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Immunoenhancing property of dietary un-denatured whey protein derived from three camel breeds in mice

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Abstract: Data have demonstrated that whey protein (WP) enhances the immune system. The aim of this study was to investigate and compare the effects of WP from three camel breeds on oxidative stress, blood lipid profile and the cytokine levels. Seventy five male mice were randomly split into five groups. The first served as a control group. The second, the third and the fourth groups were orally administrated the WP from Majaheim, Maghateer and Soffer camel breeds, respectively, at a dose of 100 mg/kg mouse body weight. The fifth group was supplemented with bovine serum albumin (BSA). Results showed similar electrophoretic patterns of the three whey proteins. WP was found to significantly inhibit the hydroperoxide and the Reactive Oxygen Species (ROS) in leukocytes, liver and skin as well as the blood cholesterol level in a time dependent manner. A significant enhancement of glutathione was revealed in WP groups. Furthermore, WP was found to significantly elevate the IL-2 with a significant time dependent enhance of IL-8. On contrast, a significant lowering effect of whey proteins on the pro-inflammatory cytokines, IL-1α, IL-1β, IL-6 and IL-10 was detected. Moreover, a mitogenic activity of WP was observed on the lymphocytes. Non-significant changes were observed in AST, ALT, creatinine and glucose level. These findings suggest that WP significantly improved the levels of the oxidative markers and the immune functions without any difference in the bioactivities of the three studied whey proteins.

Key words: whey proteins; oxidative stress; glutathione; cytokines; lymphocytes

Abbreviations: Alanine Aminotransferase (ALT); Aspartate Aminotransferase (AST); Interleukin (IL); Peripheral Blood Mononuclear Cells (PBMCs); Bovine serum albumin (BSA); Reactive Oxygen Species (ROS); Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); Whey proteins (WP).

Introduction

Scientific efforts to search for natural bioactive substances for the amelioration of diseases have led to the discovery of products with substantial bioactive properties. Components of milk including the whey protein (WP) is easily available from different sources, especially camel in Saudi Arabia. WP is believed to be the highest quality protein available, even when compared to different proteins such as egg, casein, beef, or soy (David 1999). Moreover, it was found that the un-denatured WP was more effective when compared with the denatured one (Ebaid et al. 2005), the WP hydrolysates (Akhavan et al. 2010) or bovine collagen hydrolysates (Castro et al. 2010).

WP contains all essential and non-essential amino acids and is an excellent source of glutamine and the branched-chain amino acids that are necessary for cell growth (David 1999). In addition, WP contains a number of immunomodulatory peptides that are naturally present or that are part of the primary sequence of whey proteins. These peptide sequences can be released during digestion in the gut and can also be produced by in *vitro* enzymatic hydrolysis (Gauthier et al. 2006). Normal processes to extract whey component from the other constituents lead to significant denaturing of the bioactive whey proteins components. Un-denatured whey protein isolates utilize proprietary processes to attain a protein containing over 90% un-denatured whey.

WP possesses many bio-active properties (Ballard et al. 2009) and its peptide hydrolysates derived in *vivo* and/or in *vitro* modulate various immune functions, including lymphocyte activation and proliferation, cytokine secretion, antibody production, phagocytic activity, and granulocyte and natural killer cell activity (Gauthier et al. 2006). Fractions of the WP stimulated IL-1β, IL-8, IL-6, macrophage inflammatory protein (MIP)-1α, MIP-1β, and TNF-α (Rusu et al. 2010). The pro-inflammatory cytokines IL-1β, IL-6 and TNF-α play key roles within the cytokine network. They play an important role in the mediation of inflammation and trauma and could be useful for the determination of vi-

tality and wound age (Grellnera et al. 2000; Rivat et al. 2005).

Data demonstrate that a cysteine-rich WP concentrate represents an effective cysteine delivery system for glutathione (GSH) replenishment during the immune response. The GSH antioxidant system is the principal protective mechanism of the cell and is a crucial factor in the development of the immune response by the immune cells. Animal experiments showed that the concentrates of whey protein also exhibit anticancer activity via the GSH pathway, the induction of p53 protein in transformed cells and inhibition of neoangiogenesis (Bounous & Molson 2003).

We have previously investigated the role of the un-denatured WP in the wound healing in a non-diabetic mouse model. Data of that work indicated an increased capacity of the un-denatured WP-fed animals to heal wounds comparing to those fed on casein or denatured WP (Ebaid et al. 2005). In this work the un-denatured WP was extracted from the milk of three different camel species (Majaheem, Maghateer, Soffer) to compare their immunomodulatory effects. Significant enhancement of the immune system was observed of these three proteins with a little bioactivity difference among them.

