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Tissue Granulation in Cutaneous Wound Healing is Improved by a Camel Milk Peptide in Streptozotocin-induced Diabetes in Rat Models

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ABSTRACT

Background: Diabetes alters keratinocyte proliferation and tissue granulation during wound healing, thereby delaying the healing process. Thus, we studied the correlation between keratinocyte proliferation and tissue granulation with healing stages and the impact of camel milk peptide (CMP).

Methods: Rats were grouped into wounded normal, wounded diabetic and wounded diabetic with daily treatment of CMP at a dose of 100 mg/kg of body weight. One-way ANOVA and Tukey's method were used to compare the overall effects of each treatment.

Result: Diabetes strikingly impaired Protein kinase B (Akt) and Alpha Smooth Muscle Actin (SMA-á) concentrations in the wounded tissues. Furthermore, the gene expression of KGF in wounded tissues was downregulated in diabetic rats. Conversely, CMP showed notable restoration of these parameters. Thus, granulation was enhanced compared with that in non-treated diabetic rats. In addition, the wound margin epithelia of CMP-diabetic rats showed an increase in both size and migration, with two epidermal tongues directed inward and clearly visible on both sides of the wound. However, diabetic rats showed increased extent of wound margin neoepithelia without clear epidermal tongues. These data confirmed the potential role of CMP in wound healing in diabetic rats.

Key words: Camel milk peptide, Diabetes, Tissue granulation, Wound healing.

INTRODUCTION

Diabetes is a global disease that leads to serious long-term health complications (Khajuria *et al*., 2018; Khawandanah 2019). Diabetes is a multifaceted metabolic disease that accompanies diabetes wounds in more than 20% of cases (Patel *et al*. 2019). Diabetes wounds are complications that can be immensely affected by significant morbidity and associated financial costs (Zhang *et al*. 2017). The systemic accumulation of advanced glycation end-products irreversibly alters fibroblast and endothelial cell proliferation, migration, homing, secretion and organization in granulation tissues (Van *et al*. 2016) which in turn, leads to programmed cell death. In addition, the recruitment of endothelial progenitor cells and tubulogenesis are also disturbed (Fournet *et al*. 2018). Keratinocytes play a pivotal role in tissue repair during wound healing (Wu *et al*. 1996). These cells are the key cellular components of re-epithelialization during wound healing (Hu and Lan, 2016; Chandnani *et al*., 2022). The reduction in granulation tissue mass content is a critical factor in impeding wound healing in diabetes (Yue *et al*. 1986).

Whey protein (WP) can improve wound healing in diabetic models (Ahmed *et al*. 2015) and improved TNF-á and Fas and T-autoreactive cells in T1D (Ebaid *et al*. 2014a,b). Furthermore, Ebaid *et al*. (2015) demonstrated that CMP isolated from WP accelerate wound healing in a diabetic model. Camel milk peptide (CMP) has all of the necessary nutrients both fresh and soured (Abu-Taraboush *et al*. 1998). The effectiveness of an addition to normal diabetic therapy in type 1 diabetes offers fresh hope for managing this disorder by adding a medical dietary supplement. CMP health benefits have been studied worldwide for three decades. Biochemical investigations show that insulin-like protein, lactoferrin and immunoglobulins provide CMP scientific value (Agrawal *et al*., 2013; Singh *et al*., 2019). CMP is a powerful bioactive for use as antidiabetic drugs (Anwar *et al*. 2022). This study investigated how camel milk peptides (CMP) facilitate wound healing in a diabetic rat model.

