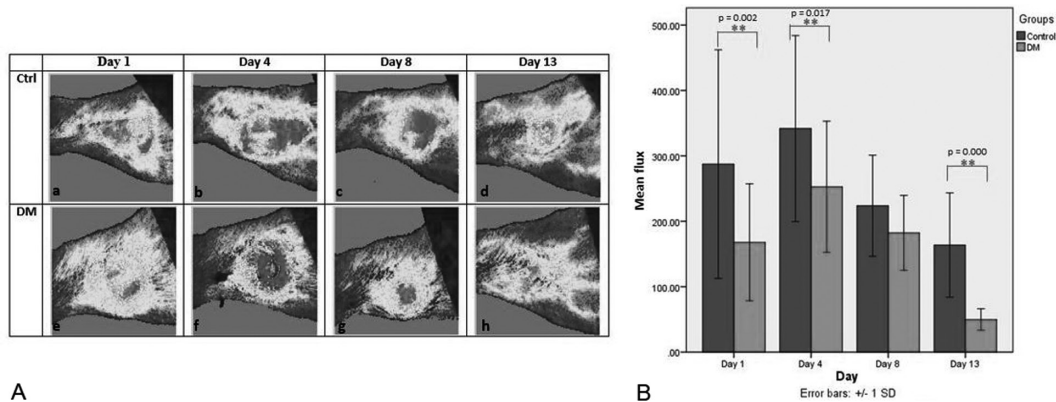

Fig. 3. Laser Doppler images of blood flow at the foot wounds and comparison of laser Doppler imaging measurements (Flux) of DM and Ctrl rats at Days 1, 4, 8, and 13 post wounding (n=6/ time point). A) The images indicated that Ctrl rats (a, b, c, and d) had more blood circulation than DM rats (e, f, g, and h) from Day 1 to Day 13 post wounding. B) DM rats had significantly lower flux values than Ctrl rats at Days 1, 4, and 13 post wounding (*P*=0.002, 0.017, and 0.000, respectively). Blood perfusion showed differences of up to 41.59%, 26.02%, and 69.51% between Ctrl and DM rats on Days 1, 4, and 13 post wounding, respectively. ** Significant difference.

and in the wound of the DM rats was significantly lower than that in Ctrl rats (Fig. 3B).

ELISA and CRP

CRP was used as a marker to compare the inflamma-

tion markers between DM and Ctrl rats post wounding (n=6/ time point) (Fig. 4). The CRP levels of DM rats (Day 4, 52.92 ± 7.62 ng/ml; Day 8, 53.03 ± 2.66 ng/ml; Day 13, 57.00 ± 5.13 ng/ml) were significantly higher than those of Ctrl rats (Day 4, 39.60 ± 5.83 ng/ml; Day
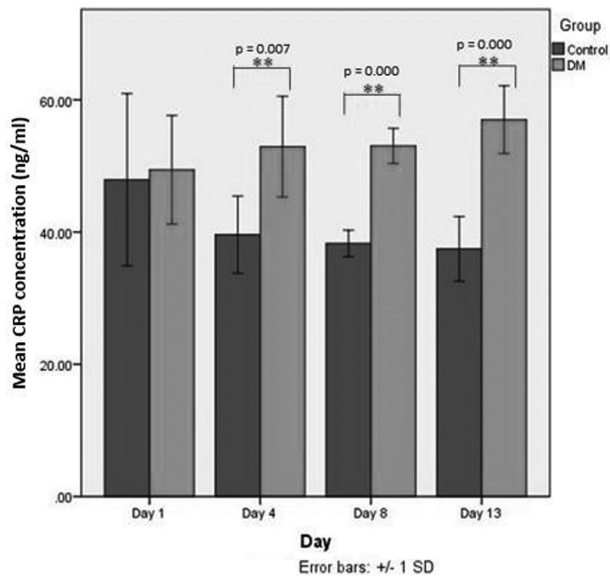
Fig. 4. Comparison of C-reactive protein (CRP) levels between the DM and Ctrl groups (n=6/ time point). The concentrations of CRP of the DM rats increased with time, while those of Ctrl rats declined. Significant differences were observed at Day 4, Day 8, and Day 13 post wounding, with the CRP concentrations of the DM rats being significantly higher than those of the Ctrl group (P=0.007, 0.000, and 0.000, respectively). The CRP level showed differences of up to 25.17%, 27.81%, and 34.28% between Ctrl and DM rats at Days 4, 8, and 13 post wounding, respectively. ** Significant difference.

