

Effect of STZ-Induced Diabetes on Spleen of Rats: Improvement by Camel Whey Proteins

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Abstract.- Diabetes is the most prevalent disease, and the number of people affected by this disease is still increasing. This study was conducted to assess the effect of whey proteins (WPs) on the spleen tissues of diabetic rats. Data showed that a significant decrease in the total body weight was recorded in diabetic animals compared with the non-diabetic control ones. The extent of this weight loss was significantly less in the WP-treated diabetic group. Relative spleen weight in the diabetic animals revealed that splenic atrophy was more pronounced compared to those of control ones. Results showed that diabetes significantly up-regulated both the reactive oxygen species FMO2 mRNA and Fas mRNA when compared with the control animals. Light microscopy showed white pulps that were greatly diffused with highly distributed trabeculae in the spleen tissue of diabetic rats. Moreover, Perl's Prussian blue staining revealed impaired phagocytic activity in diabetic animals. Interestingly, feeding diabetic animals on WPs successfully restored the histological integrity of their spleens. Furthermore, induced diabetes was found to significantly up-regulate both reactive oxygen species, FMO2 mRNA and Fas mRNA in diabetic animals compared to the control ones. In addition, WPs were found to significantly down-regulate the Fas mRNA in both control and diabetic animals. In conclusion, the current study proved that WPs restored the oxidative stability and the splenic structural integrity and activity, which may be proposed as natural candidates for the treatment of diabetes and oxidative stress.

Key words: Spleen, Whey proteins, FMO2, oxidative stress, diabetes.

INTRODUCTION

Diabetes is the most prevalent disease in the Kingdom of Saudi Arabia (KSA), where the number of people affected by this disease is still increasing. Type 1 diabetes (T1D) is caused by autoimmune destruction of insulin-producing β -cells. In this process, β -cell apoptosis involves multiple signaling cascades stimulated by interleukin-1beta (IL)-1 β , interferon-gamma (IFN- γ), and tumor necrosis factor-alpha (TNF- α) (Faloon *et al.*, 2011).

Whey proteins (WPs) contain several biologically active components, including lactoerrin, beta-lactoglobulin, alpha-lactalbumin, glycomacropptide, and immunoglobulins. Therefore, it demonstrates a range of immune-enhancing

properties in diabetes (Ebaid, 2014a). The primary mechanism by WPs is thought to exert its effects by intracellular conversion of the amino acid cysteine to glutathione, a potent intracellular antioxidant (Bounous *et al.*, 1988, 1989; Ebaid *et al.*, 2012). A number of clinical trials have successfully been performed using WPs in the treatment of cancer, HIV, hepatitis B, cardiovascular disease, osteoporosis, and as an antimicrobial agent (Marshall, 2004).

Spleen represents a large lymphatic tissue passed by re-circulating lymphocytes, which are able to promptly elicit specific T or B lymphocyte-mediated immune reactions. This study was designed to assess the effect of WP on the spleen tissues following diabetes.

Abbreviations:

CMC, Carboxymethyl cellulose; Fas, Programmed cell death-receptor; H&E, Haematoxylin-eosin; IFN- γ , Interferon gamma; IL-6, Interleukin 6; MTT, Cell proliferation assay; STZ: Streptozotocin; TNF- α , Tumour necrosis factor-alpha; WPs:

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Whey proteins.

MATERIALS AND METHODS

Preparation of un-denatured camel milk whey proteins

Whey proteins were prepared from fresh camel milk according to Ebaid *et al.* (2005). Briefly, milk was skimmed by centrifugation at 5000g for 20 min using an IEC Model K centrifuge (Boston, USA). Skimmed milk was acidified to pH 4.3 using 1M HCl. The precipitated casein was removed by centrifugation (5000g for 20 min), and the supernatant containing the whey protein was saturated with 70% ammonium sulfate and incubated overnight at 4°C. The precipitated whey proteins were collected by centrifugation and dialyzed against distilled water for 48 h at 4°C using a Spectra/Pro® Membrane, MWCO 6000-8000 Da. The obtained dialyzate was lyophilized using a Unitop 600 SL, (Virtis Company, Gardiner, New York 12525 USA) and were kept at -20°C until use. The dialyzate containing un-denatured whey proteins were freeze-dried and refrigerated until use.