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MATERIALS AND METHODS

Camel milk (Majaheem camel breed from the Alazeria farm, Najd region; GPS: 300 02 47/ 300 02 27 in Saudi Arabia)

was centrifuged at 5000 g for 20 min. The casein was obtained from centrifuged milk by acidification at pH 4.3. The supernatant WP was saturated with ammonium sulfate. Thus, the WP was then dialyzed for 48 h at 4°C using a Spectra/Pro® Membrane, MWCO 6000-8000 Da. The WP was lyophilized using a Unitop 600 SL [Virtis Company, Gardiner, USA] and stored at -20°C until use (Jakubowicz *et al*. 2014). A 2.5% WP solution (w/v) was prepared in water at pH 7.0 using 1-mol NaOH. The trypsin enzyme was added to WP in a ratio of 1/100, which was inactivated. Samples were cooled and stored in a refrigerator. The resulting fractions were tested for their bioactivity. Among them, peptide fraction 1 (CMP) was chosen for wound-healing experiments *in vivo* (Ebaid *et al*. 2015) in the present study.

Sixty male adult (about 6 months old) albino rats were purchased from the Department of Zoology, King Saud University. The rats were divided into four groups (n = 15). Two groups-the wounded normal (CN+) and the wounded diabetic (DM) were administered with the vehicle solution of PBS (1000 µL/rat/day) by gastric intubation for 7 days. The third group was the wounded diabetic rats that were treated with CMP (3kDa) daily, while the fourth group of wounded diabetic rats was treated with hydrolyzed 10 kDa at a dose of 100 mg/kg of body weight (1000 µL/rat/day) daily by gastric intubation for 2 days (n=5), 4 days (n= 5), or 7 days (n=5). The study protocol was approved by King Saud University number KSU-SE-20-40 and performed during 2021.

After anesthesia, the backs of the rats were shaved and cleaned with alcohol. Wounds were performed as previously described by Schwentker *et al*. (2002). The wounds were punched through the full thickness of the folded skin to form ~5 mm diameter circle below the shoulder of each rat with a sterile blade.

Diabetes was induced by a single dose of freshly dissolved streptozotocin (STZ, Sigma-Aldrich, USA) in citrate buffer (pH 4.5; 0.1 mol/L) at a dose of 50 mg/kg body weight into the peritoneum. Rats in the control group were injected with citrate buffer in equal volumes. After 14 days of STZ dosing, rats with blood glucose levels ed 220 mg/dl after overnight food deprivation were chosen as the diabetic rat model.

After days 1 and 7 of wounding, the blood was obtained from carotid artery for serum samples. The animals were dissected and their livers were kept at -80°C.

The levels of Akt and SMA in the serum samples of the experimental groups were determined using commercial ELISA kits (Abcam, UK), according to the manufacturer's instructions. The concentrations of Akt and SMA were determined using an Elisa reader at 450 nm.

For histology, hematoxylin and eosin and Mallory's trichrome staining was used to detect collagen and iron deposition. The tissue was examined using a Leica DMRB/E light microscope (Heerbrugg, Switzerland) in a blinded manner. The wounds were removed from four rats from each treatment group at 6 h, 24 h and at the end of the treatment duration after wounding by cutting a square area encompassing the entire wound site. The neutrophil number was determined at 20 random locations within the epidermal and dermal tissues in the wound region for each animal from each group, using a Leica Qwin 500 image analyzer.

Wounds from individual rats were digitally photographed daily. Wound size was calculated by determining the area of the wound daily as per the area of the standard circle. Wound closure was calculated as the ratio of the initial wound size to the wound area (daily after wounding). In addition, the density of newly generated blood vessels in each wound was calculated morphometrically. Five nonconsecutive tissue sections were prepared, of which three randomly selected fields of wounded tissues were photography (400X) using a camera attached to a microscope (Leica, Wetzlar, Germany).

