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ORIGINAL ARTICLE

Silymarin alleviates bleomycin-induced pulmonary toxicity and lipid peroxidation in mice

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Abstract

Context: The application of bleomycin is limited due to its side effects including lung toxicity. Silymarin is a flavonoid complex isolated from milk thistle [*Silybum marianum* L. (Asteraceae)] which has been identified as an antioxidant and anti-inflammatory compound.
Objective: This study evaluates the effect of silymarin on oxidative and inflammatory parameters in the lungs of mice exposed to bleomycin.
Materials and methods: BALB/c mice were divided into four groups of control, bleomycin (1.5 U/kg), bleomycin plus silymarin (50 and 100 mg/kg). After bleomycin administration, mice received 10 d intraperitoneal silymarin treatment. On 10th day, blood and lung samples were collected for measurement of oxidative and inflammatory factors.
Results: Silymarin led to a decrease in lung lipid peroxidation (0.19 and 0.17 nmol/mg protein) in bleomycin-injected animals. Glutathione-*S*-transferase (GST) which was inhibited by bleomycin (32.4 nmol/min/mg protein) induced by higher dose of silymarin (41 nmol/min/mg protein). Silymarin caused an elevation in glutathione (GSH): 2.6 and 3.1 μmol/g lung compare with bleomycin-injected animals 1.8 μmol/g lung. Catalase (CAT) was increased due to high dose of silymarin (65.7 μmol/min/ml protein) compare with bleomycin treated-mice. Myeloperoxidase (MPO) which was induced due to bleomycin ($p < 0.05$) reduced again by high dose of silymarin (0.51 U/min/mg protein). Bleomycin led to an increase in TNF-α and interleukin-6 (IL-6) (7.9 and 11.8 pg/ml). These parameters were reduced by silymarin ($p < 0.05$).
Conclusions: Silymarin attenuated bleomycin induced-pulmonary toxicity. This protective effect may be due to the ability of silymarin in keeping oxidant–antioxidant balance and regulating of inflammatory mediator release.

Keywords

Antioxidant enzymes, inflammation, lipid peroxidation, pulmonary damage

History

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Introduction

Oxidative stress consists of cellular and molecular abnormalities caused by overproduction of reactive oxygen species (ROS) as well as reduction in antioxidant defense (Faner et al., 2012; Jones, 2006). This process plays a key role in expansion of fibrosis in different organs such as lungs. Lungs are also outstandingly susceptible for oxidative damage as they are exposed to the highest level of oxygen. The origins of oxidative stress, which are able to induce pulmonary fibrosis, are exposure to toxicant and drugs such as tobacco, asbestos, silica, and bleomycin (Cheresh et al., 2012).

Bleomycin, a glycopeptide antibiotic extracted from *Streptomyces verticillus*, is commonly used as an adjuvant in chemotherapy protocols to control some human cancers such as germ cell tumor and Hodgkin's or non-Hodgkin's malignant lymphoma (Bayer et al., 1992; Levi et al., 1993). However, the therapeutic application of this anticancer drug is restricted because of its lung toxicity (Martin et al., 2005). Bleomycin-lung injury, which is mediated by ROS, eventually leads to pulmonary fibrosis (Mata et al., 2003). Bleomycin binds to DNA and in a reaction, which is mediated by molecular oxygen and ferrous ion, causes cleavage in backbone (Burger et al., 1981).

In addition to oxidative stress, inflammation is also evident in bleomycin pulmonary damage. Studies have demonstrated that cytokines including interleukin (IL)-1, IL-7, IL-8, IL-10, IL-12, tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) effectively take part in collagen deposition over the pulmonary fibrosis (Larki-Harchegani et al., 2013).

Silymarin is a mixture of flavonolignans derived from milk thistle [*Silybum marianum* L. (Asteraceae)] which has antioxidative and anti-inflammatory properties. Silymarin can scavenge free radicals, like hydroxyl, superoxide and

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hydrogen peroxide, reduces lipid peroxidation, and enhances SOD activity (Hogan et al., 2007; Kiruthiga et al., 2007). There is evidence that silymarin has protective properties against some toxicants such as halothane, thioacetamide, galactosamine, paladin, and carbon tetrachloride (Mukarram Shah et al., 2011). The role of silymarin in a variety of disorders in various organs like CNS, prostate, kidneys, and lung has been established (Gazak et al., 2007). Studies on humans and animals have confirmed that the natural antioxidant has no significant adverse effects (Oliveira et al., 2001).

