

Silymarin Supplementation Attenuation of the Lipopolysaccharide-Induced Metabolic Changes in Protein Malnourished Rats

Adel A Alhamdan

Health Sciences Department, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia

ABSTRACT

Introduction: Protein malnutrition increases the sensitivity to septic shock by impairing antioxidation and immune response. Based on the potent antioxidant effects of silymarin, the putative protective role of silymarin against sepsis-induced oxidative damage in protein malnourished rats was investigated. **Methods:** Adult male Wistar rats were subjected to protein malnutrition via a low-protein diet (8% protein), with and without silymarin supplementation (30 mg/kg/day) for four weeks, and compared to a control group on a 18% protein diet. At end of the experiment, the animals received intraperitoneal injections (i.p.) of 0.1 mg/kg lipopolysaccharide (LPS) derived from *Escherichia coli*, and decapitation 24 h later. Albumin, C-reactive protein (CRP), total protein (TP), alkaline phosphates (AKP ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), tumor necrosis factor- α (TNF- α), interleukine-1 β (IL-1 β) and interleukine-6 (IL-6) levels were measured in serum. In the lungs and liver, thiobarbituric acid reactive substances (TBARS) and glutathione (GSH) concentrations, superoxide dismutase (SOD), catalase (CAT), and glutathione-s-transferase (GST) activities were measured. **Results:** oxidative and inflammatory processes were significantly increased by the LPS injection and these changes were to a greater extent in the low-protein dietary group compared to control group. Silymarin supplementation in both dietary groups showed marked inhibition in these activities, although the affect was more prominent in the control group compared to the rats fed a low-protein diet. **Conclusions:** The study showed that silymarin protected against the impairment of antioxidation and immune response in protein malnourished rats, particularly in septic shock conditions.

Key words: Antioxidant, cytokines, endotoxin, protein malnutrition, silymarin

INTRODUCTION

The metabolic and biochemical impact of qualitative and quantitative changes in dietary protein intake continues to be of interest because of their metabolic implications for public health. In developing countries, protein malnutrition is one of the major health concerns,

especially its effect among children (Müller & Krawinkel, 2005). Diets in different populations were found frequently to be deficient in dietary protein levels and led to protein-malnutrition (Millward & Jackson, 2004). It is well known that a low protein intake reduces antioxidant levels, impairs immune functions and increases sensitivity

* Correspondence: Adel A Alhamdan; Email: adel@ksu.edu.sa

to opportunistic infections and septic shock (Beishuizen, Vermes & Haanen, 1998; Gernaat, Dechering & Voorhoeve, 1998). The protein malnutrition, also associated with low levels of endogenous antioxidant defense and increased oxidant burden, contributes to the dysfunction of various tissues, including the lungs and liver (Adenuga, Adebayo & Adegbesan, 2008; Pires-de-Melo *et al.*, 2009).

Septic shock, a systemic response to infection, is characterised by specific pathological events upon specific target organs (Wenzel *et al.*, 1996). All symptoms of gram-negative bacterial-induced septic shock can be mimicked by the injection of lipopolysaccharide (LPS), one of the major components of the outer membrane of gram-negative bacteria (Bradley, 1979). Despite major developments in medical therapy during the last two decades, the succession of septic shock to multiple organ dysfunction syndromes is still associated with an increase in mortality rate from 30% to 100%, with the incidence of septic shock and the associated lethality rates being vastly higher for the elderly than for younger people (Rivers *et al.*, 2001). This progression of early sepsis into septic shock involves several steps and numerous interconnected pathways (Cinel & Opal, 2009). Firstly, local inflammation provokes the release of proinflammatory mediators such as tumor necrosis factor- α (TNF- α), interleukine-1 β (IL-1 β) and interleukine-6 (IL-6). Then, these proinflammatory mediators induce the migration of leukocytes, lymphocytes, and platelets to those areas and cause endothelial damage, increase microvascular permeability, platelet aggregation, localised blood flow reduction, and ischemia/reperfusion injuries. The cumulative inflammatory response finally leads to multiple organ injuries or death (Adenuga *et al.*, 2008). The lungs are most frequently damaged of all organs during septic shock (Toklu *et al.*, 2008). The liver-gut-axis was hypothesised to be of significant importance to the

inflammatory reaction and hence for the incidence of multiple organ failure (Moshage, 1997).

Flavonoids are naturally occurring substances that possess various pharmacological actions and therapeutic applications. Silymarin is one such polyphenolic flavonoid anti-oxidant that can be isolated from the fruits and seeds of the milk thistle, *Silybum marianum* (Valenzuela & Garrido, 1994). Although the therapeutic effects of silymarin are restricted owing to its poor enteral absorption (23% to 47%), instability in gastric juices, and poor solubility. However, multiple dose treatments show potentially beneficial effects against several diseases (Wu *et al.*, 2007). It has demonstrated protective effects against oxidative peroxidation (Soto *et al.*, 2003). It is routinely used to treat chronic inflammatory liver disease and hepatic cirrhosis as a hepatoprotective agent (Karimi, Ramezani & Tahoonian, 2005). Silymarin treatment protects against the manganese-induced oxidative stress in a rat brain (Chtourou *et al.*, 2010). It can also enhance superoxide dismutase activity (Baluchnejadmojarad, Roghani & Mafakheri, 2010). Kang *et al.* (2004) report that silymarin has an inhibitory effect against endotoxin-induced interleukins production.

