# ORIGINAL ARTICLES

## Therapeutic effectiveness of certain whey proteins on lipopolysaccharide-induced oxidative stress and histopathological changes in rat liver

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## ABSTRACT

Lipopolysaccharide (LPS) is a major cell wall component of gram-negative bacteria known to stimulate the synthesis and secretion of several toxic metabolites, such as reactive oxygen species and cytokines. In this study, the effects of whey protein isolate (WPI), alpha-lactalbumin (α-LA) and beta-lactoglobulin (β-LG) were evaluated in LPS-induced oxidative stress and acute hepatic injury in rats. Animals were divided into two experiments, 24 hours- experiment in which animals were challenged with LPS (4 mg/kg, i.p.) or saline then treated orally with WPI, α-LA and β-LG (100 and 200 mg/kg) or saline and two weeks-experiment in which animals were challenged with LPS (4 mg/kg, i.p.) or saline then treated orally with WPI, α-LA and β-LG (100 and 200 mg/kg) for two weeks during which another challenging dose of LPS (2 mg/kg, i.p.) was injected on the 8th day. Results revealed extensive liver damage, as evidenced by increase in serum levels of alanine aminotransferase (ALT), and aspartate aminotransferase (AST) after LPS challenge. Also, LPS induced oxidative stress as indicated by increase in the hepatic lipid peroxidation marker malondialdehyde (MDA) and hepatic nitrite levels as well as decrease in hepatic reduced glutathione concentration (GSH). The administration of whey proteins (WPs) after LPS challenge resulted in improved liver functions as evidenced by the decline in serum AST and ALT levels. WPs reduced lipid peroxidation, and nitrite level while increased GSH concentration in hepatic tissues. Histopathological examination showed disruption of normal architecture, mononuclear cellular infiltration, and necrotic degeneration around the central veins as well as portal tracts in LPS-treated liver. After treatment with WPs, remarkable improvement in histological structure of liver tissues was observed. The study indicates that whey proteins may be a useful pharmacological agent in modulating LPS-induced oxidative stress and acute hepatic injury.

*Key words:* whey protein isolate, alpha-lactalbumin, beta-lactoglobulin, lipopolysaccharide, liver, oxidative stress, rats

## Introduction

Over the past few decades and, despite advances in antimicrobial therapy and critical care medicine, sepsis-related systemic inflammatory syndrome and multiple organ dysfunction continue to be the most common cause of morbidity and mortality in intensive care units, reaching a mortality rate of nearly 50% (Angus *et al*., 2001). Endotoxemia occurs frequently in cases of liver failure and is thought to play a role in the pathogenesis of liver disease (Nakao *et al*., 1994). LPS interaction with the hepatic phagocytes namely, Kupffer cells is usually associated with their activation and subsequently the release of a plethora of mediators (Decker, 1990), including reactive oxygen metabolites (ROM), products of lipid metabolism (Jaeschke, 2000), and nitric oxide (NO) (Zhang *et al*., 2000). In experimental models, LPS has been reported to deplete GSH stores in the liver, and mice deficient in glutathione peroxidase (GPx) exhibited enhanced susceptibility to LPS-mediated liver damage (Jaeschke *et al*., 1999). The downregulation of GSH levels by LPS involves several mechanisms, including its depletion as a consequence of oxidative stress (Sun *et al*., 2006). Thus, although the regulation of GSH during endotoxemia remains to be fully understood, the evidence available suggests that prodrugs that boost GSH levels may be of therapeutic relevance. Therefore, it is imperative to develop new drugs or strategies that can arrest the many indolent and deadly consequences of endotoxemia.

Whey protein, a by-product of the cheese-making process, constitutes ~20% of the total bovine milk protein. The components of whey include beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin, lactoferrin, immunoglobulins, lactoperoxidase enzymes, glycomacropeptides, lactose, and minerals (Walzem *et*

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*al*., 2002). Whey proteins are a cystine-rich protein source. Consumption of cystine-rich whey proteins can increase plasma GSH concentrations in humans (Kennedy *et al*., 1995), hence consumption of dietary whey proteins may provide a useful strategy to elevate intracellular GSH and protect the prostate against ROS-induced cell damage (Kent *et al*., 2003). Also, it has been shown to represent an effective and safe cysteine donor for GSH replenishment during GSH depletion in immune deficiency states (Wong *et al*., 1997). Furthermore WPC could enhance the defense system by way of reducing the GSSG to GSH through glutathione reductase (GR). Moreover, WPC, a potential antioxidant, protects cells from ethanol damage, and the protection includes its capacity to stimulate GSH synthesis (Tseng *et al*., 2006). Whey protein isolates (WPIs), their enzymatic digests, and peptide fractions (α-LA, β-LG and GMP) prepared from the enzymatic digestion stimulate the proliferation of murine-resting splenocytes in vitro (Mercier *et al*., 2004). Whey peptides may have some potential applications as supplements in the maintenance of immune health and provide some protection against infections involving bacteria, viruses, and parasites (Gauthier *et al*., 2006).