8, 38.28 ± 2.01 ng/ml; Day 13, 37.46 ± 4.88 ng/ml) (P=0.007, 0.000, and 0.000, respectively).

Histology

At Day 1 post wounding, a new epithelium was observed on the surface of the wounds in both groups (n=6/ time point) (Fig. 5A). At Day 4 post wounding, the epithelial layer of DM rats was not uniformly formed on the surface of the wound, and inflammation was observed in both groups. At Day 8 post wounding, inflammation persisted with accumulation of neutrophils between layers and in the epidermis in the foot wound of the DM rats, while this had subsided in the Ctrl rats, and granulation tissue moved down to the base of the wound. At Day 13 post wounding, inflammation in the foot wound of the DM rats finally subsided, but capillaries were not observed. On the other hand, the foot wound of the Ctrl rats was almost completely healed or was completely healed at Day 13 post wounding with capillaries observed at the base of the wound.
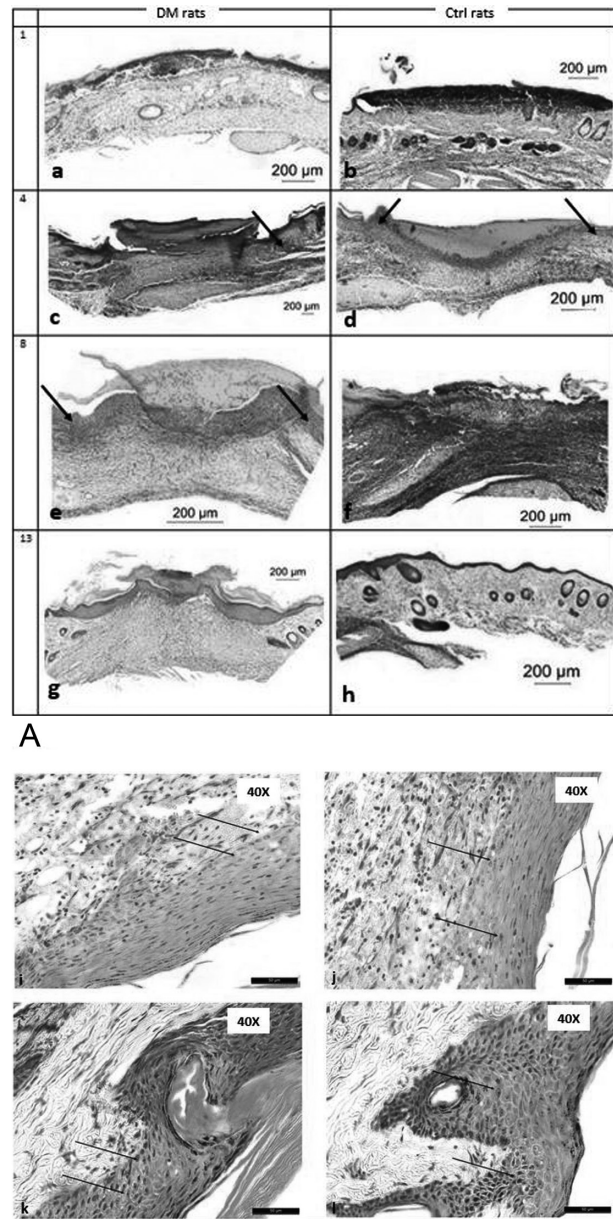
At Day 4 post wounding, the epithelial layer of Ctrl

