Supplementation with Undenatured Whey Protein During Diabetes Mellitus Improves the Healing and Closure of Diabetic Wounds through the Rescue of Functional Long-lived Wound Macrophages

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Abstract
Long and persistent uncontrolled diabetes tends to degenerate the immune system and increase the incidence of infections in diabetic patients. A serious complication of diabetes is impaired healing, which diminishes physical activity and, in some cases, leads to chronic wounds and limb amputation. Whey proteins (WPs) enhance immunity during early development and have a protective role in some immune disorders. The effect of camel WPs on wound healing in a streptozotocin-induced type 1 diabetic mice model was investigated. Sixty male mice were equally distributed into 3 experimental groups: group 1, non-diabetic control mice; group 2, diabetic mice; and group 3, diabetic mice that were orally supplemented with undenatured WP (100 mg/kg body weight/day for 1 month through oral gavage). We observed that the diabetic mice exhibited delayed wound closure characterized by a significant reduction in collagen deposition, prolonged elevation in inflammatory cytokines, aberrant activation of STAT3 and reduction in the activation of Akt and NF-κB when compared with the control mice. Moreover, in the diabetic mice, the wound-resident macrophages were dysfunctional and demonstrated increased apoptosis, a significant reduction in their phagocytotic ability, aberrant activation of STAT3 and a marked reduction in the activation of Akt. Interestingly, the supplementation of diabetic mice with WP significantly enhanced the collagen deposition, limited the inflammatory stimuli, restored the activation of STAT3, Akt and NF-κB and greatly improved the closure of diabetic wounds compared with the control mice. Most important, the supplementation of diabetic mice with WP rescued functional, long-lived wound-resident macrophages. Our data reveal the benefits of WP supplementation in improving the healing and closure of diabetic wounds.

Introduction
Type 1 diabetes is an autoimmune disease characterized by the inflammation of pancreatic islets and destruction of β cells by the immune system. Several complications are usually associated with diabetes mellitus [1]. These complications are caused by the action of free radicals [2], which damage multiple cellular components,
such as lipids, proteins and DNA. Impaired wound healing represents a severe complication of the disease, which could diminish physical activity and lead to chronic wounds and limb amputation [3]. Wound healing is a complex multi-stage process that involves distinct phases: inflammation, formation of granulation tissue, production of new structures and tissue remodeling [4]. These processes are all regulated by cytokines and growth factors modulated by systemic conditions, such as diabetes [5]. The cytokines and chemokines secreted by the skin-resident cells (keratinocytes, fibroblasts and endothelial cells) and inflammatory cells are involved in wound healing. Pro-inflammatory cytokines, including interleukins 1α and 1β (IL-1α and IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α), are thought to play important roles in wound repair, including the stimulation of keratinocyte and fibroblast proliferation, synthesis and breakdown of extracellular matrix proteins, fibroblast chemotaxis and regulation of the immune response [6]. In addition, recent reports have indicated that the dysregulation of TNF-α impairs the healing of diabetic wounds, which may involve enhanced apoptosis and decreased proliferation of fibroblasts [7]. Moreover, overexpression of IL-10, an anti-inflammatory cytokine, decreases the inflammatory response to injury and creates an environment conducive for regenerative wound healing [8].

Other important chemokines include macrophage inflammatory proteins 1α and 2 (MIP-1α, MIP-2), which have been identified as regulators of specific leukocyte accumulation at wound sites [9]. Particularly, Keratinocyte-derived chemokine (KC) and MIP-2 are believed to participate in the recruitment of neutrophils to sites of inflammation in many tissues [10]. Previous studies have demonstrated that platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) play critical roles in wound repair. They function in leukocyte chemotaxis, fibroblast and smooth muscle cell mitogenesis and extracellular matrix deposition during granulation tissue formation [6, 11]. Upon binding to cytokines and chemokines, which bind to specific receptors and contribute to the wound healing process, these chemokines activate numerous signaling pathways and transcription factors, such as the signal transducer and activator of transcription (STAT), nuclear factor-κB (NF-κB) and serine/threonine protein kinase B (PKB or AKT) [12, 13]. The wound disorders in obese C57Bl/6J mice are paralleled by the degradation of the inhibitor of NF-κB (IκB-α) in the absence of Akt activation [13].