The purpose of the current study is to evaluate the possible therapeutic effectiveness of whey protein isolate (WPI), and its major protein components such as alpha-lactalbumin (α-LA), beta-lactoglobulin (β-LG) in modulating the hepatotoxicity induced by endotoxin (LPS).

**Materials And Methods**

*Chemicals:*

Lipopolysaccharide (Escherichia coli, serotype 055:B5) was purchased from Sigma-Aldrich, Germany, whey protein isolate (WPI), and its major protein fractions; β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), were obtained from Davisco Foods International, Inc.USA, 5,5-dithi-2-nitrobenzoic acid (DTNB), reduced glutathione, thiobarbituric acid and 1,1,3,3-tetramethoxypropane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals, used throughout the experiment, were of the highest analytical grade available. Kits used to measure serum ALT, AST and hepatic nitrite were purchased from Biodiagnostic, Inc., (Egypt).

*Animals:*

Adult female Sprague–Dawley rats weighing 120-150 gm were obtained from the animal house at the National Research Center (Giza, Egypt), and fed a standard laboratory diet and tap water ad libitum. Experimental animals were housed in an air-conditioned room at 22–25 °C with a 12-h light/dark cycle. All animals received humane care and the study protocols were in compliance with institutional guidelines for the use of laboratory animals.

*Experimental design:*

After an acclimatization period of one week, forty-eight healthy female rats were randomly assigned to eight groups of six rats per group in each experiments; in 24 hours-experiment; vehicle control group received the respective vehicles only, LPS group received a single i.p dose of LPS at 4mg/kg which is well documented to induce hepatotoxicity *(Pearson et al., 1997, Cuzzocrea et al., 2006)*, WPI 100 & WPI 200 groups received a single i.p. dose of LPS, two hours later a single oral dose of WPI 100 and 200 mg/kg, respectively. α-LA100 & α-LA200 groups received a single i.p. dose of LPS, two hours later a single oral dose of α-LA 100 and 200 mg/kg, respectively. Finally, β-LG100 & β-LG 200 groups received a single i.p. dose of LPS, two hours later a single oral dose of β-LG 100 and 200 mg/kg, respectively. After 24 hours, rats were sacrificed.

In two weeks-experiment; vehicle control group received the respective vehicles only for 15 days, LPS group received an i.p dose of LPS at 4mg/kg on day 1 followed by another challenging dose of LPS at 2mg/kg on day 8, and received the oral vehicle till the end of the experimental period. WPI 100 & WPI 200 groups received LPS dosage as previously mentioned, with daily oral dose of WPI 100 and 200 mg/kg, respectively for 15 days. α-LA 100 & α-LA 200 groups received LPS dosage, with daily oral dose of α-LA100 and 200 mg/kg, respectively for 15 days. Finally, β-LG 100 & β-LG 200 groups received LPS dosage, with daily oral dose of β-LG 100 and 200 mg/kg, respectively for 15 days. At the end of the experimental period (day 16), rats were sacrificed by cervical dislocation under ether anesthesia and liver samples were collected for biochemical and histopathological examinations.

*Collection of blood samples:*

Blood samples were withdrawn from the retro-orbital vein of each animal, under light anesthesia by diethyl ether, according to the method described by Cocchetto and Bjornsson (1983). Blood was allowed to coagulate

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and then centrifuged at 3000 rpm for 15 min. The obtained serum was used to estimate the activities of ALT and AST enzymes.

*Preparation of liver samples:*

Immediately after blood sampling, animals were sacrificed by cervical dislocation and the liver tissues were rapidly removed, washed in ice-cooled saline, plotted dry and weighed. A weighed part of each liver was homogenized, using a homogenizer (Medical instruments, MPW-120, Poland), with ice-cooled saline (0.9% NaCl) to prepare 20% w/v homogenate. The homogenate was then centrifuged at 4000 rpm for 5 min. at 4°C using a cooling centrifuge to remove cell debris (Laborzentrifugen, 2k15, Sigma, Germany). The aliquot was divided into three parts; the 1st part was used for the assessment of reduced glutathione (GSH), the 2nd part was used for the assessment of lipid peroxidation (LPO) as malondialdehyde (MDA), the 3rd part was used to estimate the level of nitric oxide (NO).

*Measurement of serum liver function enzymes:*

Hepatic dysfunction was assessed by measuring the rise in serum levels of ALT and AST using commercially available kits. The results were expressed as IU/L.

