

# Investigation of Effects of Silymarin in 5-Fluorouracil Hepatotoxicity and Nephrotoxicity in Mice

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## Research Article

**Keywords:** 5-Fluorouracil, DNA damage, Hepatotoxicity, Mice, Nephrotoxicity, Silymarin.

**Posted Date:** May 11th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-448267/v1>

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# Abstract

Hepatotoxicity and nephrotoxicity are common side effects of 5-Fluorouracil (5-FU). The present study aimed to investigate the effects of Silymarin (SLY) on 5-FU induced hepatotoxicity and nephrotoxicity in mice. In our study, 10 mice in each group were randomly divided into four groups as the control group, 5-FU, SLY50+5-FU, and SLY100+5-FU group. SLY50+5-FU and SLY100+5-FU groups were administered at a dose of 50 and 100 mg/kg for seven days, respectively. 5-FU was administered at a dose of 400 mg/kg intraperitoneally on the fourth day. After the applications, the mice were decapitated under anesthesia. The liver and kidney functions which urea, creatinine, AST, ALT, and total bilirubin levels were analyzed in serum. In liver and renal tissues, MDA and GSH levels, SOD, CAT, and GR activity were determined. Also, histopathological and immunohistochemical changes were examined in liver and kidney sections. Urea, creatinine, ALT, AST, and total bilirubin levels increased 5-FU group according to control and prevented to this increases the especially high dose of SLY. 5-FU also causes histopathological and immunohistochemical changes such as degeneration, necrosis, hyperemia, DNA damage, and IL-6 increase in kidney and liver tissue. High doses of SLY prevented these changes caused by 5-FU. As a result of this study, it was determined that SLY has hepatoprotective and nephroprotective effects on 5-FU-induced liver and kidney damage in mice.

## Introduction

Cancer is an important public health problem worldwide in recent years (Siegel et al. 2019). In the treatment of cancer are applied to different strategies such as surgery, radiotherapy, and chemotherapy. Chemotherapy is a powerful therapy for cancer and antineoplastic agents. Used for this purpose, 5-Fluorouracil (5-FU) is a commonly used agent in the treatment of various malignancies such as colon, breast cancer, head and neck cancer (Akindele et al. 2018; Grem 2000). Despite a lot of advantages, 5-FU's treatment has been largely limited due to some organ toxicity, inhibition of thymine synthesis, and DNA damage (Akindele et al. 2018; Gelen et al. 2018). Considering the mechanism of action of 5-FU; It affects the S phase of the cell cycle, activates thymidine phosphorylase, thymidylate synthase inhibiting fluorodeoxyuridine. Thus, it prevents DNA synthesis, which leads to cell death (Gelen et al. 2017). The major part of 5-FU is removed by the liver and only a little portion is abolished via the kidney (Longley et al. 2003). The incidence of hepatotoxicity and nephrotoxicity induced by drug is increasing (Abdel-Daim et al 2017; Akindele et al. 2018; Ibrahim et al. 2016; Sengul et al. 2021) and anticancer agents are among of these drugs (Abdel-Daim et al. 2019; Çayır et al. 2011; Gelen et al. 2018; Gedikli and Şengül 2019; Sengul et al. 2019). Like other anticancer agents, 5-FU causes liver and kidney toxicity and function disorders, and these tissue induced oxidative stress, inflammation, and apoptosis (Ali 2012; Gelen et al. 2017; Gelen et al. 2018). Many studies have been investigated the effects of traditional and alternative therapies against the unwanted effects of chemotherapeutics (Gelen et al. 2018b; Raskovic et al. 2011; Şengül et al. 2017). Silymarin (SLY) is a plant-based flavonoid (Köksal et al. 2009) and it has been determined that SLY has some effects as antioxidant, antioxidant, immune system regulating, and anti-inflammatory (Brinda et al. 2012; De La Puerta et al. 1996; Wen et al. 2008). In previous studies, the

therapeutic and protective effects of SLY in nephrotoxicity and hepatotoxicity induced by some pharmacological agents have been determined (Bektur et al. 2016; Kandemir et al. 2017). As a result of the literature review, we determined that the effects of SLY on 5-FU-induced hepatorenal toxicity in mice have not been investigated yet. Therefore, we investigated the protective effects of SLY on 5-FU-induced hepatotoxicity and nephrotoxicity in a mouse model.