Wound-site macrophages drive wound inflammation. Recently published reports reveal that macrophage dysfunction impairs the resolution of inflammation in the wounds of diabetic mice, and dysfunctional macrophage efferocytosis increases the apoptotic cell burden at the wound site. This burden prolongs the inflammatory phase and complicates wound healing [14]. Many drugs, such as drugs that interfere with clot formation, platelet function, inflammatory responses and cell proliferation, all affect wound healing. Camel whey proteins (WPs) are comprised of a heterogeneous group of proteins that include serum albumin, α-lactalbumin, immunoglobulin, lactoferrin and peptidoglycan recognition protein [15]. Recently published data have suggested that WPs have antioxidant activity. Therefore, WP may be a therapeutic tool for oxidative stress-associated diseases [16]. WPs modulate a variety of immune functions, including the proliferation and activation of immune cells, cytokine secretion, antibody production, phagocytic activity, and granulocyte and natural killer (NK) cell activity [17]. In addition, studies have demonstrated a clear modulation of immune functions by several whey protein-derived products in vitro and in vivo [18]. Recent studies have indicated that whey increases antioxidant activity in the body, combats fatigue and inflammation, hastens healing, improves stamina and may discourage related infections because of the immune system-enhancing and natural antibiotic properties of its components [19, 20]. Recently, WP was reported to have immunomodulatory properties and to be able to increase the host defense system [21]. Moreover, anti-cancer [22] and protective effects against childhood asthma and atopic syndrome [23] have also been reported. Nevertheless, there are few studies that have investigated the influence of WPs in wound healing. The aim of this study was to investigate the potential modulatory effects of the oral administration of WPs on wound healing using a diabetic mice model.

Materials and Methods

Preparation of whey proteins

Raw milk was collected from healthy female Majaheem camels from Riyadh, Saudi Arabia, and the milk was centrifuged to remove the cream. The resulting skim milk was acidified to pH 4.3 using 1 N HCl at room temperature and centrifuged at 10,000 x g for 10 min to precipitate the casein. The resulting whey and whey proteins were mixed with ammonium sulfate to 80% to precipitate the whey proteins. The precipitated whey proteins were dialyzed against 20 volumes of distilled water for 48 h using a porous membrane with a molecular weight cut-off (MWCO) of 6,000-8,000 kDa. The dialysate containing the undenatured whey proteins was freeze-dried and refrigerated until further use.

Badr
Streptozotocin (STZ) was obtained from Sigma Chemicals Co. (St. Louis, MO, USA). STZ was dissolved in cold 0.01 M citrate buffer (pH 4.50), which was freshly prepared for immediate use (within 5 min) when needed.

Animals and experimental design

A total of 60 sexually mature 12-week-old male Swiss Webster (SW) mice weighing 25-30 g each were obtained from the Central Animal House of the Faculty of Pharmacy at King Saud University. All animal procedures were conducted in accordance with the standards set forth in the Guidelines for the Care and Use of Experimental Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the National Institutes of Health (NIH). The Animal Ethics Committee of the Zoology Department, College of Science, King Saud University approved the protocol used in this study according to the Helsinki Principles. All animals were acclimated to the metal cages in a well-ventilated room for 2 weeks prior to experimentation. The animals were maintained under standard laboratory conditions (23°C, 60-70% relative humidity and a 12-hour light/dark cycle), fed a constant and did not exceed 250 µl per dosage. The optimal dose of WP was determined in our laboratory on the basis of the LD₅₀ and several established parameters. Animals in the three groups were fed a standard commercial diet containing protein (minimum 22.0%), carbohydrates (maximum, 54.0%), fat (minimum, 4.5%), cellulose (maximum, 8.0%), minerals (maximum, 10.0%), water (maximum, 12.5%), and vitamins. Food intake (amount of food consumed by each animal) was monitored throughout the experiment period and no significant difference was observed among the different mice groups.