*Determination of hepatic reduced glutathione:*

Reduced glutathione (GSH) concentration in hepatic homogenate was determined according to the method described by Beutler *et al*. (1963). The GSH concentration was determined using a standard curve constructed with different concentrations of an authentic sample. The results were expressed as µg/g wet tissue weight.

*Determination of hepatic lipid peroxidation:*

Lipid peroxidation, as an indicator of oxidative stress, was estimated by measuring thiobarbituric acid reactive substance (TBARS) that sometimes referred to as malondialdehyde (MDA) in hepatic homogenates as previously described (Ruiz-Larrea *et al*., 1994). The amount of MDA was expressed as nmol/g wet hepatic tissue using 1,1,3,3-tetramethoxypropane as a standard.

*Determination of hepatic Nitric Oxide (NO):*

Nitric oxide was determined in rat liver homogenate (20%) using a commercial kit according to the method described by Montgomery and Dymock (1961). The Nitrite Assay Kit provides an accurate and convenient method for measurement of endogenous nitrite concentration as indicator of nitric oxide production in biological fluids. The concentration of nitrite was calculated using sodium nitrite as a standard and expressed as nmol/g wet hepatic tissue.

*Histopathological examinations:*

The specimens from the liver were taken and fixed immediately in 10% neutral buffered formalin, processed for light microscopy to get (5µm) paraffin sections and stained with: Hematoxylin & Eosin to verify histological details and Masson's trichrome staining to demonstrate the collagen fibers as described by Bancroft and Gamble (2002).

*Statistical analysis:*

The degree in variability of results was expressed as means ± standard error of means (SEM). Data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. The level of significance was accepted at $P < 0.05$.

*Results:*

*Effect of whey protein isolate (WPI), alpha-lactalbumin (α-LA) and beta-lactoglobulin (β-LG) 100mg/kg and 200mg/kg on serum ALT and AST after LPS in 24 hours-treatment:*

Injection of LPS in a dose level of 4 mg/kg, i.p. resulted in a considerable hepatic injury as assessed by elevations of serum ALT and AST (Table. 1). These markers were substantially elevated by about 102.4%, and

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235% respectively, when compared with that of saline treated animals. Administration of WPI (100 and 200 mg/kg p.o.) significantly decreased the LPS-induced elevations in serum ALT, and AST while β-LG, at only 200mg/kg dose level, was able to exert slight but significant decrease in ALT level.  As well as, α-LA (200mg/kg) exerted remarkable decrease in AST level.

**Table 1:** Effect of WPI, α-LA and β-LG on serum biochemical parameters in rats treated with LPS in 24 hours

| Groups | Parameters* | |
|---|---|---|
| | ALT (IU/L) | AST(IU/L) |
| Control | $13.07 \pm 0.65^a$ | $49.08 \pm 0.59^a$ |
| LPS | $43.75 \pm 1.11^b$ | $99.32 \pm 0.92^b$ |
| LPS & WPI 100 | $32.55 \pm 0.99^c$ | $85.47 \pm 0.55^c$ |
| LPS & WPI 200 | $38.32 \pm 1.11^d$ | $86.88 \pm 1.23^c$ |
| LPS & α-LA 100 | $38.22 \pm 0.84^d$ | $97.60 \pm 1.17^b$ |
| LPS & α-LA 200 | $35.25 \pm 0.79^{dc}$ | $93.13 \pm 0.69^d$ |
| LPS & β-LG 100 | $40.33 \pm 1.130^{be}$ | $85.02 \pm 0.75^c$ |
| LPS & β-LG 200 | $38.40 \pm 1.207^{de}$ | $88.67 \pm 1.15^c$ |

ALT, alanine aminotransferase; AST, aspartate aminotransferase.* within each column, means with different superscript letters are significantly different (P<0.05).

*Effect of whey protein isolate (WPI),alpha-lactalbumin (α-LA) and beta-lactoglobulin (β-LG) 100mg/kg and 200mg/kg on hepatic reduced GSH, MDA and NO after LPS in 24 hours-treatment:*

The effect of WP on GSH concentration in LPS-treated animals is presented in Table 2. Results showed that LPS decreased GSH content by about 20% in the liver as compared to saline-treated control rats. Treatment with WPI, α-LA and β-LG were able to significantly increase the hepatic concentration of GSH up to normal levels in 24 hours, although high GSH levels attained by whey proteins, hepatic MDA levels in all WP-treated groups were not significantly different from LPS-treated rats (Table 2). On the other hand, induction of endotoxemia by LPS resulted in a significant elevation in hepatic nitrite level by about 73% compared to the control group value. Treatment with WPI, α-LA and β-LG were able to slightly decrease the high levels of hepatic NO compared to endotoxic rats in 24 hours with exception of β-LG (200mg/kg) which exerted non-significant effect on hepatic NO (Table 2).