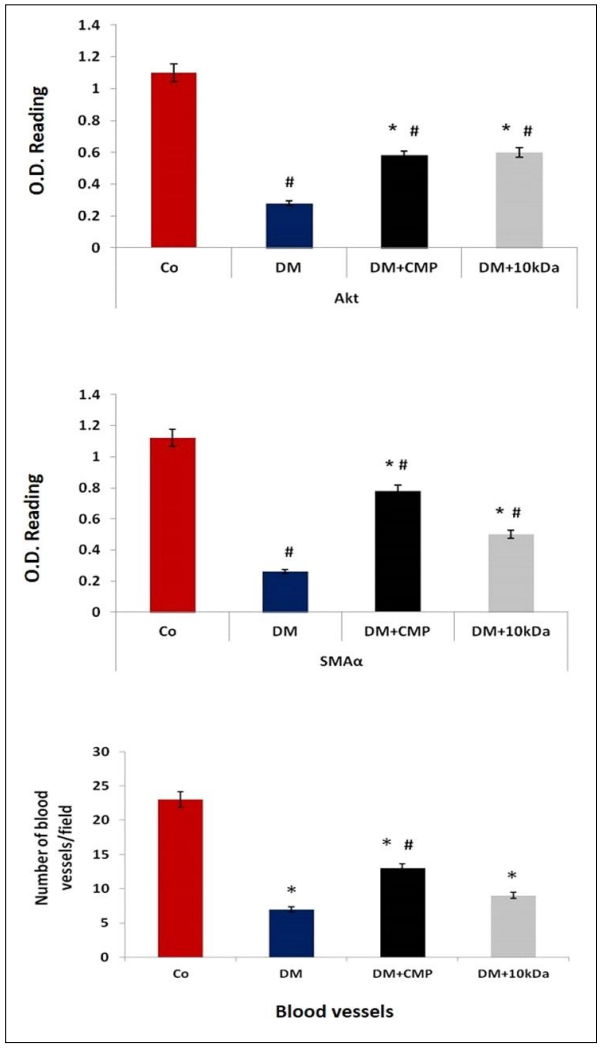

Fig 1: Bars showing the levels of Akt and alpha-smooth muscle actin (α-SMA) estimated using ELISA**.** All data are expressed as mean ± SEM. In addition, bars show the morphometric count of blood vessels in the dermal tissue during granulation. *and # indicate significance compared to the control and DM groups, respectively.

KGF gene expression was measured by RT-PCR using the QIAGEN OneStep RT-PCR kit, according to the manufacturer's instructions.

Statistical analysis was conducted using the MINITAB software (State College, PA, Version 13.1, 2002). One-way ANOVA and Tukey's method were used to compare the overall effects of each treatment. All results are expressed as mean (M)±standard deviation (SD). Only statistically significant differences ($P<0.05$) were found between groups.

RESULTS AND DISCUSSION

We treated diabetic wounds with a selected peptide (CMP) from the hydrolysates after enzymatic hydrolysis of WP. This study investigated the effect of CMP on keratinocyte proliferation and dermal granulation in diabetic rats. Our results clearly show that CMP strikingly decreases the period of wound healing in diabetic rats. Wound healing is involving the interaction among various types of cells, signaling molecules, cytokines and chemokines. Normally, the process of wound healing can be classified into five phases: granulation, wound contraction, collagen deposition, epithelialization and cicatrization.

AKT and α-SMA

Results showed that both signal molecules were decreased in the DM group compared with the non-treated control group. After CMP treatment of rats with DM (DM CMP group), the levels of both were notably restored compared to those in the diabetic group (Fig 1). Both α-SMA and Akt play an essential role in wound healing and are expressed and synthesized in smooth muscles, pericytes and myofibroblasts (Shinde *et al*. 2017). It is known that an increased α-SMA expression is sufficient to enhance fibroblast contractile activity.