Excisional wound preparation and macroscopic examination

Following diabetes induction, the mice in each group were wounded at 12 weeks of age. The mice were wounded as previously described [25]. Briefly, the mice were anaesthetized with a single i.p. injection of ketamine (80 mg/kg body weight) and xylazine (10 mg/kg body weight). The hair on the back of each mouse was shaved, and the back was cleaned with 70% ethanol. Six wounds (5 mm in diameter, 3-4 mm apart) were made on the back of each mouse by excising the skin and underlying panniculus carnosus, and the wounds were allowed to form a scab. Skin biopsy specimens were obtained from the animals at 1, 4, 7, 10, and 13 days post-wound formation. At each time point, a skin sample, which included the scab, the complete epithelial and dermal compartments of the wound margins, the granulation tissue, and parts of the adjacent muscle and subcutaneous fat tissue, was excised from each individual wound. As a control, a similar amount of skin was collected from the backs of non-wounded wild-type mice. Each wound site was digitally photographed at the indicated time intervals to determine the wound area. Changes in the wound area were expressed as a percentage of the initial wound area. At the indicated time intervals, tissue from two wounds from ten animals (n = 20 wounds) was collected for RNA, western blot and ELISA analyses.

Measurement of hydroxyproline content in the wound sites

After drying for 24 h at 120 °C, the amount of hydroxyproline, a major constituent of collagen in skin wound sites, was measured to index collagen accumulation at the wound site as previously described [26]. The hydroxyproline contents were expressed as the amount (mg) per wound.

Blood analysis

The blood glucose levels were determined using the AccuTrend sensor (Roche Biochemicals; Mannheim, Germany). Luminex (Biotrend; Düsseldorf, Germany) was used to analyze serum insulin and tumor necrosis factor (TNF-α) levels according to the manufacturer’s instructions. Serum IL-1β, IL-6 and IL-10 levels were determined in an ELISA assay using the rat Bio-Plex cytokine assay kit (Bio-Rad; Hercules, CA) according to the manufacturer’s instructions. For a complete blood count of circulating neutrophils, the mice were bled from the mandibular vein into 1.5-ml microcentrifuge tubes through heparinized tubes before and 3 days after wound formation. The blood count was measured using a multispecies hematology system according to the manufacturer’s instructions.

Biochemical analysis of wounded tissue

Measuring cytokine levels. A 2.0-mm punch biopsy at the wound site was harvested and frozen in liquid nitrogen. The specimens were homogenized in cytoplasmic lysis buffer containing protease inhibitors (Roche Diagnostics), disrupted using Fast Prep (Q-Biogene; Solon, OH, USA) and centrifuged at 5000 x g for 10 min. The protein concentration in each lysate was determined using the bicinechonic acid (BCA) protein assay kit (Pierce). The supernatants were used to determine the TNF-α, IL-6, MIP-1α, MIP-2, KC, TGF-β1 and PDGF levels using a commercial ELISA kit (R&D Systems; France) according to the manufacturer’s instructions. The data were expressed as the target molecule (picograms) per total protein (milligrams) for each sample.

Extraction of total RNA and RT-PCR. Total RNA was isolated from the skin samples using TRIzol reagent (Invitrogen Life Technologies; France) according to the manufacturer’s instructions. Prior to reverse transcription, the RNA was treated

Whey Protein Enhances the Healing of Diabetic Wounds Cell Physiol Biochem 2012;29:571-582
with RNase-free DNase I according to the manufacturer’s protocol. The cDNA was synthesized using three micrograms of total RNA and a Superscript III RT kit (Invitrogen Life Technologies; France). The unique primer sets for mouse collagen type 1 were (F) 5´-ATC ACT GCA AGA ACA GCG TAG-3´ and (R) 5´-TGT TTT CCA AAG TCC ATG TG-3´ and for β-actin were (F) 5´-TTC TAC AAT GAG CTG GTG GC-3´ and (R) 5´-CTC ATA GCT CTT CTC CAG GGA GGA-3´ based on the sequences deposited at the National Center for Biotechnology Information (NCBI). PCR was performed with 1 µl of cDNA in a reaction mix using Taq polymerase (Invitrogen Life Technologies). The PCR was linear between 20 and 35 cycles, and the conditions were optimized to allow for a semi-quantitative comparison of the results. The PCR products were separated by agarose gel electrophoresis and visualized using ethidium bromide. The digital images were acquired and quantified using NIH Image Analysis Software. The intensity of each primer product was normalized to the intensity of the β-actin primer product and expressed relative to the levels found in the injured skin of the non-diabetic control mice.