**Table 2:** Effect of WPI, α-LA and β-LG on hepatic GSH, MDA and NO in rats treated with LPS in 24 hours

| Groups | Parameters* | | |
|---|---|---|---|
| | GSH (µg/g tissue) | MDA(nmol/g tissue) | NO (nmol/g tissue) |
| Control | $77.47 \pm 0.555^a$ | $43.95 \pm 0.742^a$ | $480.7 \pm 9.258^a$ |
| LPS | $60.73 \pm 0.506^b$ | $96.02 \pm 0.913^b$ | $832.7 \pm 9.737^b$ |
| LPS & WPI 100 | $78.02 \pm 0.621^a$ | $92.25 \pm 0.921^b$ | $713.7 \pm 8.494^c$ |
| LPS & WPI 200 | $95.63 \pm 0.789^c$ | $93.23 \pm 1.813^b$ | $769.3 \pm 5.147^d$ |
| LPS & α-LA 100 | $78.45 \pm 0.622^a$ | $95.23 \pm 1.699^b$ | $766.6 \pm 7.374^d$ |
| LPS & α-LA 200 | $75.47 \pm 0.477^a$ | $94.25 \pm 1.973^b$ | $776.7 \pm 9.471^d$ |
| LPS & β-LG 100 | $77.58 \pm 0.752^a$ | $94.53 \pm 1.370^b$ | $830.3 \pm 8.635^b$ |
| LPS & β-LG 200 | $81.12 \pm 0.820^d$ | $95.38 \pm 1.310^b$ | $715.1 \pm 7.265^c$ |

GSH, reduced glutathione; MDA, Malondialdehyde; NO, nitric oxide; LPS, lipopolysaccharide; WPI, whey protein isolate; α-LA, alpha-lactalbumin; β-LG, beta-lactoglobulin.* Within each column, means with different superscript letters are significantly different (P<0.05).

*Effect of whey protein isolate (WPI),alpha-lactalbumin (α-LA) and beta-lactoglobulin (β-LG) 100mg/kg and 200mg/kg on serum ALT, AST after LPS in two weeks-treatment:*

**Table 3:** Effect of WPI, α-LA and β-LG on serum biochemical parameters in rats treated with LPS in two weeks

| Groups | Parameters* | |
|---|---|---|
| | ALT (IU/L) | AST(IU/L) |
| Control | $11.17 \pm 0.75^a$ | $30.63 \pm 0.39^a$ |
| LPS | $20.68 \pm 0.85^b$ | $59.83 \pm 0.79^b$ |
| LPS & WPI 100 | $15.17 \pm 0.61^c$ | $44.18 \pm 0.97^c$ |
| LPS & WPI 200 | $16.02 \pm 1.11^c$ | $42.83 \pm 1.1^c$ |
| LPS & α-LA 100 | $15.92 \pm 0.84^c$ | $43.83 \pm 1.1^c$ |
| LPS & α-LA 200 | $13.00 \pm 0.79^{ac}$ | $41.30 \pm 1.13^c$ |
| LPS & β-LG 100 | $18.7 \pm 0.79^b$ | $44.52 \pm 1.06^c$ |
| LPS & β-LG 200 | $14.78 \pm 0.90^{ac}$ | $43.47 \pm 1.19^c$ |

ALT, alanine aminotransferase; AST, aspartate aminotransferase.*within each column, means with different superscript letters are significantly different (P<0.05).

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The results, displayed in Table 3, showed that intraperitoneal injection of LPS (4mg/kg and 2mg/kg) significantly increased serum ALT level by 85.2% compared to control group in two weeks, while oral administration of WPI (100 mg/kg and 200 mg/kg) significantly decreased serum ALT compared to LPS group by 26.7% and 22.5%, respectively. Moreover, α-LA 200mg/kg and β-LG 200mg/kg significantly maintained the normal level of ALT within two weeks of treatment. Whey proteins treatment showed remarked significant decrease in AST level as compared to endotoxic rats.

*Effect of whey protein isolate (WPI), alpha-lactalbumin (α-LA) and beta-lactoglobulin (β-LG) 100mg/kg and 200mg/kg on hepatic reduced GSH, MDA and NO after LPS in two weeks-treatment:*

Treatment with WPI at dose level of 100mg/kg failed to exert therapeutic effect on hepatic GSH, while WPI (200mg/kg), α-LA and β-LG (100mg/kg and 200mg/kg) were able to increase GSH concentration significantly compared to LPS treated group in two weeks (Table 4). Effects of WPs on hepatic MDA varied as WPI (100mg/kg), α-LA and β-LG (200 mg/kg) failed to resist LPS induced-lipid peroxidation during two weeks, while WPI (200mg/kg), α-LA and β-LG (100 mg/kg) significantly decreased MDA level in LPS pretreated animals. Endotoxemia induced significant elevation in hepatic NO level compared to saline treated animals (Table 4), WPI and α-LA at both dose levels were able to maintain normal levels of NO. Moreover, β-LG as significantly decreased hepatic NO level compared to endotoxic rats.