Induction of diabetes

Diabetes was induced into experimental rats (*Rattus norvegicus*) by a single intraperitoneal injection of freshly dissolved STZ (60 mg/kg of body weight; Sigma, USA) in a 0.1 mol/l citrate buffer (pH 4.5) according to Li (2007). Control rats were injected with citrate buffer. Rats were screened for serum glucose levels 7 days post-injection for analysis. Rats with a serum glucose level ≥ 200 mg/dl after 2 h of glucose intake were considered diabetic (Li *et al.*, 2007) and selected for further experimental studies.

Feeding on whey proteins

Rats were supplemented with whey protein in their diet as previously described (Bonous *et al.*, 1988, 1989; Moura *et al.*, 2014) after 4 months of diabetes induction. To prepare 500 g of the diet, 5 g vitamins, 25 g mineral salts, 40 g fats, 50 g sucrose, 100 g protein (20% protein) and 280 g starch were mixed. Casein was the protein source for both the control and the diabetic groups. The un-denatured camel milk whey protein was the protein source for the WP-treated diabetic group. Diets were kept at

4°C until use (Ebaid *et al.*, 2005).

Experimental design

A total of 45 male rats (12-week-old), weighing 120-150g each, were obtained from the Central Animal House of the Faculty of Pharmacy at King Saud University. All animals were allowed to acclimatize in metal cages inside a well-ventilated room for 2 weeks prior to the experiment. Animals were maintained under standard laboratory conditions (temperature at 23°C, 60%–70% relative humidity and a 12:12h light:dark cycle) and were allowed to feed on a diet of standard commercial pellets and given water *ad libitum*. Animals were divided into three experimental groups (15 animals each): group I was daily administered 1% carboxymethyl cellulose (CMC), group II diabetic rats (DM) was supplemented with distilled water (250 μ l/rat/day) for five weeks and group III was supplemented with camel milk un-denatured WPs (DMWP) (100 mg/kg/ body weight dissolved in 250 μ l/day) for five weeks. Rats of the third group were freely supplemented with camel milk un-denatured whey proteins as a protein constituent of the diet.

Table I.- Histopathological changes in splenic tissues in experimental groups.

	White pulp depletion	Dilated blood vessels	Collagen deposition	Perl's-stained particles
CO	0	0	0.2 \pm 0.05	2.8 \pm 0.5
DM	2.1 \pm 0.4*	2.68 \pm 0.5*	2 \pm 0.7*	0.2 \pm 0.03*
DMWP	0	0.2 \pm 0.02*	0.4 \pm 0.09	2.23 \pm 0.3

CO, control; DM, diabetic; DMWP, diabetic rats supplemented with WP

* represents the significance in comparison to the control group.

Quantification of mRNA expression

RNA was extracted from the collected samples (RNA latter) using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. RT-PCR was performed using QIAGEN One Step RT-PCR kit as directed by the manufacturer's instruction manual. The desired genes were amplified using specific primers (e-oligos, Hawthorne, USA) listed in Table I. For each sample, 25 μ l reaction mixture was performed. PCR reaction was carried out using Gene-Amp 9700

thermal cycler. RT-PCR products were analyzed in 1.2 % agarose gel. PCR conditions were optimized to allow semi-quantitative comparisons of results. The levels of the three mRNA and β -actin mRNA were quantified by gel electrophoresis and densitometry. mRNA levels were normalized versus β -actin and are expressed in arbitrary units.

Histopathological study

Spleen samples from each animal were processed using light microscopy. The tissue sections were fixed in 10% neutral buffered formalin and embedded in paraffin. The paraffin sections were then stained with hematoxylin-eosin (H&E). Mallory Trichrome and Perl's Prussian staining were applied for detecting the collagen and the iron depositions in the splenic tissue, respectively. The degree of spleen damage was examined blindly using a Leica DMRB/E light microscope (Heerbrugg, Switzerland). Photographs of the sections were taken and the images were digitized using Adobe Photoshop (Adobe Systems, Mountain View, CA). Spleen histopathological changes were scored according to Dommels *et al.* (2007). A rating score between 0 (no damage) and 3 (maximal damage) was assigned for each investigated section. Sections from at least five rats were carefully investigated.

Ethical approval

Camel milk was obtained from a camel breed (Majaheem) from the Najd region (Alazeria farm; GPS: 300 02 47/ 300 02 27) in Saudi Arabia. Specific permissions were not required for activities in this private farm. This study did not involve endangered or protected species. Regarding experimental animals, all 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 and the National Institutes of Health. The study protocol (care and handling of experimental animals) was approved by the Animal Ethics Committee of the Zoology Department in the College of Science at King Saud University.

