Original Paper

Camel Whey Protein Protects B and T Cells from Apoptosis by Suppressing Activating Transcription Factor-3 (ATF-3)-Mediated Oxidative Stress and Enhancing Phosphorylation of AKT and I κ B- α in Type I Diabetic Mice

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Key Words

Apoptosis • Camel whey protein • Diabetes mellitus • Free radicals • Lymphocytes

Abstract

Background: Diabetes mellitus (DM) is associated with severe immune system complications. Camel whey protein (CWP) decreases free radicals (ROS) and modulates immune functions, but its effect on DM-impaired immune systems has not been studied. We investigated the impact of CWP on the immune system in a Type 1 diabetes mouse model. **Methods:** Three experimental groups were used: (1) non-diabetic control; (2) diabetic; and (3) CWP-treated diabetic mice. **Results:** Induction of diabetes by streptozotocin was associated with reduction of body weight and insulin level, increase in glucose level and pro-inflammatory cytokines (IL-1 β , IL-6, and TNF- α), and reduction in IL-2 and IL-4 levels. Upregulated ATF-3 expression was followed by a marked elevation in ROS levels. Lymphocytes from diabetic mice exhibited increased apoptosis through decreased phosphorylation of AKT and I κ B- α , increased infiltration of T cells in the spleen and thymus, and decreased B cell numbers in the spleen. Supplementation with CWP decreased the levels of proinflammatory cytokines, ROS, and ATF-3 expression, and increased the levels of IL-4. Treatment with CWP decreased apoptosis by enhancing the phosphorylation of AKT and I κ B- α as well as T-cell and B-cell distribution in the spleen and thymus. **Conclusions:** Our findings suggest the beneficial effects of CWP supplementation during diabetes on decreasing and orchestrating the redox status and subsequently rescuing the immune cells from exhaustion.

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Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease that follows the autoimmune destruction of insulin-producing pancreatic β -cells by auto-reactive T cells [1]. This leads to an increase in pro-inflammatory cytokines and reactive oxygen species (ROS) and a decrease in insulin secretion [2]. Likewise, T1D contributes to a defect in lymphocyte function, especially in B- and T-lymphocytes, which increases susceptibility to infection [3, 4]. Moreover, hyperglycemia increases the oxidative modification of lipids, DNA, and proteins in various tissues, which is involved in the development of diabetic complications [5]. Cytokines are mediator proteins that play a crucial role in inflammation by controlling innate and adaptive immune responses as well as tissue damage repair associated with T1D [6]. Disturbance of pro-inflammatory cytokines such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) plays an important role in immune response regulation during diabetes [7].

Activating transcription factor-3 (ATF-3) is a stress-inducible gene; its expression is associated with cellular damage, including apoptosis of pancreatic β -cells, ROS production, and high concentrations of glucose or fatty acids, which correlate to diabetic complications [8, 9]. Increased ATF3 expression in the β -cells of diabetic patients indicates upregulation of the pro-apoptotic genes [10]. IL-2, also known as T-cell growth factor, shows several immunoregulatory effects that can promote T cell-dependent immune responses [11]. We previously reported that decreased levels of IL-2 and IL-4 led to a reduction in the proliferation of lymphocytes [6]. Protein kinase B (AKT) is a serine/threonine kinase that regulates downstream transcription factors which play central roles in cell survival and protects cells against apoptosis [12, 13]. Dysregulation of AKT contributes to several diseases such as cancer and diabetes [14]. AKT/PKB, the downstream target of phosphatidylinositol 3 kinase (PI3K), is activated through phosphorylation mediated by the phosphatidylinositol-dependent kinase (PDK1), which is an important enzyme-regulated function of insulin [15, 16]. AKT is reportedly activated by insulin and other growth factors, while PI3K prevents activation of insulin [17, 18]. Nuclear factor kappa B (NF κ B) has been shown to regulate the gene expressions of numerous cytokines and chemotactic and matrix proteins that mediate inflammation, immunological responses, and cell proliferation [19]. Moreover, high activation of NF κ B by growth factors and cytokines under hyperglycemia is a potential mechanism for increased vascular dysfunction during diabetes [20, 21].