**Table 4:** Effect of WPI, α-LA and β-LG on hepatic GSH, MDA and NO in rats treated with LPS in two weeks

| Groups | Parameters* | | |
|---|---|---|---|
| | GSH (μg/g tissue) | MDA (nmol/g tissue) | NO (nmol/g tissue) |
| Control | 79.47±1.023[a] | 36.77±1.387[a] | 480.1±6.354[a] |
| LPS | 60.42±0.543[b] | 53.87±0.526[b] | 724.1±7.673[b] |
| LPS & WPI 100 | 60.42±0.543[b] | 53.47±1.466[b] | 500.6±8.951[a] |
| LPS & WPI 200 | 67.82±0.697[c] | 31.88±0.443[c] | 488.9±9.192[a] |
| LPS & α-LA 100 | 75.37±1.554[ad] | 39.00±1.079[ad] | 484.5±7.125[a] |
| LPS & α-LA 200 | 70.28±1.760[dc] | 51.28±0.828[b] | 489.3±7.826[a] |
| LPS & β-LG 100 | 67.82±1.383[c] | 43.23±0.859[d] | 602.1±9.735[c] |
| LPS & β-LG 200 | 72.92±1.089[dc] | 56.23±0.890[b] | 583.9±8.203[c] |

GSH, reduced glutathione; MDA malondialdehyde; NO, nitric oxide; LPS, lipopolysaccharide
WPI, whey protein isolate; α-LA, alpha-lactalbumin; β-LG, beta-lactoglobulin.* Within each column, means with different superscript letters are significantly different (P<0.05).

*Histopathological studies:*

Light microscopic examination of the liver of control rats revealed normal architecture of hepatic lobules with hepatocytes radiating from the central veins, with narrow sinusoids and prominent nucleus (Fig.1A). LPS showed disruption of normal architecture of hepatic lobules and deposited collagen fiber in the form of very thick fibrous septa containing numerous fibroblasts around central veins as well as portal tracts. Central veins were congested with cellular infiltration. Most hepatocytes had lost most of their cytoplasmic masses and appeared as empty spaces with, deeply stained nuclei and vacuolations. (Fig. 1. B)**.** WPI (100 mg/kg b.w.) and LPS showed preservation of nearly normal hepatic architecture. The hepatic cells appeared with congested blood sinusoid (Fig 1 C). treatment with WPI, (200 mg/kg b.w.), and LPS showed partly preserved the hepatic normal architecture (Fig. 1 D). Administration of α-LA (100 mg/kg b.w.) and LPS caused marked amelioration pronounced in normalized appearance of liver cells with strains of hepatocytes comparing with section of LPS (Fig. 1.E).

In α-LA (200 mg/kg b.w.) and LPS -treated group, the liver sections exhibited some degree of histological regeneration, reduction in most alteration induced by LPS, less sinusoids dilatation with decrease number of Kupffer cells, less pyknotic cells were observed(Fig. 1F). The hepatocytes of β-LG-treated liver tissues - (100 mg/kg b.w.) and LPS partly preserved the hepatic normal architecture. Mild degrees of hydropic changes with mild dilated congested blood sinusoids were recorded (Fig. 1 G). This improvement was more pronounced in β-LG group (200 mg/kg b.w.) and LPS treated rats compare to LPS alone. Light microscopy investigation showed normal lobular architecture with congested central veins and radiating hepatic cords and minimal inflammatory lymphocyte infiltrations were observed (Fig. 3 H).