Increased sensitivity to septic shock has been reported in protein malnourished patients (Gernaat *et al.*, 1998). Li *et al.* (2002) observed that protein malnutrition is a direct cause of increased LPS-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation and transcription levels of its downstream genes IL-1 β and TNF- α . Adenuga *et al.* (2008) suggest that partial hepatectomy exacerbates lipid peroxidation in rats with protein malnutrition compared to their controls. Natural products are known to be safe with least adverse effects on any transient body metabolisms. Silymarin is one of such products having antioxidant, anti-hepatotoxic and anti-inflammatory

properties, and also increases the activity of antioxidant enzymes including superoxide dismutase (SOD) and catalase (CAT) (Toklu *et al.*, 2008; Soto *et al.*, 2003). Based on the potent antioxidative and anti-inflammatory effects of silymarin, the protective role of silymarin supplementation against endotoxin-induced septic shock that increased pro-inflammatory markers such as TNF- α , IL-1 β and IL-6 and induced oxidative damage in lung and liver tissues was investigated by using a protein-malnutrition model in rats.

METHODS

Forty male Wistar albino rats, roughly the same age and weighing 150 to 170 g were received from the Experimental Animal Care Center (King Saud University, Riyadh, Saudi Arabia). They were maintained under controlled temperature (22 \pm 1 °C), humidity (50 to 55%), and light (12 h light/dark cycles) conditions, and were provided with Purina chow (Grain Silos & Flour Mills Organisation, Riyadh, Saudi Arabia) and drinking water *ad libitum*. All procedures, including the euthanasia procedure, were conducted in accordance with the requirements of the Institute for Laboratory Animal Research (1996) and the Experimental Animal Care Center.

Normal protein diet (NPD) with 18% protein and low protein diet (LPD) with 8% protein were formulated and prepared in our laboratory with casein as the protein source (vitamin-free, WINLAB, Lab. Chem., United Kingdom) and included sucrose and corn oil as sources of carbohydrate and fat, respectively. Every week fresh feed in powdered form was prepared and stored in a refrigerator for use.

Experimental design

After an acclimatisation period of seven days, the animals were randomly divided into five groups of eight animals and placed individually in a suppurate metabolic cage:

1. Controls were fed NPD
2. NPD fed rats were injected with LPS (NPD+LPS)
3. NPD fed rats were supplemented with silymarin (30 mg/kg/day) and injected with LPS (NPDS+LPS).
4. LPD fed rats were injected with LPS (LPD+LPS).
5. LPD fed rats were supplemented with silymarin (30 mg/kg/day) and injected with LPS (LPDS+LPS).

The body weight and food intake of the rats were recorded daily. The rats were decapitated exactly 24 h after the LPS injection. Blood samples were collected through cardiac puncture and centrifuged at 3000 revolutions per minute (rpm) for 15 minutes. Serum was separated and kept in a freezer at -20 °C prior to analysis. Lung and liver tissues were dissected, immediately dipped in liquid nitrogen for a minute and then stored at -75 °C prior to analysis.

Serum analysis

Serum albumin, C-reactive protein (CRP), total protein (TP), alkaline phosphates (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were measured using standard Bohringer Ingelheim laboratory techniques. Additionally, serum pro-inflammatory cytokines, including TNF- α , IL-6 and IL-1 β concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) kit supplied by ShangHai SenXiong Science and Technology Company. The levels were estimated following the instructions provided by the manufacturer.

GSH concentration in liver and lung

Glutathione (GSH) concentration in liver and lung tissues was measured according to the method described by Sedlak & Lindsay (1968). A cross section of the liver or lung (200 mg) was dissected and homogenised in ice-cold 0.02 M ethylenediaminetetraacetic

acid (EDTA). The aliquots of 0.5 mL of tissue homogenates were mixed with 0.2 M Tris buffer, pH 8.2 and 0.1 mL of 0.01 M Ellman's reagent, [5,5'-dithiobis-(2-nitro-benzoic acid)] (DTNB). Sample tubes were centrifuged at 3000 g at room temperature for 15 min. The absorbance of the clear supernatants was recorded using spectrophotometer at 412 nm in one centimetre quartz cells. The results were quantified as nmol/100mg wet tissue.

TBARS concentration in liver and lung

The thiobarbituric acid reactive substances (TBARS) levels in the liver and lung were measured by a TBARS assay kit following the instruction provided by the manufacturer (ZeptoMetrix Corporation). The results were given as nmol MDA/g wet tissue.

SOD activity in liver and lung

The SOD activity in tissues was assayed using the method described by Kakkar, Das & Viswanathan (1984) with the aid of nitroblue tetrazolium as the indicator. A cross-section of lung or liver (200 mg) was homogenised with 10 times (weight/volume) 0.1 sodium phosphate buffer (pH 7.4). The reagents sodium pyrophosphate buffer 1.2 mL (0.052 M) pH 8.3, 0.1 ml phenazine methosulphate (186 μ M), 0.3 mL nitro blue tetrazolium (300 μ M), and 0.2 mL nicotinamide adenine dinucleotide (NADH) (780 μ M) were added to 0.1 mL of processed tissue sample. The sample mixture was incubated for 90 min at 30 °C. To this was added 4 mL of n-butanol and 1 mL of acetic acid before being shaken vigorously. Samples were centrifuged at 4000 RPM for 10 min and the organic layer was withdrawn and absorbance was measured at 560 nm using a spectrophotometer (LKB-Pharmacia, Mark II). The SOD activity was reported as unit/min/mg of protein.

CAT activity in liver and lung

The CAT activity was measured by the method of Aebi (1983). The activity was

expressed as unit/min/mg of protein using the extension coefficient of 0.0436 mM/mg. A cross-section of liver or lung (200 mg) was homogenised in 8 mL of 0.05 M phosphate buffer at pH 7.0 and the homogenates were centrifuged at 4 °C for 15 min at 1500 g. Supernatants were removed into separate test tubes and kept on ice until the enzyme assay. Samples were measured against blanks containing 2.8 mL (1:500 volume/volume) phosphate buffer instead of a H₂O₂ (30 mM hydrogen peroxide) and 0.2 mL enzyme solution. The reaction was started by the addition of H₂O₂. The initial absorbance was expected to be A=0.500 followed by the decrease in the following 30 sec.