Statistical analysis

The statistical analysis was performed using the MINITAB software (MINITAB, State College, PA, Version 13.1, 2002). The data from the experiments were tested for normality using the Anderson Darling test, and for variance homogeneity prior to any further statistical analysis. The data were normally distributed with homogeneous variances. Thus, the one-way ANOVA statistical measure was used to determine the overall effect of each treatment. This measure was supplemented by individual comparison between the different treatments using Tukey's method for pairwise comparisons. The results were expressed as arithmetic mean (M) \pm standard deviation (SD).

RESULTS

Effect of induced-diabetes on body weight

STZ-induced diabetic rats were kept with continuous sugar monitoring before having their diet supplemented with WP for five weeks. In the untreated diabetic group a significant decrease in the total body weight (47%) was observed (Fig. 1) as a major diabetic complication-symptom compared with the control animals. In the WPs-treated diabetic group, however, the extent of this weight loss was significantly less (10% less compared to the control group) (Fig. 1).

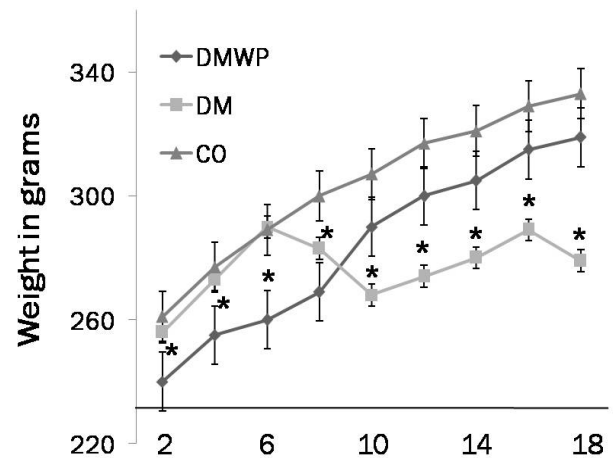


Fig. 1. Total body weights in animals of the control (CO), diabetic (DM) and diabetic rats supplemented with WP (DMWP). Values

shown are mean values \pm SD. *shows the significance in comparison to the control group.

Relative spleen weight

Relative spleen weight of the spleen in animals from the diabetic group revealed that splenic atrophy (Fig. 2) was a clear feature compared to those from the control animals.

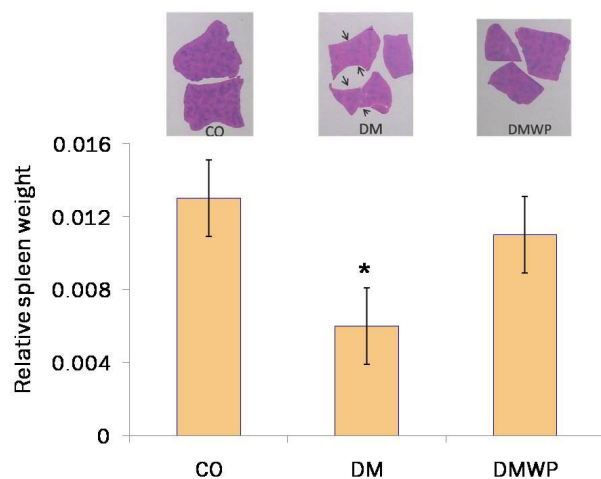


Fig. 2. Relative spleen weights in animals of the control (CO), diabetic (DM) and diabetic rats supplemented with WP (DMWP). Above each group-column, a representative morphology photo of spleen in the same group. Values shown are mean values \pm SD. * represents the significance in comparison to the control group.

Histological integrity of spleen

Representative section of the control spleen is shown in Figure 3A, D, G for comparison. Light microscopic examination of sections from the spleen of diabetic rats showed that the white pulp was greatly diffused. Mature lymphocytes in peripheral sections of the spleen were also dramatically reduced (Fig. 3B,E) with dilated blood vessels (Fig. 3H). Spleen sections from WP-treated diabetic rats, however, showed an obvious improvement. The lymphocyte population was normally distributed and there was a normal ratio relative to the red pulp (Fig. 3C,F,I).

