

# **Effect of Various Doses of Silymarin on the Oxidative Stress Induced by Busulfan Administration in the Different Organs of Rats**

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*The antineoplastic drug Busulfan can lead to impaired antioxidant defense system. Silymarin as a natural herbal product has powerful antioxidant properties. In the present study, the effects of Busulfan and Silymarin on oxidative stress system were evaluated. For the study 48 rats were prepared and fed with special ration, then they were divided to 6 groups with 8 rats in each group: (C1): no drugs, (C2): 175 mg/kg/day Silymarin for 14 days as gavage, (C3): 20 mg/kg/day Busulfan for 14 days intra peritoneal, (T1): 20 mg/kg/day Busulfan for 14 days intra peritoneal and after 2 weeks 175 mg/kg/day Silymarin for 14 days as gavage, (T2): 20 mg/kg/day Busulfan for 14 days intra peritoneal and after 2 weeks 250 mg/kg/day Silymarin for 14 days as gavage, (T3): 20 mg/kg/day Busulfan for 14 days intra peritoneal and after 2 weeks 325 mg/kg/day Silymarin for 14 days as gavage. The amount of MDA, SOD and GPX in the different tissue extracts (liver, spleen, pancreas, testes, heart and kidneys) were measured. The results of this study showed that after Busulfan treatment, the activity of SOD and GPX in different tissues was significantly decreased. Silymarin in C1 could increase the activity of SOD and GPX compared to C3. In C3, the level of MDA in different tissues compared to other the groups was significantly increased. Silymarin in C1 could decrease the level of MDA compared to C3. It can be concluded that the use of Busulfan can induce oxidative stress and Silymarin had an antioxidant role along with decreasing the lipid peroxidation.*

**Key Words:** Busulfan; Silymarin; Oxidative stress

## INTRODUCTION

Free radicals and other reactive oxygen species (ROS) are constantly formed in the human body and are also removed by endogenous antioxidant defense mechanism that acts by scavenging free radicals, decomposing peroxides and / or binding with pro-oxidant metal ion. Free radicals interact with other molecules within cells (1). This can cause oxidative damage to proteins, membranes and genes. Oxidative stress, defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses, is discussed in relation to its possible role in the production of tissue damage (1-2). Many factors such as lifestyle, drugs, toxicants, nutritional imbalances, and infections lead to oxidative stress. Busulfan is an alkylating chemotherapeutic agent used to treat chronic leukemia, ovarian cancer, lymphoma and myeloproliferative disorders and is also used before bone marrow transplantation in cancer patients (3). Busulfan has side effects on various organs including the liver, skin, bladder, nervous system and gonadal function, and is potentially carcinogenic and mutagenic. There is some evidence that Busulfan has the potential to induce free radical damage to lipid peroxidation (4). Interest in natural sources of antioxidant molecules for use in the food, beverage and cosmetic industries has resulted in a large body of research in recent years. It is well known that natural antioxidants extracted from herbs and spices have high antioxidant activity and are used in many food applications. Of these substances, the phenolic compounds, which are widely distributed, have the ability to scavenge free radicals by single-electron transfer (5-6). Silymarin is a known standardized extract obtained from seeds of *silybummarianum* and is also called "milk thistle". *Silybummarianum* is one of the family Asteraceae (7-8). Silymarin consists of a large number of flavolignans, including taxifolin, silychristin, silydianin, silybin A, silybin B (silybin or silybinin is the most active component), isosilybin A and isosilybin B (9-11). It has been reported as having multiple pharmacological activities including antioxidant, hepatoprotectant and anti-inflammatory agent, antibacterial, antiallergic, antimutagenic, antiviral, antineoplastic, antithrombotic agents, and vasodilatory actions (12). Asghar and Masood (8) suggested that *Silymarin* may be used in preventing free radical-related diseases as a dietary natural antioxidant supplement (8). It is suggested that many of these properties may be related to the antioxidant and free radical scavenging activity of Silymarin. Silymarin has been said to be at least ten times more potent in antioxidant activity than vitamin E (13). It increases glutathione in the liver by more than 35% in healthy subjects and by more than 50% in rats (14). Glutathione is responsible for detoxifying a wide range of hormones, drugs, and chemicals. High levels of glutathione in the liver increase its capacity for

detoxification. Silymarin also increases the level of the important antioxidant enzyme superoxide dismutase in cell cultures (15). Silymarin inhibits the synthesis of leukotrienes, mediators of inflammation (16).