GST activity in liver and lung

The glutathione-s-transferase (GST) activity in tissues was measured by the method of Habig, Pabst & Jakoby (1974). The reaction mixture consisted of 0.067 mM GSH, 0.067 CDNB, 0.1 M phosphate buffer (pH 6.0) and 0.1 ml of post-mitochondrial supernatant in a total volume of 3 ml. Absorbance was read at 340 nm for a duration of 10 min at a frequency of every 30 sec by an optical plate reader. The enzyme activity was calculated as mmol CDNB were conjugate formed min⁻¹ mg⁻¹ protein using a molar extinction coefficient of $9.6 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$.

Statistical analysis

All data were presented as mean \pm standard deviation (SD). The data were evaluated by a one-way analysis of variance (ANOVA) using GraphPad InStat program (version 3.06) and the differences between means were assessed using the Student-Newman-Keuls (SNK) method. The differences were considered statistically significant at $P < 0.05$.

RESULTS

Serum albumin, CRP and total protein

Serum albumin concentration was significantly decreased with LPS challenge in all dietary groups compared to the

control. Silymarin supplementation corrected the albumin inhibition-induced by LPS in both dietary groups compared to their corresponding dietary groups without silymarin supplementation, but this was only statistically significant in the NPDS+LPS group. The albumin values were found to be significantly lower in LPDS+LPS group compared to NPDS+LPS group (Figure 1-A). CRP level was significantly elevated by the LPS injection in all dietary groups compared to the control. The elevated CRP levels were significantly reduced in silymarin supplemented groups compared to their corresponding dietary groups. The LPS-induced CRP levels were found to be significantly higher in the LPD group compared to the NPD fed rats, and the silymarin inhibition effect was found to be less in the LPD fed rats compared to the NPD fed animals (Figure 1-B). Total protein concentration was significantly decreased after LPS administration in all dietary groups compared to the control (Figure 1-C). After the LPS challenge, silymarin supplementation to NPD (NPDS+LPS) significantly increased total protein concentration compared to the corresponding dietary group (NPD+LPS).

Serum enzymes

Serum enzymes including the activities of ALP, AST and ALT were examined as indicators of hepatic injury. In all dietary groups, enzymatic activities were significantly increased by the endotoxin injection compared to the controls. Silymarin supplementation prevented the increased level of ALP in both dietary groups compared to the corresponding dietary groups without silymarin supplementation (Figure 2-A). Silymarin showed a similar effect against the LPS-induced increase in AST and ALT concentrations (Fig. 2-B & C), but this was only statistically significant in the NPDS+LPS group. However, the enzymatic activities were significantly

higher in the LPD fed rats compared to the NPD fed rats 24 h after the LPS challenge. The silymarin protection against the hyper-enzymatic activities induced by the LPS was found to be lower in the LPD fed rats (LPDS+LPS) compared to the NPD fed rats (NPDS+LPS).

Serum cytokines

Unsurprisingly, pro-inflammatory cytokines, TNF- α , IL-1 β and IL-6 levels all increased significantly in LPS injected groups compared to the control group (Figures 3-A, B & C). The extent of the increased levels of the proinflammatory cytokines were about 40% higher in the LPD+LPS group compared to the NPD+LPS group. Silymarin supplementation reduced the impact of LPS on the pro-inflammatory cytokines and reduced their levels significantly in both dietary groups compared to their corresponding dietary groups without silymarin supplementation.

Oxidative and enzymatic markers in the lung

In the lung, TBARS concentrations were significantly increased after LPS injection in both dietary groups. Silymarin supplementation showed significant protection against the elevation induced by the LPS in both the dietary groups compared to their corresponding dietary groups without silymarin. GSH levels were significantly decreased by LPS injection and the values were corrected significantly by silymarin supplementation in both dietary groups compared to their corresponding untreated groups. In LPS challenged groups, SOD, CAT and GST activities significantly decreased compared to the control group. Silymarin administration significantly enhanced these activities in the NPDS+LPS group. The LPDS+LPS group showed silymarin's positive effect against LPS damage, but this