Histopathological changes in splenic tissues in different groups are summarized in table 1. Statistical analysis of the histopathological changes of the splenic tissues revealed that diabetes significantly induced depletion in the white pulp, a

dilation in the blood vessels, an increase of the collagen deposition and a depletion in the iron particles detected in the red pulp. WP, on the other hand, was found to significantly restore these parameters to the normal levels when compared to the control tissues.

Total count of the circulatory lymphocytes

In order to address whether the leukocytes are escaped from the splenic tissues to the circulatory blood, they have been counted in the peripheral blood of diabetic animals. Interestingly, diabetes was found to down regulate the circulatory lymphocytes (Fig. 4).

Collagen deposition in spleen

Representative section of the control spleen is shown in Figure 5A,D for comparison. It was also observed that the spleens of diabetic rats exhibited an increase in capsule thickness with highly distributed trabeculae and a relatively high degree of fibrosis (Fig. 5B,E). In contrast, sections from the diabetic rats treated with WPs showed normal capsule thickness and a normal distribution of the splenic trabeculae (Fig. 5C,F).

Erythrophagocytic activity in spleen

Representative section of the control spleen is shown in Figure 6A,D for comparison. Perl's Prussian blue staining of sections from the diabetic group revealed impaired phagocytic activity (Fig. 6B,E) but WP was found to restore the erythrophagocytic activity (Fig. 6C,F), with the distribution of blue granules being similar to the control group.

Expression of FMO2 and Fas mRNA by RT-PCR

Figure 6 shows RT-PCR expression of the reactive oxygen species FMO2 (A) and cell death receptor Fas (B) genes in the control, diabetic (DM) and whey proteins-treated rat (DM-WP) spleens. Results showed that diabetes significantly up-regulated both the reactive oxygen species FMO2 mRNA and Fas mRNA in comparison of the control animals. Although whey proteins partially down-regulated FMO2 gene, it still significantly high in comparison to the control rats. However, whey proteins were found to significantly down regulate

the Fas mRNA in comparison to both the control and the diabetic animals.