Based on this evidence, the aim of this study is to determine whether Busulfan has an oxidative effect after administration and whether different doses of Silymarin can protect different organs from Busulfan oxidative-induced damage.

## **EXPERIMENTAL**

### *Animals and Experimental design*

This study was performed at the Animal House of School of Veterinary Medicine, Shiraz University, Iran. Forty-eight Wistar male rats ( $250\pm 20$ ) were housed in wire cages at  $22\pm 1^\circ\text{C}$  under a 12-h light-dark cycle with 30% humidity and fed a standard diet and water.

All chemicals and drugs (Busulfan and Silymarin) were purchased from Sigma-Aldrich and used without further purification. The rats were randomly divided into six equal groups of eight rats in each group as follows: Group 1 (C1) - control group (received nothing). Group 2 (C2) - control Silymarin (Sigma, Aldrich, USA), 175 mg/kg/day Silymarin was given by gavage for 14 days. Group 3 (C3) - anemic control group, received 20 mg/kg/day (i.p.) Busulfan (Sigma, Aldrich, USA) for 14 days. Group 4 (T1) - experimental group 1 received 20 mg/kg/day Busulfan (i.p.) for 14 days and after 2 weeks of 175 mg/kg/day Silymarin was gavage for 14 days. Group 5 (T2) - 2 groups received 20 mg/kg/day dose Busulfan (i.p.) for 14 days and after 2 weeks of 250 mg/kg/day Silymarin was gavage for 14 days. Group 6 (T3) - 3 groups received 20 mg/kg/day dose Busulfan (i.p.) for 14 days and after 2 weeks of 325 mg/kg/day Silymarin was gavage for 14 days. At the end of the treatment period the rats were anesthetized by using ether then killed; after sacrifice the peritoneal cavity was opened through a lower transverse abdominal incision and their organs were promptly collected. The organs collected were testis, spleen, kidneys, liver, pancreas and heart and were used for determination of organ SOD, GPX activity and MDA concentration. Small pieces of each organ were then transferred to a sterile vessel containing phosphate buffer 0.025M (pH 7.4) solution. Then the organs were immediately ground to make a tissue homogenate (1g/4ml). The homogenates were centrifugated at 750g for 15 minutes and the supernatant was collected as a tissue extract for biochemical analysis.

### *Animal ethics*

This experiment was accomplished under the approval of the State Committee on Animal Ethics, Shiraz University, Shiraz, Iran. The recommendations of European Council Directive (86/609/EC) of November 24, 1986 regarding the standards in the protection of animals used for experimental purposes were also followed.

### *Biochemical analysis*

SOD activity was measured with a commercial kit (RANSOD kit, Randox Com, UK). Tissue homogenates glutathione peroxidase (GPX) enzyme activity was estimated by Ransel spectrophotometric kit (RANSEL, Randox Laboratories Ltd. UK). Tissue homogenates malondialdehyde (MDA) was measured using the colorimetric method described by Placer (17) based on the reaction of thiobarbituric

acid (TBA) with MDA, one of the aldehyde products of lipid peroxidation. The absorbance of the MDA-TBA adduct thus produced was measured spectrophotometrically at 532 nm. The results were expressed in terms of the mmol of malondialdehyde (MDA) / mg of tissue homogenates total protein.

### *Statistical analysis*

Data were analyzed by one-way analysis of variance followed by Duncan's multiple range test using SPSS software package, version 18. The values are Mean±SEM for eight rats in each group. P value ≤0.05 was considered as significant and included in the study.

## **RESULTS**

### *The tissue SOD activity manifestations*

The lowest activity of SOD of liver was seen in C3 group as compared to the C1 group ( $p < 0.05$ ). The activity of SOD in C2 group, T1, T2 and T3 group showed significant increase compared to the C3 group ( $p < 0.05$ ). The lowest activity of SOD of spleen was seen in C3 group compared to the C1 group ( $p < 0.05$ ). The activity of SOD in C2 groups has increased significantly in comparison to C3 group ( $p < 0.05$ ). The activity of SOD of pancreas in C3 group showed a significant decrease compared to other groups ( $p < 0.05$ ). The lowest activity of SOD of testis was seen in C3 group as compared to the other groups ( $p < 0.05$ ). The activity of SOD in C2 group and T1, T2 and T3 group increased compared to the C3 group but not to C1 group. The lowest activity of SOD of testis was seen in C3 group compared to the other groups ( $p < 0.05$ ). The other groups (C1, C2, T1, T2 and T3) showed a significant increase compared to C3 group ( $p < 0.05$ ). The activity of SOD of kidney in C3 group showed a significant decrease compared to other groups ( $p < 0.05$ ) (Table 1).