Material and methods

Preparation of whey proteins

Raw camel milk was collected from healthy she-camels (three camel breeds, Majaheim, Maghateer, Soffer) from Riyadh area, Saudi Arabia, and then centrifuged for cream removing. The obtained skim milk was acidified to pH 4.3 using 1N HCl at room temperature and centrifuged at 10,000 g for 10 min. to precipitate casein. Resultant whey containing whey proteins was saturated with ammonium sulphate to final saturation 80% for whey proteins precipitation. The precipitated whey proteins were dialyzed against 20 folds of distilled water for 48 h through molecular-porous membrane MWCO. 6000–8000. The dialysate containing un-denatured whey proteins was freeze-dried, then kept refrigerated till use.

Preparing diets for experimental mice

To prepare 500 g of the diet, 5 g vitamins, 25 g mineral salts, 40 g fats, 50 g sucrose, 100 g protein (casein) and 280 g starch were mixed (20% protein by weight of total diet). The diet was kept at 4 °C until use. All mice groups fed this diet.

Animal groups

Seventy five male of laboratory mice weighing 25–30 g were obtained from the Central Animal House, Faculty of Pharmacy, King Saud University. All animal procedures were in accordance with the standards set forth in the guidelines for the care and use of experimental animals by the Committee Control for Purpose of Supervision of Experiments on Animals (CPCSEA) and the National Institutes of Health (NIH) protocol. The study protocol was approved by the Animal Ethics Committee of the Zoology Department, College of Science, King Saud University. Animals were allowed to acclimatize in metal cages inside a well-ventilated room for 2 weeks prior to the experiment. They were maintained under standard laboratory conditions-temperature 23 °C, relative humidity 60–70% and a 12-hour light/dark cycle, and were fed a pellet diet prepared in the lab as mentioned above, and water. Seventy five male mice were randomly split into five groups. The first served as a control group. The second, the third and the fourth groups were independently orally supplemented with the un-denatured whey protein from Majaheim (Second group, WPA), Maghateer (Third group, WPB) and Soffer (Fourth group, WPC), respectively, at a dose of 100 mg/kg body weight daily for 6 days. The fifth group was orally supplemented with the bovine serum albumin (BSA) at the same dose (100 mg/kg body weight daily for 6 days). The dose of the WP was applied in the current study according to Bounous (2000).

Blood samples, plasma and PBMCs

Animals were anesthetized with pentobarbital (60 mg/kg body weight) and samples (blood, liver and skin) were obtained 2, 4 and 6 days post supplementation of whey proteins. Whole blood was drawn from the abdominal aorta in heparinized tubes. Heparinized venous blood was centrifuged at 800 g for 10 min., and plasma was stored at –20 °C until analysis. After plasma isolation, half of the obtained blood cells were subjected to peripheral blood mononuclear cell (PBMCs) isolation using a Ficoll gradient method. Isolated mononuclear leukocytes were subjected to the measurement of the ROS as mentioned below.

Estimation of glutathione

Glutathione (GSH) assay was carried out on tissue as previously described (Clark et al. 2010). Liver was removed and gently rinsed in physiological saline. The fresh organ weights was recorded, then was frozen until use. A 10% (w/v) homogenate of each frozen tissue was prepared. Glutathione concentrations were measured by adding 100 µl of supernatant to 400 µl PBS [containing 200 mM MCB and 2 U ml^{-1} glutathione S-transferase (per 100 µl)]. Glutathione concentrations were then determined by measuring the absorbance of the reaction after 1 min at 340 nm using an UV Visible Spectrometer (Ultrospec 2000, Pharmacia Biotech). Glutathione standards were measured concurrently to obtain a standard curve that was used to calculate GSH concentrations in samples. Results were expressed as µg GSH/g tissue. Five replicates of each experiment were carried out for performing statistical comparisons of GSH activities between controls and treatments in each case were performed using Minitab statistical program as detailed below.

ROS measurement

ROS levels were determined using 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA) (Beyotime Institute of Biotechnology, Haimen, China). Supernatant from skin and liver homogenates, and the mononuclear leukocytes were directly treated with 10 µM H2DCF-DA dissolved in 1 ml PBS at 37 °C for 20 min. The fluorescence intensity was monitored using an excitation wavelength of 488 nm and an emission wavelength of 530 nm (Shimada et al. 2011).

Hydroperoxide levels

Blood levels of hydroperoxide were evaluated using a free-radical analytical system (FRAS 2, Iram, Parma, Italy). This colorimetric test takes advantage of the ability of hydroperoxide to generate free radicals after reacting with transitional metals (Wolff et al. 1994).

Plasma glucose and lipid profile
Blood glucose levels were measured with the glucose oxidase method (Chan et al. 2007) using BioMerieux kits (France). Lipid profiles were determined colorimetrically with BioMerieux kits and a standard assay method. Cholesterol levels were evaluated using the cholesterol esterase method (Allain et al. 1974).

Determination of serum ALT, AST, and creatinine levels
Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine were measured using commercial kits (Labtest Diagnostica, Brazil) according to the manufacturer's instructions.