In light of above knowledge, the present study evaluates the protective activity of silymarin following oxidative stress and inflammation induced by bleomycin in mice.

Materials and methods

Animals and groups

Pathogen-free BALB/c mice (20–25 g) were purchased from Pasteur Institute, Tehran, Iran. The mice were kept in temperature-controlled room at 25 °C and on a standard diet and water. All experimental procedures were approved by the Medical Ethics Committee of Tehran Medical University. Twenty-eight mice were randomly divided into four groups ($n = 7$) including control (intratracheal saline), bleomycin (intratracheal bleomycin), bleomycin plus silymarin (50 and 100 mg/kg b.w).

After anaesthetization, a single dose of bleomycin (1.5 U/kg in normal saline) was administered intratracheally to mice. In silymarin groups, silymarin (Sigma, St. Louis, MO) was dispersed in normal saline +0.5% w/v methylcellulose and following the bleomycin administration, treated intraperitoneally for 10 consecutive days.

Sample collection

The entire animal group was slightly anesthetized on day 10 after bleomycin administration, blood was drawn by cardiac puncture and serum sample was immediately separated. Lung tissue was also quickly removed and processed for homogenization and fractionation.

Lung lipid peroxidation assay

Formation of thiobarbituric acid-reactive substances (TBARS) was measured in lung homogenates (20% w/v) by the method described by Buege and Aust (1978). Briefly, the tissue was homogenized in 100 mM potassium phosphate buffer (pH 7.4) and the homogenate was centrifuged for 5 min at 3000 *g*. One milliliter of supernatant was mixed with 2 ml of the TBA–TCA–HCl reagent (Thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA) and vortexed thoroughly. The samples were kept in a boiling water bath for 15 min and then allowed to cool at room temperature. The samples were then centrifuged at 3000 *g* for 10 min and the absorbance of supernatant was measured at 535 nm by a spectrophotometer. The MDA levels was determined from the extinction coefficient at 535 nm ($\varepsilon = 1.56 \times 10^5\,M^{-1}\,cm^{-1}$).

Lung reduced glutathione (GSH) assay

The level of lung GSH was measured according to the method of Sedlak and Lindsay (1968). In short, tissue samples were homogenized in 0.02 M EDTA, added an equal volume of 10% TCA to the homogenate, vortexed for 15 min, and centrifuged at 10 000 rpm for 10 min at 4 °C to obtain protein free supernatant. About 0.5 ml of Ellman's reagent (19.8 mg of DTNB in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2 M, pH 8.0) were added to 1.0 ml of supernatant and the absorbance was measured spectrophotometrically at 412 nm.

Measurement of lung glutathione-*S*-transferase (GST) activity

GST activity was assayed according to the method of Habig and Jakoby (1981). In this assay 1-chloro-2,4-dinitrobenzene (CDNB) was used as the substrate. The change in absorbance was monitored spectrophotometrically at 340 nm for 3 min. The enzymatic activity was expressed as units/(min mg proteins) using a molar extinction coefficient of $9600\,M^{-1}\,cm^{-1}$.

Measurement of catalase (CAT) activity

CAT activity was determined using a kit (Cayman, Ann Arbor, MI) according to the manufacturer's instructions. The absorbance was measured at 540 nm and the standard curve was used to calculate the results.

Measurement of lung myeloperoxidase (MPO) activity

In MPO activity, assay of 4-aminoantipyrine/phenol solution was used as the MPO substrate. The absorbance at 510 nm was recorded using a UV–visible spectrophotometer. One unit of enzyme activity was defined as the amount causing degradation of 1 μmol hydrogen peroxide in 1 min at 37 °C. The enzyme activity was expressed as U/g tissue (Wei & Frenkel, 1993).

Measurement of serum cytokines (TNF-α and IL-6)

The level of cytokines TNF-α and IL-6 in serum was determined by enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, UK) according to manufacturers' instruction.