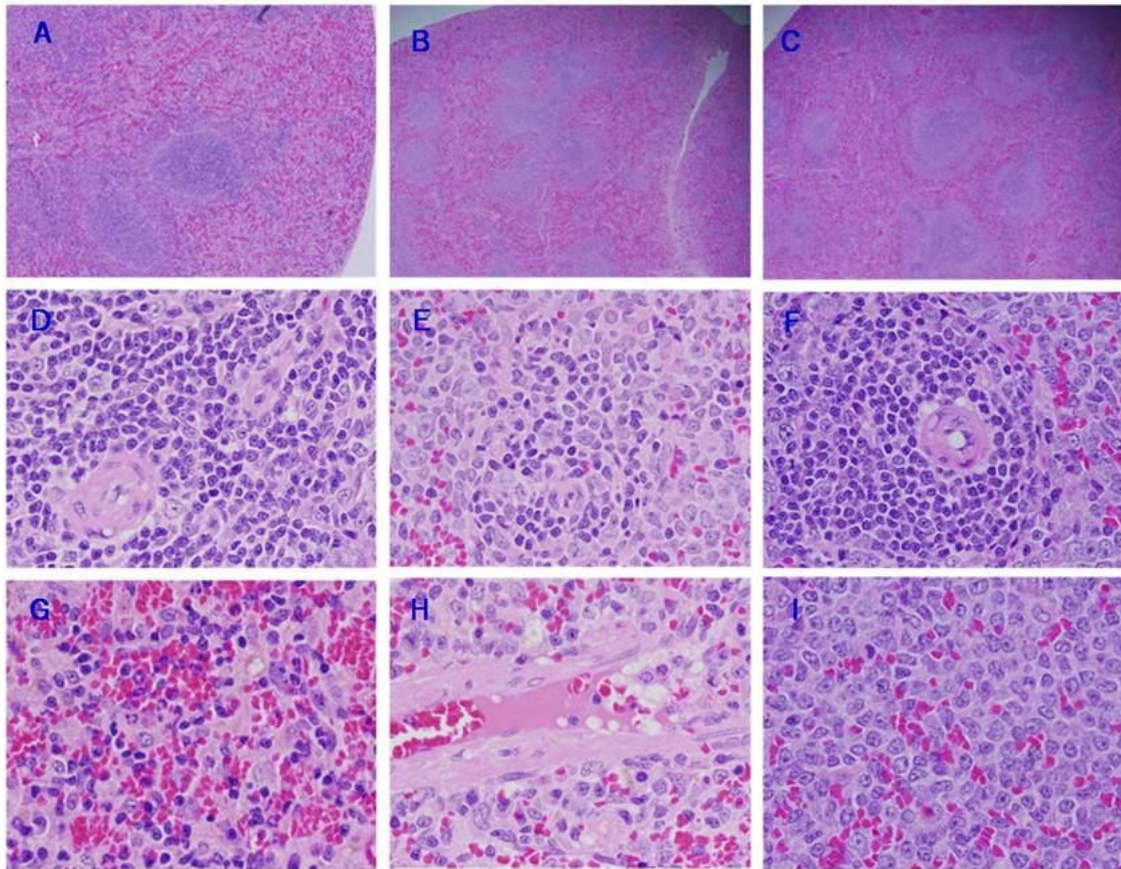


Fig. 3. Representative spleen histological sections stained with H&E of control rats (A, D, G) diabetic rates (B, E, H) and diabetic rats supplemented with whey proteins (C, F, I) showing the general architecture of the spleen during diabetes and after treatment with whey protein. Magnification: A, B, C = 200x; D, E, F, G, H, I = 400x.

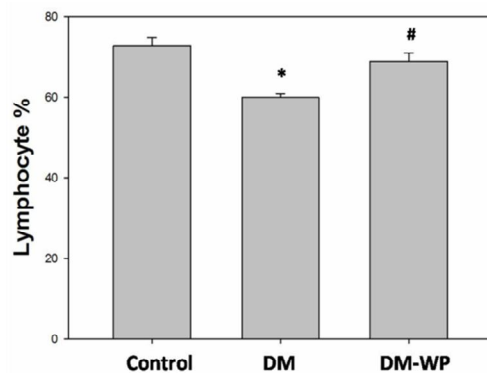


Fig. 4. The lymphocyte percentage in the peripheral blood in the control, diabetic (DM) and diabetic supplemented with whey proteins (DMWP) animals. * shows the significance in comparison to the control group. # represents

the significance in comparison to the diabetic group.

DISCUSSION

Diabetes decreases immune response capacity, including the suppression of immune cell function, atrophy of immune organs (Ebaid, 2014a, Abu-Taweel *et al.*, 2013). Many studies have been performed to assess the potential utility of natural products as immunomodulatory agents to enhance host responses to disease (Krifa *et al.*, 2013). This study is reporting the adverse pathological effects of diabetes on one of important immune organs, spleen and the treatment effect of the natural product WP.

For oxidative stress, FMO2 expression was estimated by RT-PCR. It is one of the responsible

genes for regulating the production of ROS. Here, FMO2 was significantly up-regulated in diabetic rats, compared to controls. WP was found to

