Marmara Pharmaceutical Journal 19: 52-59, 2015
DOI: 10.12991/mpj.2015198610

ORIGINAL RESEARCH

Investigation of the effect of silymarin on the liver in experimental sepsis

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ABSTRACT

The aim of the study is to investigate the possible protective effect of silymarin (extract of *Silybum marianum*) on the liver of septic rats was at early stage of sepsis. Polymicrobial sepsis was induced by the cecal ligation and perforation (CLP) technique. Sepsis and sepsis + silymarin treated groups received vehicle or silymarin (50 mg/kg, orally). The rats were decapitated 6 h after the CLP procedure. Protein, glutathione, lipid peroxidation levels, tissue factor activity and some enzyme activities were determined and polyacrylamid gel electrophoresis was carried on liver samples. TNF-a, IL-1b and IL-6 were determined in blood samples. Glutathione levels significantly increased and tissue factor activity significantly decreased in both sepsis and sepsis+silymarin treated groups when compared with control. Superoxide dismutase activity significantly decreased in sepsis+silymarin treated group when compared control and sepsis groups. No significant difference was found in electrophoresis protein bands. Blood proinflammatory cytokine levels were significantly decreased in silymarin treated sepsis group when compared with sepsis group. The effect of silymarin was not apparent since oxidative damage was not obvious in liver consequently it can be suggested that some protective mechanisms related with glutathione in liver may be involved in early stage sepsis.

Key Words: Sepsis, Silymarin, Liver, Tissue factor, Oxidative stress

1. INTRODUCTION

The incidence of sepsis is expected to rise during the next decade owing to the aging population, a growing immunosuppressed population, the increased use of invasive catheters and prosthetic materials, and the growing problem of antimicrobial resistance (1-3).

Sepsis causes disruption of homeostasis through a currently uncontrollable cascade of excessive inflammation and coagulation with impaired fibrinolysis that contributes to an inflammatory response, microvascular hypoperfusion, organ dysfunction, and increased mortality. The magnitude of disruption in homeostasis is influenced by the virulence of the causative pathogens and the host's response to the infection (3-7).

Sepsis can occur as a result of infection at any body site, including the lung, abdomen, skin or soft tissue, or urinary tract (4). Bacteria are the pathogens most commonly associated with the development of sepsis, although fungi, viruses, and parasites can cause sepsis. The pathophysiology of sepsis is initiated by the outer membrane components of

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both gram-negative organisms (lipopolysaccharide [LPS], lipid A, endotoxin) and gram-positive organisms (lipoteichoic acid, peptidoglycan). These outer membrane components are able to bind to the CD14 receptor on the surface of monocytes. By virtue of the recently described toll-like receptors, a signal is then transmitted to the cell, leading to the eventual production of the proinflammatory cytokines tumor necrosis factor-alpha (TNF-alpha) and interleukin-1 (IL-I). These cytokines have a direct toxic effect on tissues (1,4).

Some disturbances in coagulation system can be seen in sepsis. Tissue factor (TF), coagulation FIII, is expressed on the surfaces of the endothelium and of monocytes. TF leads to the production of thrombin, which is a pro-inflammatory substance itself. Fibrinolysis is also impaired during the septic process. IL-1 and TNF-alpha lead to the production of plasminogen activator inhibitor-1, a potent inhibitor of fibrinolysis (4,5).

The extracts of the flowers and leaves of *Silybum marianum* (St. Mary's thistle, milk thistle) have been used for centuries to treat liver, spleen, and gallbladder disorders (8). Silmarin is an flavonolignan mixture that most of the clinical studies were carried out. The main constituents are silibinin, isosilibinin, silicristin, and silidianin (9). One of the important issues about silymarin is that it may be accepted as a safe herbal product, since no health hazards or side effects are known in conjunction with the proper administration of designed therapeutic dosages (10). Furthermore, its antioxidant, anti-inflammatory, and anticarcinogenic properties were demonstrated in the studies conducted with silymarin against oxidative stress, inflammatory responses, and benzoil peroxide-induced tumor promotion in mice (11, 12).