### *The tissue GPX activity manifestations*

The activity of liver GPX in C1 and C2 group increased remarkably compared to other groups ( $p < 0.05$ ). The highest activity of spleen GPX was seen in the C1 in comparison to other groups ( $p < 0.05$ ). The lowest activity of GPX of pancreas was seen in C3 group compared to the other groups and C2 group has increased significantly compared to C1, T1, T2 and T3 groups ( $p < 0.05$ ). The testis GPX activity of C3 group showed significant decrease compared to group which received different doses of Silymarin ( $p < 0.05$ ). The GPX activity of heart in C2 group compared to C3 group and C1 group had a significant increase ( $p < 0.05$ ). The highest kidney activity of GPX belonged to C1 and C2 groups compared to the other groups and C3 group showed a significant decrease compared to C1 and C2 groups ( $p < 0.05$ ) (Table 2).

### *The tissue MDA level manifestation*

The results indicated that the highest MDA level of liver, spleen, pancreas, testis, heart and kidney was seen in C3 group compared to other groups ( $p < 0.05$ ) (Table 3)

**Table 1.** Effect of Busulfan and Silymarin administration on changes in SOD activity profile (Mean±SEM). Dissimilar letters in each column indicate significant differences between the different groups (p<0.05).

Tissues	Liver	Spleen	Pancreas	Testis	Heart	Kidney
<b>Groups</b>						
<b>(T1)</b>	41.54 ±7.71 <sup>b</sup>	36.97±1.85 <sup>ab</sup>	35.17±4.96 <sup>b</sup>	39.50±4.19 <sup>b</sup>	60.58±3.76 <sup>b</sup>	46.55±6.48 <sup>b</sup>
<b>(T2)</b>	43.86 ±7.51 <sup>b</sup>	47.00±3.72 <sup>bc</sup>	39.05±3.12 <sup>bc</sup>	47.82±3.28 <sup>b</sup>	57.70±9.16 <sup>b</sup>	52.55±6.98 <sup>b</sup>
<b>(T3)</b>	42.00±1.95 <sup>b</sup>	58.09±2.45 <sup>cd</sup>	41.87±3.60 <sup>bc</sup>	48.24±3.07 <sup>b</sup>	57.37±3.36 <sup>b</sup>	58.05±3.23 <sup>b</sup>
<b>(C3)</b>	18.67±1.94 <sup>a</sup>	31.32±3.34 <sup>a</sup>	18.23±3.30 <sup>a</sup>	23.99±3.33 <sup>a</sup>	23.16±2.24 <sup>a</sup>	13.42±1.39 <sup>a</sup>
<b>(C2)</b>	50.92±7.66 <sup>b</sup>	63.91±5.95 <sup>d</sup>	51.70±6.49 <sup>c</sup>	47.00±3.79 <sup>b</sup>	66.88±4.61 <sup>b</sup>	58.58±5.79 <sup>b</sup>
<b>(C1)</b>	40.86±3.82 <sup>b</sup>	55.40±4.20 <sup>cd</sup>	49.07±5.95 <sup>bc</sup>	43.08±5.04 <sup>b</sup>	56.88±4.44 <sup>b</sup>	48.58±5.38 <sup>b</sup>

**Table 2:** Effect of Busulfan and Silymarin administration on changes in GPX activity profile (Mean±SEM). Dissimilar letters in each column indicate significant differences between the different groups (p<0.05).