Camel whey protein (CWP) comprises 20–25% of the total camel milk proteins [22]. It also contains a different group of proteins including serum albumin, α -lactalbumin, immunoglobulin, lactoferrin (LF), peptidoglycan recognition protein, lactoperoxidase, and lysozyme [23, 24]. CWP has antioxidant activity due to the existence of cysteine or glutamylcysteine [25]. Whey protein (WP) modulates immune functions such as the activation and proliferation of lymphocytes, cytokine secretion, production of antibodies, phagocytic activity, and granulocyte and natural killer (NK) cell activity [26]. It also stimulates IL-1 β , IL-6, and TNF- α [27]. Moreover, WP stimulates lymphocytes and increases phagocytosis and secretion of immunoglobulin A (IgA) from Peyer's patches [27, 28]. We previously reported that CWP improves immunity during early life and protects against immune disorders during diabetes [29–31]. However, the effects of CWP on the diabetic immune system and its underlying mechanisms have not been studied. In this study, we aimed to investigate the effect of CWP on the diabetic immune system in a streptozotocin (STZ)-induced type I diabetic mouse model.

Materials and Methods

Preparation of camel whey proteins

Raw camel milk was collected from healthy female camels from Marsa Matrouh, Egypt and centrifuged to remove the cream. The resulting skim milk was acidified to pH 4.3 using 1N HCl at room temperature and

centrifuged at 10,000 $\times g$ for 10 min to precipitate the casein. The resulting CWP was mixed with ammonium sulfate to precipitate the CWP. The precipitated CWP was dialyzed against 20 volumes of distilled water for 48 h using a porous membrane with a molecular weight cut-off (MWCO) of 6000–8000 kDa. The dialysate containing the undenatured CWP was freeze-dried and refrigerated until further use [30].

Streptozotocin

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

Experimental design and doses

Forty-five adult male mice weighing 25–30 g were purchased from the Institute of Theodor Bilharz, Cairo, Egypt. Mice were housed in cages and kept at a room temperature at $25 \pm 5^\circ\text{C}$ under a normal 12 h light/12 h dark cycle. They had *ad libitum* access to pelleted diet and water for one week for acclimatization. All experimental protocols used on animals were performed according to regulations set by the Institutional Animal Care and approved by Assiut University. All animal procedures were also performed in accordance Declaration of Helsinki with the guidelines for the care and use of experimental animals that was established by NIH protocol as previously described [31, 32]. We made an effort to minimize animal distress and to reduce the number of animals used in this study. After one week of acclimatization, the mice were randomly assigned to three groups each contains 15 mice: control, diabetic, and diabetic treated with CWP. The mice of group 2&3 were rendered diabetic with an intraperitoneal injection (i.p.) of three consecutive doses of STZ (60 mg /kg body weight) in 0.01 M citrate buffer (pH 4.5). Mice in the control group were injected with only the vehicle (0.01 M citrate buffer, pH 4.5). After 4 d, glucose levels were measured. Diabetes was determined as >220 mg/dl. After two weeks of injection with STZ, control non-diabetic mice were orally supplemented with distilled water (250 μl /mouse/d for one month by oral gavage); group 2 diabetic mice with distilled water (250 μl /mouse/d for one month by oral gavage); and group 3 diabetic mice with CWP (100 mg/kg body weight dissolved in 250 μl /d for one month by oral gavage).

Blood collection and analysis

Whole blood was collected from the abdominal aorta and immediately transferred to heparinized tubes. The blood was centrifuged at $3,000 \times g$ for 20 min using a bench top centrifuge (Anke TGL-16B) to remove red blood cells and recover plasma. The plasma samples were separated, collected using dry Pasteur pipettes, and stored at -20°C until further use. After plasma isolation, peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll gradient method. Freshly isolated PBMCs were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and HEPES for at least 4 h prior to staining with antibodies and preparation for flow cytometry analysis. Blood glucose level was determined using the Rightest® Blood Glucose Monitoring System GM100 (Bionime). Insulin level was analyzed by Luminex assay (Biotrend, Düsseldorf, Germany) according to the manufacturer's instructions.

Measuring reactive oxygen species (ROS) level

Plasma levels of ROS were assessed using 2, 7-dichlorodihydrofluoresce in diacetate (H2DCF-DA) (Beyotime Institute of Biotechnology, Haimen, China). The oxidation of 2'-7' dichlorofluorescein (H2DCF) to 2'-7'dichlorofluorescein (DCF) has been used extensively to quantitate H_2O_2 levels. The oxidation of H2DCF by ROS converts the molecule to 2', 7' dichlorodihydrofluorescein (DCF), which is highly fluorescent. DCF fluorescence was assessed using 498 nm for excitation and 522 nm for emission [33].