The classical wound healing process involves the proliferation of local dermal fibroblasts, followed by an increase in α-SMA, modulation of myofibroblasts and

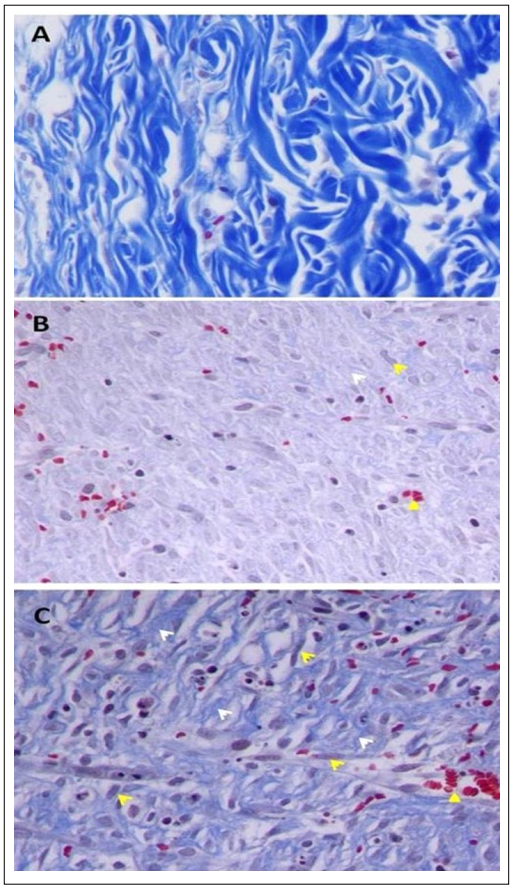

Fig 2: Representative skin sections on day 7 post-wounding showing granulation in the wounded tissue (Mallory's trichrome stain; X400) of normal non-wounded (A), wounded diabetic (B) and wounded diabetic rats treated with CMP (C). Collagen fiber deposition (white arrows) by dermal fibrocytes (yellow arrow) and new blood vessels (yellow triangle) into the dermis of wounded skin seven days post-wounding.

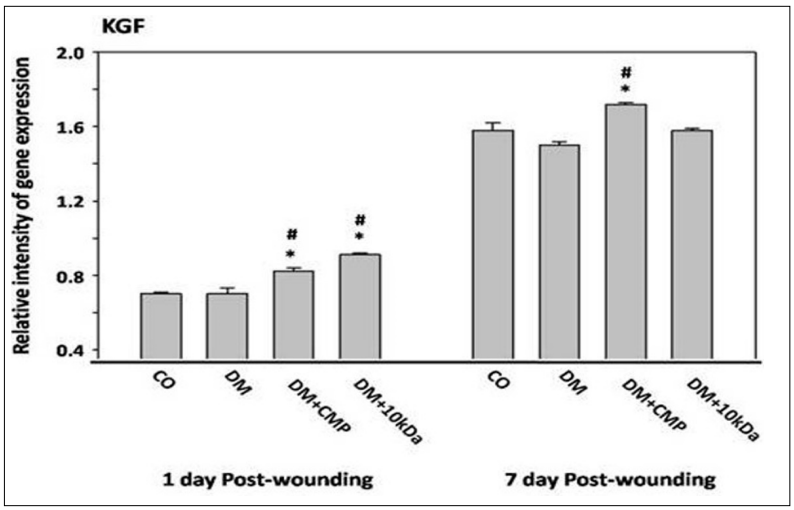

Fig 3: The Expression profiles of keratinocyte growth factor (KGF). Values shown are mean±SD. * and # indicate significance compared to the control and DM groups, respectively.

reorganization of the extracellular matrix, resulting in skin regeneration (Darby *et al*. 2014). Our results showed that á-SMA levels were significantly depleted in the diabetic group compared to the non-diabetic control group 24 h post-wounding. A similar trend was reported by Kim *et al*. (2014). The depletion of α-SMA in diabetic rats may be due to prolonged oxidative stress Pannirselvam *et al*. (2003). In patients with diabetes, pro-inflammatory macrophages do not transition into anti-inflammatory phenotypes, thereby halting the tissue repair process around the affected area (Chandnani *et al*. 2022). This elongates the inflammatory phase and slows down the resolution phase, which triggers growth factors and the consequent tissue repair.