ELISA assay for the plasma cytokine profile
The levels of IL-1α, IL-β, IL-10, IL-2, IL-6, and IL-8 were estimated in sera of different groups according to the manufacturers' instructions (SABiosciences). The optical densities (OD) are measured using the molecular devices 405 nm. The detection limits are set according to the log-log correlative coefficient of the standard curve.

Polyacrylamide gel electrophoresis (SDS-PAGE)
Whey protein samples were separated using a polyacrylamide gel as previously described (Laemmli 1970). Briefly, gel (final concentration): 12.5% acrylamide; 0.33% bisacrylamide; 0.37 M Tris-HCl buffer, pH 8.8; 0.1% SDS; 0.03% ammonium persulphate; and 0.1% N,N,N′,N′-tetramethylenediamine. Stacking gel (final concentration): 4.5% acrylamide, 0.12% bisacrylamide, 0.125 M Tris-HCl buffer pH 6.8; 0.1% SDS; 0.09% ammonium persulphate; and 0.1% N,N,N′,N′-tetramethylenediamine. Running buffer: 50 mM Tris, 0.196 M glycine and 0.1% SDS (wt/vol), pH 8.3. After the electrophoresis run (18 mA/1.5 mm thickness gel at 10°C) for approximately 6 h the gels were marked with Coomassie brilliant blue R-250 over night (0.2% in 45:45:10 methanol:water:acetic acid) and destaining with water:methanol:acetic acid (65:25:10). Prestained molecular weight marker solution (broad range, Sigma) was used contained: bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehydes-3-p-dehydrogenase (36 kDa), carbonic anhydrase, bovine (29 kDa), trypsinogen, bovine pencrease (24 kDa), soybean trypsin inhibitor (20 kDa), Bovine milk α-lactalbumin (14.2 kDa).

Statistical analysis
Statistical analysis was undertaken using MINITAB software (MINITAB, State College, PA, Version 13.1, 2002). Data from experiments were first tested for normality using Anderson Darling test, and for variances homogeneity prior to any further statistical analysis. Data were normally distributed, and variances were homogeneous, thus, One-way ANOVA was used to determine overall effects of treatments followed by individual comparison using Tukey's Pairwise comparison.

Results

SDS-PAGE of three whey proteins shows similar electrophoretic pattern
Nutritional and functional characteristics of whey proteins are related to the structure and biological functions of these proteins. In SDS-PAGE, the aim was to determine the protein pattern-difference among the three whey proteins. Difference should explain the variation in mode of action of each one. The SDS-PAGE

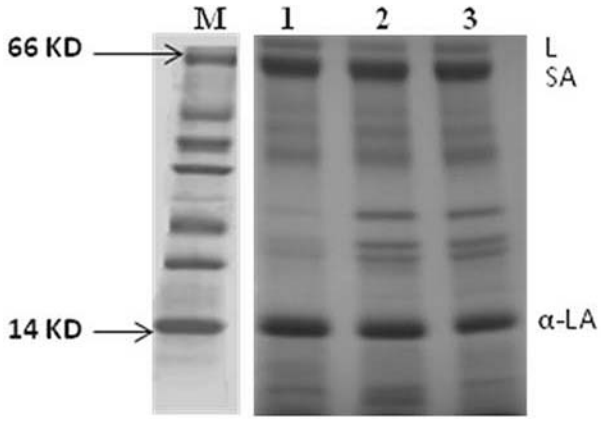

Fig. 1. Electrophoretic pattern of the whey proteins from three camel breeds. M – protein marker (66, 45, 36, 29 24 20 14.2 kDa); 1 – Majaheim breed; 2 – Maghateer breed; 3 – Soffer breed; L – Lactoferrin; SA – Serum Albumin, α-LA – α-Lactalbumin. Mice were supplemented with WP (groups 2–4) at a dose of 100 mg kg^{-1} of body weight. Electrophoresis shows no difference among the three whey proteins in the protein distribution pattern. Five mice from each group were killed two days intervals (2, 4 and 6 days post treatment).

analysis of these three whey proteins revealed a similar electrophoretic pattern which indicated a relative structure of the protein and carbohydrate components (Fig. 1). At the whey protein from the three camel breeds, lactoferrin, serum albumin and α-Lactalbumin are distributed at molecular weights of about 80, 66 and 14 kDa, respectively, as previously described (Redwan & Tabll 2007; Farah 1986; Elagamy et al. 2008; Elagamy et al. 1996).

