The effect of silymarin on the liver in thermal burn injury

Evren YEMİSEN, Aysen YARAT, Tugba TUNALI AKBAY, Hale TOKLU, Göksel ŞENER

ABSTRACT
Hepatic homeostasis and metabolism are essential for survival in critically ill and severely burned patients. Silymarin, the major component of thistle milk has been shown to have hepatoprotective effect. In this study the effect of silymarin on the liver of burned rats was investigated. Wistar Albino rats were exposed to 90°C bath for 10 seconds to induce skin burn. Silymarin either locally (30 mg/kg) applied on 4 cm² area or locally+systemically (50 mg/kg p.o) was administered after burn and repeated twice daily. Rats were decapitated 48 h after injury and blood was collected for tumor necrosis factor-α (TNF-α) and lactate dehydrogenase (LDH) activity. In liver tissue samples, total protein, tissue factor activity (TF) and activities of carbonic anhydrase (CA), glutathione-S-transferase (GST), catalase (CAT), superoxide dismutase (SOD), alkalen and acid phosphatase (ALP, ACP respectively) enzymes were determined. In addition polyacrylamid gel electrophoresis was carried on liver tissue samples. Burn caused a significant increase in blood TNF-α and LDH levels. Total protein levels and ALP activity increased and SOD activity decreased in the liver tissue at 48h after burn. Both local and systemic silymarin treatment significantly reversed this parameters except ALP. Silymarin treatment significantly increased ALP activity. Local silymarin treatment increased, CAT and ACP activities and decreased TF activity compared to control and burn group; and increased CA activity compared to systemic+local silymarin treatment. Addition of systemic silymarin treatment to local silymarin treatment reversed these effects of local treatment to control group levels. Non significant differences were found between protein bands obtained in electrophoresis. Minor liver damage was obvious 48 h after thermal skin burn. Both local and systemic silymarin treatment were effective to reverse the effects of burn induced liver injury.

Key Words: Burn, liver enzymes, liver tissue factor activity, SDS-polyacrylamide gel electrophoresis, silymarin

INTRODUCTION
Thermal trauma, one of the most common problems faced in the emergency room, may cause damage to multiple organs distant from the original burn wound and may lead to multiorgan failure (1). Following burn injury all tissues subject to ischemia and consequently, during burn shock resuscitation, reperfusion occurs. The role of oxygen radicals, neutrophils and endothelial cells in ischemic insult or organ failure is well documented (2, 3).

The liver plays an important role in the body’s response to thermal injury. It is the principal organ responsible for producing acute-phase proteins and modulating the systemic inflammatory response (4,5). Following thermal injury, the acute-phase response brings about the activation of the coagulation and complement cascades, granulocytes, and monocytes/macrophages as well as platelets (6-10) and induces the liver to synthesize and release proteins that exert effects on a variety of tissues (1, 4, 5).
Burn injury induces also many pathological changes in the human body, leading to alterations in pharmacokinetic parameters such as bioavailability, protein binding, volume of distribution and clearance (11). The pharmacokinetic parameters following burn injury vary between the acute phase of injury and the second hypermetabolic phase beyond the initial 48 hours of thermal injury (12). The effects of systemic and local antioxidant treatment on severe burn have been extensively studied (13). The results showed that prevention of local tissue damage is an important issue since skin burn may cause damage to multiple organs distant from the original burn wound and may lead sepsis and multiple organ failure (14).

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. In the 1960s the biologically active molecules of the seed and fruit extracts were isolated, and the chemical structures were elucidated. The isolation led first to a mixture that was named silymarin, and it was this flavonolignan mixture, with that most of the clinical studies were carried out. Flavanoids are naturally occurring substances that possess various pharmacological actions and therapeutic applications. Some of these, due to their phenolic structures, have antioxidant effect and inhibit free radical-mediated processes (15-17).

Based on these findings, we investigated the putative protective role of local and systemic (oral) silymarin treatment against burn-induced oxidative and degenerative damages on the liver at the late phase of burn.

MATERIALS AND METHODS

Animals and Protocol for the Induction of Burn

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.

The rats were divided into the following four groups of six rats (three males and three females) in each; 1- vehicle-treated sham operated control group, 2- vehicle treated burn group , 3- local silymarin-treated burn group and 4- local+oral silymarin-treated burn group

Under brief ether anesthesia, the dorsum of the rats was shaved, and exposed to 90 °C water bath for 10 sec, which resulted in partial-thickness second-degree skin burn involving 30% of the total body surface area. All the burned animals were then resuscitated with physiological saline solution (10 ml/kg, subcutaneously, s.c.). In order to rule out the effects of anesthesia, the same protocol was applied in the control group, except that the dorsums were dipped in a 25 °C water bath for 10 sec.