Western blot analysis. The skin and wound tissue biopsies were homogenized in lysis buffer (1% Triton X-100, 137 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 10 mM NaF, 2 mM Na3V3O4, 5 mM ethylenediaminetetra-acetic acid, 1 mM phenylmethylsulfonylfluoride, 5 ng/ml aprotonin, 5 ng/ml leupeptin and 20 mM Tris/HCl, pH 8.0), and the lysates were prepared as previously described [27]. Fifty micrograms of total protein from the skin lysates was analyzed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. Antibodies directed against phosphorylated signal transducer and activator of transcription (pSTAT-3) (Y705), phospho-Akt (S473) (Cell Signaling; Frankfurt, Germany), inhibitor of nuclear factor κB (IκB)-α (Santa Cruz; Heidelberg, Germany) and β-actin (Sigma) were used in combination with horseradish peroxidase-conjugated secondary antibodies, and the proteins were visualized using enhanced chemiluminescence (ECL, Supersignal Westpico chemiluminescent substrate; Perbio, Bezons, France) detection system. The ECL signal was detected on Hyperfilm ECL. To quantify the band intensities, the films were scanned, saved as TIFF files and analyzed using NIH Image software.

Isolation of Wound Macrophages from Polyvinyl Alcohol (PVA) Sponges. Subcutaneously implanted polyvinyl alcohol (PVA) sponges were used as model for the role of macrophages in the wound healing studies that addressed inflammation [28]. The model is best suited for acute studies because the sponge elicits a foreign body response and results in increased cell accumulation and fibrosis after 4 weeks of implantation. For short-term studies, this approach represents a reproducible and biologically valid model for the study of acute healing responses. The isolation of wound macrophages was conducted as previously described [14]. Sterile circular (8 mm) PVA sponges were subcutaneously implanted on the backs of the mice at the site of the excisional wounds. Briefly, the mice were anesthetized and two midline 1-cm incisions were made with a scalpel. Small subcutaneous pockets were created by blunt dissection (two pockets per animal). Two PVA sponges were inserted into each pocket. The incisions were closed with skin staples (9 mm) or sutures (3-0 SurgilineTM). The animals were returned to clean cages to monitor their recovery. The PVA sponges were harvested on the designated day, and a single-wound cell suspension was generated from the sponges by repeated compression. The cell suspension was filtered through a 70-mm nylon cell strainer (BD; Canada) to remove any sponge debris. The isolated cells were collected and washed twice in PBS. For the macrophage isolation, magnetic cell sorting was performed using mouse anti-CD11b-tagged microbeads (Miltenyi Biotec; Auburn, CA). This procedure yielded a greater than 95% pure population of wound macrophages, which was determined by flow cytometry. No major differences were observed in the cell characteristics and activities of the PVA sponge-derived cells and closed-incision derived cells. It was difficult to isolate wound macrophages at one day after wound generation; therefore, the wound macrophages were isolated at 4, 7, 10 and 13 days post-wound formation. The macrophages were cultured in R-10 medium (RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 µg/ml streptomycin, 100 U/ml penicillin and 2 mM Ultra Glutamine).

Analysis of wound macrophage proprieties. The macrophages were seeded onto 6-well plates and cultured in RPMI 1640 media containing 10% heat-inactivated bovine serum for 24 h under standard culture conditions. The caspase-3 activity was monitored by flow cytometry analysis, and apoptosis was measured using the Annexin V-FITC binding assay. The phosphorylation of AKT, STAT3 and IκBα was investigated as previously described [14]. Briefly, apoptosis was induced in thymocytes using 5 mM dexamethasone for 12 h, and yielded greater than 95% phosphatidyl serine (PS) positive cells, which is one of the key mechanisms of apoptotic cell recognition by macrophages and apoptotic thymocytes. Prior to co-culture with macrophages, apoptotic thymocytes were labeled with a fluorescent cell-tracker reagent (CellTrackerTM Orange CMTMR; Molecular Probes). The wound macrophages were seeded onto 8-well chambered slides. The apoptotic-labeled thymocytes were added to each chamber at a 1:10 macrophage: thymocyte ratio, and the phagocytosis assays were performed for 1 h at 37°C. For the co-culture studies, shorter incubation times (10-15 min) were used in the adherence assay, and longer (45-60 min) co-culture periods were used for the phagocytosis assays. The macrophages were extensively washed to remove non-phagocytosed cells. The cells were fixed with 4% paraformaldehyde and stained using F4/80-FITC. The imaging was performed using a fluorescence microscope (thymocytes, red; macrophages, green or phase contrast). Approximately 50-100 macrophages from each well were counted to determine the number of phagocytosed thymocytes, and the data were expressed as the “phagocytic index,” which is defined as the total number of apoptotic cells engulfed per macrophage present in the field of view. This approach enabled the normalization of the data against the macrophage number.
Statistical analysis