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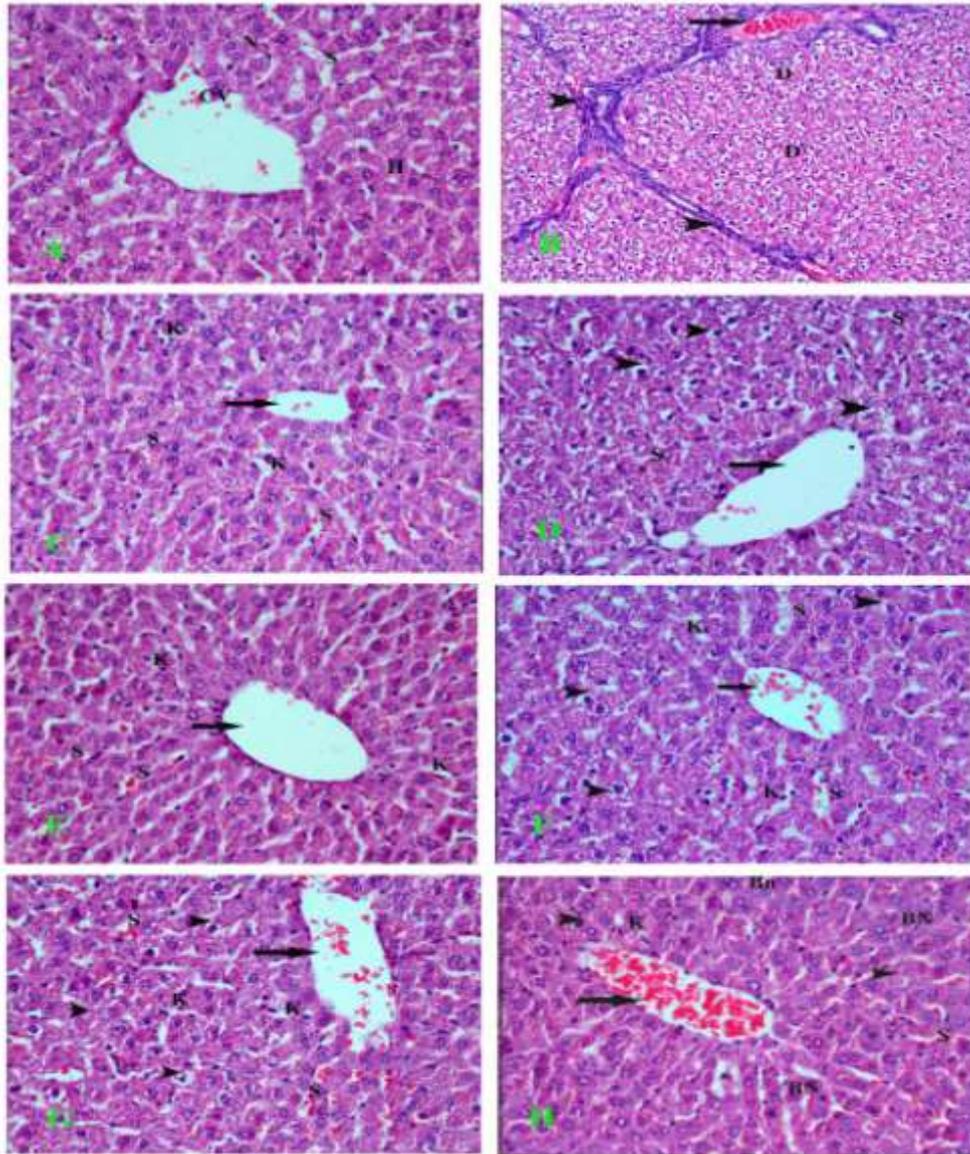


**Fig. 1.A:** A photomicrograph of the control liver of rat with central vein (CV) and surrounding hepatocytes (H), sinusoids (S) and nucleus (N). **(B)** Rat administration LPS showing disarrangement of normal hepatic cells disruption of normal architecture of hepatic lobules and deposited collagen fiber in the form of very thick fibrous septa containing numerous fibroblasts in between hepatic lobules, around central veins as well as portal tracts (arrow head). Central veins were congested (long arrow), degenerated hepatocytes(D)with, deeply stained nuclei. **(C)** Rat administration (WPI) at dose (100 mg/kg b.w.) and LPS showing noticeable improvement, dilatation sinusoid (S) and activated of Kuppfer cells (K). Pyknotic cells (PK) and mitotic figure (M) were observed. **(D)** Rat administration (WPI) at dose (200 mg/kg b.w.) and LPS showing mild improvement with signs of degeneration of hepatocytes is still present with pyknotic cells (PK). **(E)** Rat administration (α-LA) at dose (100 mg/kg b.w.) and LPS showing improvement in the liver tissue with few pyknotic cells. **(F)** Rat administration (α-LA) at dose (200 mg/kg b.w.) and LPS showing mild improvement in the liver tissue with some of degeneration of hepatocytes is still present with pyknotic cells (PK). Dilatation and heamorraghe in blood sinusoids was observed. **(G):** Rat administration (β-LG) -at dose (100 mg/kg b.w.) and LPS showing mild improvement in the liver tissue with dilatation and heamorraghe in blood sinusoids, and activated of Kuppfer cells (K) Note binucleated hepatocytes (BN) and pyknotic cells (PK). **(H):** Rat administration (β-LG) -at dose (200 mg/kg b.w.) and LPS showing normalization of liver tissue is observed, although there is degeneration of hepatocytes (long arrow) and activated of Kuppfer cells (K). Note binucleated hepatocytes (BN) and pyknotic cells (PK). (**H & E X 400**)).

