J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 940-948, 2024
Doi: 10.33483/jfpau.1443799

**ORIGINAL ARTICLE / ÖZGÜN MAKALE**

# SILDENAFIL DECREASED TNF-α AND IL-6 LEVELS IN CD-INDUCED ACUTE TOXICITY

## *SİLDENAFİL CD İLE İNDÜKLENEN AKUT TOKSİSİTEDE TNF-α VE IL-6 DÜZEYLERİNİ DÜŞÜRÜR*

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## ABSTRACT

**Objective:** *This study aimed to evaluate the effects of sildenafil (SIL) on inflammation and histopathological changes in cadmium (Cd)-induced toxicity in female rats.*
**Material and Method:** *Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α) levels were measured to assess the degree of inflammation. Histopathological changes in the liver, lungs and kidneys were also assessed.*
**Result and Discussion:** *SIL significantly reduced the cellular release of TNF-α and IL-6, which have been implicated in the pathogenesis of Cd-induced tissue damage. When SIL was administered alone, it showed histopathological effects similar to the control group. However, it was found that co-administration of SIL with Cd prevented portal vein dilation and central vein enlargement in the liver, prevented necrosis in kidney tissue, but did not affect the lung. Although SIL has variable protective effects on tissues, our results are in support of the idea that the use of SIL in tissue damage management can be investigated for its efficacy in modulating oxidative stress-induced proinflammatory cytokine activation in vivo and ultimately help prevent Cd-induced tissue damage. Our study has shown that SIL can reduce Cd-induced acute toxicity in rats. SIL may be use as a protective agent against toxicity of heavy metals.*
**Keywords:** *Cadmium, liver enzymes, oxidative stress, sildenafil, tissue damage*

## ÖZ

**Amaç:** *Bu çalışmada, dişi sıçanlarda kadmiyum (Cd) kaynaklı toksisitede sildenafilin (SIL) inflamasyon ve histopatolojik değişiklikler üzerindeki etkilerinin değerlendirilmesi amaçlanmıştır.*
**Gereç ve Yöntem:** *İnflamasyon derecesini değerlendirmek için interlökin-6 (IL-6) ve tümör nekroz faktörü-alfa (TNF- α) seviyeleri ve karaciğer, akciğer ve böbreklerdeki histopatolojik değişiklikler*

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**Submitted / Gönderilme** : 27.02.2024
**Accepted / Kabul** : 08.07.2024
**Published / Yayınlanma** : 10.09.2024

This article has been derived from PhD thesis of Ahmet Hüsamettin Baran.

*değerlendirilmiştir.*
**Sonuç ve Tartışma:** *SIL, Cd ile indüklenen doku hasarının patogenezinde rol oynayan TNF-α ve IL-6'nın hücresel salınımını önemli ölçüde azaltmıştır. SIL tek başına uygulandığında, kontrol grubuna benzer histopatolojik etkiler göstermiştir. Bununla birlikte, SIL'in Cd ile birlikte uygulanmasının karaciğerde portal ven genişlemesini ve merkezi ven büyümesini önlediği, böbrek dokusunda nekrozu önlediği, ancak akciğeri etkilemediği bulunmuştur. SIL'in dokular üzerinde değişken koruyucu etkileri olmasına rağmen, sonuçlarımız doku hasarı yönetiminde SIL kullanımının in vivo oksidatif stres kaynaklı proinflamatuar sitokin aktivasyonunu modüle etmedeki etkinliğinin araştırılabileceği ve nihayetinde Cd kaynaklı doku hasarını önlemeye yardımcı olabileceği fikrini desteklemektedir. Çalışmamız, SIL'in sıçanlarda Cd kaynaklı akut toksisiteyi azaltabildiğini göstermiştir. SIL, ağır metallerin toksisitesine karşı koruyucu bir ajan olarak kullanılabilir.*
**Anahtar Kelimeler:** *Doku hasarı, kadmiyum, karaciğer enzimleri, oksidatif stres, sildenafil*

## INTRODUCTION

Cadmium (Cd) is a carcinogenic heavy metal that is commonly found in the earth's crust and has significant harm to human, animal and plant health [1]. As a result of industrial activities Cd, which spreads as a contaminant to air, water and soil, causes serious health problems on living organisms due to its toxicity and bioaccumulation in the ecosystem [2]. The mechanisms that trigger oxidative stress and initiate the inflammatory process underlie the nephrotoxicity, hepatotoxicity and lung toxicity observed as a result of environmental and occupational exposure to Cd [3-6].