To investigate possible oxidative and degenerative changes induced by sepsis and the putative protective role of oral silymarin treatment in the liver, protein, glutathione (GSH), lipid peroxidation (LPO) levels, TF activity and some enzyme activities such as carbonic anhydrase (CA), glutathion-S-transferase (GST), catalase (CAT), superoxide dismutase (SOD), alkalen phosphatase (ALP), acid phosphatase (ACP) were determined. In addition polyacrylamid gel electrophoresis was carried on liver samples.

2. MATERIALS AND METHODS

2.1 Animals and Protocol for the Induction of Sepsis

Wistar albino rats of both sexes, weighing 250 to 300 g, were fasted for 12 h, but allowed free access to water before the experiments. The animals were kept in individual wire-bottom cages, in a room at a constant temperature (22 ± 2°C) with 12-h light and dark cycles, and fed standard rat chow. The study was approved by the Marmara University School of Medicine Animal Care and Use Committee (19.02.2007-70.2006.mar).

Table 1 : Tumor necrosis factor-alpha (TNF-a), interleukin-1b (IL-1b) and interleukin-6 (IL-6) levels in serum samples of all groups (n=6 per group)

Groups	TNF-a (pg/mL)	IL-1b (pg/mL)	IL-6(pg/mL)
Control	3.6 ± 0.6	2.5 ± 0.5	2.63 ± 0.5
Sepsis	24.0 ± 1.3 ***	51.2 ± 2.6 ***	27.6±2.4***
Sepsis +Silymarin	6.13 ± 5.4 +++	15.6 ±2.1 ***,+++	7.26 ± 1.9 +++
$P_{(ANOVA)}$	0.0001	0.0001	0.0001

Values are given mean ± Standard deviation. ***p<0.001: significantly different from control group; +++p<0.001: significantly different from sepsis group.

The rats were divided into the following three groups of six rats (three males and three females) each; 1- vehicle-treated sham operated control (C) group, 2- vehicle treated cecal ligation and perforation (CLP) group (sepsis group) and 3- silymarin-treated CLP (Sepsis+ Silymarin) group.

Polymicrobial sepsis was induced by the CLP technique described previously (13,14).

Under brief ether anesthesia, a midline laparotomy was made using minimal dissection and the cecum was ligated just below the ileocaecal valve with 3-0 silk, so that intestinal continuity was maintained. The antimesentric surface of the cecum was perforated with an 18 gauge needle at two locations 1 cm apart and the cecum was gently compressed until fecal matter was extruded. The bowel was then returned to the abdomen and the incision was closed. At the end of the operation, all rats were resuscitated with saline, 3 ml/100 g body weight given subcutaneously. Postoperatively, the rats were deprived of food, but had free access to water for the next 6 hours until they were killed. The sham-operated groups were given a laparotomy, and the cecum was manipulated, but not ligated or perforated.

2.2 Silymarin Treatment

Silymarin (Mikrogen Pharmaceuticals), used in this study is a dried extract of *Silybum marianum*. Silymarin was suspended in %0.5 methyl cellulose. Sham-operated (control) and sepsis groups received vehicle or silymarin (50 mg/kg, orally) 10 days prior to the sepsis induction and a single dose immediately after the operation. The rats were

Table 2: Protein, glutathione (GSH), lipid peroxidation (LPO) levels, tissue factor (TF) activity and activities of carbonic anhydrase (CA), glutathion-S-transferase (GST), catalase (CAT), superoxide Dismutase (SOD), alkalen phosphatase(ALP), acid phosphatase (ACP) enzymes in liver tissue samples of all groups (n=6 per group)

