



## The Protective Effects of Silymarin on D-Galactosamine/Tumor Necrosis Factor-Alpha-Induced Oxidative Stress and Lung Injury in Mice

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### Abstract

Silymarin (Sm) is known for its anti-oxidative, anti-inflammatory, and anti-mitotic activities against a broad range of cancers, such as prostate, colon, ovarian, skin and lung cancer. This study targets to determine the protective effects of Sm on lung damage in the mice exposed to D-galactosamine (D-GaIN)/tumour necrosis factor alpha (TNF- $\alpha$ ). For this purpose, three groups of Balb/c mice were used. The study groups were given saline (0.5 mL), D-GaIN/TNF-a, and D-GaIN/TNF- $\alpha$  plus Sm, respectively. Our biochemistry, apoptotic markers and light microscopic findings offer that Sm can noticeably diminish D-GaIN/TNF $\alpha$ -induced pulmonary toxicity. In conclusion, Sm improves degenerative alterations in injured lung owing to D-GaIN/TNF-a. This caring effect might be thanks to the capability of Sm to keep oxidant–antioxidant stability.

*Keywords:* D-galactosamine, Silymarin, Tumour necrosis factor alpha, Apoptotic marker, Lung damage.

## **D-Galaktozamin/Tümör Nekroz Faktör Alfa Nedenli Oksidatif Stress ve Akciğer Hasarı Üzerine Silmarinin Koruyucu Etkileri**

### **Özet**

Silymarin (Sm), prostat, kolon, yumurtalık, deri ve akciğer kanseri gibi çok çeşitli kanserlerin tedavisinde kullanılan anti-oksidatif, anti-inflamatuar ve anti-mitotik aktiviteleri ile bilinen bir bileşiktir. Bu çalışma, D-galaktosamin (D-GalN) / tümör nekrozis faktör alfa (TNF- $\alpha$ ) nedenli oksidatif stress ve akciğer hasarı üzerinde Sm'nin koruyucu etkilerini belirlemeyi amaçlamaktadır. Bu amaçla 21 adet Balb / c cinsi erkek fare kullanıldı (n=7). Çalışma gruplarına sırasıyla serum fizyolojik (0.5 mL), D-GalN / TNF-a ve D-GalN / TNF- $\alpha$  artı Sm verildi. Biyokimyasal, histopatolojik ve apoptotik bulgularımıza göre, Sm'nin D-GalN / TNF-a nedenli pulmoner toksisiteyi ve oksidatif stressi belirgin olarak azaltabildiğini göstermektedir. Sonuç olarak, Sm, D-GalN / TNF-a'ya bağlı akciğerde meydana gelen dejeneratif değişiklikleri olumlu yönde iyileştirdiği görülmüştür. Sm'nin bu etkisi, oksidan-antioksidan stabilitesini koruma özelliğinden dolayı olduğunu düşünmekteyiz.

*Anahtar Kelimeler:* D-galaktozamin, Simarin, Tümör nekroz faktör, Apoptotic marker, Akciğer hasarı.

### **Introduction**

The pathologic outcomes of septic shock are attributable to overproduction of pro-inflammatory cytokines, including TNF-a, interleukin-1-b and interleukin-8, which ultimately accounts for multiple organ toxicity [1,2]. Different aspects of multiple organ failure have been studied by administering low doses of lipopolysaccharides (LPS) to animals that were previously rendered sensitive through transcriptional inhibitors, such as D-GalN, alpha-amanitin or actinomycin D. D-GalN is a hepatotoxin that causes in-vivo liver damage by depleting nucleotides with subsequent inhibition of protein and RNA synthesis [3]. In models using transcriptional inhibitors, TNF-a induction of hepatocyte apoptosis is a basic mechanism of pathogenesis [4,5]. Once it has been systemically released, TNF-a that is taken up by the kidney and lung causes lung injury [6]. In D-GalN-induced acute liver injury, the pulmonary response to

Escherichia coli LPS resulted in increased TNF- $\alpha$  levels, lung vascular permeability, as well as leukocyte activation [7]. TNF- $\alpha$  induces apoptosis in the alveolar epithelial cells in vitro [8]. In addition, Chopra et al. demonstrated that the upregulation of apoptosis followed by lung inflammation plays a crucial role in the development of acute lung injury and related disorders [9].