Measuring cytokine levels

Plasma cytokine profile was determined in samples that were stored at -80°C . The levels of cytokines (IL-1 β , IL-2, IL-4, IL-6, and TNF- α) were determined by ELISA using a Bio-Plex Mouse Cytokine Assay Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Western blot analysis

Prior to Western blot analysis, lymphocytes were incubated in prewarmed RPMI-1640 without serum. Whole-cell lysates in RIPA buffer and equal amounts of the total cellular protein (60 μg) were subjected to SDS-PAGE and blotted onto a nitrocellulose membrane (Millipore, Bedford, MA, USA). After primary

antibodies recognizing anti phospho-AKT (p-AKT), total-AKT (pan-AKT), anti phospho-I κ B- α (p-I κ B- α), total-I κ B- α (pan-I κ B- α), anti ATF-3 and β -actin (Cell Signaling, UK) were diluted at 1:1000 in 1X TBS with 0.1 % Tween-20 and 5% bovine serum albumin (BSA), the membrane was incubated overnight with a primary antibody on an orbital shaker. Following overnight incubation, the primary antibody was removed and the membrane was washed three times in washing buffer for 5 min each. A horseradish peroxidase (HRP)-labeled secondary antibody (Cell Signaling, UK) was diluted to 1:1000 and applied to the membrane for 1 h at room temperature on an orbital shaker. The membrane was then washed three times in washing buffer for 5 min, followed by a single wash in distilled water for 5 min. The antigens were visualized using chemiluminescence (ECL, SuperSignal West Pico Chemiluminescent Substrate; Perbio, Bezons, France) and exposure to X-ray film (Amersham Biosciences, France). The ECL signal was specifically recorded on ECL hyper-film. To quantify the band intensities, the films were scanned, saved as TIFF files, and analyzed using NIH ImageJ software [34, 35].

Flow cytometry analysis

The percentage of lymphocytes undergoing apoptosis was determined by flow cytometry. Dead cells were identified using the Trypan blue exclusion test. To distinguish between viable, early apoptotic, and late apoptotic cells, the cells were washed and incubated in PBS containing 30% human AB serum (4°C for 30 min) prior to staining with Annexin V-FITC and propidium iodide (PI) (15 min at 25 °C) using a commercial kit according to the manufacturer's instructions (Abcam, Canada). The cells were analyzed by flow cytometry using a FACS Calibur flow cytometer (BD-Pharmingen) within 1 h of staining, and the percentage of cells undergoing apoptosis was determined [36, 37].

Immunohistochemical detection of T and B cells

Paraffin sections of thymus and spleen samples fixed in formal alcohol were cleared in xylene, rehydrated in graded ethanol (100%–70%), washed in PBS for 5 min, and blocked for 60 min in 2% BSA in PBS. Sections were incubated with primary antibodies: anti-CD3 (pan T cells) & anti-CD20 (pan B cells) in BSA for 60 min at room temperature, then washed in PBS with three changes for 5 min each. The sections were then incubated for 45 min with biotinylated secondary antibody and washed in PBS for 5 min. Next, the sections were incubated for 45 min with AB enzyme reagent and washed in PBS for 5 min. The sections were incubated in 1–3 drops peroxidase substrate for 30 s–10 min, and then washed in deionized water for 5 min. Sections were counterstained with hematoxylin for 3 min and mounted. Photographs of the sections were taken; the images were digitized using Adobe Photoshop (Adobe Systems, Mountain View, CA). The numbers of anti-CD3 or anti-CD20-stained cells were determined at 20 random locations in the section taken from five animals from each group using a Leica Qwin 500 image analyzer [3].

Statistical analysis

The data were tested for normality (using an Anderson-Darling test) and variance homogeneity prior to further statistical analysis. The data were normally distributed and are expressed as the means $\pm$ SEM (standard error of the mean). Significant differences between groups were analyzed using one-way analysis of variance (for more than two groups) followed by Tukey's post-test using Graph Pad Prism software version 5. Differences were considered statistically significant at * $P < 0.05$ for the diabetic group vs. control mice; * $P < 0.05$ for the diabetic + CWP group vs. control mice; and # $P < 0.05$ for the diabetic + CWP group vs. diabetic mice.