Statistical analysis

The experimental results are presented as mean ± standard deviation (SD). Following the assurance of normal distribution of data, a one-way analysis of variance (ANOVA) with the Tukey *post hoc* test was done in SPSS (17.0) (SPSS Inc., Chicago, IL) (p values < 0.05).

Results

The influence of silymarin treatment on lung lipid peroxidation, formation of thiobarbituric acid reacting substances (TBARS), revealed that the levels of TBARS were elevated in bleomycin-injected mice (~92%). Silymarin treatment to bleomycin-administered mice resulted in a significant decrease in TBARS ($p < 0.05$).

As shown in Table 1, lung GSH was depleted due to bleomycin administration (83%); while, silymarin treatment leads to a significant increase in pulmonary GSH

Table 1. Effects of silymarin on pulmonary oxidative parameters following bleomycin exposure.

Groups	MDA (nmol/mg protein)	GSH (μmol/g lung)	GST (nmol/min/mg protein)	Catalase (μmol/min/ml protein)
Control	0.14 ± 0.01	3.3 ± 0.4	48.2 ± 3.5	71.9 ± 3.5
BLM	0.27 ± 0.02^{a}	1.8 ± 0.2^{a}	32.4 ± 2.8^{a}	51.5 ± 3.1^{a}
BLM + Silymarin 50	0.19 ± 0.02^{b}	2.6 ± 0.4^{b}	38 ± 3.3	60.0 ± 4.8
BLM + Silymarin 100	0.17 ± 0.01^{b}	3.1 ± 0.2^{b}	41 ± 2.6^{b}	65.7 ± 2.5^{b}

Data are presented as means ± SD ($n = 7$ mice/group). BLM, bleomycin.
[a]$p < 0.05$ versus control group.
[b]$p < 0.05$ versus BLM group.

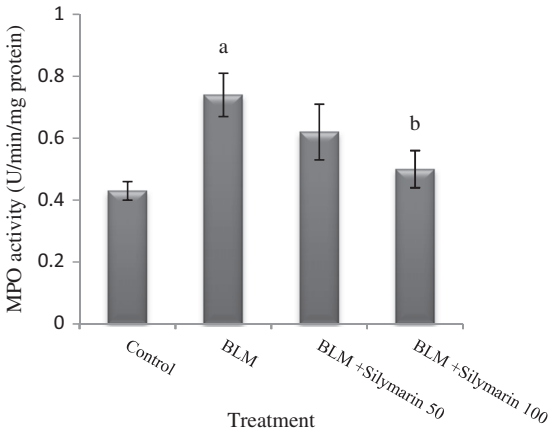

Figure 1. Effect of silymarin on pulmonary MPO activity in bleomycin-exposed mice. BLM, bleomycin. MPO activity is expressed as U min/mg of protein. Results are mean ± SD for 7 mice/group. [a]$p < 0.05$ versus control group. [b]$p < 0.05$ versus BLM group.

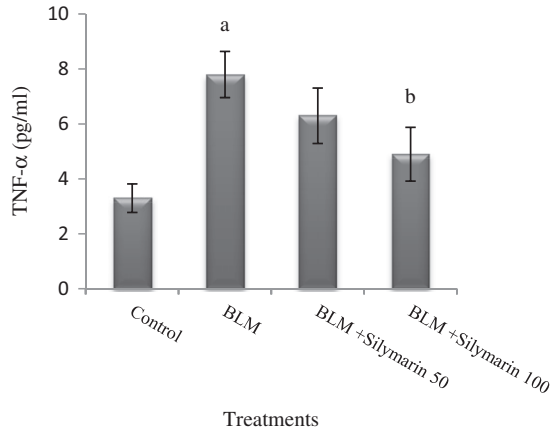

Figure 2. Effects of silymarin in serum tumor necrosis factor-alpha (TNF-α) level in animal exposed to bleomycin. BLM, bleomycin. Results are mean ± SD for 7 mice/group. [a]$p < 0.05$ versus control group. [b]$p < 0.05$ versus BLM group.

content ($p < 0.05$). Our results showed that GSH almost reached to normalcy in rats undergoing high dose silymarin treatment.