Tissues	Liver	Spleen	Pancreas	Testis	Heart	Kidney
<b>Groups</b>						
<b>(T1)</b>	6.97±0.59 <sup>a</sup>	11.08±0.98 <sup>a</sup>	6.67±0.61 <sup>b</sup>	6.28±0.77 <sup>bc</sup>	8.76±0.78 <sup>ab</sup>	10.53±0.54 <sup>ab</sup>
<b>(T2)</b>	6.72±0.55 <sup>a</sup>	12.86±1.00 <sup>ab</sup>	6.58±0.50 <sup>b</sup>	6.88±0.63 <sup>c</sup>	8.85±1.29 <sup>ab</sup>	11.92±0.80 <sup>b</sup>
<b>(T3)</b>	7.03±0.62 <sup>a</sup>	13.07±3.35 <sup>ab</sup>	7.36±0.27 <sup>b</sup>	6.89±0.60 <sup>c</sup>	13.27±2.27 <sup>bc</sup>	10.81±0.75 <sup>ab</sup>
<b>(C3)</b>	8.91±1.05 <sup>a</sup>	14.23±0.82 <sup>ab</sup>	3.01±0.17 <sup>a</sup>	4.13±0.21 <sup>a</sup>	4.72±0.54 <sup>a</sup>	8.57±0.61 <sup>a</sup>
<b>(C2)</b>	15.39±1.08 <sup>b</sup>	18.90±0.94 <sup>b</sup>	12.91±0.92 <sup>d</sup>	5.07±0.27 <sup>ab</sup>	17.45±3.98 <sup>c</sup>	15.80±1.17 <sup>c</sup>
<b>(C1)</b>	15.43±0.89 <sup>b</sup>	27.80±4.61 <sup>c</sup>	10.52±0.68 <sup>c</sup>	5.36±0.34 <sup>abc</sup>	10.12±0.68 <sup>ab</sup>	15.58±0.89 <sup>c</sup>

**Table 3:** Effect of Busulfan and Silymarin administration on changes in MDA profile (Mean±SEM). Dissimilar letters in each column indicate significant differences between the different groups (p<0.05).

<b>Tissues</b>	<b>Liver</b>	<b>Spleen</b>	<b>Pancreas</b>	<b>Testis</b>	<b>Heart</b>	<b>Kidney</b>
<b>Groups</b>						
<b>(T1)</b>	5.70±0.03 <sup>a</sup>	5.71±0.02 <sup>a</sup>	5.72±0.02 <sup>a</sup>	5.75±0.03 <sup>a</sup>	5.71±0.02 <sup>a</sup>	<b>5.71±0.02<sup>a</sup></b>
<b>(T2)</b>	5.70±0.02 <sup>a</sup>	5.71±0.02 <sup>a</sup>	5.73±0.03 <sup>a</sup>	5.70±0.03 <sup>a</sup>	5.70±0.02 <sup>a</sup>	<b>5.69±0.03<sup>a</sup></b>
<b>(T3)</b>	5.81±0.03 <sup>a</sup>	5.83±0.02 <sup>a</sup>	5.82±0.03 <sup>ab</sup>	5.84±0.04 <sup>a</sup>	5.82±0.03 <sup>ab</sup>	<b>5.83±0.03<sup>ab</sup></b>
<b>(C3)</b>	9.33±0.22 <sup>b</sup>	9.47±0.22 <sup>b</sup>	9.39±0.23 <sup>c</sup>	9.40±0.23 <sup>b</sup>	9.35±0.21 <sup>c</sup>	<b>9.41±0.22<sup>c</sup></b>
<b>(C2)</b>	5.79±0.03 <sup>a</sup>	5.81±0.02 <sup>a</sup>	5.80±0.03 <sup>ab</sup>	5.82±0.04 <sup>a</sup>	5.79±0.03 <sup>ab</sup>	<b>5.80±0.03<sup>ab</sup></b>
<b>(C1)</b>	5.98±0.03 <sup>a</sup>	5.99±0.04 <sup>a</sup>	6.03±0.02 <sup>b</sup>	6.00±0.04 <sup>a</sup>	6.01±0.03 <sup>b</sup>	<b>6.00±0.03<sup>b</sup></b>