Cadmium exposure causes an oxidant/antioxidant imbalance in the organism and increases peroxide formation. This leads to the production of high levels of hydroperoxides and disruption of lipid, carbohydrate and protein metabolism. On the other hand, it has been shown to increase the levels of biological indicators such as tumor necrosis factor alpha (TNF-α) and interleukins which are responsible for inflammation, apoptosis and tumor development [1-6].

The phosphodiesterase 5 (PDE5) enzyme is a cyclic nucleotide phosphodiesterase found in many tissues, including neurons, smooth muscle cells, and the brain. It neutralizes the second messenger 3′-5′ cyclic guanosine monophosphate (cGMP), by hydrolyzing it to guanosine monophosphate (GMP) [7]. Sildenafil (licensed as Viagra®) is a selective and potent PDE5 inhibitor, extending the half-life of endogenous cGMP and expanding corpus cavernosum vascular smooth muscles. As a PDE5 inhibitor, SIL is now frequently used to treat pulmonary hypertension and erectile dysfunction [8,9]. In some recent studies, there have been suggestions that the use of SIL may have anti-inflammatory and antioxidant activities [10-12]. However, the protective effect of SIL against multi-organ damage in rats has not been adequately studied.

The aim of this study was to evaluation of the effect of the use of SIL on acute lung, liver and kidney damage induced by a single dose of Cd administered to female rats, and its anti-inflammatory potential, using interleukin-6 (IL-6) and TNF-α as inflammation markers and histopathological analysis of the tissues.

## MATERIAL AND METHOD

Although different exposure routes of Cd are mentioned in literature reviews, human intoxication generally occurs orally. In experimental models, the intraperitoneal route is a preferred one to induce acute Cd toxicity [13-16]. The route of administration and dosage of SIL in our study were established on the basis of previous studies [17-19].

All animals received humane care according to the guidelines established by the Committee for the Institutional Use and Care of Laboratory Animals of the University of Fırat, and the same committee approved the experimental protocol (Ethical Approval Number; 2016/151). Female Sprague-Dawley rats (180-220g) were obtained from Fırat University Laboratory of Experimental Animals. The animals were fed a standard pellet diet. Water was provided ad libitum. They were kept in plastic cages at a temp of 21 ± 2°C with a 12-hour light/dark period and an air humidity of 30% to 70%. The rats were divided into four experimental groups. All groups contained seven animals. Group I: 0.5 ml/rat of saline solution

was injected i.p. on the 7th day to the Control group. Group II: 3.7 mg/kg single dose of Cd was injected i.p. on the 7th day to the Cd group. Group III: SIL group was administered with 5 mg/kg/day of SIL in drinking water for 10 days. Group IV: Cd+SIL group was given 5 mg/kg/day of SIL drinking water during 10 days and a single i.p. injection of 3.7 mg/kg of Cd on day 7 (Figure 1). To finish experimenting, the animals were under anesthesia by i.p. administration of 50 mg/kg ketamine and 5 mg/kg xylazine and liver, lungs and kidneys removed for further analysis. Tissue samples had a storage temperature of -80°C. To prevent deformation of the fragile molecules, an experimental environment was prepared with ice water before removing the lung, kidney and liver tissues from the -80°C deep freezer, and all procedures were performed in this environment. Lung, kidney and liver tissues were removed, weighed on a precision balance and placed in plastic tubes at a weight of approximately 100 mg. The tubes were homogenized with a homogenizer after the addition of 1 ml of phosphate buffer and sonication. After centrifugation, supernatants were collected in Eppendorf tubes and used to measure biochemical parameters.