The formation of blood vessel and collagen fibers

The number of new blood vessels in the wounded region was remarkably reduced in diabetic rats compared to that in normal rats, but CMP notably activated angiogenesis and the construction of dermal constituents (Fig 1).

Mallory's trichrome showed that diabetes disturbed the wounded tissues. Collagen deposition in the dermal tissues of diabetic rats treated with CMP appeared to be similar to

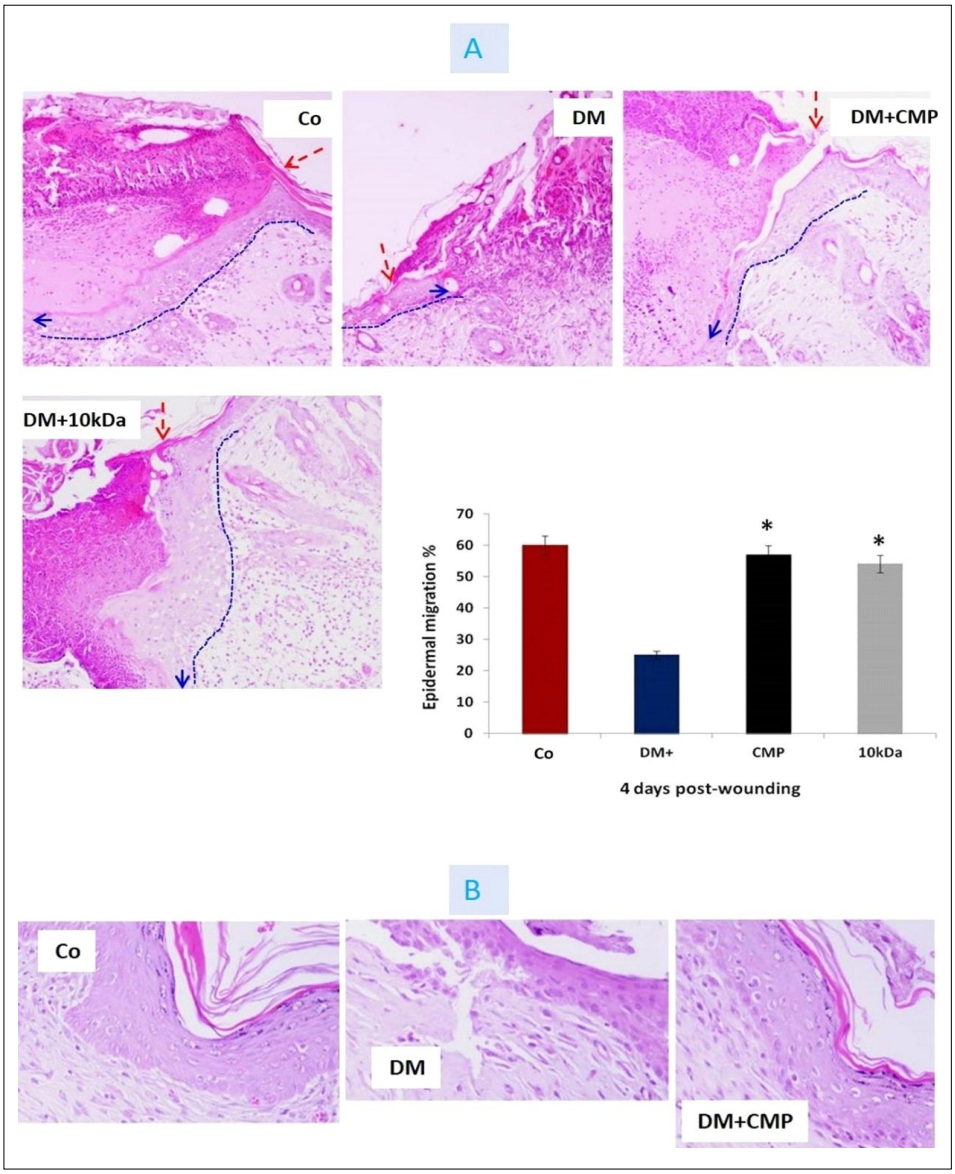

Fig 4: A: Representative skin sections on day 2 post-wounding showing the migration of proliferating epidermal cells (H&E; X100). The epidermal tongues directed toward the entire wound region (blue arrow) are underlined with the blue dotted line from the wound start point (red arrow). In addition, the histogram shows the percentage of epidermal tongue migration toward the entire wound relative to the whole wound. Values shown are the mean±SD, where * indicates significance in comparison to the DM+ group. B: Representative skin sections on day 8 post wounding showing the thickness of proliferating epidermal cells (H&E; X400).

that in normal tissues. In CMP-treated rats, a moderate number of collagen fibrils and bundles were organized more regularly than in the untreated diabetic rats, which tended to be asymmetrically distributed. Dermal regeneration in rats supplemented with CMP was characterized by fibroblasts and well-developed symmetry (Fig 2).

Keratinocyte proliferation and epidermal migration

KGF is a small signaling molecule that binds to fibroblast growth factor receptor Helsten *et al*. (2015). In this study, CMP upregulated the KGF gene compared to both normal and diabetic rats (Fig 3).