WP supplementation improves oxidative stress and increases glutathione
Similarity of the electrophoretic distribution clearly indicated similar bioactivity as expected. A significant inhibition of the oxidative stress parameters were observed in similar behaviors for the three whey proteins (Figs 2, 3). We monitored changes of the ROS in the isolated leukocytes, liver and skin homogenates. We observed a significant decrease of ROS in isolated leukocytes, and liver and skin homogenates in the three WP groups comparing to control and BSA groups. Notably, the decreased effect in the three WP groups was approximately similar in a clear time dependent manner (Fig. 2). Moreover, hydroperoxide level was measured in the whole blood samples. Statistical analysis revealed that the three mice groups (WPA, WPB, WPC) supplemented with WP from three camel ecotypes showed a significant decrease in the level of hydroperoxide comparing to the control mice. This decrease was time dependent (Fig. 3). Interestingly, glutathione was significantly elevated ($P < 0.05$) in WP supplemented mice in a time dependent manner comparing to the BSA and control mice (Fig. 3).

WP improves blood lipogram, and liver and kidney functions
Blood glucose, and liver and kidney functions have been measured to monitor any adverse effects of the whey

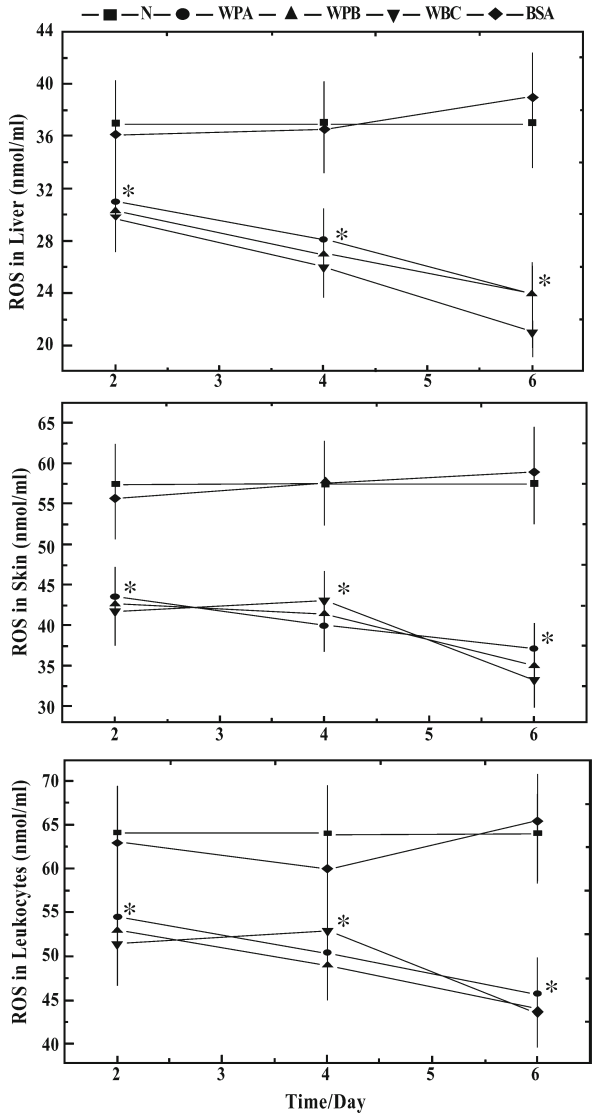

Fig. 2. ROS levels in leukocytes, skin and liver from the five groups of male mice ($n = 15$), the control group (N), three whey protein groups (WPA, WPB, WPC) and BSA group. Five mice from each group were killed in two days intervals (2, 4 and 6 days post treatment). Results are expressed as means ± SEM. * $P < 0.05$.

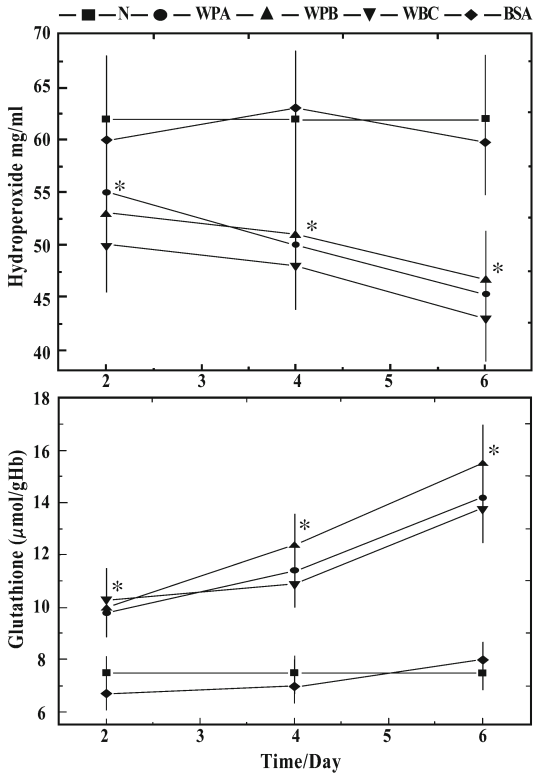

Fig. 3. Hydroperoxide of the whole blood and the liver total glutathione levels from five groups of male mice ($n = 15$), the control group (N), three WP groups (WPA, WPB, WPC) and BSA group. Five mice from each group were killed in two days intervals (2, 4 and 6 days post treatment). Results are expressed as means ± SEM. * $P < 0.05$.