Results

Characteristics of the diabetic animal model and complications of diabetes before and after CWP treatment

We first monitored changes in body weight, blood glucose, and insulin levels in all groups throughout the experimental period. STZ caused marked hyperglycemia that was detectable as a significant decrease in body weight and insulin levels and a marked increase in blood glucose levels compared to control animals ($n = 5$, * $P < 0.05$) (Table 1). Interestingly, Supplementation of diabetic animals with CWP partially restored the levels of blood glucose and insulin and body weight compared to diabetic untreated animals (Table 1).

Table 1. Impact of STZ-induced diabetes and CWP treatment on blood glucose levels, insulin levels, and body weight. Body weight, blood glucose, and insulin levels were measured in three groups of mice throughout the experimental period. The pooled data for five mice from each group are expressed as the mean value for each parameter $\pm$ SEM. * $P < 0.05$ for diabetic vs. control mice and * $P < 0.05$ for diabetic + CWP vs. control mice (ANOVA with Tukey's post-test)

Groups	Glucose (mg/dl)	Insulin (ng/mL)	Body weight (g)
Control	111.2 $\pm$ 7.3	11.6 $\pm$ 0.81	31.33 $\pm$ 1.09
Diabetic	517.9 $\pm$ 46.2*	3.6 $\pm$ 0.66*	27.26 $\pm$ 0.82*
Diabetic+CWP	383.5 $\pm$ 60.8*#	5.6 $\pm$ 0.56*#	27.92 $\pm$ 1.03+

The impact of CWP supplementation on levels of proinflammatory cytokines in diabetic mice

Using ELISA, we measured the levels of pro-inflammatory cytokines IL-6, IL-1 β , and TNF- α . Accumulated data from five individual mice from each group showed elevated levels of pro-inflammatory cytokines in the diabetic mice compared to control samples (Fig. 1). In particular, diabetic mice exhibited elevated levels of pro-inflammatory cytokines IL-6 (Fig. 1A), IL-1 β (Fig. 1B), and TNF- α (Fig. 1C) compared to control non-diabetic animals. However, pro-inflammatory cytokine levels were significantly decreased in the plasma of diabetic mice treated with CWP ($n = 5$).

CWP treatment modulates ATF-3 expression in PBMCs and levels of ROS, IL-2, and IL-4 in plasma of diabetic mice

The pro-inflammatory response mediates activation of transcription factors such as ATF-3, leading to the induction of oxidative stress, which can impair the immune system. Therefore, we investigated the expression of ATF-3 in the three groups of mice. A representative immunoblot illustrating one of the five experiments is shown in Figure 2A. Immunoblots for ATF-3 and total actin (loading control) levels in the PBMCs isolated from control non-diabetic, diabetic, and CWP-treated diabetic mice are also illustrated. The diabetic mice exhibited a prominent elevation in the expression of ATF-3 compared to control non-diabetic mice. Additionally, the expression of ATF-3 was clearly decreased in CWP-treated diabetic mice, as the immunoblots showed a very faint band compared to that of diabetic

Fig. 1. CWP supplementation decreased pro-inflammatory cytokines during T1D. The levels of pro-inflammatory cytokines (IL-6, IL-1 β , and TNF- α) were measured in three groups of mice using ELISA. The results are presented as cytokine levels (pg) per mg of plasma in control non-diabetic (open bars), diabetic (closed black bars), and CWP-treated diabetic (hatched bars) animals. Data are expressed as the mean $\pm$ SEM ($n = 5$). * $P < 0.05$ for diabetic vs. control mice; * $P < 0.05$ for diabetic +CWP vs. control mice; and # $P < 0.05$ for diabetic + CWP vs. diabetic mice (ANOVA with Tukey's post-test).