Silymarin Treatment

Silymarin either locally (30 mg/kg) applied on 4 cm² area or locally+oral (50 mg/kg p.o) was administered after burn and repeated twice daily.

Rats were decapitated 48 h after injury and blood was collected for tumor necrosis factor-α (TNF-α) and lactate dehydrogenase (LDH) activity. In liver tissue samples, protein, tissue factor (TF) activity and activities of carbonic anhydrase (CA), glutathione-S-transferase (GST), catalase (CAT), superoxide dismutase (SOD), alkalen and acid phosphatase (ALP, ACP) enzymes were determined. In addition polyacrylamid gel electrophoresis was carried on liver tissue samples.

Biochemical assays

Serum TNF-α and LDH analysis

Serum TNF-α and LDH were quantified according to the manufacturer’s instructions and guidelines using enzyme-linked immunosorbent assay (ELISA) kits specific for the rats (Biosource International, Nivelles, Belgium).

Livers Tissue Analysis

Liver tissue total protein, TF activity and CA, GST, CAT, SOD, ALP, ACP enzyme activities were determined by the methods of Lowry (18), Ingram (19), Vepoorte (20), Habig (21), Aebi (22), Mylorie (23), Walter (24) respectively. Electrophoretic examination of liver proteins was carried out by Laemmli SDS-polyacrylamid gel electrophoresis (25).

Statistical Analysis

Statistical analysis was done using SPSS 10.0. All data are expressed as means ± S.D. Kolmogorov-Smirnov test, Friedman test, Mann-Whitney-U test and Spearsman correlation analysis were used.

RESULTS

Burn caused a significant increase in blood TNF-α and LDH levels (Table 1). Total protein levels and ALP activity increased and SOD activity decreased in the liver tissue at 48h after burn (Table 2). Both local and systemic silymarin treatment significantly reversed this parameters except ALP. Silymarin treatment significantly increased ALP activity. Local silymarin treatment increased, CAT and ACP activities and decreased TF activity compared to control and burn group; and increased CA activity compared to systemic+local silymarin treatment. Addition of systemic silymarin treatment to local silymarin treatment reversed these effects of local treatment to control group levels. Non significant differences were found between protein bands obtained in electrophoresis (Fig1).

Table 1: Blood TNF-α and LDH levels

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Burn group</th>
<th>Burn Treatment Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Local silymarin</td>
<td>Local+oral silymarin</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>4.00± 1.58</td>
<td>23.20 ± 5.63</td>
<td>10.00±3.39b 7.60±2.07b</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>2514±316,80</td>
<td>4238±375,90</td>
<td>2854±475,90b 2666±429,20b</td>
</tr>
</tbody>
</table>

Values are given means±standard deviation; a p<0.01 significantly different from control group; b p<0.01 significantly different from burn group
**Table 2**: Activities of glutathione-S-transferase (GST), catalase (CAT), superoxide dismutase (SOD), carbonic anhydrase (CA), tissue factor (TF), acid phosphatase (ALP), alkaline phosphatase (ACP) enzymes and protein levels in liver tissue samples of all groups (n=6 per group)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control</th>
<th>Burn</th>
<th>Local Silymarin</th>
<th>Local+Oral Silymarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST (U/mg protein)</td>
<td>0,46±0,4</td>
<td>0,42±0,8</td>
<td>0,47±0,7</td>
<td>0,49±0,2</td>
</tr>
<tr>
<td>CAT (U/mg protein)</td>
<td>30,06±9,4</td>
<td>36,81±6,7</td>
<td>52,88±6,1</td>
<td>40,22±9,1</td>
</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td>1,21±0,7</td>
<td>0,84±0,2</td>
<td>1,08±0,8</td>
<td>0,96±0,2</td>
</tr>
<tr>
<td>CA (U/mg protein)</td>
<td>0,23±0,26</td>
<td>0,24±0,29</td>
<td>0,26±0,36</td>
<td>0,21±0,25</td>
</tr>
<tr>
<td>TF (sec)</td>
<td>181,5±15,7</td>
<td>188,5±27,5</td>
<td>316,6±51,3</td>
<td>180,8±21,0</td>
</tr>
<tr>
<td>ACP (U/mg protein)</td>
<td>33,48±3,01</td>
<td>31,91±3,60</td>
<td>41,34±4,65</td>
<td>39,08±4,23</td>
</tr>
<tr>
<td>ALP (U/mg protein)</td>
<td>0,89±0,18</td>
<td>1,32±0,29</td>
<td>1,97±0,42</td>
<td>1,59±0,21</td>
</tr>
<tr>
<td>Total protein (mg/ mg tissue)</td>
<td>217,5±17,6</td>
<td>253,7±9,9</td>
<td>210,3±7,9</td>
<td>214,8±8,4</td>
</tr>
</tbody>
</table>

Values are given mean±standard deviation. *p<0.05 and **p<0.01: significantly different from control group; *p<0.05 and **p<0.01: significantly different from burn group; *p<0.05 and **p<0.01: significantly different from burn+local and oral silymarin treatment groups. Since the clotting time is inversely proportional to the TF activity, the lengthening of the clotting time is a manifestation of decreased TF activity.