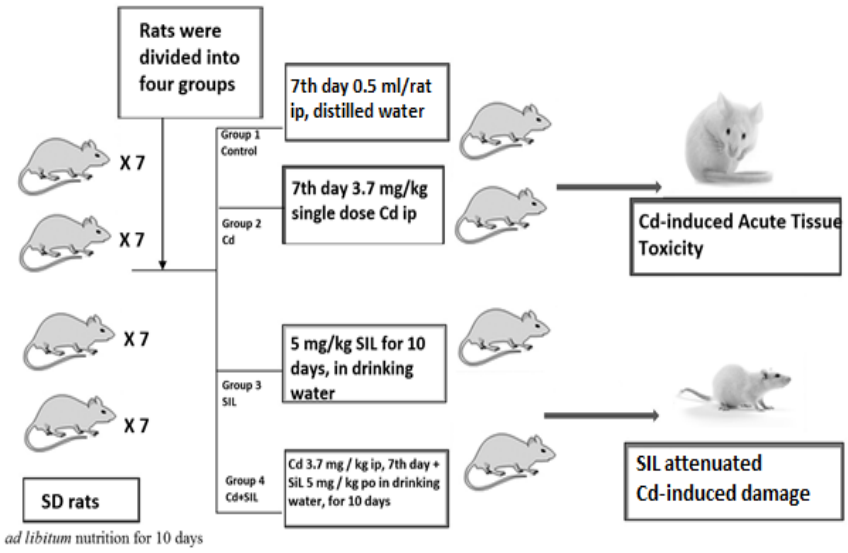


**Figure 1.** Scheme of the experimental design

This experimental protocol was designed taking into account similar studies [20-23]. The fact that Cd accumulates mainly in the kidney and liver, where metallothionein levels are high, dictated the choice of tissues analyzed. Furthermore, Cd hepatotoxicity is the main cause of acute Cd lethality and the liver is the main target organ of toxicity after acute Cd poisoning [24].

**Cytokine Analysis**

Enzyme-linked immunosorbent assay (ELISA) kits obtained from Shanghai Yehua Biological Technolgy were used for IL-6 and TNF-α determinations. For this, Samples previously kept at -20°C were allowed to come to room temperature. The kits were removed from 4°C and kept at room temperature for 30 min. Chromogen B in the kits was kept in a closed box as it is light sensitive. Sera were centrifuged at 2500 g for 20 min. Supernatants were separated. Dilution of standard solutions was started. For this, 120 µl of the original standard in the kit was taken and mixed with 120µl of standard dilute in an ependorf tube. Then 120 µl of the mixture was taken and mixed with the same amount of standard dilution each time. Samples and solutions were then loaded onto the ELISA plate. 50 µl of standards were loaded and 50 µl of streptomycin HRP was added. The blind portion was left blank and 40 µl serum and 10 µl TNF-α antibody for TNF-α measurement and IL-6 antibody solution for interleukin 6 were added. Finally, 50 µl streptavidin-HRP was added and the layer was covered. Shaken gently and incubated at 37°C for 1 hour. The washing solution was prepared by mixing 1/30 (v/v) with distilled water and applied 5 times for 30 s to all wells in the incubated layers. For color formation, 50 µl of chromogen A was first added to all wells. Then 50 µl of chromogen B was added and shaken

carefully. 37°C incubation was allowed for 10 min. In the meantime, the assay was performed in the dark to avoid light exposure. After incubation, 50 µl of the stop solutions were added and read immediately at 450 nm. The data were calculated in the coordinate plane according to the standards. Results were expressed in pg/ml for TNF-α and IL-6.