Fig. 2. CWP supplementation decreased expression of ATF-3 and levels of ROS and increased the levels of IL-2, and IL-4 in diabetic mice. (A) Western blot analysis was performed, and immunoblots for ATF-3 and total β -actin from one representative experiment are shown for a control non-diabetic, diabetic, and CWP-treated diabetic mouse. (B) The accumulated, normalized data from five independent experiments are shown for ATF-3 expression of control non-diabetic (open bars), diabetic (closed black bars), and CWP-treated diabetic animals (hatched bars). The values represent the mean $\pm$ SEM. Free radical levels (ROS) were measured throughout the experimental period. The pooled data for five mice from each group are expressed as the mean value for each parameter $\pm$ SEM. Levels of IL-2 and IL-4 were measured in the three groups of mice during the experimental period. Pooled data for five mice from each group are expressed as the mean value for each parameter $\pm$ SEM. Levels of IL-2 and IL-4 were measured in the three groups of mice during the experimental period. Pooled data for five mice from each group are expressed as the mean value for each parameter $\pm$ SEM. * $P < 0.05$ for diabetic vs. control mice; * $P < 0.05$ for diabetic + CWP vs. control mice; and # $P < 0.05$ for diabetic + CWP vs. diabetic mice (ANOVA with Tukey's post-test).

mice. The collected results of five individual mice per group are shown for the normalized expression of ATF-3 to total actin levels. Diabetic mice showed marked overexpression of ATF-3 compared to control non-diabetic mice (Fig. 2B), while diabetic mice supplemented with CWP showed a significant amelioration in ATF-3 expression, to nearly its normal expression value.

Upregulation of ATF-3 expression in diabetic has been associated with an increase in plasma levels of ROS and a decrease in IL-2 and IL-4 levels. Therefore, we measured the levels of ROS, IL-2, and IL-4 in plasma using ELISA. The accumulated data from five individual mice from each group are shown in Figures 2C and 2D. The diabetic mice exhibited a significant elevation in plasma ROS compared to the control non-diabetic mice (* $P < 0.05$). Interestingly, CWP-treated diabetic mice exhibited a significant restoration of ROS levels compared to untreated diabetic mice (# $P < 0.05$), while diabetic mice showed decreased levels of IL-2 and IL-4 (Fig. 2D) compared to control non-diabetic animals. In addition, CWP-treated diabetic mice showed a significant restoration of IL-4 levels compared to untreated diabetic animals (# $P < 0.05$). However, CWP-treated diabetic mice showed non-significant elevation of IL-2 levels compared to untreated diabetic animals.

Treatment of diabetic mice with CWP restores impaired PI3K/AKT and NF κ B/I κ B- α signaling pathways in PBMCs in T1D.

We investigated whether impaired lymphocyte proliferation in diabetic mice is associated with alteration in PI3K/AKT and NF κ B/I κ B- α signaling pathways. The phosphorylation of

Fig. 3. Influence of CWP supplementation on phosphorylation of AKT and I κ B- α during T1D. (A) Western blot analysis was performed, and immunoblots for p-AKT and total AKT from one representative experiment are shown for a control non-diabetic, diabetic, and CWP-treated diabetic mouse. (B) The accumulated, normalized data from five independent experiments are shown for AKT phosphorylation from control non-diabetic (open bars), diabetic (closed black bars), and CWP-treated diabetic animals (hatched bars). (C) Immunoblots for p-I κ B- α and total I κ B- α from one representative experiment are shown for a control non-diabetic, diabetic, and CWP-treated diabetic mouse. (D) The accumulated normalized data from five independent experiments are shown for I κ B- α phosphorylation from control non-diabetic (open bars), diabetic (closed black bars), and CWP-treated diabetic animals (hatched bars). * $P < 0.05$ for diabetic vs. control mice and # $P < 0.05$ for diabetic + CWP vs. diabetic mice (ANOVA with Tukey's post-test).

AKT and I κ B- α was inspected in PBMCs isolated from control non-diabetic, diabetic, and CWP-treated diabetic mice. Immunoblots for p-AKT and pan-AKT (loading control) in control non-diabetic, untreated diabetic, and CWP-treated diabetic mice are shown in Figure 3A. We observed that diabetic mice exhibited marked reduction in phosphorylation of AKT compared to control non-diabetic mice. The phosphorylation of AKT was clearly increased in the CWP-treated diabetic mice, as the immunoblots showed very clear bands compared to those for untreated diabetic mice. The accumulated data from five individual mice per group are shown for the normalized phosphorylation of AKT to the total AKT (Fig. 3B). Immunoblots for p-I κ B- α and pan-I κ B- α (loading control) in control non-diabetic, untreated diabetic, and CWP-treated diabetic mice are shown in Figure 3C. We observed that diabetic mice exhibited markedly decreased phosphorylation of I κ B α as compared to control non-diabetic mice. CWP-treated diabetic mice exhibited significant restoration of I κ B- α phosphorylation, as shown by the clear bands in their immunoblots compared to those of untreated diabetic mice. The accumulated data from five individual mice per group are shown for the normalized phosphorylation of I κ B α to the total I κ B- α level (Fig. 3D). We found that vehicle-treated diabetic mice exhibited significant reduction phosphorylation of I κ B- α compared to control and CWP-treated diabetic mice. Most importantly, when the diabetic mice were treated with CWP, they exhibited a significant restoration of I κ B- α phosphorylation.