A significant reduction in GST activity was occurred following bleomycin treatment, compared with control mice (48%), whereas high dose of silymarin treatment led to a significant induction in enzyme activity ($p < 0.05$). Although low dose of silymarin caused an increase in GST activity it was not significant.

Changes in serum CAT activity showed that enzyme activity, which was inhibited due to bleomycin (~92%), was induced again following a high dose of silymarin injection (Table 1).

MPO activity is used as an index for neutrophil infiltration. As shown in Figure 1, the MPO activity was induced significantly via bleomycin administration in compare with controls (~70%). However, enzyme activity was suppressed by the higher dose of silymarin ($p < 0.05$).

The data obtained from cytokines revealed that a dramatic increase in serum TNF-α level occurred following bleomycin exposure (Figure 2). Our results show that silymarin administration at a low dose failed to reduce this inflammatory factor ($p > 0.05$), while treatment of bleomycin-injected rats with high dose of silymarin resulted in a significant depletion of serum TNF-α (~60%).

It was found that serum IL-6 was elevated significantly due to bleomycin comparing with the control group ($p < 0.05$). However, IL-6 decreased in mice subjected to silymarin treatment (Figure 3).

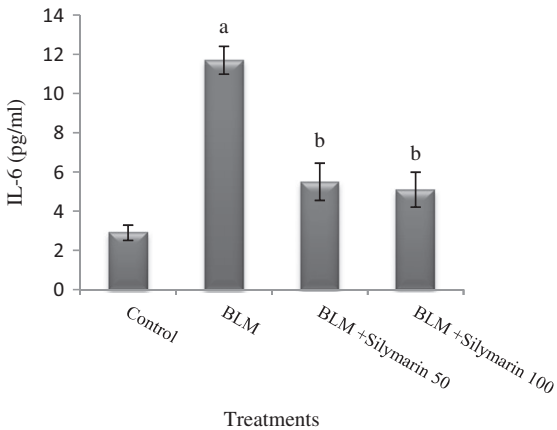

Figure 3. Effects of silymarin treatment in serum interleukin-6 (IL-6) level in mice exposed to bleomycin. BLM, bleomycin. Results are mean ± SD for 7 mice/group. [a]$p < 0.05$ versus control group. [b]$p < 0.05$ versus BLM group.

Discussion

The lung injury following bleomycin administration was defined as changes in some parameters such as a significant elevation in lung lipid peroxidation, MPO activity, TNF-α and IL-6 levels, depletion in GSH content and GST and CAT suppression.

Overproduction of reactive oxygen species as well as decrease in antioxidant capability of the cell leads to tissue oxidative damage. Lipid peroxidation along with the changes

in radical scavengers is evidence of oxidative stress occurrence (Khan, 2006).

The generation of free radicals led to lipid peroxidation and production of by-products such as MDA which is a marker of lipid peroxidation (Janero, 1990; Marnett, 1999). Bleomycin exposure caused a significant elevation in pulmonary MDA level compared with control mice which demonstrates improved lipid peroxidation in mice. Our results are in accordance with other studies that have reported lipid peroxidation by exposure to bleomycin (Kara et al., 2010; Sener et al., 2007; Yildirim et al., 2005). Sener et al. (2007) demonstrated that bleomycin lung injury was accompanied by a significant depletion in pulmonary GSH level as well as GST and glutathione peroxidase activity. In this study, they found that administration of resveratrol, an antioxidant agent, can ameliorate oxidative injury and fibrosis due to bleomycin.

GSH (γ-glutamylcysteinylglycine) is an endogenous non-enzyme antioxidant, which is responsible for scavenging of free radicals and preserves the redox status. In addition, GSH plays a central role in catalytic cycles of antioxidant enzymes, such as glutathione peroxidase, glutathione reductase (GR), and GST (Townsend et al., 2003). The data obtained from GSH levels following bleomycin treatment showed that bleomycin depleted pulmonary content of GSH (Table 1). Depletion in GSH content following oxidative stress can be due to suppression of the enzymes, which participate in GSH synthesis and/or GR activity. Some studies have indicated that loss of GSH will straightly influence the activity of the GR, which is responsible for generation of GSH from GSSG (Tobwala et al., 2013).