## Material And Methods

### Animals

We used 40 male mice in our study. The weight of the mice was chosen to be 30-40 g on average. Mice were obtained from Atatürk University Medical Experimental Research and Application Center. Animals were subjected to standard feeding conditions. The necessary permission for the study was obtained from Atatürk University Rectorate Animal Experiments Local Ethics Committee (Protocol number: 2019/17).

### Experimental design

In our study, the nephrotoxicity and hepatotoxicity model was formed by 5-FU (400 mg/kg, intraperitoneal (i.p.), three dose starting from the fourth day) and SLY (50 and 100 mg/kg, 7 days) were administered intraperitoneally. The animals were divided into 4 groups. Separated groups and application methods are as follows.

**Group I (Control)** was i.p. applied 1 ml of propylene glycol (0,25 ml)+saline (0,75 ml) for 7 days

**Group II (5-FU)** was i.p administered to Propylene glycol (0,25 ml)+saline (0,75 ml) for 7 days and three doses of 5-FU (dose of 400 mg/kg) were injected starting on day 4th of the administration.

**Group III (SLY50+5-FU)** was given SLY (50 mg/kg) and injected 5-FU (400 mg/kg, three-dose) starting on day 4th of the experiment.

**Group IV (SLY100+5-FU)** was injected SLY (100 mg/kg) and given 5-FU (400 mg/kg, three-dose) starting on day 4th of the experiment.

At the end of the applications, mice in all groups were weighed and then intracardiac blood samples were taken under anesthesia. The mice were decapitated after blood samples were taken. The liver and kidney tissues of all mice in the experimental groups were removed and weighed. The right kidney of mice was placed in 10% formaldehyde for histopathological and immunohistochemical examinations. Left kidneys of mice were washed with cold phosphate buffer and frozen with liquid nitrogen. It was stored at -20 °C until biochemical studies were carried out.

### Analysis of liver and renal function parameters

The blood samples taken from the experimental groups were centrifuged at 3500-4000 rpm in a cooled centrifuge at 4 ° C for 12 minutes. Serum samples were taken into tubes. It was stored at 80 ° C until analysis. Serum urea, creatinine, ALT, AST, and total bilirubin levels were determined using the Randox IV Monaco-Auto-Chemistry-Analyzer.

### **Preparation of liver and renal homogenates**

Liver and kidney tissues obtained from experimental groups were homogenized to 5 microns in a Tissue Lyser II (Qiagen) with liquid nitrogen on the day of analysis. Tissues were weighed specifically, then diluted 1:20 with phosphate buffer (pH 7.4). Subsequently, samples were homogenized in Tissue Lyser II. After homogenization, the homogenates were centrifuged at 3000 rpm for 20 minutes at 4°C and the supernatant was used for ELISA analysis.

### **Determination of lipid peroxidation level (MDA) and antioxidant enzyme (SOD, GSH, CAT, and GR) activities**

Malondialdehyde (MDA) and glutathione (GSH) levels and superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) activities in liver and kidney homogenates were measured by using commercial ELISA kits (Ylbiont, Shanghai, China).

### **Histopathological and immunohistochemical examination**

Hematoxylin-eosin (HE), 8-Hydroxy-2'-deoxyguanosine (8-OHdG) and interleukin-6 (IL-6) immunohistochemical staining will be performed in liver and kidney tissues. According to histopathological findings, the sections were evaluated none (-), mild (+), moderate (++) and severe (+++). Again, according to their immunopositivity, none (-), mild (+), moderate (++) , severe (+++) and very severe (++++ ) were evaluated.

### **Statistical analysis**

The results of our study were evaluated statistically. The results were given as  $X \pm SD$ . The quantitative values were statistically analyzed in SPSS 20.00 statistical data program. Then, one-way ANOVA was evaluated by the Tukey test.  $p < 0.05$  was considered significant.