Supplementation with CWP during T1D decreases apoptosis of blood PBMCs

We next evaluated the number of lymphocytes circulating in the blood and their tendency to undergo apoptosis in the three groups of animals. PBMCs isolated from control non-diabetic, diabetic, and CWP-treated diabetic mice were stained with PI/Annexin V and analyzed by flow cytometry to determine the percentages of viable cells (lower left quadrant), early apoptotic cells (lower right quadrant), and late apoptotic cells (upper right quadrant) (Fig. 4A). The data from one representative experiment are presented in the dot plot. The percentage of apoptotic PBMCs (early and late apoptotic) was 21.2% in the control non-diabetic group and increased markedly to 62.8% in the diabetic group, while the percentage

Fig. 4. CWP supplementation decreased apoptosis of PBMCs in diabetic mice. Viable, apoptotic, and necrotic PBMCs were differentiated by flow cytometry based on their PI/Annexin V staining pattern. (A) A dot plot of one representative data set from five independent experiments. (B) Pooled data from five experiments expressed as the mean percentage of apoptotic PBMCs cells $\pm$ SEM for PBMCs isolated from control mice (open bars), diabetic mice (closed black bars), and diabetic mice treated with CWP (hatched bars). * $P < 0.05$ for diabetic vs. control; # $P < 0.05$ for diabetic + CWP vs. diabetic; * $P < 0.05$ for diabetic + CWP vs. control (ANOVA with Tukey's post-test).

of apoptotic PBMCs decreased to 36% in CWP-treated diabetic mice. The pooled data for five individual mice from each group indicate that treatment of the diabetic mice with CWP significantly (* $P < 0.05$) rescued PBMCs from apoptosis (Fig. 4B).

CWP supplementation suppresses T cells in the thymus during T1D

Short life span and apoptosis induction in the circulating T cells could reflect abnormalities in their maturation in the thymus gland. We therefore investigated the distribution of T cells in the thymus gland of the three animal groups. One representative experiment demonstrated that thymus section from control (Fig. 5A), diabetic (Fig. 5B) and CWP-treated diabetic mice (Fig. 5C) were stained with anti-CD3 antibody to realize the T cells distribution. These results revealed that the number of T cells was increased in the thymus cortex of diabetic animal as compared to the control non-diabetic animal. On the other hand, the number of T cells in diabetic mice supplemented with CWP was reduced in the thymus cortex compared to diabetic treated with distilled water. Quantification analysis of CD3+ T cells (Fig. 5D) from five different animals from each group confirmed that diabetic animals exhibited a significant increase in the number of CD3+ T cells in the thymus cortex as compared to control non-diabetic animals. Nevertheless, when the diabetic mice were supplemented with CWP, the numbers of CD3+ T cells were significantly decreased in the thymus cortex as compared to diabetic mice.

CWP supplementation increases B cells and suppresses T cells in the spleen during T1D

Because apoptosis induction in the circulating lymphocytes alters their migration to the lymphoid organs, we therefore investigated the distribution of T and B cells in the