## DISCUSSION

### *Tissue SOD and GPX alteration*

Tissue SOD results indicate that SOD activity of different organs in C3 (liver, spleen, pancreas, testis, heart and kidney) is significantly decreased due to Busulfan administration and this event shows that Busulfan could induce oxidative stress and reduction of SOD as the first antioxidant defense for neutralization of ROS produced by Busulfan. It was evident from data that SOD activity in C2 group was remarkably increased as compared to rats treated with only Busulfan (C3). Also, different doses of Silymarin could increase SOD activity that has been decreased by Busulfan. But there was no dose dependent manner. The results indicate the protective antioxidant effect of Silymarin on oxidative stress induced by Busulfan. Tissue GPX activity in the control group treated with Busulfan was lower than control and Silymarin control groups. This observation shows oxidative potential of Busulfan. Except for liver and spleen, in other organs administration of different doses of Silymarin in combination with Busulfan could increase the activity of GPX. This case may be due to different content of SOD in organs other than GPX. Another reason is probably that tissues responses to treatment are different from each other. Treatment with only Silymarin could increase GPX activity and the antioxidant effect of Silymarin is also indicated. Some experimental studies confirm our study. Das et al. (13) examined the efficacy of Silymarin on the immunomodulatory activity and vascular function in mice with liver abnormalities induced by chronic ethanol consumption. They found that chronic ethanol consumption increased MDA and glutathione S transferase (GST) and decreased the activity of catalase and SOD and GPX and Silymarin treatment reduced MDA and GST and elevated SOD, GPX and catalase. So they suggested that Silymarin can effectively ameliorate ethanol induced oxidative challenges (13). Similarly in Tzeng et al's (18) study, it was indicated that Silymarin (200 mg/Kg, 3 times a day) could decrease the activity of plasma AST and ALT and increase the activity of liver SOD and GPX in comparison to mice that received CCl<sub>4</sub> intraperitoneally. CCl<sub>4</sub> could induced hepatic fibrosis and increased the plasma AST and ALT level and decreased the liver SOD and GPX [23]. Our findings are in agreement with Sajedianfard et al. (19). Their study showed that in diabetic rats, high therapeutic doses of Silymarin had a major effect on serum antioxidant factors. They revealed that Silymarin plays a role by increasing antioxidant enzymes level (19-20). Some chemotherapeutic agents induce oxidative stress as a mechanism for killing cancer cells. However, certain chemotherapeutic agents induce oxidative stress in nontargeted tissues and thereby lead to "normal tissue injury." Although chemotherapy improves the survival rates of cancer patients, oxidative stress-mediated impairment of normal tissues is a significant side effect and decreases the quality of life of patients (21). So far there have been no studies about the effect of Busulfan on the body fluid or tissue SOD and GPX activity.

### *Tissue MDA alteration*

The present study shows that the MDA level of different organs (liver, spleen, pancreas, testis, heart and kidney) in the Busulfan control group was significantly increased as compared to other groups. In the present study the elevated tissue MDA level as a marker of oxidative stress and decreased antioxidant enzymes activity simultaneously in rats treated only with Busulfan indicated the pro-oxidant role of Busulfan in producing ROS and lipid peroxidation (22). This point is in agreement with the findings of Hosseini Ahar et al. (23). Their study on body and testicular weight and serum malondialdehyde (MDA) enzyme in rat was performed following the use of Busulfan. They concluded that the use of Busulfan can reduce body and testicular weight, and increase serum MDA and there could be side effects in reproduction process (23). It has been shown that combined treatment of Busulfan, Melatonin and liver growth factor reduces the Busulfan side effects (24), such as the study conducted by Ray et al. (22). They explored the protective effects of water extract of *Spirulina platensis* (blue green algae) on Busulfan-induced lipid peroxidation using goat liver as the lipid source and found lipid peroxidation induction capacity of Busulfan and the antiperoxidative potential of water extract of *Spirulina platensis* (blue green

algae) on Busulfan-induced lipid peroxidation (22). In another investigation Ray et al. revealed that ascorbic acid could suppress the drug induced lipid peroxidation such as Busulfan to a significant extent (4). Some studies have shown that Silymarin's protective effect was also reflected on preserving the integrity of the plasma membrane and it seems Silymarin can affect  $Ca^{2+}$  modulation as an essential role in hepatoprotection produced by Silymarin (25-26).

## **CONCLUSION**

In conclusion, the present data demonstrated that Busulfan as an anticancer drug along with its therapeutic effects has side effects like other drugs and some of these effects could be due to its pro-oxidative effect in inducing oxidative stress. On the other hand, Silymarin as an ancient herbal drug could be a useful antioxidant agent to protect against oxidative damages induced by Busulfan and could be considered as an alternative treatment for protecting the side effects of Busulfan.

## **CONFLICT OF INTEREST STATEMENT**

We declare that we have no conflict of interest.

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