## **Results**

### **Effects of SLY on body, liver, and kidney weights in 5-FU-toxicity**

The live weights of the mice were similar among to groups. Liver weights reduced to 5-FU group so far as control but this decreasing not statistically significant. Liver weights of the SLY10+5-FU group were higher than the 5-FU group ( $p < 0.05$ ). Also, were similar among to groups (Table 1).

**Table 1.** The body, liver, and renal weights in experimental groups (<sup>a,b</sup>:  $p < 0.05$ ).

Experimental Groups	Body weight (g)	Liver weight (g)	Renal weight (g)
Control	37,4±2,5 <sup>a</sup>	1,8±0,13 <sup>ab</sup>	0,43±0,05 <sup>a</sup>
5-FU	34,1±1,4 <sup>a</sup>	1,6±0,10 <sup>a</sup>	0,43±0,04 <sup>a</sup>
SLY50+5-FU	34,5±2,1 <sup>a</sup>	1,8±0,13 <sup>ab</sup>	0,46±0,04 <sup>a</sup>
SLY100+5-FU	36,3±2,1 <sup>a</sup>	1,9±0,11 <sup>b</sup>	0,45±0,0,06 <sup>a</sup>

### Effects of SLY on liver enzymes and renal function parameters

Serum ALT, AST, total bilirubin, urea, and creatinine levels were markedly increased in the 5-FU and SLY50+5-FU groups according to control. In the SLY100+5-FU group lower levels of these parameters by comparison to 5-FU and SLY50+5-FU groups (Table 2).

**Table 2.** Liver and kidney function parameters of mice in experimental groups ( a,b: p <0.01; a,c; b,c: p <0.05).

Experimental Groups	ALT (IU/L)	AST (IU/L)	Total Bilirubin (mg/dl)	Urea (mg/dl)	Creatinine (mg/dl)
Control	33,2±4,7 <sup>a</sup>	134,1±9,7 <sup>a</sup>	1,2±0,3 <sup>a</sup>	35,6±3,2 <sup>a</sup>	0,32±0,05 <sup>a</sup>
5-FU	46,8±4,9 <sup>b</sup>	241,1±20,8 <sup>b</sup>	2,2±0,4 <sup>b</sup>	64,3±6,3 <sup>b</sup>	0,49±0,08 <sup>b</sup>
SLY50+5-FU	44,8±2,7 <sup>b</sup>	170,1±21,3 <sup>c</sup>	1,8±0,2 <sup>b</sup>	48,9±8,9 <sup>c</sup>	0,43±0,05 <sup>b</sup>
SLY100+5-FU	35,8±3,1 <sup>a</sup>	158,1±25,1 <sup>ac</sup>	1,3±0,2 <sup>a</sup>	38,4±5,2 <sup>ac</sup>	0,35±0,03 <sup>a</sup>

### Effects of SLY on liver MDA and GSH levels

MDA levels were significantly higher in 5-FU and SLY50 + 5-FU groups compared to control, lower in SLY50 group than 5-FU group, but there was no significant difference (p> 0.05). MDA level was higher than control in SLY100 + 5-FU group. However, it is lower than the 5-FU group (Figure 1A). Also, liver GSH levels decreased significantly in the 5-FU and SLY50 + 5-FU groups up to the control and SLY100 + 5-FU groups (Figure 1C).

### Effects of SLY on liver SOD, CAT, and GR activities

Liver SOD, CAT, and GR activities were markedly lower in the 5-FU group in comparison with control. These enzyme activities increased in the SLY50+5-FU and SLY100+5-FU groups according to the 5-FU group (p<0.05) and these effects of SLY were dose-dependent and higher doses of SLY more significantly prevented the 5-FU-induced reduction in antioxidant enzyme activities (Figure 1B, 1D, 1E).

## Effects of SLY on renal MDA and GSH levels

MDA levels in 5-FU and SLY50+5-FU groups had significantly higher according to the control and were lower in the low dose group of SLY than 5-FU group ( $p \leq 0.05$ ). MDA level in SLY100+5-FU group reduced according to 5-FU (Figure 2A). Renal GSH levels reduced significantly in the 5-FU and SLY50+5-FU groups so far as to control and SLY100+5-FU groups (Figure 2C).