The data were tested for normality (using Anderson-Darling test) and homogeneity variances prior to further statistical analysis. The data were normally distributed and are expressed as the mean ± SEM (standard error of the mean). The statistical differences between the groups were analyzed using one-way analysis of variance (for more than two groups) followed by Tukey’s post-test using SPSS software version 17. The data are expressed as the mean ± SEM. The differences were considered statistically significant at *P< 0.05 for diabetic vs. control; †P< 0.05, diabetic + WP vs. control; ‡P< 0.05, diabetic + WP vs. diabetic. (D) Hydroxyproline content, an index of collagen accumulation at the wound site was determined. Values represent mean ± SEM. (E) RT-PCR analysis was performed to measure the expression of collagen type I at the wound sites. Representative data from three independent experiments in each group are shown.

Results

Camel WP administration improves wound closure in diabetic mice

We evaluated the macroscopic changes at the skin-excision wound sites in the control mice, diabetic mice and diabetic mice supplemented with WP. Pictures were taken on day 0 immediately after the injury. The wound sites exhibited a similar morphology in all 3 experimental groups on day 1 post injury, while the wounds in the control and diabetic mice supplemented with WP were similarly closed at 13 days post injury. In contrast, the diabetic mice exhibited delayed wound closure. A representative experiment is shown (Fig. 1A). The accumulated data for the changes in the percentage of wound closure at each time point compared with the original wound area from 10 individual mice in each group are shown in Fig. 1C. These results revealed that wound closure and healing were accelerated in the diabetic mice supplemented with camel WP compared with the diabetic mice, which exhibited delayed wound closure. To optimize the parameters and conditions of the animal models during the experiments, blood glucose levels in the 3 groups of mice were monitored before
and throughout the indicated time points post-wound formation (Fig. 1B). The glucose levels in the WP-treated diabetic group were significantly lower than the diabetic group and were higher than the control group. The increased collagen content in the extracellular matrix is a characteristic change observed in the proliferative phase of the wound healing process. Because hydroxyproline is a major constituent found almost exclusively in collagen, we used the hydroxyproline content as an indicator of collagen type I at the wound sites. The accumulated data from 10 individual mice in each group demonstrated that the hydroxyproline content was significantly decreased in diabetic mice with decreased wound closure compared with the control mice (Fig. 1D). Compared with the control mice, there was less collagen accumulation at the wound sites in the diabetic mice, which confirmed the presence of a delayed healing process. However, the diabetic mice supplemented with camel WP exhibited a significant restoration of hydroxyproline content compared with diabetic mice (Fig. 1D). These results suggest that collagen production was enhanced with the oral administration of WP. The expression of collagen type 1 was significantly upregulated in the excisional wound tissues of the diabetic mice supplemented with WP as determined by RT-PCR at days 1, 4, 7, 10 and 13 post injury compared with the diabetic mice. A representative experiment from three independent experiments is shown in Fig. 1E.

The effect of WP supplementation during diabetes on the plasma levels of pro- and anti-inflammatory cytokines

We monitored the plasma levels of different cytokines that control immune cell function during wound healing in the three groups of mice. The accumulated data from 10 individual mice from each group is shown (Fig. 2). We observed that the level of pro-inflammatory (TNF-α, IL-1β and IL-6) and anti-inflammatory (IL-10) cytokines peaked at 4 days post injury (Fig. 2A-D). In the diabetic mice, we observed aberrant and significantly elevated levels of TNF-α, IL-1β and IL-6 compared with the control and WP-treated diabetic mice from 4 to 13 days post injury, which indicated a prolonged pro-inflammatory phase during the healing of diabetic wounds. In contrast, the level of IL-10 was significantly decreased in the diabetic mice compared with the control and WP-treated diabetic mice from 4 to 13 days post injury. Thus, WP supplementation during diabetes significantly restored the levels of TNF-α, IL-1β, IL-6 and IL-10.