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*Discussion:*

Hepatic dysfunction after sepsis is a frequent event that is characterized by loss of synthetic function, hepatocellular necrosis, and release of inflammatory mediators such as TNF-α, interleukin-1β, interleukin-6, and NO (Zhang *et al.*, 2000) . The endotoxin induces a strong pro-inflammatory response initiated by a cascade of molecular events leading to NF-kB activation and resulting in multiple organ failure syndrome (Zhang and Ghosh, 2000). The harmful effects of LPS are, in part, due to its ability to induce an oxidative stress status characterized by depletion of endogenous antioxidant enzyme activities such as SOD, CAT and GPx. The pro-oxidant action of the endotoxin is due to its ability to induce excessive ROS and RNS accumulation leading to cellular injury by impairment of vital macromolecules as protein and lipid (Sebai *et al.*, 2010). The present study demonstrates that LPS-induced endotoxemia resulted in impairment in liver functions that was manifested by increased serum ALT and AST levels. Endotoxemia also induced oxidative stress that was revealed by increased hepatic MDA, a marker of lipid peroxidation, and hepatic NO levels as well as decline in hepatic GSH. Post-treatment with WPI and its major protein fractions; α-LA (20-25% of total whey protein) and β-LG (50-55% of total whey protein) (Marshall, 2004) attenuated almost all LPS deleterious effects.

The hepatic injury following LPS administration is well established by the elevated levels of serum enzymes indicating cellular leakage and loss of functional integrity of hepatic membrane (Hagar, 2009, Sebai *et al.*, 2010, Mohamadin *et al.*, 2011). This correlates with our results, which showed remarkable increase in serum levels of ALT and AST in LPS-treated rats during 24 hours and moderate but significant increase during two weeks. Increased activities of serum hepatic markers suggested the extensive liver injury induced by LPS through its free radical generation mechanism, which in turn has the ability to cause hepatic damage resulting in increased leakage of cellular enzymes (Sener *et al.*, 2005).

Consistent with the involvement of oxidative stress after LPS exposure, GSH has been shown to play an important role in the susceptibility to LPS-induced liver injury (Payabvash *et al.*, 2006). In experimental models including the present study, LPS has been reported to deplete GSH stores in the liver, and mice deficient in GPx exhibited enhanced susceptibility to LPS-mediated liver damage (Jaeschke *et al.*, 1999). Consistent with these findings, the exogenous administration of GSH has been shown to decrease LPS-induced systemic inflammatory response and mortality (Sun *et al.*, 2006). Nevertheless, it has been proposed that antioxidants which maintain the concentration of reduced GSH may restore the cellular defense mechanisms, block LPO and thus protect against the oxidative tissue damage (Mohamadin *et al.*, 2011). In accordance with previous fact, our results also verify that WPI, α-LA and β-LG at 100 and 200 mg/kg, orally administered 2 hours after LPS insult, were capable in maintaining normal levels of hepatic reduced GSH in 24 hours-experiment, also in two weeks-experiment WPI, α-LA and β-LG treatment attenuated the depletion of hepatic GSH compared to endotoxic rats, with superior effect of α-LA (100mg/kg) which was able to regain the normal levels of GSH in liver tissues of endotoxic rats. A large body of evidence suggested that WP has an antioxidant activity probably depending on the abundance of cysteine in WP or on the presence of glutamyl-cysteine groups which are found in other food proteins. Therefore, WP may be considered as a possible therapeutical tool in oxidative stress correlated diseases (Balbis *et al.*, 2009). Furthermore, experimental studies indicate that plasma glutamate concentration decreases during sepsis with increased consumption of glutamate (Poeze *et al.*, 2008). Although cysteine has generally been considered limiting for glutathione synthesis, the availability of glutamine appears to be important for the regeneration of glutathione stores during experimental conditions of severe hepatic injury as glutamine provides the glutamate portion of glutathione and attenuates the rapid fall in hepatic glutathione stores that occurs after acetaminophen toxicity (Hong *et al.*, 1992). Whey proteins (WP) are particularly rich in cysteine (cys) and in glutamine (glu) and therefore potentially capable of increasing the organism's antioxidant defences via synthesis of glutathione (Traverso *et al.*, 2010).

WPI, α-LA and β-LG couldn't alleviate LPS-induced lipid peroxidation at both dose levels in 24 hours despite compensating depletion of GSH, while in two weeks experiment WPI (200 mg/kg) and α-LA (100mg/kg) normalized hepatic MDA level as well as β-LG (100 mg/kg) significantly resisted LPO as indicated by reduction in hepatic MDA and thereby supported their antioxidant activity (Brown *et al.*, 2004), which is mostly due to high cystein content, and chelating activity for heavy metal ions that may catalyze formation of ROS (Hurrell *et al.*, 1989).