significantly restore FMO2 mRNA expression to the

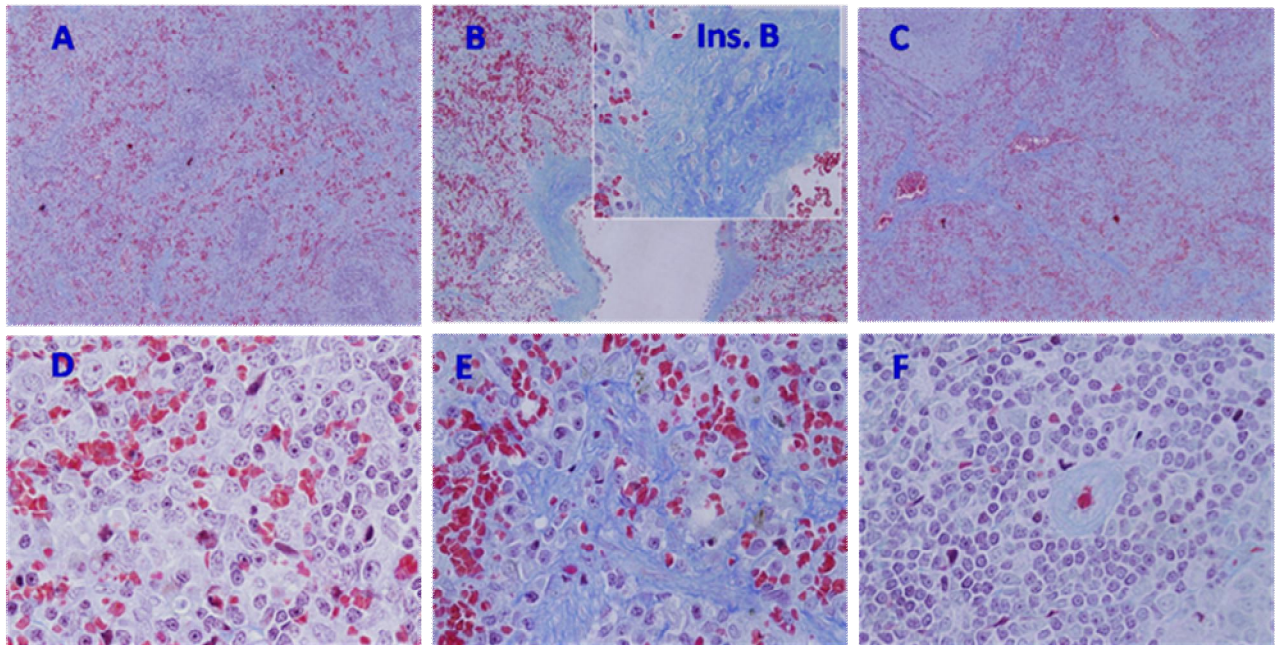


Fig. 5. Histological structure of spleen stained with Mallory Trichrome. Control rats (A, D) diabetic rats (B, E) and diabetic rats supplemented with whey proteins (C, F) showing the collagen deposition in the splenic tissue. The insert (Ins. B) is magnified from B ($\times 400$). Magnification: A, B, C = 200x; D, E, F = 400x.

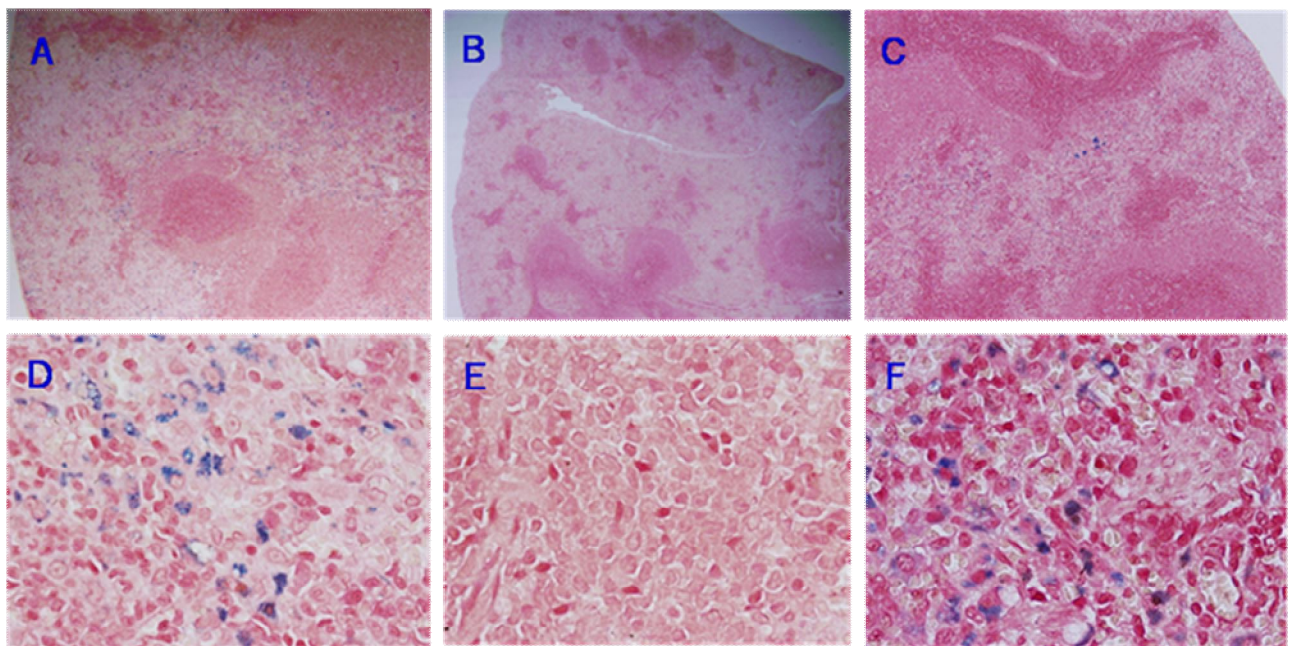


Fig. 6. Histological structure of spleen stained with Perl's Prussian blue of control rats (A, D), diabetic rat (B, E) and diabetic rats supplemented with whey proteins (C, F) showing the erythrophagocytic activity in the splenic tissue. Magnification: A, B, C = 200x; D, E, F = 400x.