## Effects of SLY on renal SOD, CAT, and GR activities

Renal SOD, CAT, and GR activities were significantly lower in the 5-FU group in comparison with others groups. These enzyme activities increased in the SLY50+5-FU and SLY100+5-FU groups according to the 5-FU group ( $p > 0.05$ ) and these effects of SLY were dose-dependent (Figure 2B, 2D, 2E).

## Histopathological findings

When the liver and kidney tissues of the control group were examined to histopathologically, it was observed that they were in normal histological structures (Figure 3-4 A). In 5-FU groups, especially in the acinar region of the liver and hepatocytes were observed to severe degeneration, necrosis, and vascular hyperemia (Figure 3B). In the liver sections of SLY50+5-FU group, moderate degeneration, mild necrosis, and hyperemia in the vessels were detected in hepatocytes (Figure 3C). When the liver tissues of SLY100+5-FU group were examined histopathologically, mild degeneration in the hepatocytes and mild hyperemia in the vessels were observed (Figure 3D). The renal tubular epithelium of mice in the 5-FU group were observed severe hydropic degeneration and coagulation necrosis and were detected severe hyperemia in the glomerular and interstitial vessels (Figure 4B). The renal tubular epithelium of the SLY50+5-FU group was detected moderate degeneration, necrosis, and severe hyperemia in interstitial and glomerular vessels (Figure 4C). When the kidney tissues of the SLY100+5-FU group were examined histopathologically, mild degeneration in the tubular epithelium and mild hyperemia in the interstitial and glomerular vessels were determined (Figure 4D). A significant difference ( $p \leq 0.05$ ) was detected when compared with the 5 FU groups (Table 3).

## Immunohistochemical findings

When liver and kidney tissues of the control group were examined immunohistochemically, 8-OHdG and IL-6 expression in liver tissues (Figure 5-6A) and kidney tissues (Figure 7-8A) were negative. Severe cytoplasmic 8-OHdG expression was detected in hepatocytes in the liver, acinar region, in the 5-FU group (Figure 5B). Also, severe IL-6 expression was observed in the liver at sinusoidal and portal intervals (Figure 6B). In the 5-FU group was viewed severe cytoplasmic 8-OHdG expression in kidney tubular epithelium (Figure 7B), severe IL-6 expression in the glomerulus, intertubular intervals, and vascular circumference (Figure 8B). In the SLY50+5-FU group were observed moderate cytoplasmic 8-OHdG expression in liver hepatocytes (Figure 5C) and in this group was detected moderate IL-6 expression in the sinusoidal spaces, vascular environment and portal spaces (Figure 6C). The SLY50+5-FU group were determined moderate cytoplasmic 8-OHdG expression in the tubular epithelium (Figure 7C), moderate IL-6

expression in glomeruli, intertubular intervals, and vascular circumference (Figure 8C). In livers of SLY100+5-FU group were determined mild cytoplasmic 8-OHdG expression in the tubular epithelium (Figure 5D), sinusoidal intervals and in the portal region was detected to IL-6 expression at mild level (Figure 6D). In the SLY100+5-FU group, in the kidney tissues were observed 8-OHdG expression in the cytoplasmic mild level in the tubular epithelium (Figure 7D), and were determined to mild IL-6 expression in the glomerulus, intertubular intervals, and vascular circumference (Figure 8D). A significant difference ( $p \leq 0.05$ ) was detected when compared with the control group. Immunohistochemical findings are summarized in table 3.