proteins. Non-significant changes ($P < 0.05$) were observed in AST, ALT and creatinine in whey protein groups comparing to control and BSA groups (Table 1). In addition, whey proteins regulated glucose level at a range of approximately that of the control mice group. WP was found to significantly ($P < 0.05$) decrease the total blood cholesterol level compared with the control and BSA groups (Table 1). The decrease effect on the blood cholesterol level was in a time dependent manner in almost all mice of the whey protein groups.

Whey proteins change the cytokine profile and increase lymphocyte count

The effect of three whey components on cytokine expression profiles has been investigated. Significant enhancement of the immune system was observed by the three WP. Results revealed a significant and a time dependent lowering effect of whey proteins on the proinflammatory cytokines, IL-1α, IL-1β, IL-10 (Fig. 4) and IL-6 (Fig. 5). In addition, WP was found to significantly ($P < 0.05$) elevate the T cell growth factor (IL-2) (Fig. 5). Moreover a significant elevation of IL-8 ($P < 0.05$) in a time dependent manner was recorded in groups supplemented with WP (Fig. 5). A mitogenic bioactivity of WP was observed on the total leukocytes count, and in particular the lymphocyte count with little changes in other blood parameters as shown in Table 2.

Discussion

It is evident that amino acids incorporated in the WP can influence the immune response (Bounous et al. 1989; Belokrylov et al. 1992). Bioactive components include lactoferrin, lactoperoxidase, glycomacropeptide, BSA, various growth factors and immunoglobulins exhibit anticancer, antiviral, antibacterial, and antifungal activity (Kawasaki 1993; Marchetti 1994; Wong & Watson 1995). Here, we found that WP clearly improved the different immune functions compared with the BSA.

Oxidative stress takes place due to an imbalance between the production of ROS and the protection by cellular antioxidants (Shen et al. 2011). The blood level of hydroperoxides and the ROS level in leukocytes, liver

Table 1. Liver (AST, ALT) and kidney (creatinine) functions, blood glucose level and plasma lipid profile of five groups of male mice ($n = 15$), the control group, WP groups (WPA, WPB, WPC) and BSA group.

Parameters	Groups	2 days	4 days	6 days
AST (U ml^{-1})	Control	44 ± 4.1	44 ± 4.1	44 ± 4.1
	WPA	45 ± 4.6	47.3 ± 4.9	43 ± 4.1
	WPB	43 ± 4.2	46.4 ± 4.4	44.5 ± 4
	WPC	46 ± 4.4	44 ± 3.8	46.7 ± 3.9
	BSA	46 ± 4.45	43 ± 3.8	46 ± 4.55
ALT (U ml^{-1})	Control	56 ± 4.6	56 ± 4.6	56 ± 4.6
	WPA	54 ± 4.2	57.6 ± 4.65*	52 ± 5.1
	WPB	55.5 ± 5.1	59.1 ± 5*	55.1 ± 4.7
	WPC	57 ± 4.75*	54.4 ± 4.9	55.1 ± 4.5
	BSA	53 ± 4.9	57 ± 5.9	58 ± 5.4
Creatinine (dg ml^{-1})	Control	3.75 ± 0.27	3.75 ± 0.27	3.75 ± 0.27
	WPA	3.71 ± 0.31	3.65 ± 0.30	3.5 ± 0.34
	WPB	3.83 ± 0.36*	3.7 ± 0.32	3.4 ± 0.34
	WPC	3.9 ± 0.38*	3.58 ± 0.33	3.45 ± 0.3
	BSA	3.9 ± 0.33*	3.6 ± 0.35	3.85 ± 0.36*
Blood Cholesterol (mg/100 ml)	Control	44 ± 3.9	44 ± 3.9	44 ± 3.9
	WPA	45.6 ± 4.2	40 ± 3.6*	36.1 ± 3.2*
	WPB	43.9 ± 4.3	39 ± 3.3*	33.6 ± 3.1*
	WPC	41.2 ± 3.7*	37.3 ± 3.1*	34.3 ± 3.4*
	BSA	46.4 ± 4.4	43 ± 3.6	46 ± 4.2
Blood Glucose (mg dl^{-1})	Control	133 ± 9.6	143 ± 9.2	142 ± 8.6
	WPA	139 ± 9.1*	150 ± 8.6*	145.6 ± 7.7
	WPB	143 ± 8.8*	148.6 ± 8.1	138.4 ± 7.1*
	WPC	137 ± 8.7*	139 ± 7.9	149 ± 8.2*
	BSA	143 ± 10.1*	135 ± 9.3	141 ± 8.7

Explanations: Results are expressed as means ± SEM; * $P < 0.05$.