Fig. 2. The alterations in the plasma levels of pro- and anti-inflammatory cytokines during wound healing. The levels of plasma pro-inflammatory (TNF-α, IL-1β and IL-6) and anti-inflammatory (IL-10) cytokines were measured in the 3 groups of mice using ELISA before (Day 0) and on the indicated days post-wound formation. The results are presented as the cytokine levels (pg) per ml of plasma and are expressed as the mean ± SEM (n=10). *P< 0.05, diabetic vs. control; +P< 0.05, diabetic + WP vs. control; #P< 0.05, diabetic + WP vs. diabetic (ANOVA with Tukey’s post-test).
Table 1. The effect of WP supplementation during diabetes on wound tissue cytokines. The cytokine levels in the wound tissue collected from the 3 groups of mice on 1, 4, 7, 10 and 13 days post-wound formation were measured using ELISA. The cytokine levels from the control skin (Day 0, one hour before wounding) were also measured. The data were tested for normality and homogeneity variances prior to statistical analysis. The data were normally distributed and calculated as the cytokine levels (pg) per mg of tissue. The data are presented as the mean ± SEM. *P< 0.05, diabetic vs. control; +P< 0.05, diabetic +WP vs. control; #P< 0.05, diabetic +WP vs. diabetic (ANOVA with Tukey’s post-test).

The effects of WP supplementation on wound-tissue cytokines with diabetes

An ELISA assay was performed to measure the levels of cytokines, chemokines and growth factors, which play important roles in wound healing, in the excisional wound tissues collected from the 3 groups of mice on days 1, 4, 7, 10 and 13 after wound formation. Day 0 represented one hour before wound formation (non-wounded skin tissue). The accumulated data from 10 individual mice from each group revealed aberrant elevations in the levels of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in the diabetic mice compared with the control group. The diabetic mice supplemented with WP showed a partial restoration of the levels of pro-inflammatory cytokines (Table 1). Similarly, the chemokine levels (KC, MIP-1α, and MIP-2) were significantly prolonged and elevated in the diabetic mice compared with the control mice. The WP-treated diabetic mice exhibited partially restored chemokine levels compared with the control and diabetic mice. In contrast, the levels of IL-10, PDGF and TGF-β were significantly decreased in the diabetic mice when compared with the control mice, and supplementation with WP partially restored these levels in the diabetic mice, suggesting that WP decreased the excess pro-inflammatory milieu in diabetic wounds.

The WP-treated diabetic mice exhibited a modulation in the activation of wound-tissue STAT3, Akt and NF-κB signaling pathways

The activation of transcription factors, such as Akt, STAT3 and NF-κB, which are regulators of the inflammation and wound healing processes, was investigated in the excisional wound tissues from the three groups of mice. The immunoblots of one representative experiment showed phosphorylated Akt (Fig. 3A), STAT3 (Fig. 3C) and IκBα, the negative regulator of NF-κB (Fig. 3E) in non-wounded (Day 0) and wounded (at 1, 4, 7, 10 and 13 days) skin in the 3
groups of mice. Total Akt, STAT3 and IκBα were used as equal loading controls. We found that phosphorylation of Akt and IκBα were significantly reduced in the diabetic mice throughout the complete period of wound healing when compared with the control mice. Moreover, the WP-supplemented diabetic mice displayed enhanced phosphorylation of Akt and IκBα, which peaked at 1 and 4 days post injury and was similar to the levels observed in the control mice. In contrast, aberrant and sustained phosphorylation of STAT3 was observed in the wound tissue from diabetic mice, which was unlike the STAT3 phosphorylation observed in the control and WP-treated diabetic mice (Fig. 3C). The accumulated data from 10 individual mice in each group were expressed as the normalized average of phosphorylated protein/total relevant protein ± SEM. After excisional wounding, we observed that the phosphorylation of Akt peaked at day 1 post injury and exhibited a 4-fold increase in the WP-supplemented diabetic mice when compared with the diabetic mice (Fig. 3B). Similarly, the phosphorylation of IκBα peaked at 4 days post injury, and the WP-supplemented diabetic mice exhibited significantly elevated (3-fold) IκBα phosphorylation when compared with the diabetic mice (Fig. 3F), which suggests that the WP reverted the impaired Akt and IκBα phosphorylation during the healing of diabetic wounds. Nevertheless, aberrant and continuous phosphorylation of STAT3 was observed in the wound tissue of the diabetic mice, which was different from the control and WP-supplemented diabetic mice (Fig. 3D) and confirmed that prolonged pro-inflammatory signals delay the healing of diabetic wounds.