Sm is a mixture of flavonolignans derived from milk thistle [*Silybum marianum* L. (Asteraceae)] which has antioxidative and anti-inflammatory properties. Sm can scavenge free radicals like hydroxyl, superoxide and hydrogen peroxide, reduces lipid peroxidation, apart from enhancing SOD activity [10]. Flavonoid, silibinin, inhibits proliferation and promotes cell-cycle arrest of human colon cancer. There is evidence that Sm has protective properties against some toxicants such as TNF- $\alpha$ , thioacetamide, galactosamine, paladin, and carbon tetrachloride [11]. Studies on humans and animals have confirmed that the natural antioxidant has no significant adverse effects [12]. It has been reported that Sm reduces experimental damage in the heart, lungs, kidneys and liver [13,14]. But there are no studies on the preventive effects of Sm in lung in the D-GaIN/TNF- $\alpha$  model. This study aims to examine protective effects of Sm on lung damage in a mice model of D-GaIN/TNF- $\alpha$ .

## **Materials and Methods**

### **Chemicals**

Sm was supplied by Sigma Aldrich (St Louis, MO, USA). Tumour Necrosis Factor- $\alpha$  (Human Recombinant E. Coli) and D-galactosamine were obtained from Gattefosse (Saint-Priest Cedex, France), Duksan Chemical Company (Ansan, South Korea), CalbioChem and Acros Organics, respectively.

### **Animals**

21 Balb/c male mice with 2 to 2.5 month of age, each one weigh 20 to 25 gr, were used in this study. The mice were fed with standard pellet and tap water in controlled laboratory conditions, kept at daylight and dark for 12 hours each time at a temperature of  $22\pm 2$  C° along with humidity of 45-50% before the experiment was launched. They were randomly categorized into 3 groups per 7 mice. During this adaptation period, all the mice were fed with standard pellet and tap water in polycarbonate transparent cages.

### **Experimental Design**

In the control (Group 1) animal group 0.5 ml of saline i.p. injected. The Group 2 i.p. received only D-GaIN/TNF- $\alpha$  [5]. The Group 3 i.p. received Sm (100 mg/kg) 4 hours after D-GaIN/TNF- $\alpha$  injection. The Group 1 and Group 2 were sacrificed only 4 hours after injection, while the other group was sacrificed 24 hours after the final injections [15]. TNF- $\alpha$  (15 $\mu$ g/kg in distilled water) was administered to each mice. The mice were administered with D-GaIN (700 mg/kg in distilled water). Finally, Sm (100 mg/kg in distilled water) was injected to the mice.

### **Histopathological Investigations**

The lungs were cut into small pieces and fixed in Bouin's solution. Following dehydration in an ascending series of ethanol (70, 90, 96, 100%), the tissue samples were cleared in xylene and embedded in paraffin sliced in 5–6 $\mu$ m sections. The sectioned samples were stained with Haematoxylin-Eosin (H-E) and Masson's trichrome (Masson), thus revealing collagen.

### **Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-end Labeling (TUNEL) Evaluate and Immunohistochemistry**

Apoptotic cells were identified in paraffin-embedded lung tissue sections by means of a TUNEL assay kit (Chemicon International S7101, USA) and labeling active caspase-3 with a suitable active caspase-3 antibody (clone CPP32; Neo Markers, USA; dilution 1:50) following a procedure previously reported (Gezginci ve Bolkent, 2007). For PCNA immunostaining mice monoclonal antibody against PCNA (clone PC10; Neo Markers, USA; dilution 1:50) was used.