Table 2. Blood parameters from five groups of male mice ($n = 15$), the control, WP (WPA, WPB, WPC) and BSA groups.

Parameters	Groups	2 days	4 days	6 days
Hb (g dl^{-1})	Control	13.6 ± 0.8	13.9 ± 0.2	14 ± 0.6
	WPA	13.8 ± 1	13.9 ± 1	14.4 ± 1.2
	WPB	13.8 ± 1	14.1 ± 1.1	14.3 ± 1.3
	WPC	13.8 ± 0.7	14 ± 1.3	14.5 ± 1.5
	BSA	33.9 ± 3.3	13.5 ± 1.2	13.7 ± 1.3
Hematocrite (%)	Control	34.5 ± 2.1	35.5 ± 2.7	34.5 ± 3
	WPA	34.55 ± 2.5	35.1 ± 3.2	36 ± 3.4
	WPB	35 ± 3.1	35.4 ± 3.5	37.3 ± 3.6*
	WPC	34.9 ± 2.2	35.3 ± 2.9	34.8 ± 3.1
	BSA	33.9 ± 3.3	34 ± 3.1	35.4 ± 3.5
Lymphocytes ($\times 10^3$ mm^{-3})	Control	5.9 ± 0.55	5.7 ± 0.6	5.8 ± 0.7
	WPA	6.4 ± 0.6	6.6 ± 0.7*	7 ± 0.80*
	WPB	6.5 ± 0.54	6.7 ± 0.6*	7.3 ± 0.75*
	WPC	6.7 ± 0.7*	6.8 ± 0.65*	6.9 ± 0.70*
	BSA	5.7 ± 0.51	5.8 ± 0.53	6.1 ± 0.59
WBCs ($\times 10^3$ mm^{-3})	Control	9.9 ± 0.8	9.75 ± 0.7	9.65 ± 0.5
	WPA	10.5 ± 1.1	11 ± 10*	12 ± 1.1*
	WPB	10.7 ± 1.3*	10.9 ± 0.9*	12.6 ± 1.3*
	WPC	10.6 ± 0.9*	9.85 ± 0.9	13 ± 1.25*
	BSA	10 ± 1.2	9.8 ± 0.8	10 ± 0.9
Neutrophils ($\times 10^3$ mm^{-3})	Control	4 ± 0.34	4 ± 0.5	4 ± 0.65
	WPA	4.5 ± 0.4	4.1 ± 0.35	5.1 ± 0.45
	WPB	4.3 ± 0.42	4.1 ± 0.4	4.8 ± 0.4*
	WPC	4.4 ± 0.45	3.9 ± 0.37	4.75 ± 0.5
	BSA	3.7 ± 0.37	3.9 ± 0.35	4.2 ± 0.32
RBCs ($\times 10^3$ mm^{-3})	Control	5.6 ± 0.48	5.9 ± 0.6	5.9 ± 0.85
	WPA	6 ± 0.53	5.8 ± 0.5	6 ± 0.55
	WPB	6.4 ± 0.58	5.9 ± 0.45	5.8 ± 0.5
	H WPC	6.2 ± 0.6	6 ± 0.55	6.2 ± 0.6
	BSA	5.5 ± 0.51	5.4 ± 0.4	5.5 ± 0.48

Explanations: Results are expressed as means ± SEM; * $P < 0.05$.