Groups Parameters	Control	Sepsis	Sepsis+Silymarin	P(Anova)
GSH(mg/mg protein)	0.57 ± 0.10	1.14 ± 0.33**	0.98 ± 0.24*	**0.003**
GST(U/mg protein)	0.56 ± 0.11	0.63 ± 0.12	0.63 ± 0.17	**0.62**
LPO(nmolMDA/mg protein)	0.38 ± 0.09	0.35 ± 0.10	0.30 ± 0.07	**0.33**
CAT(U/mg protein)	30.15 ± 4.11	35.17 ± 6.24	33.22 ± 2.09	**0.18**
SOD(U/mg protein)	1.27 ± 0.06	1.24 ± 0.06	1.04 ± 0.06***,$^{+++}$	**0.0001**
CA(U/mg protein)	0.54 ± 0.06	0.51 ± 0.08	0.52 ± 0.11	**0.86**
TFactivity(sec)	210.67 ± 26.60	330.33 ± 48.03***	294.83 ± 20.58**	**0.0001**
ACP(U/g protein)	40.27 ± 5.01	46.60 ± 6.50	49.42 ± 7.99	**0.08**
ALP(U/g protein)	2.01 ± 0.98	2.63 ± 0.92	3.03 ± 0.78	**0.17**
Protein (total) (mg/g tissue)	195.80 ± 18.41	204.21 ± 10.19	207.24 ± 23.74	**0.55**

Values are given mean ± Standard deviation. *p<0.05, **p<0.01 and ***p<0.01: significantly different from control group; $^{+}$p<0.05, $^{+++}$p<0.01: significantly different from sepsis group. Since the clotting time is inversely proportional to the TF activity, the lengthening of the clotting time is a manifestation of decreased TF activity.

decapitated 6 h after the CLP procedure, and liver tissue samples were immediately taken and stored at

-70 ^{o}C. Afterwards, protein, GSH, LPO levels, TF activity and CA, GST, CAT, SOD, ALP, ACP enzyme activities were determined. In addition polyacrylamid gel electrophoresis was carried on liver samples. Serum samples were evaluated for TNF-α, IL-1β, and IL-6.

2.3 Biochemical assays

2.3.1 Serum TNF-α, IL-1β, and IL-6 analysis

Serum TNF-α, IL-1β, and IL-6 were quantified according to the manufacturer's instructions and guidelines using enzyme-linked immunosorbent assay (ELISA) kits specific for the previously mentioned rat cytokines (Biosource International, Nivelles, Belgium).

2.3.2 Livers Tissue Analysis

Liver tissue protein, LPO, GSH levels, TF activity and CA, GST, CAT, SOD, ALP, ACP enzyme activities were determined by the methods of Lowry (15), Ledwozyw (16), Beutler (17), Ingram (18), Vepoorte (19), Habig (20), Aebi (21), Mylorie (22), Walter (23) respectively. Electrophoretic examination of liver proteins was carried out by Laemmli SDS-polyacrylamid gel electrophoresis (24).

The TF activity was measured by Quick's one-stage prothrombin time determination test (18). In one-stage prothrombin time determination test, a commercial tissue thromboplastin (tissue factor) is mixed with equal amounts of plasma and Ca++. A firm clot forms in a short time, the time being dependent on the potency of the tissue thromboplastin used. We modified the above test by replacing commercial tissue thromboplastin with the tissue homogenates we prepared. Since the clotting time is inversely proportional to the TF activity, the lengthening of the clotting time is a manifestation of decreased TF activity.

2.4 Statistical Analysis

Statistical analysis was done using SPSS 11.5. All data are expressed as means ± S.D. Groups of data were compared with an analysis of variance (ANOVA) followed by Bonferroni and Dunnet's test. Pearson correlation analysis was used to investigate the relations between the parameters. Values of p<0.05 were considered as significant (25).

3. RESULTS

In serum samples , TNF-a, IL-1b and IL-6 levels significantly increased in sepsis group when compared with control group. The rise in these serum levels were significantly abolished with silymarin treatment (Table 1).

In early stage of sepsis oxidative damage was not obvious in liver tissue. GSH levels significantly increased and TF activity significantly decreased in both sepsis and sepsis+silymarin treated groups when compared with control. SOD activity significantly decreased in sepsis+silymarin treated group when compared control and sepsis groups. Other biochemical parameters were not changed significantly (Table 2).

No significant differences were found between protein bands obtained in electrophoresis (Fig. 1). There was a negative significant correlation between GSH and TF activity ($r=-0.69$, $p<0.01$); positive significant correlation between ACP and CA activity and ALP activity ($r=0.53$, $p<0.05$; $r=0.61$, $p<0.01$).