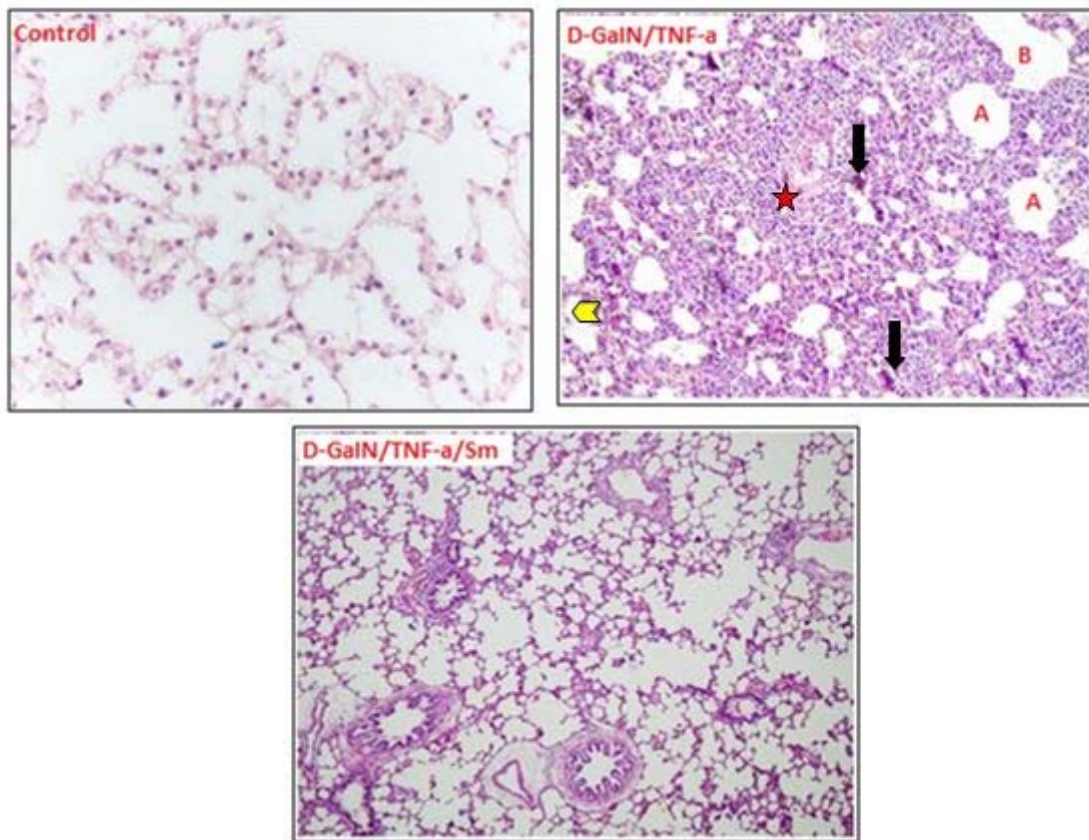
### **Statistical Analysis**

A package software version of SPSS 12.0 for windows was used when assessing the data obtained in the present study. The differences observed for serum GSH and plasma MDA levels in the groups were assessed via one-way ANOVA. The numerical value ( $p$ ) for the difference was deemed significant if it was  $p < 0.05$ .

## Histological Results

### Sm prevents lung damage due to D-GaIN/TNF-a

The lungs of the control mice exhibited a normal structure. In the lungs of the mice given D-GaIN / TNF-a, infiltration zones around the pulmonary vein, edema in the subpleural locations and increased leukocytes in some bronchioles with alveolar expansion. The lungs of the mice given D-GaIN and TNF-a plus Sm showed a general improvement in deformed morphological structure with a decrease in edema (Figure 1).