### Histopathological Analysis

Tissue samples were fixed in 10% buffered neutral formalin and embedded in paraffin. 5-micron thick sections were cut from each paraffin block using a fully automated rotary microtome (Leica Biosystems RM2245 Semi-Automated Rotary Microtome) and stained with hematoxylin-eosin. Preparations were then examined by light microscopy (Leica Dm 200 Led Light Microscope) and photographed using an Olympus 3E03784 camera.

### Statistical Analysis

Statistical analysis was performed using the INSTAT automated software package (GraphPad Prism Inc., San Diego, CA, USA). All values are expressed as mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) has been used when comparing differences between groups, and Tukey post hoc test was used when comparing paired groups. Statistically significant was defined as a P value of <0.05.

## RESULT AND DISCUSSION

The biochemical findings related to all groups studied are summarized in Table 1. There are no significant differences in inflammation markers in SIL group compared to the control group. Management of Cd increased TNF-α levels in the liver (1.6- fold), lung (2.0-fold), and kidney (2.1-fold) tissues. SIL treatment significantly inhibited Cd-induced TNF-α increase in the liver (20.2%), lung (36.8%), and kidney (34.7%) tissues. The IL-6 levels were higher in the liver (1.9-fold), lung (1.8-fold), and kidney (1.4-fold) tissues in Cd group when compared to the control. SIL treatment significantly decreased Cd-induced IL-6 increase in the liver (33.1%), lung (24.7%), and kidney (14.7%) tissues. The results of the analysis of TNF-α and IL-6 biomarkers are shown in Table 1. TNF-α, liver (ANOVA F= 22.8, p= 0.0036), lung (ANOVA F= 33.228, p= 0.001), and kidney (ANOVA F= 66.28, p= 0.001). IL-6, liver (ANOVA F= 85.95, p=0.0009), lung (ANOVA F= 24.62, p=0.0079) and kidney (ANOVA F= 36.14, p= 0.0027).

**Table 1.** Effect of SIL on inflammation markers in Cd induced toxicity

| Groups | | Control | SIL | Cd | SIL + Cd |
|---|---|---|---|---|---|
| TNF-α (pg/ml) | Liver | 59.39±7.62 | 56.2±5.48 | 94.61±14.52*** | 75.49±8.97** |
| | Lung | 36.78±7.43 | 34.81±6.72 | 75.13±10.24*** | 47.53±9.26*** |
| | Kidney | 43.37±6.25 | 41.89±5.62 | 89.14±9.74*** | 58.24±7.21*** |
| IL-6 (pg/ml) | Liver | 230.1±24.13 | 218.32±28.93 | 430.16±32.15*** | 287.92±25.16*** |
| | Lung | 118.25±20.16 | 111.44±21.25 | 209.15±27.87*** | 157.42±25.63** |
| | Kidney | 167.3±12.35 | 153.27±13.27 | 228.01±17.29*** | 194.52±14.62** |

Abbreviations: TNF-α, tumor necrose factor; IL-6, interleukin-6. Values are represented as mean±SEM. **Significant change in comparison with control at P <0.01; ***Significant change in comparison with control at P <0.001

Lung, liver and kidney tissues were examined for inflammation and necrosis. As a result of the histopathological examination of the kidney, lung and liver tissues of the control group, a normal histopathological appearance was found. Histopathological examinations of the kidney, lung and liver tissues of the SIL group revealed normal histopathological features similar to the control group. Histopathological examination revealed of kidney, lung and liver tissues of the Cd group; enlargement of the portal (Figure 2C1) and central vein (Figure 2C2) and necrosis of single cells were observed (Figure 2C3). Inflamed areas and hemorrhage were detected in the lung tissue (Figure 3C). Focal