4. DISCUSSION

In this study, the CLP method was used as the sepsis animal model because its biphasic clinical manifestations and early (hyperdynamic) and late (hypodynamic) stages mimic those in humans. It has been demonstrated that the early stage persisted from 2 to 10 hours and the late stage occurs at 16 to 20 h after CLP (26). Therefore early stage of sepsis (6 hr) was chosen for preventing the organ damage at the beginning of sepsis. Sepsis is associated with the development of progressive damage in multiple organ systems remote from the locus of infection (27). There is an increasing evidence that oxidative stress has an important role in the development of sepsis-induced multiorgan failure (28). As the liver is an important organ affected by oxidative stress both in early sepsis or late sepsis and it plays an important role and it is a major organ responsible for the initiation of multiple organ dysfunction (29-32).

In the present study the possible oxidative and degenerative changes induced by sepsis and the putative protective role of oral silymarin treatment in the liver were investigated by determining liver protein, GSH, LPO levels, TF activity and activities of some liver enzymes.

During infection or after experimental induction of sepsis, TNF, IL-1, or IL-6, TF expression is rapidly up-regulated on circulating monocytes and endothelial cells. On the other hand, cytokines are known to peak up to this time point, causing free radical formation and oxidative tissue injury (27). The early effects of sepsis and antioxidant treatment on liver tissue were investigated in the present study. Consistent with the others, our data also showed the significantly increase in serum cytokines levels within hours following CLP, while these increases were attenuated by silymarin treatment (29, 30, 33). Leng-Peschlow have demonstrated that the anti-inflammatory effect of silymarin is through the inhibition of leukotrien production, which is known as a potent inflammatory mediator (29). Furthermore, IL-1b, a proinflammatory cytokine, which is increased during inflammatory processes and plays a crucial role in the development of the LPS-induced sepsis, was inhibited by silymarin treatment (33).

In the present study, the liver LPO, SOD, GST, ALP, and protein levels of sepsis group were not significantly

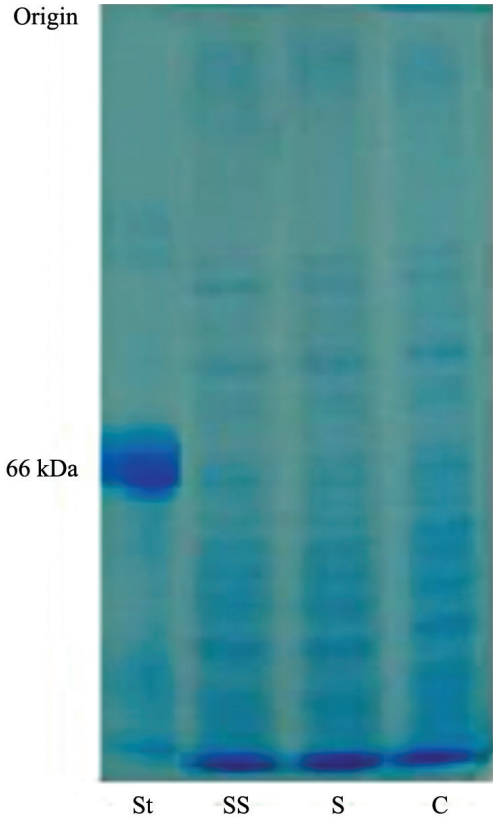

Figure 1: Samples of liver SDS-PAGE

St: Standard-Bovine Albumine, C: Control group,
S: Sepsis group,
SS: Sepsis+Silymarin group

different from those of controls. These result are compatible with early sepsis studies (26, 30, 34). Although hepatic failure during sepsis is generally believed to be late complication of pulmonary and renal failure (35). Wang and Chaudary reported that the hepatocellular dysfunction occurs as early as 1.5 h after the onset of sepsis and is further depressed during the progression of sepsis (36). In the present study liver ACP and CA activity were investigated for the first time and non significant differences were found in these parameters between sepsis and control groups. This finding supports that hepatic failure is not occurred early stage of sepsis.