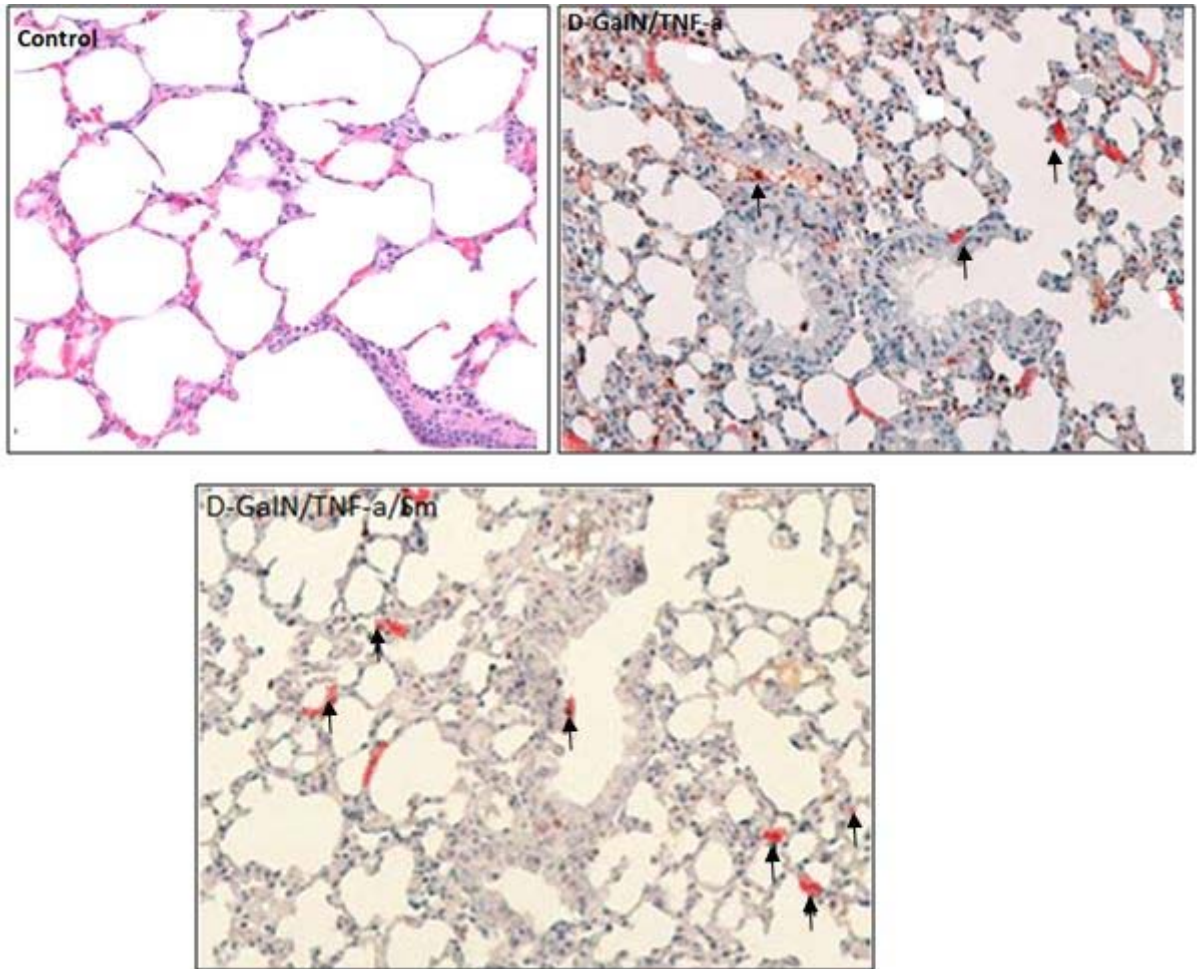
**Figure 1.** Lung structure of the control mice, D-GaIN/TNF-a administered mice and D-GaIN/TNF-a/Sm treated mice in sections stained with HE: A alveol; Bronchiole; B Oedema; Arrowheads, swelling pneumocytes, \* infiltration area.

### TUNEL assays and caspase-3

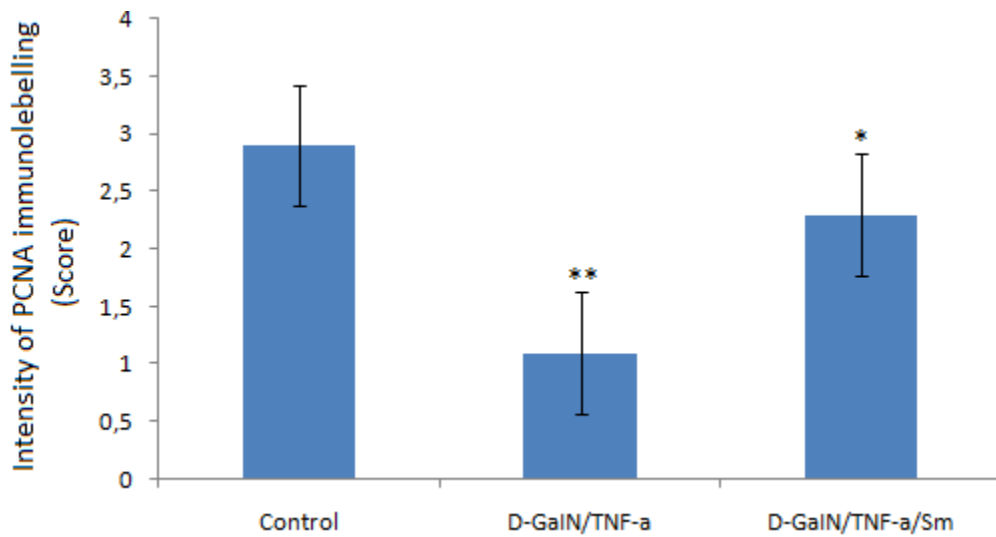
No apoptotic cells were present in the lungs of the mice in any group, and no caspase-3 immunostaining was observed.