necrosis areas were detected in the kidney tissue (Figure 4C). It was observed that Cd toxicity had the potential to cause multiple organ damage in all three groups. The results of the histopathological examination of the SIL+Cd group; necrosis in the liver tissue (Figure 2D), chronic inflammation and hemorrhage in the lung tissue (Figure 3D), and normal histopathological appearance in the kidney tissue (Figure 4D) were observed. Cd administration caused significant pathological changes in female rat liver (Figure 2C1, 2C2, 2C3). Co-administration of SIL with Cd had no effect on necrosis formation, but reversed portal vein dilation and central vein enlargement caused by Cd (Figure 2D). Cd administration caused significant pathological changes in female rat lung compared to the control group (ANOVA F = 12.25, p = 0.0077) (Figure 3C). The administration of cadmium resulted in significant pathological changes in the kidney tissue in comparison with the control group (Figure 4C). Co-administration of SIL with Cd prevented necrosis caused by Cd when given alone (Figure 4D). When the three tissues were compared, it was determined that SIL caused a partial improvement in liver histopathology, had no positive effect on lung tissue, and reversed the effect of Cd in kidney tissue. The protective effect of SIL was most pronounced in kidney tissue.

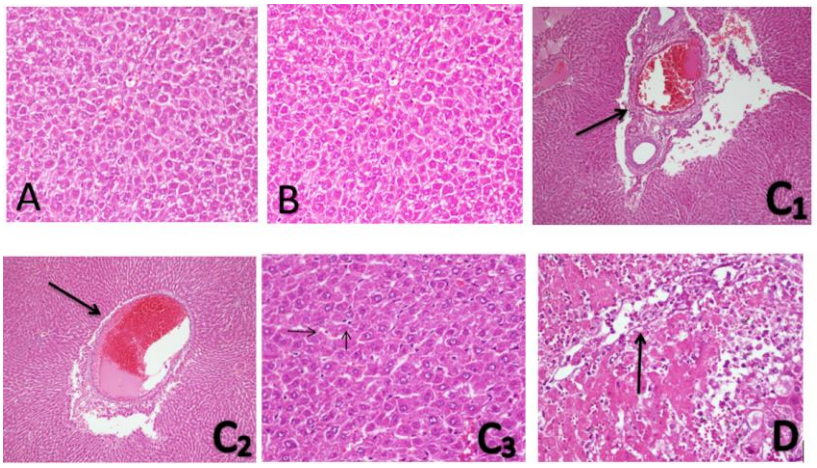


**Figure 2.** Liver tissue histopathology examination

A) Liver-control; Normal histopathological appearance in liver tissue B) Liver-SIL; Normal histopathological appearance in liver tissue C1) Liver-Cd; Portal vein dilation in liver tissue C2) Liver-Cd; Central vein enlargement in liver tissue C3) Liver-Cd; single cell necrosis in liver tissue D) Liver-SIL+Cd; Necrosis in liver tissue

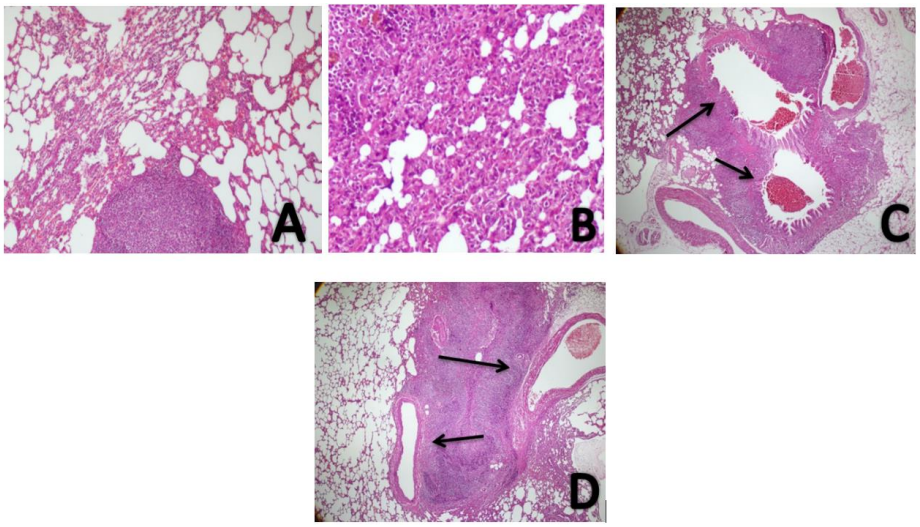


**Figure 3.** Lung tissue histopathology examination

A) Lung-control; Normal histopathological appearance in lung tissue B) Lung-SIL; Normal histopathological appearance in lung tissue C) Lung-Cd; Chronic inflammation, hemorrhage in lung tissue D) Lung-SIL+Cd; Chronic inflammation, hemorrhage in lung tissue