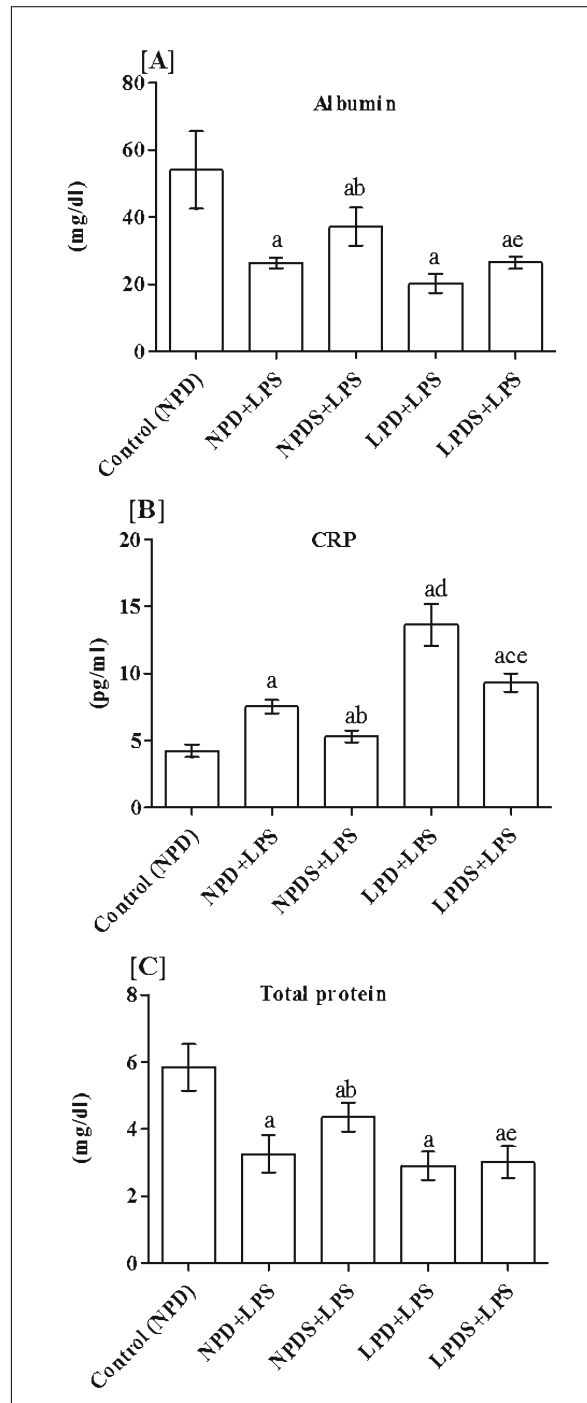


Figure 1. The effect of silymarin on LPS-induced changes in serum levels of albumin [A], CRP [B] and total protein [C] in rats fed NPD or LPD

The results are presented as mean \pm SD (n=6). One-way ANOVA and SNK multiple comparisons test were used. $P \leq 0.05$ was considered statistically significant. 'a' all groups compared with NPD, 'b' NPD+LPS vs NPDS+LPS, 'c' LPD+LPS vs LPDS+LPS, 'd' NPD+LPS vs LPD+LPS, and 'e' NPDS+LPS vs LPDS+LPS.

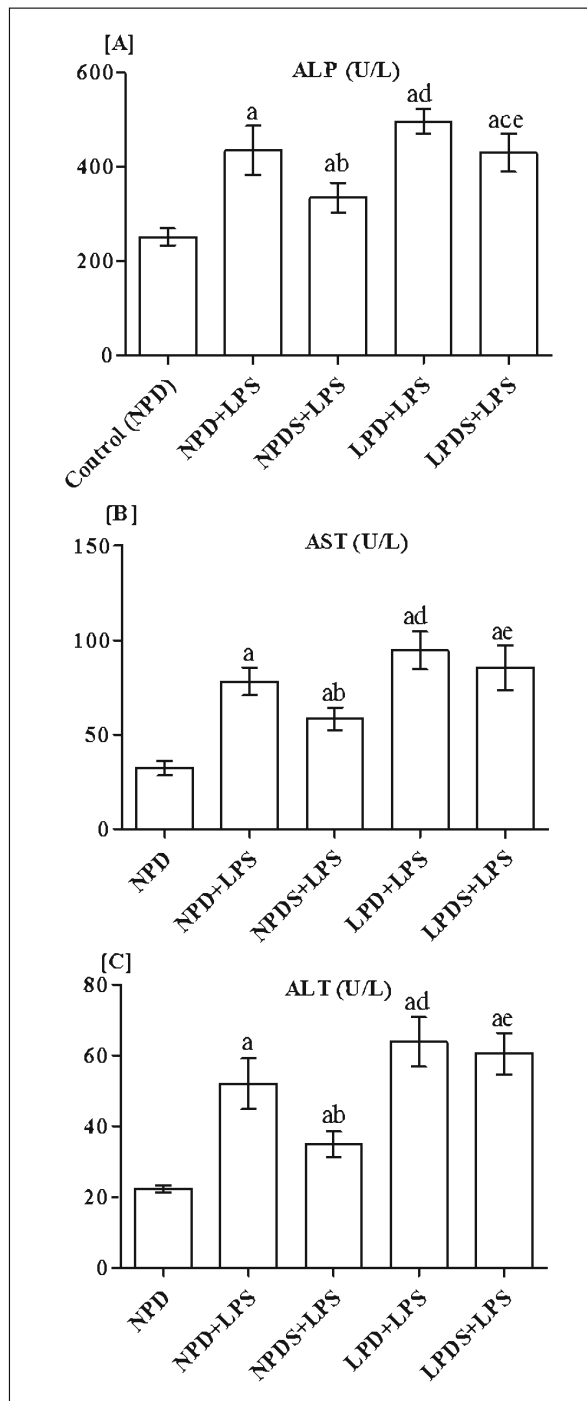


Figure 2. The effect of silymarin on LPS-induced changes in serum enzymatic activities of ALP [A], AST [B] and ALT [C] in rats fed NPD or LPD

The results are presented as mean±SD (n=6). One-way ANOVA and SNK multiple comparisons test were used. P≤0.05 was considered statistically significant. 'a' all groups compared with NPD, 'b' NPD+LPS vs NPDS+LPS, 'c' LPD+LPS vs LPDS+LPS, 'd' NPD+LPS vs LPD+LPS, and 'e' NPDS+LPS vs LPDS+LPS.