Fig. 5. Representative histology images of DM rats and Ctrl rats (n=6/ time point). A) H&E staining showed that inflammation persisted with accumulation of neutrophils in the foot wounds of DM rats until Day 13 post wounding (c and e), while inflammation in Ctrl rats subsided after Day 4 post wounding. Compared with Ctrl rats, no capillaries were observed in the wounds of DM rats at Days 8 and 13 post wounding. Note: Arrows indicate the locations of neutrophils. B) Representative histology images of DM rats (image e in Fig. 5A) and Ctrl rats (image d in Fig. 5A) at 40× magnification. H&E staining at 40× showed that accumulation of neutrophils (arrows) at Day 4 in the DM rats showing neutrophil that persisted up to Day 8 post wounding (images k and l); in contrast to the Ctrl rats (images i and j) that shows earlier progression to the proliferative phase with less neutrophil accumulation. **Arrows indicate the locations of neutrophils.
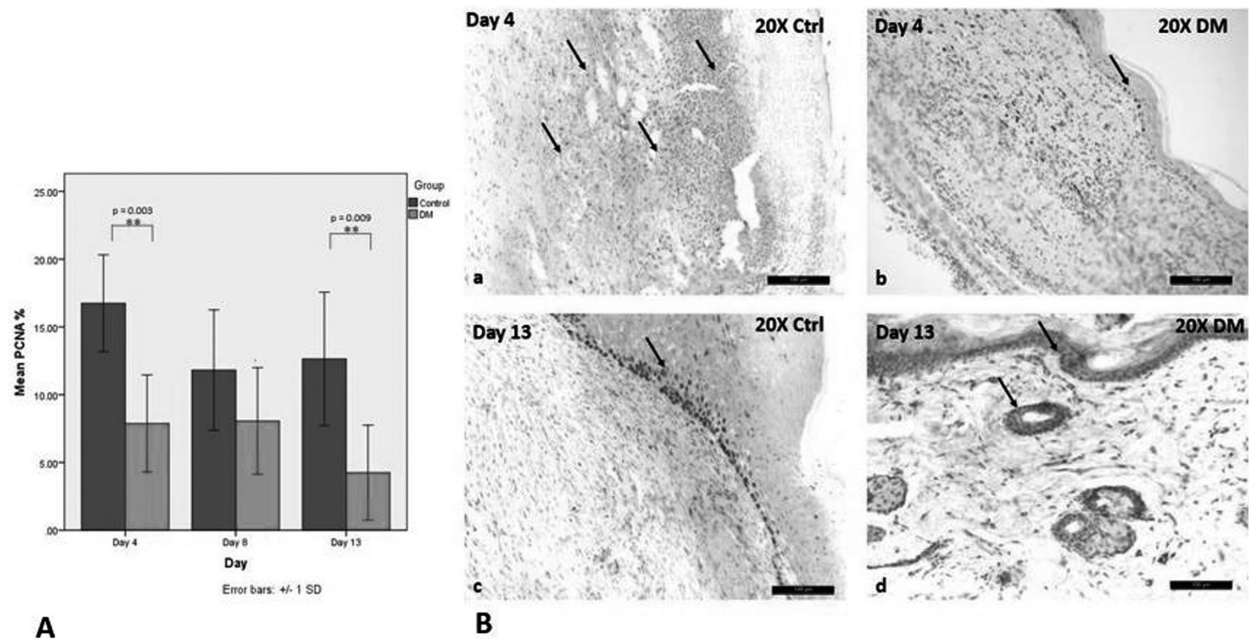
Fig. 6. Comparison of PCNA immunohistochemistry between the DM and Ctrl groups (n=6/ time point). A) The PCNA expression of the Ctrl group was generally higher than that of the DM group, with significant differences at Day 4 and Day 13 (*P*=0.003 and 0.009, respectively). PCNA expression showed differences of up to 52.98% and 66.45% between Ctrl and DM rats at Days 4 and 13 post wounding, respectively. **Significant difference. B) Representative images of PCNA immunohistochemistry stains at 20× for the Ctrl (a and c) and DM (b and d) rats at Day 4 and Day 13. Note: Arrows indicate PCNA expression.

rats was more completed than that observed in the DM rats. A crust/scab was observed on the wound surface of the Ctrl rats. At Day 8 post wounding, scar tissue was more visible in Ctrl rats than in DM rats, and the granulation tissue moved down to the base of the wound. Inflammation was still observed in DM rats at Day 8 post wounding, while inflammation had subsided in Ctrl rats, and a dry scab was observed on the surface of the wound (Fig. 5B).