**Figure 1**: Samples of liver SDS-PAGE

Columns: (A) burn+local+oral silymarin treatment, (B) burn+local silymarin treatment, (C) burn, (D) control, (St) Standard-Bovine Albumin

**DISCUSSION**

Following burn injury, increasing evidences implicated reactive oxygen species (ROS) as causative agents of local and systemic damage. ROS are first generated from the burned skin (26). Activated mononuclear phagocytes (27) and neutrophils (28,29) sequestered in systemic organs would also be the source of ROS. It is well known that ROS give rise to damage to proteins, lipids, nucleic acids and other biological macromolecules. In addition, lipid peroxides generated from burned skin are released into the serum and diffuse into some organs causing subsequent organ injury (30). After thermal injury, uncontrolled ROS production is, at least in part, responsible for the local wound edema and systemic pathophysiological changes. The effects of systemic and local antioxidant treatment on severe burn have been extensively studied (31). The prevention of local tissue damage is an important issue since skin burn may cause damage to multiple organs distant from the original burn wound and may lead sepsis and multiple organ failure (14).

In the present study serum TNF-α and LDH markedly increased following skin burn. Silymarin treatment reduced serum levels of the proinflammatory cytokine TNF-α and LDH. These findings suggest that silymarin, appears to have a protective role in the burn-induced injury. It was demonstrated that some phytochemicals, including silymarin, are known to suppress cancer cell proliferation, inhibit growth factor signaling pathways, induce apoptosis, and inhibit NF-kappaB (32). Chang et al. who studied the mechanism of the inhibition of NF-kB, demonstrated that silymarin inhibited TNF-α-induced calcium-dependent NF-kappaB activation irrespective of its antioxidant effect (33). However, since proinflammatory cytokines are triggered by ROS, antioxidants may contribute the inhibition of cytokine release by the inhibition of radicals. Although we did not evaluate the NF-kB, it can be speculated that inhibition of proinflammatory cytokine, TNF-α may cause the inhibition of NF-kB. Furthermore, silymarin was also shown to modulate immune response, by augmenting synthesis of anti-inflammatory cytokines, such as IL-10, IL-12 (34,35).

Alteration of liver function tests is extremely common following major burns. An incidence as high as 50% has been described when there is transient elevation of the aspartate aminotransferase, alanine aminotransferase, and particularly the ALP (1). The precise etiology of these changes is not known, but they usually are benign in character and resolve spontaneously. Liver ALP activity was determined in the present study and it increased significantly in burn group consistent with other burn studies (1,36). Both silymarin treatments did not reduce this increase.

After severe burn, hepatic protein synthesis shifts from hepatic constitutive proteins, such as albumin, prealbumin, transferrin, and retinol-binding protein, to acute phase proteins (5) which serve as mediators of the inflammatory process, function as transport proteins, and participate in burn wound healing (10,37). In consistency, in the present study liver total protein level was found to be significantly increased in burn group at 48 h after burn. Both silymarin treatment reversed this effect. However no significant differences were found between protein bands obtained in electrophoresis.

SOD enzyme is widely distributed in various tissues, but it can be found especially in the liver (38). SOD catalyzes the dismutation of two superoxide radicals to O2 and H2O2 (39). Liver SOD activity was found to be significantly decreased in burn group in this study. The decrease of SOD activity in the liver has been shown in thermal burn studies (40, 41, 42). In our study silymarin treatments significantly decreased SOD activity in liver.

In a recent study by Ramakrishnan et al. (43), investigating the mechanism underlying the protective effects of silymarin in hepatic carcinogenesis, it was demonstrated that a number of
endogenous antioxidants, including GSH, SOD, CAT, glutathione peroxidase (GPs), glutathione reductase (GR), and glucose-6-phosphate dehydrogenase (G6PD) can be induced by silymarin, and that this chemically mediated upregulation of cellular defenses is accompanied by a markedly increased resistance to hepatic cell injury elicited by ROS (43). Silymarin also reduces the increase of hepatic stellate cells and TGF-β1 production in the CCl4-treated rats suggesting that silymarin prevents hepatic fibrosis through suppression of inflammation and hypoxia in the hepatic fibrogenesis (44). Moreover, oral silymarin administration has been shown to reduce lipid peroxidation of brain tissues in acetaminophen induced toxicity by enhancing antioxidant enzymes (45). On the other hand silymarin improving antioxidant status in blood and liver, positively affects plasma lipoprotein profile in an experimental model of dietary induced hypertriglyceridemia (46).