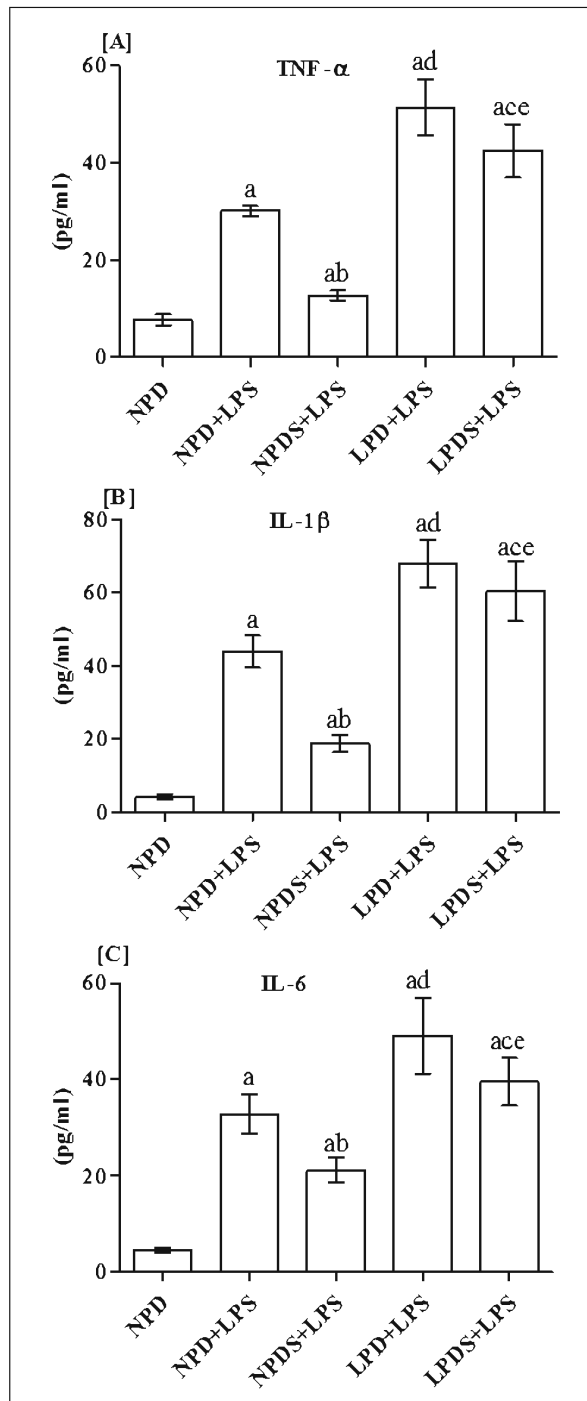


Figure 3. The effect of silymarin on LPS-induced changes in serum levels of pro-inflammatory cytokines including TNF- α [A], IL- β [B] and IL-6 [C] in rats fed NPD or LPD. The results are presented as mean \pm SD (n=6). One-way ANOVA and SNK multiple comparisons test were used. $P < 0.05$ was considered statistically significant. 'a' all groups compared with NPD, 'b' NPD+LPS vs NPDS+LPS, 'c' LPD+LPS vs LPDS+LPS, 'd' NPD+LPS vs LPD+LPS, and 'e' NPDS+LPS vs LPDS+LPS.

was not statistically significant (Table 1).

Oxidative and enzymatic markers in the liver

LPS also caused significant elevation in liver TBARS levels. Silymarin supplementation to the NPD+LPS and LPD+LPS groups significantly lowered the increased levels of TBARS compared to their corresponding untreated dietary groups. GSH levels significantly decreased in the LPS-injected groups compared to the controls, and this inhibition was significantly corrected by the silymarin in NPD-fed rats compared to the corresponding untreated dietary group. In LPS challenged groups, SOD, CAT and GST activities significantly decreased in the liver compared to the control group. Silymarin supplementation significantly brought back these activities to normal levels in the NPD-fed rats, but not in the LPD-fed rats. In the LPD-fed rats, silymarin enhanced SOD, CAT and GST activities after LPS injection, but the effect was not statistically significant. These activities were found to be significantly higher in the NPDS+LPS group compared to the LPDS+LPS group (Table 2).

DISCUSSION

LPS-induced septic shock by activating macrophages, initiating lymphocyte differentiation and blood coagulation, and inducing oxidative stress and proinflammatory molecule release from macrophages, leading to tissue damage, organ failure, and death (González-Renovato *et al.*, 2013). The pro-inflammatory biomarkers TNF- α , IL-1 β , and IL-6 are thought to be involved in the pathogenesis of septic shock with LPS. In accordance with complement activation, the cytokine stimulation of circulating and resident immune cells and endothelial cells results in elevated accumulation of reactive oxygen species (ROS) and reactive nitrogen species, such as superoxide anion and NO (von Dessauer *et al.*, 2011).

Antioxidant defense mechanisms of the organism have developed to restrict the levels of reactive oxidants and the harm they induce. In addition to the protective effects of endogenous antioxidant defense mechanisms, supplementation of exogenous nutritional antioxidants seems to be of great significance (Sandoval *et al.*, 1997).

The LPD has controversial effects on body weight, although this is usually associated with an increase in food intake (Aparecida de França *et al.*, 2009); some researchers report decreases (Chamson-Reig *et al.*, 2009) compared to an isocaloric NPD. In this study, body weights of rats decreased in the LPD fed group from week one, and they became significantly less by the end of week four compared to the NPD fed rats, and the dietary intake of the LPD group was higher than in the NPD (results are not reported).

The increase in the blood level of the positive acute-phase protein, CRP, gives an indication of the degree of the response to inflammation, trauma, or infection (Volanakis, 2001). Negative acute-phase proteins are those proteins, which decrease their concentrations after inflammation, such as albumin and transferrin (Fleck, 1989). This study showed that the LPS challenge significantly increased serum CRP and decreased albumin and total protein levels, and that silymarin supplementation inhibited the CRP elevation in both the dietary groups, although the LPS effect on CRP was found to be significantly higher in the LPD fed rats compared to the NPD fed rats. This might be due to protein malnutrition, which reduces antioxidant levels, impairs immune functions and increases sensitivity to opportunistic infections and septic shock (Li, Quan & Bray, 2002). Silymarin has a regulatory action on cellular and mitochondrial membrane permeability, and is associated with an increase in membrane stability against xenobiotic

Table 1. The effect of silymarin on LPS-induced changes in lung TBARS and GSH levels, and SOD, CAT and GST activities of rats fed NPD or LPD