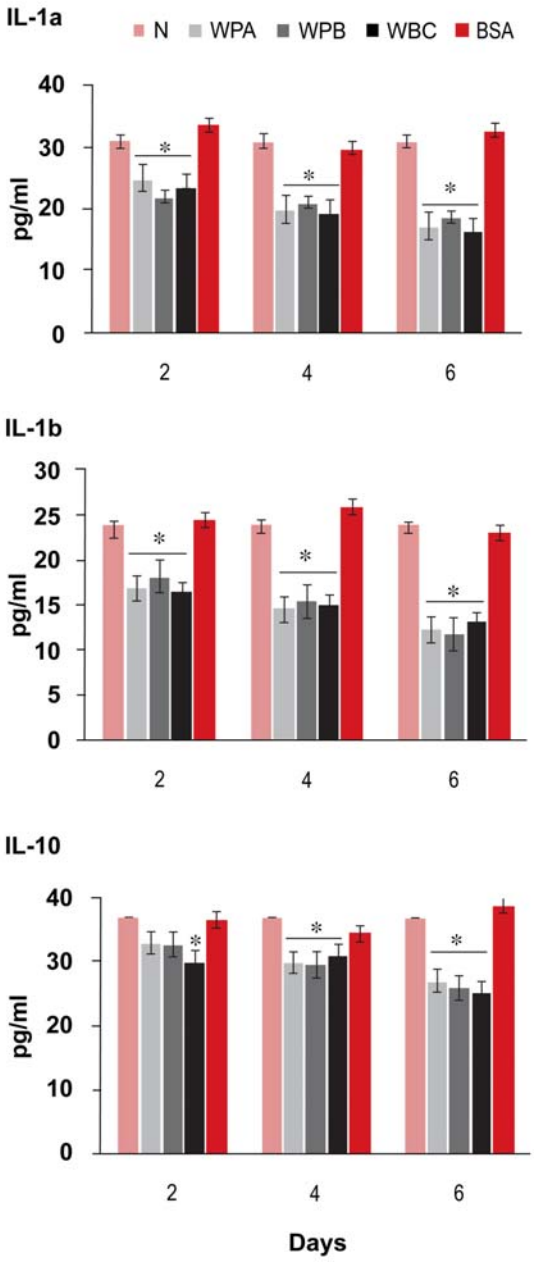

Fig. 4. ELISA estimation of plasma cytokine levels (IL-1α, IL-β, IL-10) from five groups of male mice, the control group (N), three WP (WPA, WPB, WPC) and BSA groups. The three whey proteins showed a significant down-regulation of all tested inflammatory cytokines, IL-1α, IL-β, IL-10, in a time dependent manner. Five mice from each group were killed in two days intervals (2, 4 and 6 days post treatment). Results are expressed as means ± SEM. * $P < 0.05$.

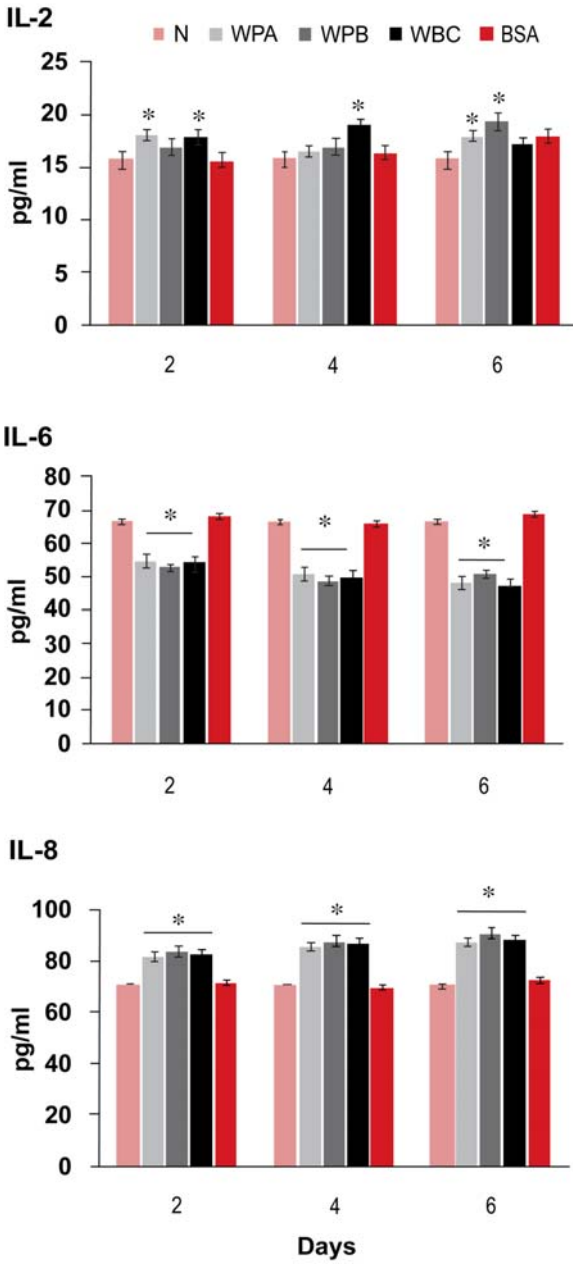

Fig. 5. ELISA estimation of plasma cytokine levels (IL-2, IL-6, IL-10) from male mice groups, the control group (N), three WP (WPA, WPB, WPC) and BSA groups. WP was found to significantly up-regulate the levels of IL-2 and IL-8, and significantly down-regulated the level of IL-6. Five mice from each group were killed in two days intervals (2, 4 and 6 days post treatment). Results are expressed as means ± SEM. * $P < 0.05$.

and skin tissues were significantly suppressed by WP what indicates a potential antioxidant role of WP. Previous studies confirmed the active roles of this protein in iron transport and in the cytotoxic defenses of neutrophils, and in scavenging free radicals (Kawasaki 1993; Marchetti 1994; Wong & Watson 1995). Antioxidants play a vital role in maintaining immunity and protection against free radical damage in the human body. A significant enhancement of glutathione level was recorded in WP supplemented mice in this study. Un-denatured WP is a potent inducer of glutathione (Bounous 2000). Glutathione is an intracellular tripeptide vigorously binds damaging free radical molecules that would otherwise harm the cell. Many studies confirmed the role of glutathione, which is increased by the dietary WP, as a powerful antioxidant system (Lands et al. 1999). The efficiency of cysteine in increasing the glutathione level is greater when it is delivered in the WP than as free cysteine (Bounous et al. 1989).