Immunohistochemistry (PCNA)

Immunohistochemistry of PCNA was performed to evaluate cell proliferation during the inflammation and proliferation phases of the wound healing process (n=6/ time point). The PCNA expression levels of Ctrl rats (Day 4, 16.74 ± 3.57%; Day 13, 12.64 ± 4.92%) were significantly higher than those observed in DM rats (Day 4, 7.87 ± 3.57%; Day 13, 4.24 ± 3.50%) (*P*=0.003 and 0.009, respectively) (Fig. 6).

Discussion

This study aimed to characterize the wound healing process of a full-thickness excision open foot wound in an n5-streptozotocin (n5-STZ)-induced type 2 DM rat model. Our results revealed that the foot wounds of DM rats had lower blood circulation, lower PCNA expression levels, and prolonged inflammation than those of Ctrl rats. The systemic marker CRP was also significantly elevated in the blood of DM rats than those of Ctrl rats. These pieces of evidence suggested that hyperglycemia delayed wound healing through significantly reduced blood circulation, elevated inflammation and CRP, and reduced cell proliferation in wounds.

The neonatal n5-STZ-induced DM rats used in this study showed significant weight loss and high glucose levels beginning at 5 weeks old. The blood glucose levels of all DM rats were higher than 300 mg/dl as recommended in past literatures, which classified the rats as having severe hyperglycemia [50]. Similar to the findings observed in Takada's group in their type 2 DM n5-STZ-induced rat model, the DM rats lost a significant amount of weights as early as week 7 with increased food and water intakes, polyphagia, polydipsia, polyuria, and hyperglycemia [46].

The foot wounds of the DM rats healed significantly slower than those of the Ctrl rats, with a significant difference at Day 8 post wounding. The delayed foot wound

healing in the DM rats might be due to significantly lower blood circulation in wounds. This was supported by our results indicating that the foot wounds of the DM rats had significantly lower flux values than those in the Ctrl rats at Days 1, 4, and 13 post wounding, as laser Doppler imaging gives a direct measure of the microcirculatory flow in terms of flux beneath the skin surface [33]. Furthermore, the laser Doppler imaging results showed that inflammation in the foot wounds of DM rats persisted at Day 13 post wounding, while the inflammation had subsided significantly in the Ctrl rats at that time point. Altered blood flow and a dysfunctional inflammatory state might be associated with abnormal chemokine expression in DM wounds, as reported by Galiano *et al.* with their 6 mm circular full-thickness wound in the dorsal skin of the back in type 2 DM *db/db* mice [13]. The lower flux values in the foot wounds of the DM rats in our study may be due to the minimal to no capillaries observed in the H&E sections at Days 8 and 13 post wounding. According to Ferguson's group, narrowing or occlusion of the blood vessels was observed within the edge of foot ulcers in humans with DM [4]. Thus, hyperglycemia might have induced microvascular complications [39] by altering angiogenesis and extracellular matrix (ECM) structure [23] resulting in lower blood flow in the wounds.

The delayed foot wound healing of the DM rats might also be due to significantly elevated inflammation in the DM rats. Our data demonstrated significantly higher CRP circulating in the blood of the DM rats than in the blood of the Ctrl rats. This is because CRP is a systemic marker of inflammation [16] and is positively associated with hyperglycemia independently [37]. Hyperglycemia might have elevated CRP in the blood of DM rats, to inducing endothelial dysfunction as in rat DM models [19]. Furthermore, the continuously elevated CRP level during the post wounding process was equivalent to the existence of chronic inflammation [18] in patients with type 2 DM in clinical settings [12]. Our H&E sections further showed that inflammation persisted with accumulation of neutrophils between the layers and in the epidermis in the foot wounds of DM rats until Day 13 post wounding, while those of Ctrl rats subsided after Day 4 post wounding. Excessive neutrophils persisting in the wounds of DM rats can contribute to delayed wound healing and the development of chronic wounds [51], but further assessments are needed to show the activities of neutrophils wounds, as we did not examine

them in detail in this study. High inflammatory responses were reported in the foot wounds of DM rats previously, as high leukocytosis and abscesses were found at the wound gaps of full-thickness wounds on the upper back of mice [5]. Furthermore, the wounds of the DM rats at Day 8 post wounding in our study showed a lack of orientation resulting in a nonuniform wound contraction at Day 13 post wounding. According to Darby's group, cell migrations were inhibited or retarded in full-thickness excisional skin wounds on the back of type 2 DM mice with a lack of orientation of fibroblasts compared with control wounds at Day 7 post wounding [8].