Groups	TBARS (nmol/g)	GSH (nmol/100 mg)	SOD (U/mg of protein)	CAT (U/mg of protein)	GST (mmol/mg of protein)
NPD	138.08±11.38	63.07±9.98	8.40±0.76	6.57±0.84	53.62±5.17
NPD + LPS	205.60±16.84 a	43.51±5.14 a	6.15±0.67 a	4.43±0.55 a	35.22±3.46 a
NPDS + LPS	161.24±18.25 ab	54.78±5.90 ab	7.27±0.35 ab	5.55±0.43 ab	42.80±3.26 ab
LPD + LPS	210.62±18.21 a	36.32±4.66 a	5.47±0.55 a	4.12±0.50 a	28.56±5.88 ad
LPDS + LPS	181.43±15.63 ac	46.38±4.25 ace	5.88±0.31 ae	4.56±0.37 ae	30.68±4.02 ae

The results are presented as mean±SD (n=6). One-way ANOVA and SNK multiple comparisons test were used. P<0.05 was considered statistically significant. 'a' all groups compared with NPD, 'b' NPD+LPS vs NPDS+LPS, 'c' LPD+LPS vs LPDS+LPS, 'd' NPD+LPS vs LPD+LPS, and 'e' NPDS+LPS vs LPDS+LPS.

Table 2. The effect of silymarin on LPS-induced changes in liver TBARS and GSH levels, and SOD, CAT and GST activities of rats fed NPD or LPD

Groups	TBARS (nmol/g)	GSH (nmol/100 mg)	SOD (U/mg of protein)	CAT (U/mg of protein)	GST (mmol/mg of protein)
NPD	255.11±20.54	177.52±14.10	5.42±0.41	7.35±0.53	85.32±5.14
NPD + LPS	373.15 ±29.70 a	131.92 ±6.98 a	3.64±0.64 a	5.26±0.62 a	57.57±13.27 a
NPDS + LPS	307.39±21.76 ab	152.80±5.81 ab	4.97±0.46 ab	6.69±0.40 ab	78.18±7.00 ab
LPD + LPS	428.39±21.76 ad	119.20±13.99 ad	2.64±0.53 ad	3.95±0.49 ad	48.67±8.04 a
LPDS + LPS	385.56±53.88 ace	127.64±13.47 a	3.08±0.48 ae	4.56±0.58 ae	58.50±4.88 ae

The results are presented as mean±SD (n=6). One-way ANOVA and SNK multiple comparisons test were used. P≤0.05 was considered statistically significant. 'a' all groups compared with NPD, 'b' NPD+LPS vs NPDS+LPS, 'c' LPD+LPS vs LPDS+LPS, 'd' NPD+LPS vs LPD+LPS, and 'e' NPDS+LPS vs LPDS+LPS.

injury (Münter, Mayer & Faulstich, 1986). These actions along with anti-peroxidative property make silymarin a suitable candidate for the treatment of iatrogenic and toxic liver diseases. The results of this study concur with others who have found that protein malnourished patients have increased sensitivity to septic shock (Gernaat *et al.*, 1998). Additionally, the results showed that silymarin significantly decreased the hepatic enzymes (AST and ALT) activities only in the NPD-fed rats. These results substantiate the hypothesis that protein malnutrition may lead to dysfunction of the liver cells (Kravchenko & Tutel'ian, 1990).

Clinical and experimental studies report that protein malnutrition increases plasma proinflammatory cytokines, such as TNF- α , IL-1 and IL-6 (Ling *et al.*, 2004) and increases sensitivity to septic shock (Gernaat *et al.*, 1998). This study found similar results in which LPS significantly increased the proinflammatory cytokines in all dietary groups; however, the effects of LPS were found to be significantly higher in the protein malnourished rats compared to rats fed the normal-protein diet. Silymarin supplementation showed protection against the LPS-induced elevation in those pro-inflammatory markers. It is well established that pro-inflammatory cytokines play a crucial role in the development of LPS-induced septic shock (Kang *et al.*, 2004). Increased sensitivity to septic shock has been reported in protein malnourished patients (Gernaat *et al.*, 1998). In an experimental study, Li *et al.* (2002) showed that mice fed a protein-restricted diet resulted in a significant increase in LPS-induced NF- κ B activation and transcription levels of its downstream genes, IL-1 β and TNF- α . Sauerwein *et al.* (1997) reported that plasma concentrations of the inflammatory cascade mediators including IL-6, CRP and the soluble receptors of TNF- α (sTNFR-p55 and sTNFR-p75) are greater in protein malnourished children particularly

in those suffering with kwashiorkor. As shown in this study, the protein malnourished animals fed the LPD and challenged with LPS had increased levels of the proinflammatory markers, including TNF- α , IL-1 β and IL-6, and that silymarin acted as an anti-inflammatory compound. Silymarin has been shown to have an anti-inflammatory effect and an inhibitory action on TNF- α expression and ornithine decarboxylase activity (Kang *et al.*, 2004; Zi, Mukhtar & Agarwal, 1997; Agarwal *et al.*, 1994).

Endotoxin-induced activation of macrophages and cytokines following the subsequent formation of reactive oxygen and nitrogen species has central pathogenic importance in various inflammatory diseases, including septic shock (Bian & Murad, 2001). The lung is the most frequently identified organ damaged in septic shock. However, the precise mechanism of lung injury-induced by LPS under different dietary conditions remains unclear and requires further elucidation. In this study, LPS induced oxidative damage in lung and liver tissues was found, as evidenced by increased lipid peroxidation (TBARS) with a concomitant decrease in endogenous antioxidant GSH levels in the LPD model. In this study, septic shock induced a significant increase in lung and liver TBARS levels along with a depletion of tissue GSH levels, which might indicate the enhancement of lipid peroxidation as a result of an impaired antioxidant defense mechanism. Since silymarin treatment in this study was associated with the preservation of GSH and inhibition of lipid peroxide level, it appeared that silymarin attenuates toxicity through its antioxidant effects. Present data showed that silymarin significantly ameliorated LPS-induced TBARS increase and GSH decrease in both the tissues of rats fed with NPD or LPD, although, GSH levels were depleted and TBARS levels were increased to a greater extent in the LPDS+LPS group compared to the NPDS+LPS group. Protein malnutrition