Fig. 3. Supplementation with WP affects the phosphorylation of wound tissue STAT3, Akt and IκBα. The immunoblots of representative data from three independent experiments are shown for phosphorylated Akt (A), STAT3 (C) and IκBα (E) in non-wounded (Day 0) and wounded (at 1, 4, 7, 10 and 13 days post injury) skin in the 3 groups of mice. The total amount of the relevant proteins, Akt, STAT3 and IκBα, are shown as loading controls (B, D, F). The data from 10 individual mice in each group are expressed as the means of normalized phosphorylated protein/total relevant protein ± SEM. *P< 0.05, diabetic vs. control; +P< 0.05, diabetic +WP vs. control; #P< 0.05, diabetic +WP vs. diabetic.
WP supplementation in diabetic mice restored the impaired functional activity of wound tissue macrophages

Macrophages play central roles in the wound healing process. Because it was difficult to isolate wound tissue macrophages on day 1 post injury, we assessed the effect of WP supplementation during diabetes on the activities of wound tissue macrophages at 4, 7, 10 and 13 days post wound injury using flow cytometry analysis. The histograms from a representative experiment for scoring active caspase 3-stained cells are shown in Fig. 4A. We observed a marked decrease in the percentage of
apoptotic macrophages in the diabetic mice supplemented with WP (36 %) compared with the diabetic mice (74 %). However, the percentage of apoptotic macrophages in the diabetic group was elevated when compared with the control mice (11%) (Fig. 4A). In addition, the Annexin V binding assay demonstrated consistent apoptosis of macrophages (Fig. 4B). The bar graphs show the accumulated data from 10 individual mice from each group. This data revealed that the percentage of apoptotic wound macrophages was significantly decreased by 2-fold in the diabetic mice supplemented with WP when compared with diabetic mice, which suggests that WP supplementation during diabetes sustains long-lived wound macrophages. The functional activity of the wound-tissue macrophages was monitored using the Phosflow assay and flow cytometry. The phosphorylation of Akt and STAT3 was assessed as indicators of the activation state of the macrophages. We observed an increase in the percentage of macrophages exhibiting p-Akt in the WP-supplemented diabetic mice (59 %) when compared with the diabetic mice (39 %) (Fig. 4C). However, in the control group, the percentage of macrophages exhibiting p-Akt was 82%. The bar graphs represent the accumulated data from 10 individual mice from each group. This data demonstrated that the percentage of wound-tissue macrophages expressing p-Akt in the WP-supplemented diabetic mice was significantly higher than in the diabetic mice, which suggests a role for WP supplementation in enhancing the survival and activity of wound macrophages. Unlike p-Akt, STAT3 was aberrantly phosphorylated (98 %) in diabetic mice compared with the control (43%) and WP-supplemented diabetic (66%) groups (Fig. 4D). The bar graphs represent the accumulated data from 10 individual mice from each group. Our data revealed that the percentage of wound-tissue macrophages expressing p-STAT3 in the WP-supplemented diabetic mice was significantly decreased when compared with the diabetic mice throughout the time points, which suggests a role for WP in suppressing inflammatory stimuli and improving the function of wound macrophages. Finally, to monitor the actual functional activity of wound-tissue macrophages, the phagocytic index (engulfment of apoptotic cells by wound macrophages) was calculated at 4, 7, 10 and 13 days post injury for the 3 groups of mice. As shown in Fig. 4E, the wound macrophages exhibited a significant increase in the phagocytic index in diabetic mice supplemented with WP when compared with the diabetic mice throughout the interval time points.