In addition, thermal injury has a direct impact on hepatic metabolism: the rate of phase I metabolism decreases, which is thought to be related to the oxygen-derived free radicals released during the course of injury, whereas phase II metabolism is unimpaired and may possibly increase (12). Altered phase I and phase II metabolism are not only markers for liver injury but may compound liver damage as administered drugs can not be adequately cleared.

GSTs are a family of phase II enzymes that detoxify metabolites and foreign compounds by conjugating them with GSH. Although GSTs are widely distributed in many organs, liver cytosolic GSTs are considered most important in this detoxification process. In addition to their conjugation properties, GSTs also exibit some peroxidase activity, which provide protection against reactive oxygen species and peroxidized lipid products. GSTs contribute to liver homeostasis and are released into plasma after liver injury. Pham et al. found reduction in expression of specific GST enzymes after burns and found correlation with reduced GST activity. They suggested that altered GST expression may contribute to liver damage after systemic injury (47). In the present study, GST activity did not significantly change after burn injury and silymarin administration also did not alter the results. The decreases in SOD activity and increases in ALP activity that were not accompanied by significant changes in other liver enzyme activities such as GST, CAT, CA and ACP, may indicate the presence of minor damage in liver tissue at48 h after thermal burn injury. Local silymarin treatment increased, CAT and ACP activities compared to control and burn group; and increased CA activity compared to systemic+local silymarin treatment. Addition of systemic silymarin treatment to local silymarin treatment reversed these effects of local treatment to control group levels.

The present study, for the first time, investigates the effect of silymarin on liver TF activity. No significant change was found in TF activity in liver samples following burn injury. This may also support minor liver damage at 48 h after burn. TF, known as thromboplastin or Factor III, is an important coagulation factor that initiates extrinsic blood coagulation with FVII. It is not actively found in the blood but it is the cell component of the membranes (48). It has been shown that some tissues and fluids of the body have TF activities (49-54). Thrombotic and fibrinolytic mechanisms are activated after burn injury, with the extent of activation correlated to the severity of the thermal injury (5). The liver produces most of the factors involved in coagulation and fibrinolysis that help to maintain the balance between thrombin deposition and removal (1). On the other hand, TF activity decreased significantly in rats treated with local silymarin administration in the present study. Locally administered silymarin decreased the activity more effectivly than locally + orally administered silymarin. This finding was also another evidence that silymarin has protective effects on liver. Addition of systemic silymarin treatment to local silymarin treatment reversed these effects of local treatment to control group levels. The effect of local + oral administration of silymarin on liver TF activity need further investigation.

CONCLUSION

Minor liver damage was obvious 48 h after thermal skin burn. Silymarin treatment was effective to reverse the damages seen after burn injury.

Termal yanıkta silymarinin karaciğer üzerinde etkisi

ÖZET

Karaciğer yanık hastaları için hayati öneme tashır. Meryamana dikeninin ana maddesi olan silymarinin karaciğer üzerindeki koruyucu etkisi gösterilmiştir. Bu çalışmada termal deri yanıklı sıçanlardaki termodinamik silymarinin karaciğer üzerine etkisi araştırıldı. Wistar-Albino türü sıçanlar 90°C deki şarkıya 10 saniye temas ettirilerek deri yanığı oluşturuldu. Silymarin hem lokal (30 mg/kg) olarak hemde lokal + oral yolla (50 mg/kg) yanık oluşturulan sıçanlara 2 gün (48 saat) süre ile günde 2 kez uygulandı. 48 saat sonra elde edilen protein bantları poliamilid jelyelektroforezi uygulandı. 48 saat sonra yanık grubunda TNF-α ve LDH seviyelerinde artış, kadosinsuz protein ve ALP aktivitesinde artma, SOD aktivitesinde azalmaya neden oldu. Lokal ve oral silymarin tedavisi bu parametrelerdeki artışları pektroksoru ALP hariç geri döndürdü, silymarin tedavisi ALP aktivitesine arttırdı. Lokal silymarin tedavisi CA aktivitesine ve DF aktivitesini kontrol grubuna göre arttırdı. CA aktivitesini de lokal+oral silymarin tedavisi uygulanan gruba de arttırdı. Lokal silymarin tedavisi sistemik silymarin tedavisi ile desteklenmesi lokaal silymarin tedavisinin neden olduğu bu etkileri düzeltti ve kontrol grubu düzelyerine indirdi. Elektroforez ile elde edilen protein bantları arasında anfali farklılıklar tespit edildi. Termal yanıkların 48 saat sonra karaciğerde minor hasar meydana geldi. Silymarin tedavisi bu hasar geri dönümede etkili oldu.

Anahtar kelimeler: Karaciğer doku faktörü aktivitesi, Karaciğer enzimleri, SDS-poliakrilamid jely elektroforezi, Silymarin, Yanık
REFERENCES