itself reduced rats' liver GSH levels by around 65% compared to those that are well-nourished (Ling *et al.*, 2004). These changes are similar to responses observed during inflammation. Hepatic GSH levels have been shown to inversely correlate with the levels of NF- κ B activation in the liver, leading to an increase in the transcription of IL-1 β and TNF- α (Sies, 1999). In this study, LPS affected the levels of TNF- α , IL-1 β , IL-6 and albumin to a greater extent in malnourished rats compared to well-nourished rats challenged with LPS. Thus, it is reasonable to consider that the reduction in GSH concentration in the lung and liver induced by dietary protein depletion might be one of the important contributing factors in the activation of a systemic inflammatory response in septic rats.

The SOD activity analysis in the lung and liver demonstrated a significant decrease after the LPS challenge in both the dietary groups. Silymarin supplementation significantly ameliorated the activity only in the NPD fed rats. One mechanism by which extracellular SOD might modulate neutrophil inflammation is by reducing cytokine release from macrophages. These findings suggest that extracellular SOD should be considered as an anti-inflammatory enzyme as well as a major antioxidant (Bowler & Crapo, 2002). Catalase is also an important enzyme that reduces H₂O₂ to H₂O, removing a key intermediate in the hydroxyl radical (OH•) and acid hypochlorous formation. In this study, CAT activity decreased in the LPS challenge groups, particularly in the LPD-fed rats, whilst in the silymarin supplemented groups, it was increased significantly only in the NPD-fed group compared to the corresponding animals without silymarin supplementation.

CONCLUSION

In conclusion, these findings provide evidence supporting the role of silymarin against the impairment of antioxidation

and immune response in protein-malnourished rats, particularly in septic shock conditions. The protective effect of silymarin can be attributed, at least in part, to its ability to balance oxidant-antioxidant status and to regulate the inflammatory mediators, particularly in a malnourished condition, in the treatment of multi-organ failure due to septic shock.

ACKNOWLEDGEMENT

This study was made possible by financial support provided by the King Saud University.

Conflict of Interest

There were no conflicts of interest in the reporting of the outcomes of this study.

REFERENCES

- Adenuga GA, Adebayo OL & Adegbesan BO (2008). The response of liver lipid peroxidative and antioxidant defense systems of protein undernourished rats to liver regeneration. *Ind J Clin Bioch* 23(1): 29-32.
- Aebi H (1983). Catalase. In *Methods of Enzymatic Analysis*. Bergmeyer H (ed.). Verlag Chemie, Weinheim 3: 273-277.
- Agarwal R, Katiyar SK, Lundgren DW & Mukhtar H (1994). Inhibitory effect of silymarin, an anti-hepatotoxic flavonoid, on 12-O-tetradecanoyl-phorbol-13-acetate-induced epidermal ornithine decarboxylase activity and mRNA in SENCAR mice. *Carcinogenesis* 15(6): 1099-1103.
- Aparecida de França S, Dos Santos MP, Garófalo MA, Navegantes LC, Kettelhut ID, Lopes CF & Kawashita NH (2009). Low protein diet changes the energetic balance and sympathetic activity in brown adipose tissue of growing rats. *Nutrition* 25(11-12): 1186-1192.
- Baluchnejadmojarad T, Roghani M & Mafakheri M (2010). Neuroprotective effect of silymarin in 6-hydroxydopamine hemiparkinsonian rat: Involvement of estrogen receptors and oxidative stress. *Neuroscience Letters* 480(3): 206-210.