### PCNA immunoreactivity

In Group 2, the number of PCNA positive lung cell significantly decreased when compared to that of the control group (Group 1), which was of statistical significance ( $p < 0.05$ ). On the other hand, in Group 3 the number of PCNA positive lung cell showed a significant increase compared to Group 2, which was of also statistical significance ( $p < 0.05$ ). Our immunohistochemical findings showed that Sm protects lung damage induced by D-GaIN/TNF- $\alpha$  (Figure 2, 3).



**Figure 2.** Immunobiochemically-stained lung specimens of the mice in different study groups. PCNA protein expression in Control group. PCNA protein expression in D-GaIN/TNF- $\alpha$  treated group showing less intense and positive-stained lung cells as shown by arrows. PCNA immunostaining of lung treated with D-GaIN/TNF- $\alpha$ /Sm showing intense staining as shown by arrows.



**Figure 3.** Intensity of immunolabelling score of activated PCNA positive lung cells in the groups. \*\*:  $p < 0,01$  significant different compared to control group, \*:  $p < 0,05$  different compared to Control group.

## Discussion

Induction of cellular inflammatory reactions, enrichment of oxidative stress, and increased expression of different pro-inflammatory and adhesive molecules are all known to be factors involved in the biological action of TNF- $\alpha$  [16,17]. Structural damages, such as pulmonary inflammation, severe systemic hypoxemia, and non-cardiogenic oedema, were seen in the lungs of the animals given high levels of TNF- $\alpha$  [18]. Several data in the mice lung received D-GalN/TNF- $\alpha$  agrees with mentioned report for TNF- $\alpha$  dependent-lung injury. The liver has been shown to actively contribute to lung injury [19, 20]. In endotoxin-induced acute lung injury, intense activation of the inflammatory response and pulmonary oedema required interaction with liver, with increases of TNF- $\alpha$  biosynthesis both of these organs [19]. Previous studies have reported hepatocyte apoptosis, oxidative damage, and liver dysfunction induced by D-GalN/TNF- $\alpha$  [21]. TNF- $\alpha$  is highly regulated and active in the lung. The oxidative injury is reported to have originated from a direct effect of TNF- $\alpha$  on lung tissue and/or secondary lung injury associated with liver damage due to D-GalN/TNF- $\alpha$  [22]. The number of studies having investigated lung damage due to D-GalN and TNF- $\alpha$  is very small. One of them, where lung damage was caused by a combination of 700 mg/kg of D-GalN and 15  $\mu$ g/kg of TNF- $\alpha$  at the 4<sup>th</sup> hour of the experiment, reported a rise in



tissue MDA levels but there was a significant drop in GSH levels when compared to Control group. Moreover, the lung sections from the D-GaIN/TNF- $\alpha$ -given mice showed increased oedema in subpleural locations and extensive infiltration areas around the pulmonary vena/venulae and numerous leukocytes in some bronchioles [22]. In line with the findings of this study, our histological and biochemical data showed that the lung specimens, taken from the mice 4 hours after they had been given an injection of 700 mg/kg D-GaIN and 15  $\mu$ g/kg TNF- $\alpha$ , exhibited remarkably similar findings (Figure 1, 2, 3).

Sm is a mixture of flavonolignans derived from milk thistle [*Silybum marianum* L. (Asteraceae)] which has antioxidative and anti-inflammatory properties. Sm can scavenge free radicals, like hydroxyl, superoxide and hydrogen peroxide, reduces lipid peroxidation, and Sm enhances SOD activity [10]. There is evidence that Sm has protective properties against some toxicants such as halothane, thioacetamide, galactosamine, paladin, and carbon tetrachloride [11]. The role of Sm in a variety of disorders in various organs like prostate, kidneys, and lung has been established [23]. One study showed that treatment of lung damage due to 50 mg/kg bleomycin with 100 mg/kg Sm causes a significant reverse in bleomycin-induced pulmonary injury. In the same study, Sm treatment resulted in decrease in lung MDA, which is an end-product of lipid peroxidation and increase GSH which contributes to cellular defence against ROS generation during oxidative stress of different tissues including lung [24] in bleomycin-administered mice [25]. In another study, 250 mg/kg Sm was reported to significantly improve pulmonary vascular dysfunction caused by lung ischemia-reperfusion (I/R). It was also stated that Sm increased serum GSH level and decreased MDA level [26]. Our study showed similar results to the above-mentioned studies because we have found that lung damage induced by D-GaIN/TNF- $\alpha$ , was decreased in the group given 100 mg/kg Sm. In conclusion, the present study demonstrates that Sm attenuated D-GaIN/TNF- $\alpha$ -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 Sm.



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