Wounded tissues from the diabetic rats were disturbed on day 2 after wounding (Fig 4), whereas those of the CMP-diabetic rats appeared similar to the normal tissues. Four days after injury, wound areas in diabetic rats showed an increased margin of wound neoepithelia without obvious epidermal tongues. Conversely, the wound margin epithelia of CMP-diabetic rats showed an increase in both size and migration with two epidermal tongues directed inward, visible on both sides of the wound. The migration of epithelial cells on the edges of the wound in diabetic rats displayed a moderate degree of wound closure on the fourth day after wounding. However, the wounds of CMP-diabetic rats were completely re-epithelialized (Fig 4).

In the diabetic group, the proliferative activity of epidermal cells was delayed throughout the observation period. CMP enhanced the proliferation activity behaving similarly to the normal rat group and peaked on the seventh day after wounding and decreased thereafter.

Our study demonstrated a restoring effect of the CMP-treated diabetic group on á-SMA levels. Such effects may account for the enhanced tissue regeneration and improvement of wound healing. This is supported by the ability of CMP to reduce oxidative stress in the wound region (Ebaid *et al*. 2011). Normally, the first type of cell migrating to the wound site is neutrophils, which eliminate microorganisms followed by their consumption by macrophages (M2), ceasing the level of inflammation at the wound site (Yavuz *et al*. 2022). The cessation of inflammatory