Accordingly, WP regulated and kept the blood glucose level at normal values and significantly decreased the total cholesterol levels. Akhavan et al. (2010) found

that dairy protein ingestion, when consumed with carbohydrates, reduces glucose. A significant increase in arterial elasticity index and a significant improvement in glucose and lipid metabolism were observed in patients who treated with antioxidants (Shargorodsky et al. 2010). Additionally, the beneficial effect of antioxidant supplementation on LDL oxidation has been demonstrated (Plotnick et al. 1997; Devaraj et al., 2000). An inverse correlation was demonstrated between blood lipid profile and T-cell proliferative capacity in dogs, where the reduction in total blood cholesterol, LDL and non-HDL-cholesterol levels was correlated with an increase in T-cell proliferation (Nunes et al. 2008). Another study revealed that the improvement in T-cell function during diabetes was probably partially a result of the reduction in total blood cholesterol and LDL (Han et al. 2003).

A mitogenic effect of WP was observed on the total leukocytes, and in particular the lymphocyte count in the present study. Similarly, it was previously found that WP play the role of a lymphocytic mitogen (Belokrylov et al. 1992; Middleton et al. 2004). This is because WP contains substantially cysteine which is the potential factor that controls the lymphocyte number in the blood. Moreover, Wong and Watson (1995) found that response of T cells to the WP was significantly higher than to the concanavalin A which is a T cell mitogen. Mercier et al. (2004) showed that the in *vitro* proliferation of murine spleen lymphocytes is stimulated by WP which possesses many bio-active properties (Ballard et al. 2009). In addition, Rusu et al. (2010) found that WP hydrolysates modulate various immune functions including lymphocyte activation and proliferation.

More importantly, the plasma level of IL-2 (a T cell growth factor required for the survival and proliferation of T cells) was significantly increased in the WP fed groups. Elevation of IL-2 led to the increase in lymphocyte count which clearly appeared in the differential count of the current study. Most probably, the increase in lymphocyte count was due to the proliferation of T-cells but not B-cells. This suggestion is partially accepted since, WP was found to significantly decrease the IL-6 which is a major stimulator of B-cell proliferation. On contrast to our results, Artym & Zimecki (2005) found that lactoferrin promote the differentiation of T and B cells from their immature precursors. IL-6 is mainly secreted by antigen-activated T-cell and is originally identified as B-cell differentiation factor (Akira et al., 1990). WP supplemented mice did not challenged by an antigen in the current study, and this can explain the contradiction of these results with those obtained by Artym & Zimecki (2005). Thus, WP did not promote the differentiation of B cells from their immature precursors in the current study. An improvement of T cell-mediated immune functions, such as increase in IL-2 production and the lymphocyte proliferative response, provides important evidence of enhancement of immune functions (Han et al. 2003). This maintained an efficient T cell immune response in the WP groups. We have previously found that increase in the levels of both IL-2 and IL-7 was accompanied by a potential T cell function (Badr et al. 2011a, b).

WP was also found to significantly and time-dependently elevate IL-8 in the current study, as it was previously found by Ustunol & Wong (2010). Shinoda et al. (1996) showed that lactoferrin stimulate the release of neutrophil-activating IL-8 from human polymorphonuclear leukocytes. Results revealed a significant and a time dependent lowering effect of whey proteins on the pro-inflammatory cytokines, IL-1α, IL-1β and IL-10. Thus, WP showed an anti-inflammatory effect on the inflammatory cytokines. Similarly, Artym & Zimecki (2005) proved an inhibition effect of lactoferrin on the pro-inflammatory cytokines.

Our results indicated potential effects of the three tested WP on immune functions without any considerable difference among their bioactivities. A significant restoration of the oxidative markers with a stimulation of glutathione by WP were proved in this study. In addition, a significant time dependent up-regulation of IL-2 and IL-8 levels was stimulated by WP. On the other hand, a down-regulation of IL-1α, IL-1β, IL-10 and IL-6 characterized the behavior of the three whey proteins. Findings of this study suggest that whey protein may serve as an alternative source of antioxidants for prevention of many injuries caused by ROS and/or mediated by the inflammatory cytokines. Thus, this study may provide critical insight into future nutritional intervention strategies designed to enhance the anti-inflammatory and the anti-oxidants protection. This strategy would show whether this WP has a significant clinical impact. Based on the current and previous studies (Ebaid et al. 2005, 2011) we hypothesized that un-denatured WP can have a significant role on the progress of wound healing process in diabetic models, and thus, such work has been initiated.

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