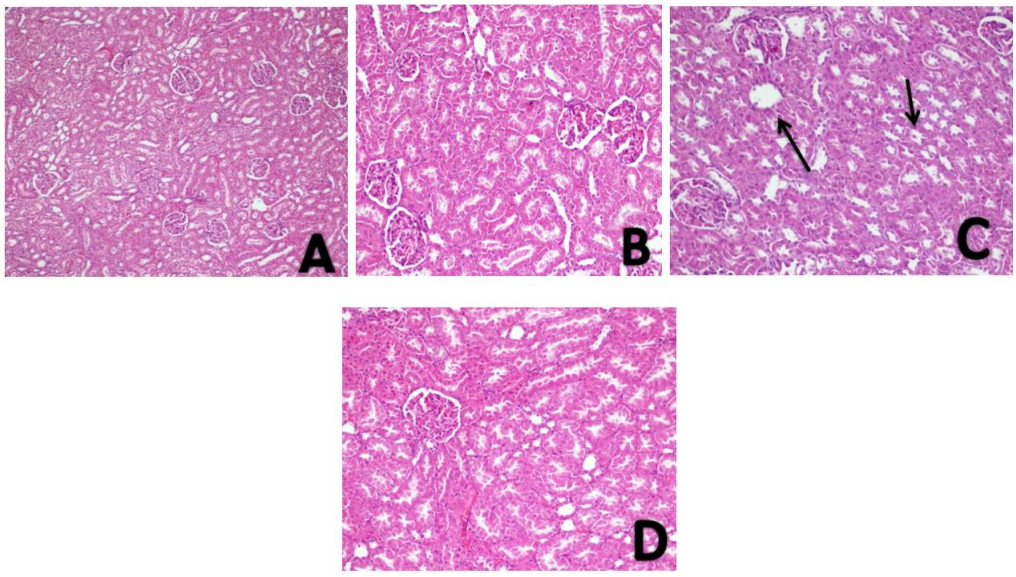


**Figure 4.** Kidney tissue histopathology examination
A) Kidney-control; Normal histopathological appearance in kidney tissue B) Kidney-SIL; Normal histopathological appearance in kidney tissue C) Kidney-Cd; Focal necrosis in kidney tissue
D) Kidney-SIL+Cd; Normal histopathological appearance in kidney tissue

Some researchers have suggested that Cd toxicity varies according to gender differences. In a study examining the impact gender differences on liver and kidney accumulation of cadmium [25–27], they found that significantly higher Cd is accumulated in the liver tissues of female rats 1 hour after subcutaneous Cd injection compared to males. They observed that Cd levels in female rats remained high for at least 10 days [25]. Therefore, we preferred to use female rats in our study.

On the basis of the results of our study, we have the following suggestions treatment with SIL may reduce the inflammatory response and prevent multiple organ damage to liver, lung and kidneys due to Cd in the rat model. As a result of histopathological evaluations, we detected focal necrosis in rat kidney tissue, chronic inflammation in lung tissue, hemorrhage, portal vein dilatation, dilation of the central vein and single cell necrosis in liver tissue. We found that SIL significantly reduced the severity of Cd damage partly in the liver tissue but mostly in the kidney tissue. Inflammation development is accompanied by inflammatory cell infiltration and inflammatory cytokine release. Cd resulted in a significant increase in the levels of IL-6 and TNF-α in the all tissues. SIL significantly reduced inflammation in the group where Cd and SIL were administered together.