In the present study, LPS administration in rats resulted in remarkable increase in hepatic nitrite level, as a measure of NO production, which was attenuated by WPI, α-LA and β-LG treatment in 24 hours, except for β-LG (100mg/kg). Further, WPI and α-LA at both dose levels attained normal levels of hepatic NO in two weeks meanwhile, β-LG was able to exert significant reduction in NO level compared LPS-treated rats. Sustained production of NO after LPS challenge may cause hepatocellular injury either directly or indirectly. NO, a free radical with high spontaneous chemical reactivities, can react with superoxide anion to generate hydroxyl radical and peroxynitrite, the more potent reactive oxygen metabolite, which are important mediators of oxidative stress and NO-mediated cell injury in many pathological states (Obata, 2002, Hsu *et al.*, 2004). Blocking peroxynitrite, a powerful and potent pro-apoptotic and pro-inflammatory mediator, would yield better

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outcomes. This hypothesis stems from the fact that removal or antagonism of ONOO⁻ in endotoxic shock will not deplete homeostatic levels of NO produced from iNOS but rather only attenuate the harmful effects of NO overproduced from iNOS (which are mediated by ONOO⁻) (Salvemini *et al*., 1998). Although the mechanisms by which WPI, α-LA and β-LG attenuated the LPS-induced increase in hepatic NO cannot be delineated from the results of this study. Further, our data showed that WPI, α-LA and β-LG significantly decreased LPS-induced NO production; this might be contributed to the attenuation of LPS-induced oxidative stress through enhancing hepatic GSH pool.

Eventually, histopathological examination of the current investigation revealed the following; the hepatic histo-architecture of the LPS treated rats resulted in necrotic changes and inflammatory cell infiltration, which basically supported the alterations observed in biochemical assays. It might be due to the formation of highly reactive radicals because of oxidative threat induced by LPS (Zhao *et al*., 2008). Consistent with present study after administration of LPS (2mg/kg) to rats, PMNs accumulate in the liver sinusoids within 1 hr, and multifocal, midzonal hepatic necrosis is prominent by 6 hr. Polymorphonuclear leukocyte (neutrophil) depletion protected against liver injury after administration of either a small (2 mg/kg) or a large (8 mg/kg) dose of LPS (Hewett and Roth, 1995). The mechanism for this injury is complex and involves the interaction of numerous inflammatory cells and soluble mediators (Hewett and Roth, 1993). Kupffer cells release cytotoxic mediators, such as ROS, and pro-inflammatory mediators, such as cytokines and chemokines. Cytokines prime and activate neutrophils to promote their recruitment into the hepatic vasculature. If chemotactically stimulated, neutrophils extravasate and adhere to parenchymal cells, which induces necrotic cell death through release of reactive oxygen, such as superoxide anion ($O_2^{\bullet-}$) and proteases (Jaeschke *et al*., 2002). Administration of WPI, α-LA and β-LG retained the histological alterations induced by LPS quite appreciably. This can be attributed to their antioxidant and chelating activities, which significantly reduced the oxidative threat leading to reduction of pathological changes and restoration of normal physiological functions (Mohamadin *et al*., 2011). In 24 hours treatment, WPI showed relatively mild improvement in liver histology compared to α-LA and β-LG which improved hepatic architecture significantly compared to LPS treated livers. Whereas the two weeks experiment, hepatic tissue of LPS (4mg/kg and 2mg/kg, i.p.) during 2 weeks showed disarrangement of normal hepatic cells with hepatic necrosis, vacuolated and fatty degenerated hepatocytes with increase eosinophility of hepatocytes. Further, inflammatory cell infiltrations were observed around the portal areas and in blood sinusoids, with thin fibrous tissue from the portal tract. The portal vein is congested with an increase in Kupffer cells and the pyknotic nuclei are clearly visible. Treatment with WPI showed moderate improvement in liver histology of endotoxic rats, while α-LA and β-LG showed almost normal appearance, the effect that was enhanced at high dose level. As shown by previous studies, that WP bears a numbers of beneficial properties including antioxidant activity, anti-inflammatory and immunomodulatory effects (Kent *et al*., 2003, Middleton *et al*., 2003, Rutherfurd-Markwick *et al*., 2005, Yamaguchi *et al*., 2009, De Carvalho-Silva *et al*., 2012), all of which could account for the observed therapeutic effects afforded by WPI, α-LA and β-LG in this study.

*Conclusion :*

Our results demonstrated that administration of WPI, α-LA and β-LG can provide new therapeutic intervention for endotoxic-liver insult induced oxidative damage as evidenced by decreased liver marker enzymes (ALT, AST), LPO, NO, increased the antioxidant, GSH. And eventually, WPs improved hepatic histopathologic picture. These effects of WPs could be due to its antioxidant nature, which may include free radical scavenging properties and its anti-oxidant promoting activity. However, despite the potent effects of WPI, α-LA and β-LG on LPS-induced oxidative stress and the hepatic improvement they afford, the possibility of their clinical application still needs further investigation.

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