Important and different result of the present study was the significant increase of the liver GSH levels in sepsis group than those of controls. The GSH level is altered in many inflammatory conditions. A fall in GSH concentration has been reported in human diseases such as HIV infection and trauma (37, 38). In animal models, during the initial phase of septic shock (during the first hours after infection), tissue GSH concentrations are decreased whereas GSSG

concentrations are increased (39, 40). In contrast, during the first days following infection, tissue GSH levels are elevated in treated animals compared with controls (41,42). However, in the later stages of infection, depletion of tissue GSH levels occurs (43,44).

Variations in glutathione levels during oxidative conditions may result from modification in synthesis and/or loss. The infectious and inflammatory state, as well as nutritional status, modulate glutathione tissue levels (41, 45, 46). However, the mechanisms that lead to these metabolic modifications are still poorly understood.

Numerous studies have shown that tissue and plasma GSH concentrations are depleted during either protein deficiency or food restriction (46,41).

Tateishi et.al. have suggested that turnover of the hepatic GSH was much faster in fasted rats than in refed ones and the degradation rate of hepatic GSH appears to be involved in regulating the hepatic GSH level (47).

Breuille et al (43) have reported that in spite of strong anorexia, GSH concentration of septic rats was greater than pair-fed rats in nearly all tissues, only blood GSH level was decreased, liver GSH concentrations were even greater than the values that had measured in healthy fed rats and suggested that the inflammatory challenge overcomes the influence of food restriction. Others have described the unchanged or increased GSH status in different tissues 24 and 48 h after induction of stress in animal models (41, 46, 48). It has been suggested that the organism is able to improve GSH status through an increase in GSH concentration in response to infection, that may represent a mechanism to protect against oxidative damage. Indeed, a higher rate of mortality has been observed in rats exposed to hyperoxia and GSH depletion (49).

The increased total liver GST activity has been suggested as an accelerated loss of GSH from the body by the excretion of reduced GSH conjugates, to protect cells against toxic compounds (48). In the present study there was non-significant increase in GST activity. High GSH levels found in the septic rats may be thought as GSH synthesis rate is enough to support increased utilization rate. In Toklu et al study (50), which the experimental condition was the same with ours, tissue GSH levels were found to be decreased while MDA levels and MPO activity were found to be increased both in the lung and the brain tissues due to CLP. These results also support that liver is affected by sepsis later than brain and lung.

Silymarin is a polyphenolic flavonoid extracted from the milk thistle that has a strong antioxidant activity and exhibits cytoprotective, anti-inflammatory, and anticarcinogenic effects (9, 50-53) and is used clinically to treat chronic inflammatory liver disease and hepatic cirrhosis. It is also useful in toxin-induced liver toxicity, including Amanita phalloides mushroom poisoning. Hepatoprotection can be attributed to its antioxidant properties by scavenging free radicals and increasing intracellular concentration of GSH (54). Thus, silymarin decreases production of superoxide anion radicals and NO. Furthermore, several reports indicate that tissue injury, induced by various stimuli, is coupled with GSH depletion (55).

In the present study, silymarin treatment significantly decreased liver SOD activity. The decrease in SOD activity may be attributed to the metabolism of silymarin.

TF is a transmembrane procoagulant glycoprotein and a member of the cytokine receptor superfamily. It is important for both normal homeostasis and the development of many thrombotic diseases (4,5,56). Recent studies conducted in our laboratory showed that several tissues (lung, heart, aorta, kidney, liver, spleen, pancreas, brain) have different TF activity (57,50) and it changes according to the diet or systemic diseases. TF, used for measuring prothrombin time is not a stabile protein. Its activity can easily be changed by the changed membrane composition, heating, changing pH, or the lipid peroxidation of membrane due to oxidative stress (58-60). Although TF activity was studied in various animal models, it is still unclear which mechanisms regulate the TF activity during sepsis (61).