Similar to our study, Fang et al. also investigated the protective role of a single i.p. dose of SIL administration in the inflammatory acute lung injury model induced by sodium taurocholate and performed histopathological analysis together with evaluation of the inflammatory parameters such as IL-6 and TNF-α. They observed low levels of lung damage and inflammation in rats treated with SIL [8]. In another study, SIL administration in the model of lung ischemia-reperfusion injury has been shown to reduce inflammation and formation of reactive oxygen species [28]. In addition to these findings investigating the effect of SIL and febuxostat on doxorubicin-induced nephrotoxicity in rats, the effect of SIL (5 mg/kg; p.o.) administration for 21 days on nephrotoxicity, oxidative stress markers, TNF-α and inflammatory mediators were examined.

SIL and/or febuxostat application with doxorubicin has led to a significant reduction in the nephrotoxicity markers and inflammatory mediators, and the return of oxidative stress biomarkers to their normal values. In addition, SIL and febuxostat improved the histological changes caused by doxorubicin [17]. Furthermore, investigating the protective role of SIL on the kidneys, SIL has been shown to reduce renal tubular damage and apoptosis in an experimental model of cisplatin-induced nephrotoxicity [29]. Similar to our study, the protective role of SIL against hepatic fibrosis induced by bile duct ligation in rats was investigated and it was found that SIL administration significantly reduced

high TNF-α values [18]. In many studies investigating the effects of SIL on inflammation, histopathological evaluations have found that SIL has a protective effect by reducing inflammation [30-32].

In our study, unlike previous studies [33,34], SIL did not have a hepatotoxic effect on the liver when administered alone or together with Cd, but it did not prevent the formation of necrosis in the liver. Neutrophil infiltration and proinflammatory cytokines like TNF-α and IL-6 are often associated with Cd-induced toxicity. Although the role of TNF-α and IL-6 in both pathological and physiological situations has not been fully elucidated, the decrease in levels of these cytokines after tissue damage is suggestive of the involvement of cellular repair mechanisms. Whereas, when inflammatory cytokines are produced in abundance, pathological conditions such as dermal toxicity, liver toxicity, rheumatoid arthritis, atherosclerosis, kidney and lung toxicity can occur [35-37]. The increase in the levels of TNF-α and of IL-6 observed in Cd-treated rats in our study indicates the initiation of inflammation leading to cellular damage, and the decrease in levels with SIL administration suggests that repair mechanisms are induced.

SIL has been shown to have protective ability in exposure to various inflammatory stimuli. However, the curative effect of SIL on Cd-induced tissue damage and the mechanism of this effect have not been studied. In this trial, we have demonstrated for the first time that protective effect of PDE5I SIL against tissue damage by an inhibitory effect on IL-6 and TNF-α activity in Cd-induced acute toxicity.

Based on the biochemical and histopathological findings of our study, we can suggest that SIL has a protective effect on the liver, lung and kidney against acute toxicity induced by Cd. SIL reverses Cd-induced increases in IL-6 and TNF-α levels, thereby exerting its anti-inflammatory effect. It also prevents cell death by inhibiting inflammation and has a protective effect as confirmed by liver and kidney histopathological analysis. Therefore, we concluded SIL may be use as a protective agent against toxicity of heavy metals.

## AUTHOR CONTRIBUTIONS

Concept: A.H.B., G.Y.; Design: A.H.B., G.Y.; Control: A.H.B., G.Y.; Sources: A.H.B.; Materials: A.H.B., G.Y.; Data Collection and/or Processing: A.H.B., A.B., A.B.U., Ö.A.S., G.Y.; Analysis and/or Interpretation: A.H.B., A.B., A.B.U., Ö.A.S., G.Y.; Literature Review: A.H.B., G.Y.; Manuscript Writing: A.H.B.; Critical Review: A.H.B., G.Y.; Other: -

## CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

## ETHICS COMMITTEE APPROVAL

Ethical approval of this study was obtained from the Committee for the Institutional Use and Care of Laboratory Animals of the University of Fırat (Ethical Approval Number; 2016/151).

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