**Discussion**

A major complication of diabetes mellitus is a deficiency in wound repair from inadequate collagen deposition and a failure to form a sufficient amount of extracellular matrix, angiogenesis, prolonged inflammation, altered host response, a tendency for vascular abnormalities, decreased growth factor production, and alterations in the functions of wound cells [29, 30]. Therefore, several attempts have been made to understand the underlying defects in wound healing. This study may introduce a new avenue for the therapeutic intervention of skin wounds in diabetic patients through the oral administration of camel WP. Our previous studies have demonstrated that WP supplementation during diabetes enhances cytoskeletal rearrangement and chemotaxis in B and T cells, which improves the immune response in diabetic mice [31]. Little is known regarding the effects of WP on impaired wound healing during diabetes. We monitored the macroscopic changes and percentage of wound closure that reflects the effects of wound contraction and healing. We observed that the macroscopic changes and rate of wound closure were significantly reduced in the diabetic mice supplemented with WP compared with diabetics. The rapid wound closure in the WP-treated diabetic wounds may be attributed to increased collagen synthesis and angiogenesis. Our results are consistent with the results of Wang et al. [32], who reported that WP promoted significant wound healing after skin incisions and had significant wound healing potential in the uterus following caesarean section in rats. In our study, we observed that defective wound repair in the diabetic mice was associated with a reduced hydroxyproline content and expression of collagen type 1 that increased after WP treatment. Similarly, Zhang et al. [33] observed that impaired wound healing in diabetic rats is associated with decreased hydroxyproline content. A previous study indicated that the impaired collagen deposition in acute wounds in Type 1 Diabetes is potentially due to decreased fibroblast proliferation [34]. The enhanced level of hydroxyproline and collagen type 1 likely strengthens the regenerated tissue in the diabetic mice supplemented with WP. Interestingly, we observed that WP treatment improved wound healing in the diabetic mice because it significantly decreased the elevated pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and increased IL-10 in the plasma and wounded tissues. This finding limits prolonged inflammation and indicates the mechanism underlying the enhanced immune response and improved wound healing.
in diabetic mice. The previous study supports our results and indicated that lactoferrin could regulate levels of TNF-α and IL-6, which would decrease inflammation and mortality [35]. Several studies have focused on the critical roles of chemokines, such as MIP-1α, MIP2, and KC, during tissue repair processes [36, 37]. We also observed that WP treatment expedited wound healing in diabetic animals by the significant increase of MIP-1α, MIP2, and KC in the wound area. Mori et al. [25] published similar results that several chemokines have chemotactic activities against neutrophils and macrophages, such as MIP-1α and MIP2, and their expression can be upregulated by other pro-inflammatory cytokines, such as IL-1β. Because growth factors, such as PDGF and TGF-β, are important for wound repair processes [38], we determined whether growth factors were present in the wound sites. Our data showed that PDGF and TGF-β were significantly increased after WP treatment when compared with diabetic mice. TGF-β induces the formation of contractile bundles in normal wound fibroblasts [39]. Kao et al. [40] recently published similar observations that fibrocyte injections could accelerate wound healing through the upregulation of growth factors (TGF-β, PDGF-A, and FGF-7) in an experimental diabetic mouse model. Our results have shown that the phosphorylation of Akt and IkBα in wound tissue was enhanced in diabetic mice supplemented with WP, whereas Akt and IkBα phosphorylation was faint or absent in the diabetic mice. In contrast, the aberrant phosphorylation of STAT3 was obvious in the wound tissues of the diabetic mice. These data are consistent with published results that show diabetes-induced wound healing disorders are associated with an absence of Akt and IkBα activation as well as aberrant activation of STAT3 [13]. Moreover, another study revealed that activation of the PI3K-Akt-mTOR signaling pathway may represent a novel clinical intervention strategy to accelerate the healing of debilitating and life-threatening wounds [41]. Consistent with these results, a previous study reported an increase in STAT3 activity associated with wound healing [42] and aberrant STAT3 activation during impaired wound healing [43]. Wound-tissue resident macrophages play central roles in the wound healing process, including the host defense, regulation of inflammation, removal of apoptotic cells, and support of cell proliferation and tissue restoration following injury [44]. Impaired functions of macrophages in diabetic wound tissue is a key element for delayed and abnormal wound closure. Using the annexin V binding assay to measure indicators of apoptosis, we observed a marked decrease in the percentage of apoptotic macrophages in the WP-treated diabetic mice when compared with the diabetic mice. Elevated levels of apoptotic macrophages augmented the inflammatory response in diabetic wounds [14]. Moreover, the observed decrease in the aberrant activation of STAT3 clearly demonstrated the additional effects of WP supplementation during diabetes on wound tissue macrophages and the subsequent attenuation of the inflammatory stimuli, which enhanced the phosphorylation of Akt and generated long-lived functional wound-tissue macrophages. The impaired phagocytic activity of macrophages in diabetic wounds may be associated with elevated apoptotic cell counts or increased oxidative stress. The phagocytic index of wound macrophages was significantly increased after WP treatment. A previous study reported that whey peptides possess immunomodulatory activities, such as lymphocyte stimulation and increased phagocytosis [45]. Our data reveal the benefits of WP supplementation in improving the healing and closure of diabetic wounds and the underlying mechanisms.

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