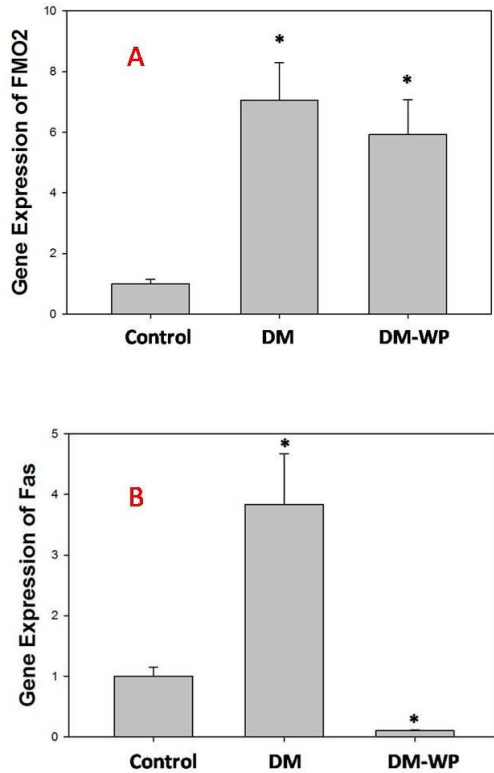


Fig. 7. RT-PCR expression of the reactive oxygen species FMO2 (A) and cell death receptor Fas (B) genes in the control, diabetic (DM) and whey proteins-treated rat (DM-WP) spleens. *represents the significance in comparison to the control group. # shows the significance in comparison to the diabetic group.

normal. At the protein concentration level, we previously found that WP significantly decreased the levels of malondialdehyde, nitric oxide and reactive oxygen species (ROS) (Ebaid *et al.*, 2011, 2013). Previous studies have shown that WP plays an active role in iron transport (Marchetti, 1994), in the cytotoxic defense (Kawasaki, 1993), and in scavenging free radicals (Wong and Watson, 1995) via enhancement of glutathione (Bounous and Batist, 1989). Therefore, WP is able to reduce the effects of oxygen radicals by increasing glutathione.

Here we found that lymphocyte numbers are dramatically declined in both peripheral blood and

spleen in diabetic rats. This indicates that lymphocytes are stressed by diabetic toxicity starting with high levels of free radicals, increasing the levels of pro-inflammatory cytokines and ending by programmed cell death. Members of the TNF superfamily causes recruitment of death domain such as Fas can lead to the activation of a signal transduction pathway that induces apoptosis. This suggestion is proved by the high expression of one of the important cell death receptors, the Fas gene by quantitative analysis of PCR. Thus, results demonstrated that diabetes may capable of inducing splenocytic apoptosis mediated by the Fas/FasL pathway in rats, which could be the potential mechanism underlying the immunotoxicity of hyperglycemia. These results are in accordance with the previous results that showed that diabetic complications and oxidative stress induce cell apoptosis via Fas up-regulation (Sainio-Pöllänen *et al.*, 1998; Park *et al.*, 2013).

This can clearly explain the damage in the spleen tissues of the diabetic rats. Regulating apoptosis of lymphocytes is an effective strategy for treatment of lymphocyte-mediated diseases (Wang *et al.*, 2013). Here we found that WP down-regulated the Fas mRNA expression in the diabetic rats treated with WP. Meanwhile, WP was previously found to demonstrate a mitogenic biological activity to lymphocytes, especially, B cells (Ebaid, 2014b). In addition, we previously found that WP restored the high levels of both IL-1b, TNF-a in diabetic rats supplemented with WP (Ebaid *et al.*, 2011). Therefore, the improvement of the lymphocyte population in the white pulp of the WP-treated diabetic rats may be due to the inhibition cascade of the programmed cell death pathway.

The decreased iron accumulation in the spleen suggested that its export was not normal in splenic macrophages of diabetic rats. WP was found to restore the iron deposition to the approximately the normal level, indicating improvement of erythrophagocytic activity. Collectively, results of microscopic examination of spleen suggested that WP could enhance the immune response. We

conclude from these studies that WP exhibited an immunomodulator effect which could be ascribed, in part, to its cyto-protective effects of splenocytes via its anti-oxidant capacity (glutathione) and regulating one of the most important programmed cell death receptor, Fas.

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