spleen of the three animal groups. One representative experiment demonstrated that spleen sections from control (Fig. 6A), diabetic (Fig. 6C) and CWP-treated diabetic (Fig. 6E) mice were stained with anti-CD3 antibody to investigate T cell distribution. Our data demonstrated that the number of T cells was increased in both red and white pulps of the diabetic animal as compared to the control non-diabetic animal. However, supplementation of diabetic animal with CWP decreased the number of T cells in both red and white pulp as compared to the diabetic animal. In the same context, spleen sections from control (Fig. 6B), diabetic (Fig. 6D) and CWP-treated diabetic (Fig. 6F) mice were stained with anti-CD20 to investigate the B cells distribution. These results represented a clear reduction in the number of B cells in both red and white pulps of the diabetic animal as compared to the control non-diabetic animal. In contrast, CWP-treated diabetic animal exhibited an increase in the number of B cells in both red and white pulp as compared to the diabetic animal. Quantification analysis of CD3+ T cells (Fig. 6G) and CD20+ B cells (Fig. 6H) from five different animals from each group confirmed that diabetic mice exhibited a significant increase in the numbers of spleen homing CD3+ T cells and significant decrease in the number of splenic CD20+ B cells as compared to control non-diabetic animals. Interestingly, when the diabetic mice were supplemented with CWP, the numbers of CD3+ T cells were significantly decreased and the numbers of CD20+ B cells were significantly increased in the spleen as compared to diabetic mice.

Discussion

Natural antioxidant CWP enhances the proliferation of immune cells and hastens the wound-healing process during diabetes in experimental animal models [30, 31, 38, 39].

Here, CWP treatment improved immune systems in diabetic mice at a rate similar to that of non-diabetic control mice and faster than that of untreated diabetic mice. The impaired immune system in diabetic mice was associated with an increase in blood glucose levels, a decrease in insulin levels, and a decrease in body weight, which were partially reversed by CWP treatment. Likewise, CWP stimulated insulin release and lower blood glucose levels in type 1 diabetic rats [39, 40]. It also decreased elevated levels of IL-1 β , IL-6, and TNF- α in diabetic mice. Lactoferrin can regulate the levels of TNF- α and IL-6, which

Fig. 5. Effect of CWP on number and distribution of T cells in the thymus during T1D. Immunohistochemical analysis revealed the number and distribution of CD3+ T cells (arrow) in the thymus (Immunoperoxidase X400). One representative experiment is shown for the thymus section of control animal (A), diabetic animal (B) and CWP-treated diabetic animal (C). Values shown (D) are the mean count of the CD3+ T cells $\pm$ SEM in control mice (open bars), diabetic mice (closed black bars), and CWP-treated diabetic mice (hatched bars). * $P < 0.05$ for diabetic vs. control; # $P < 0.05$ for diabetic + CWP vs. diabetic; * $P < 0.05$ for diabetic + CWP vs. control (ANOVA with Tukey's post-test) (n=5).

Fig. 6. CWP supplementation altered the number and distribution of T and B cells in the spleen during T1D. Immunohistochemical analysis revealed the number and distribution of CD3⁺ T cells (arrow) in the spleen (Immunoperoxidase X400). One representative experiment is shown for the spleen section of control animal (A), diabetic animal (C) and CWP-treated diabetic animal (E). Similarly, one representative experiment is shown for the number and distribution of CD20⁺ B cells (arrow) in the spleen (Immunoperoxidase X400) of control (B), diabetic (D) and CWP-treated diabetic mouse (F). Values shown are the mean count of the CD3⁺ T cells (G) and CD20⁺ B cells (H) $\pm$ SEM in control mice (open bars), diabetic mice (closed black bars), and CWP-treated diabetic mice (hatched bars). * $P < 0.05$ for diabetic vs. control; # $P < 0.05$ for diabetic + CWP vs. diabetic; * $P < 0.05$ for diabetic + CWP vs. control (ANOVA with Tukey's post-test).

decrease inflammation and mortality [41]. However, unlike the levels of IL-2, IL-4 levels were significantly increased in the CWP-treated diabetic.

In the present study, increased expression of ATF-3 expression in diabetic has been associated with an increase in plasma levels of ROS and a decrease in IL-2 and IL-4 levels. Additionally, CWP treatment significantly down-regulated the expression of ATF-3 in diabetic mice as compared to diabetic untreated mice. It has been shown that ATF-3 is highly expressed in vascular endothelial cells in atherosclerotic lesions and is implicated in cell death via apoptosis [42, 43]. In this context, demonstrated that upregulated expression of ATF-3 in diabetic wounds plays a vital role in the oxidative stress-mediated impairment of cell differentiation and wound healing [44]. In addition, ATF-3 participates in the oxidative stress-induced tubulogenic differentiation of endothelial cells by changing the expression levels of cell cycle regulators [45]. Most studies of the effects of whey protein on cancer have employed bovine whey protein. A study in 1990 in an animal model of colon carcinoma induced by injecting mice with 1,2-dimethylhydrazine (DMH) revealed that WP decreased tumor burden compared with mice fed a casein or Purina diet [46]. The animals fed the diet supplemented with CWP exhibited 0% mortality, whereas the animals fed casein or Purina exhibited 33% mortality [46]. In this context, it has been concluded that WP in milk had a significant role as an antitumor agent by providing cysteine (a substrate for GSH synthesis) to enhance GSH synthesis in numerous tissues and, consequently, the detoxification of free radicals during carcinogenesis [47]. Nevertheless, these studies on bovine WP focused only