In our study liver tissue of septic rats had low TF activity compared to control group. On the other hand the significant negative correlation was there between TF activity and GSH levels in general and in control group. The decrease in TF activity may demonstrate the decreased tendency of clot formation and my protect the liver against thrombus risk by the means of different mechanism. In the Toklu et al study which has similar experimental conditions to ours, the increase of brain TF activity and decrease of lung TF activity have been found in sepsis group compared to control (50). Since vascular beds have specific properties, the dysfunction of different organ systems in severe sepsis may exert heterogenic response.

The decrease in TF activity may be because of DIC. Experimental studies suggests that DIC formation occurs in the first 4 hours of sepsis (62). According to the report about that anticancerogenic and antiinflammatory effects of silymarin were due to the inhibiting kinases and activating NF-kB (53, 55). We expected to find the decrease in TF activity in silymarin given sepsis group because of the decrease in TF expression. However we did not find any

significant difference between sepsis and silymarin treated sepsis groups in point of TF activity. This finding may also show that liver is not affected in early stages of sepsis as TF factor can be contributed to the tissue damage. The significant negative correlation found between TF and ALP in sepsis group may also indicate oxidative damage was not obvious in early stage sepsis. The significant positive correlation found between TF and CA in silymarin treated sepsis group may indicate the effect of silymarin nötralizing the TF activity via changing pH.

5. CONCLUSION

Significant increase in liver GSH levels and significant decrease in liver TF activity and no significant changes found in all other liver parameters 6 hours after sepsis induction by CLP may indicate that some protective mechanisms related with TF in liver may be involved in early phase of sepsis.

Significant decrease in liver SOD activity and non-significant decrease in liver GSH levels after receiving silymarin 10 days prior to the sepsis induction and a single dose immediately after the operation, may due to the metabolism of silymarin rather than protective effect of silymarin on the liver. Further studies are necessary to point out the mechanisms of early stage sepsis.

Acknowledgment

The authors are grateful to Prof.Dr.Ahmet Dirican from University of Istanbul, for statistical analysis. This study was supported by a grant from Scientific Research Project Department of Marmara University (Projects No: SAG-D-010208-0019.

Silmarinin Karaciğer Üzerine Etkisnin Deneysel Sepsis Modelinde Incelenmesi

ÖZET

Çalışmanın amacı, sepsisin erken safhasında silymarin (*Silybum marianum* ekstresinin ana bileşeni) nin septik sıçanların karaciğeri üzerindeki etkisini araştırmaktır. Polimikrobiyal sepsis, çekal ligasyon ve perforasyon (CLP) tekniği ile oluşturuldu. Sepsis ve sepsis + silymarin gruplarına çözücü (%0.5 methyl cellulose) veya silymarin (oral, 50 mg / kg) verildi. Sıçanlar CLP işlemden 6 saat sonra dekapite edildi. Protein, glutatiyon, lipid peroksidasyon düzeyleri, doku faktörü aktivitesi ve bazı enzim aktiviteleri tayin edildi ve karaciğer örneklerine poliakrilamid jel elektroforezi uygulandı. Kan örneklerinde TNF-a IL-1b ve IL-6 tayin edildi. Sepsis ve sepsis + silymarin gruplarında kontrol grubuna göre glutatyon düzeyleri anlamlı dercede arttı, doku faktörü aktivitesi ise anlamlı derecede azaldı. Sepsis + silymarin grubunda süperoksit dismutaz aktivitesi kontrol ve sepsis gruplarına göre anlamlı derecede azaldı. Elektroforezde elde edilen protein bantları karşılaştırıldığında gruplar arasında anlamlı fark bulunmadı. Kan proinflamatuvar sitokin düzeyleri silmarin ile tedavi edilen sepsis grubunda sepsis grubuna göre anlamlı derecede azaldı. Karaciğerde oksidatif hasarın belirgin olmaması nedeniyle silmarinin etkisi tam olarak tespit edilemedi. Ancak sepsisin erken evresinde karaciğerde glutatyon ile ilgili bazı koruyucu mekanizmaların aracılık ettiği ileri sürülebilir.

Anahtar kelimeler: Sepsis, Silmarin, Karaciğer, Doku Faktörü, Oksidatif Stress

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