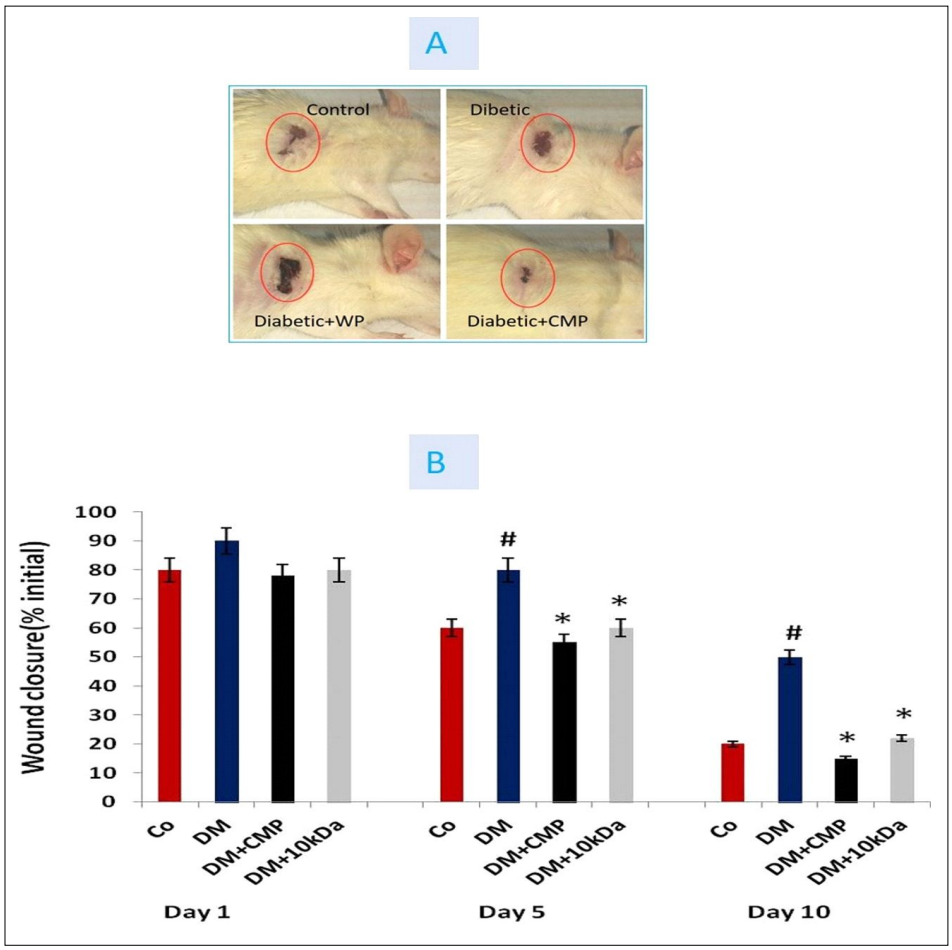

Fig 5: A: Wound morphology of the different groups during wound closure. B: Percentage of wound closure rate in control (CO), diabetic (DM), diabetic treated with CMP from hydrolysate whey protein (DM+CMP) and diabetic treated with 10 kDa hydrolysate whey protein (DM+10 kDa) groups**.** Values shown are the mean±SD. *and # indicate significance compared to the control and DM groups, respectively.

mediator aggression is essential for promoting the wound healing process (Ebaid *et al*. 2015) showed that the level of TNF-α was strikingly restored to normal in DM treated with CMP. Thus, blocking TNF-α and other inflammatory mediators leads to the transition of proinflammatory macrophages to anti-inflammatory phenotypes, releasing and triggering growth factors required for promoting the wound healing process (Voll *et al*. 1997). These findings can explain the normal secretion of growth factors, such as á-SMA and Akt, needed in the proliferation stage of wound healing on day 7th-day post-wounding in CMP-rats. This is supported by our current results, which showed a normally generated collagen fiber with a normal distribution in dermal tissues.

Hyperglycemia leads to osmotic diuresis and subsequent decrease in oxygenation and perfusion. Diabetic fibroblasts and endothelial cells may undergo mitochondrial damage, making them prone to apoptosis. It impairs the cellularity and perfusion of granulation tissue. In addition, the recruitment of endothelial progenitor cells and tubulogenesis are hampered under such conditions. The histological analysis in this study suggests that CMP enhances the recruitment of endothelial cells and promotes tubulogenesis to assist fast wound healing.

The rate of wound closure

It was observed that the wound contraction process induced by α-SMA and collagen formation in dermal tissues was achieved in the control, diabetic rats treated with CMP. Conversely, untreated diabetic rats showed open wounds 7 d post-wounding. The percentage of diabetic rats that showed wound closure was remarkably lower than that of control rats. However, CMP recovered the wound closure rate in diabetic rats compared to that in control rats (Fig 5).

CONCLUSION

CMP was observed to promote the restoration of neovascularization and keratinocyte migration in treated diabetic rats and enhanced wound closure. It does so by promoting the recruitment of endothelial progenitor cells, growth factors (Akt and α-SMA), keratinocytes and angiogenesis, consequently leading to the re-epithelialization needed for wound closure.

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Conflict of interest: None.

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