- Beishuizen A, Vermes I & Haanen C (1998). Endogenous mediators in sepsis and septic shock. *Ad Clin Chem* 33: 55-131.
- Bian K & Murad F (2001). Diversity of endotoxin-induced nitrotyrosine formation in macrophage-endothelium-rich organs. *Free Rad Biol Med* 31(4): 421-429.
- Bowler RP & Crapo JD (2002). Oxidative stress in airways: is there a role for extracellular superoxide dismutase? *Am J Respir Crit Care Med* 166(12 Pt 2): S38-S43.
- Bradley SG (1979). Cellular and molecular mechanisms of action of bacterial endotoxins. *Annu Rev Microbiol* 33: 67-94.
- Chamson-Reig A, Thyssen SM, Hill DJ & Arany E (2009). Exposure of the pregnant rat to low protein diet causes impaired glucose homeostasis in the young adult offspring by different mechanisms in males and females. *Exp Biol Med* 234(12): 1425-1436.
- Chtourou Y, Fetoui H, Sefi M, Trabelsi K, Barkallah M, Boudawara T, Kallel H & Zeghal N (2010). Silymarin, a natural antioxidant, protects cerebral cortex against manganese-induced neurotoxicity in adult rats. *Biometals* 23(6): 985-996.
- Cinel I & Opal SM (2009). Molecular biology of inflammation and sepsis: a primer. *Crit Care Med* 37(1): 291-304.
- Fleck A (1989). Clinical and nutritional aspects of changes in acute-phase proteins during inflammation. *Proc Nutr Soc* 48(3): 347-354.
- Gernaat HB, Dechering WH & Voorhoeve HW (1998). Mortality in severe protein-energy malnutrition at Nchelenge, Zambia. *J Trop Pediatr* 44(4): 211-217.
- González-Renovato ED, Alatorre-Jiménez M, Bitzer-Quintero OK, Sánchez-Luna S, Flores-Alvarado LJ, Romero-Dávalos R, Romero L, Hernández-Andalón JJ, Ortiz GG & Pacheco-Moisés FP (2013). Effect of Nutrisim© on endotoxic shock induced by lipopolysaccharide from *Escherichia coli*: 0111:b4 in rats: Structural study of liver, kidney and lung. *J Clin Exp Pathol* 4 (1): 1-5.
- Habig WH, Pabst MJ & Jakoby WB (1974). Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* 249(22): 7130-7139.
- Institute for Laboratory Animal Research. (1996). Guide for the care and use of laboratory animals. (National Research Council). Washington, DC: National Academy of Sciences.
- Kakkar P, Das B, & Viswanathan PN (1984). A modified spectrophotometric assay of superoxide-dismutase. *Ind J Biochem Biol* 21(2): 130-132.
- Kang JS, Jeon YJ, Park SK, Yang KH, Kim HM (2004). Protection against lipopolysaccharide-induced sepsis and inhibition of interleukin-1beta and prostaglandin E2 synthesis by silymarin. *Biochem Pharmacol* 67(1): 175-181.
- Karimi G, Ramezani M & Tahoonian Z (2005). Cisplatin nephrotoxicity and protection by milk thistle extract in rats. *Evid Based Complement Alternat Med* 2(3): 383-386.
- Kravchenko LV & Tutel'ian VA (1990). Dietary protein as a modulator of the activity of enzymes metabolizing xenobiotics UDP glucuronosyltransferase and glutathione transferase. *Vopr Pitan* 6: 64-68.
- Li J, Quan N & Bray TM (2002). Supplementation of N-acetylcysteine normalizes lipopolysaccharide-induced nuclear factor kappaB activation and proinflammatory cytokine production during early rehabilitation of protein malnourished mice. *J Nutr* 132(11): 3286-3292.
- Ling PR, Smith RJ, Kie S, Boyce P & Bistrrian BR (2004). Effects of protein malnutrition on IL-6-mediated signaling in the liver and the systemic acute-phase response in rats. *Am J Physiol- Regul Integr Comp Physiol* 287(4): R801-R808.
- Millward DJ & Jackson AA (2004). Protein/energy ratios of current diets in developed and developing countries compared with a safe protein/energy ratio: implications for recommended protein and amino acid intakes. *Public Health Nutr* 7(3): 387-405.
- Moshage H (1997). Cytokines and the hepatic acute phase response. *J Pathol* 181(3): 257-266.
- Müller O & Krawinkel M (2005). Malnutrition and health in developing countries. *CMAJ* 173(3): 279-86.

- Münter K, Mayer D & Faulstich H (1986). Characterisation of a transporting system in rat hepatocytes. Studies with competitive and non-competitive inhibitors of phalloidin transport. *Biochemical Biophysical Acta* 860(1): 91-98.
- Pires-de-Mélo IH, Wanderley Dos Reis F, Luz LS, Paz ST, Silva HJ, Souza SL & Leandro CG (2009). Short- and long-term effects of a neonatal low-protein diet in rats on the morphology of the larynx. *Nutrition* 25(7-8): 855-860.
- Rivers E, Nguyen B, Havstad S, Ressler J, Muzzin A, Knoblich B, Peterson E, Tomlanovich M & Early Goal-Directed Therapy Collaborative Group (2001). Early goal-directed therapy in the treatment of severe sepsis and septic shock. *N Engl J Med* 345(19): 1368-1377.
- Sandoval M, Zhang XJ, Liu X, Mannick EE, Clark DA, Miller MJ (1997) Peroxynitrite-induced apoptosis in T84 and RAW 264.7 cells: Attenuation by L-ascorbic acid. *Free Radic Biol Med* 22(3): 489-495.
- Sauerwein RW, Mulder JA, Mulder L, Lowe B, Peshu N, Demacker PN, van der Meer JW & Marsh K (1997). Inflammatory mediators in children with protein-energy malnutrition. *Am J Clin Nutr* 65(5): 1534-1539.
- Sedlak J & Lindsay RH (1968). Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem* 25(1): 192-205.
- Sies H (1999). Glutathione and its role in cellular functions. *Free Radic Biol Med* 27(9-10): 916-921.
- Soto C, Recoba R, Barrón H, Alvarez C & Favari L (2003). Silymarin increases antioxidant enzymes in alloxan-induced diabetes in rat pancreas. *Comp Biochem Physiol C Toxicol Pharmacol* 136(3): 205-212.
- Toklu HZ, Tunali-Akbay T, Velioglu-Ogunc A, Ercan F, Gedik N, Keyer-Uysal M, Sener G (2008). Silymarin, the antioxidant component of *Silybum marianum*, prevents sepsis-induced acute lung and brain injury. *J Surg Res* 145(2): 214-222.
- Valenzuela A & Garrido A (1994). Biochemical bases of the pharmacological action of the flavonoid silymarin and of its structural isomer silibinin. *Biol Res* 27(2): 105-112.
- Volanakis JE (2001). Human C-reactive protein: expression, structure, and function. *Mol Immunol* 38(2-3): 189-197.
- von Dessauer B, Bongain J, Molina V, Quilodrán J, Castillo R, Rodrigo R (2011). Oxidative stress as a novel target in pediatric sepsis management. *J Crit Care* 26(1):103.e1-7.
- Wenzel RP, Pinsky MR, Ulevitch RJ & Young L (1996). Current understanding of sepsis. *Clin Infect Dis* 22(3): 407-413.
- Wu JW, Lin LC, Hung SC, Chi CW, Tsai TH (2007). Analysis of silibinin in rat plasma and bile for hepatobiliary excretion and oral bioavailability application. *J Pharm Biomed Anal* 45(4): 635-641.
- Zi X, Mukhtar H & Agarwal R (1997). Novel cancer chemopreventive effect of a flavonoid antioxidant silymarin: inhibition of mRNA expression of an endogenous tumor promoter TNF alpha. *Bioch Biophys Res Commun* 239(1): 334-339.