to investigate the levels of GSH rather than other mechanisms. Interestingly, it has been shown that camel milk lysozyme has greater anti-bacterial activity than lysozyme from other types of milk [48].

The present study suggests an anti-inflammatory effect of CWP on diabetic mice via restoration of ROS levels. Inconsistently, the hypoxia attributed to diabetes has been shown to increase early inflammatory response by increasing inflammatory mediator levels and ROS production [49]. Increased ROS can damage cellular components such as lipids, proteins, and DNA [50, 51]. It has been shown that the therapeutic utilization of WP in many oxidative stress-associated diseases has recently been reported [25]. LF displays the most potent antiviral activity of all components present in milk [52-54]. LF can react with various molecules to elicit antibacterial [55], antiviral [48], antioxidant, immunomodulatory and anti-inflammatory activities [56] and to function as a growth factor [57]. LF exhibits antiviral activity against a broad spectrum of viruses, including RNA and DNA viruses such as cytomegalovirus, hepatitis C virus (HCV), hepatitis B virus (HBV), herpes simplex virus (HSV), HIV, simian rotaviruses and adenoviruses [58]. Most interestingly, the content of LF in CWP is higher than that in WPs from other animals, suggesting its important role against HCV [59, 60]. Camel lactoferrin (CLF) not only inhibits HCV (genotype 4a) entry into HepG2 cells and peripheral blood mononuclear cells (PBMCs) but also suppresses viral replication in infected cells [53]. Additionally, it has been demonstrated that the inhibitory effect of CLF on HCV (genotype 4a) was two-fold higher than that of human, sheep and bovine LF [60]. Most importantly, CLF has been postulated to have hepatoprotective activity because can improve imbalances in the levels of Th1/Th2 cytokines [61].

Our data demonstrate the down-regulation in the phosphorylation of AKT and $\text{I}\kappa\text{B-}\alpha$ in diabetic mice, which is up-regulated by CWP supplementation. Increased phosphorylation of AKT is directly associated with increased glucose metabolism rate [62]. In the present study, CWP appeared to improve insulin signaling and glucose metabolism via regulation of the PI3K/AKT signaling pathway. The PI3K/AKT signaling pathway plays a crucial role in insulin signaling, glucose metabolism, and lymphocyte migration and proliferation, making it an important therapeutic target for the treatment of diabetes [63]. We observed an obvious reduction in apoptosis in the lymphocytes of CWP-treated diabetic mice as compared to diabetic mice. The number of T cells was increased in the red and white pulp of the spleen and cortex of the thymus. Consistent with our findings, increased apoptosis in lymphocytes can be attributed to increased inflammatory response during diabetes [64]. By contrast, the number of B cells decreased in diabetic mice, and CWP reversed this process. These results are consistent with the finding that CWP stimulates the proliferation of lymphocytes in B cells rather than T cells in the spleen [65]. We previously reported that WP treatment in diabetic mice enhances innate immunity by improving B and T cell chemotaxis efficiency [29-31]. This finding confirms that CWP is associated with stimulation of B cells and suppression of T cells during T1D.

Abbreviations

Activating transcription factor-3 (ATF-3), camel whey protein (CWP), interleukin (IL), protein kinase B (AKT), reactive oxygen species (ROS), streptozotocin (STZ), tumor necrosis factor- α (TNF- α), type 1 diabetes (T1D).

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Disclosure Statement

All authors have read and agreed the contents of the manuscript and approved the submission. The authors declare no conflicts of interest, state that the manuscript has not been published or submitted elsewhere, state that the work complies with the Ethical Policies of the Journal and state that the work has been conducted under internationally accepted ethical standards after relevant ethical review.

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