GST is another antioxidant enzyme, which contributes in cellular defense against ROS generation during oxidative stress of different tissues including lung (Day, 2008; Quinlan et al., 1994). As indicated in Table 1, a significant suppression occurs in GST activity of mice subjected to bleomycin exposure (48%).

In addition to GST, we observed that CAT activity was inhibited due to bleomycin administration. CAT is a free radical scavenging enzyme, which is expressed in various tissues such as lung alveolar epithelial cell and inflammatory cells. This enzyme can reduce H_2O_2-mediated activation of fibroblasts in idiopathic pulmonary fibrosis (Kliment & Oury, 2010). Moreover, it has been shown that CAT administration to asbestos-exposed mice inhibits pulmonary fibrosis through reducing H_2O_2 generation in Rac 1-activated inflammatory cells (Murthy et al., 2009).

The most important sources of TNF-α production are activated monocytes, fibroblasts, and endothelial cells. TNF-α and IL-6, which are the pro-inflammatory factors, are increased by bleomycin administration (Figures 2 and 3). It was also revealed that TNF-α expression increases in lung of mice after bleomycin treatment (Piguet et al., 1989). The role of TNF-α during fibrosis process was further established when it was specified that anti-TNF-α noticeably inhibited bleomycin-induced pulmonary fibrosis by down-regulation of pro-fibrotic factors like TGF-β and MCP-1 (Chen et al., 2007). MPO, which is frequently found in neutrophil granulocytes, release in response of different stimulatory factors (Kettle et al., 1997). We observed prominent lung MPO activity in bleomycin treated animal, demonstrating enhanced PMN activity.

The protective effects of silymarin have been demonstrated against some compounds such as thioacetamide, halothane, and carbon tetrachloride (Mukarram Shah et al., 2011). There is strong evidence that silymarin can accomplish its protective actions through various mechanisms like antifibrotic, anti-inflammatory, and antioxidant activity (Chen et al., 2009; Nazemian et al., 2010; Singh & Agarwal, 2004; Yang et al., 2003).

In this study, we showed that treatment of bleomycin-administered animals with silymarin causes a significant reverse in bleomycin-induced pulmonary injury. Silymarin treatment resulted in decrease in lung MDA, which is an end product of lipid peroxidation in bleomycin-administered mice. Free radical generation via cell membrane damage can leads to lipid peroxidation product elevation. Silymarin can modulate lipid peroxidation by regulating membrane permeability and increasing the stability of membrane integrity (Pandey & Sahni, 2011).

Besides, silymarin administration efficiently restored the depleted levels of GSH in animals subjected bleomycin injection. Silymarin as a natural antioxidant can compensate the cellular glutathione content by preserving the GSH homeostasis (Pradeep et al., 2007).

The antioxidant enzymes including CAT and GST which was initially suppressed by bleomycin, induced again due to silymarin treatment. These data corroborate the previously detected pulmonary GSH reduction and lipid peroxidation. Our findings about antioxidant enzymes are in consonant with a prior study which reported that silymarin induce the activity of CAT and superoxide dismutase (Soto et al., 2003).

As evidenced in this study, silymarin depressed the elevated pro-inflammatory cytokines in response to bleomycin. It has been demonstrated that silymarin modulates the inflammatory reactions through up-regulation of anti-inflammatory cytokines, like IL-10, IL-12 as well as suppression of leukotrien formation, which is a potent inflammatory mediator (Meeran et al., 2006; Schumann et al., 2003).

Increased pulmonary MPO activity as the results of bleomycin was effectively inhibited via silymarin treatment. The depression of cytokine release by silymarin is related to infiltration of neutrophil suppression, since MPO activity diminishes in lung tissue.

The reversal of oxidative and inflammatory parameters suggests that the protective mechanism of silymarin involves the inhibition of inflammatory and oxidative mediators.

In conclusion, the present study demonstrates that silymarin attenuated blepmycin-induced pulmonary injury. Although the exact mechanism underlying these actions is unclear, this effect can be attributed to the antioxidant along with anti-inflammatory properties of silymarin. Future studies should focus on molecular mechanisms involved in the modulatory action of silymarin on bleomycin pulmonary damage.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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