Lower blood circulation, elevated inflammation, and elevated CRP level in DM rats might have reduced/inhibited cell proliferation in the wound, as reflected by reduced PCNA expression levels. Our findings showed that the PCNA expression levels in the wounds of DM rats were significantly reduced at Days 4 and 13 post wounding, indicating disrupted epidermal proliferation [5]. Similar to the findings in Ferguson's study, increased acute inflammatory cells, absence of cellular growth, and migration in foot ulcer wounds were found in humans with DM [11]. The severe hyperglycemic nature [45] of the n5-STZ model [50] might have indirectly impaired and reduced/inhibited cell proliferations [47] by elevating inflammation markers such as CRP.

No significant difference in flux was observed on Day 8 post wounding, which might be due to collagen accumulation in Ctrl rats at the end of the proliferation stage, at which time the density of the blood vessels is diminished and granulation tissue gradually matures to produce a scar [48]. However, the significant difference observed at Day 13 post wounding might be due to the alternation of blood flow and angiogenesis through hyperglycemia as observed by Galiano *et al*. in type 2 DM mice [13] as their health conditions exacerbated. The significant difference in PCNA expression at Day 13 post wounding might also be due to the severe condition of hyperglycemia in the DM rats causing a significant drop in PCNA expression levels from Day 8 to Day 13, whereas the PCNA expression level of the Ctrl rats were consistent at Days 8 and 13 post wounding.

An open full-thickness excision wound on the foot of an n5-STZ-induced animal is a better wound model to study DM wound healing because the model itself can mimic severe DM in humans with a high incidence of developing foot ulcers [25]. It also recognized as a good animal wound model that exhibits high resemblance to

clinical observations and can be reproduced and evaluated for epithelialization, contraction, inflammation, and angiogenesis [14]. The shape of the excision wound induced in this study has better advantages over other wound models for studying wound contraction and epithelialization on closure [9]. A circular excision wound (6–8 mm diameter) created by biopsy punch in animals [32, 42] was usually used in past literatures, but Montandon *et al.* observed that contraction in such wound in animals was slower than in the case of square or stellate-shaped wound due to its lack of extensibility or to the adherence of the surrounding skin [30]. Since human wounds tended to heal by reepithelialization (primary intention) [2] and contraction (secondary intention) [36], a square wound is more recommended by Montandon *et al.* for wound contraction and epithelialization studies in animals [30]. A rectangular wound was created in this study, with contraction due to centrifugal pulling of the skin at the periphery [21, 38] modifying its shape to be similar to a square wound as described by Montandon *et al.,* making it also preferable for the study of wound contraction and epithelialization than a circular wound in animals. Finally, a wound on the foot is more preferred than one on the back in animals where a high hair density might cause exaggeration of the rate of reepithelialization, especially if the wound is a partial-thickness wound rather than a typical full-thickness wound [9]. Nevertheless, the wound model used in this study has limitations due to the differences in tissue architectures and immune responses between humans and rats [10], but the characterizations and advantages listed above showed that this model mimics much better wounds in humans with type 2 DM than other types of open wound animal model. The characteristics of this wound model also matched with the clinical observations of a DM foot ulcer.

In conclusion, an open full-thickness excision foot wound in n5-STZ-induced type 2 DM rats had lower blood circulation, lower PCNA expression levels, elevated CRP, and prolonged inflammation compared with that in Ctrl rats. All this confirmed that the foot wound in the DM rats was characterized by a disorder in the inflammatory and proliferative phases of the healing process [41] attributed to hyperglycemia. Therefore, this open foot wound model in n5-STZ-induced rats provides a good approach to study the process of delayed wound healing under DM conditions.

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