**Table 3.** Scoring of histopathological and immunohistochemical findings of liver and kidney tissues

	Parameters	Control	5-FU	SLY50+5-FU	SLY100+5-FU
Liver	Degeneration in hepatocytes	-	+++	++	+
	Necrosis in hepatocytes	-	+++	+	-
	Hyperemia in the vessels	-	+++	+++	++
	8-OHdG expression	-	+++	++	+
	IL-6 expression	-	+++	++	+
Kidney	Degeneration in tubules epithelium	-	+++	++	+
	Necrosis in tubules epithelium	-	+++	+	-
	Hyperemia in the vessels	-	+++	+++	++
	8-OHdG expression	-	+++	++	+
	IL-6 expression	-	+++	++	+

## Discussion

5-FU is a widely used chemical with a chemotherapy effect. However, it has been determined by previous studies that 5-FU use has some side effects (Gelen et al. 2021; Sengul et al. 2021b). Some of these side effects are hepatotoxic and nephrotoxic effects (Gelen et al. 2017; Gelen et al. 2018). There are numerous studies in which various natural compounds have been applied to counteract the side effects of such agents on tissues. (Gelen et al. 2018; Şengül et al. 2017). One of these agents is SLY, which is stated to have anti-inflammatory and antioxidant properties and is a flavonoid. Therefore, in this study, we investigated the effects of SLY on oxidative stress and inflammation apoptosis on tissue damage in hepatorenal toxicity caused by 5-FU.

Increases in some enzymes such as AST and ALT in serum are special markers for liver damage and indicate hepatic cell damage (Drotman and Lawhorn 1978). The reason for this increase is that the enzymes in question are released from the cells into the circulation as a result of the increase in the

permeability of the membrane of the damaged cell (Lee et al. 2008). For this reason, an increase in ALT and AST levels in serum is accepted as a marker of hepatic cell damage (Bülbül et al. 2018; Turk et al. 2018). Serum urea and creatinine levels are biomarkers of kidney function, and serum levels increase when kidney tissue is damaged. (Chahdoura et al. 2018; Dağ et al. 2018; Sengul et al. 2021). According to the data we obtained in our study, liver urea, creatinine, ALT, AST, and total bilirubin increased significantly in the 5-FU group compared to the control group. These values decreased in the SLY group.

As a result of some studies, it has been shown that the increased ROS level will damage cells (Kara et al. 2016). Again, many studies have reported that oxidative stress plays a very important role in 5-FU-induced toxicity and nephrotoxicity (Rashid et al. 2014). Experimental application of 5-FU can induce DNA damage and apoptosis in the cell, causing excessive free radicals (Xia et al. 2016). Studies have indicated that SLY has the function of scavenging free radicals (Vaid and Katiyar 2010). Therefore, SLY may play a protective role in 5-FU-induced oxidative stress in liver and kidney tissues. MDA content is an important indicator of lipid peroxidation and is also a diagnostic index that helps diagnose cell oxidative damage (Zhang et al. 2014). GSH is a non-enzymatic antioxidant and because it prevents free radical formation; It has been stated in previous studies that it plays an important role in defense against ROS (Liu et al. 2008; Loro et al. 2012). It has been determined by studies that antioxidant enzymes such as SOD, GR, and CAT also have protective effects against the harmful effects of ROS on the organism (Davis et al. 2001; Ma et al. 2007; Xia et al. 2016). For this reason, in this study, we determined the MDA, GSH levels and SOD, GR, CAT activities in kidney and liver tissues obtained from the experimental groups and evaluated them among the groups. The results showed that a particularly high dose of SLM (100mg/kg) prevented 5-FU-induced oxidative damage in kidney and liver tissue and protected these tissues from oxidative damage by increasing SOD, CAT, and GR activity and GSH levels. Besides, SLM (100 mg/kg) can protect kidney and liver tissue by reducing the MDA content. The underlying mechanism may include SLM attenuated ROS formation in renal and hepatic tissue cells. Some studies in recent years show that antioxidants have a certain protective effect on organ toxicity caused by anti-carcinogenic substances. The data we obtained in our study are similar to previous studies like this one.

8-OHdG is considered to be one of the most important markers of DNA damage caused by oxidative stress (Cadet et al. 2003; Stepniak and Karbownik-Lewinska 2016). Hydroxyl radicals resulting from oxidative stress lead to hydrogenation of the nucleic acid causing 8-OHdG (Cadet 2016). In previous a study, it was reported that DNA damage was intensified in rat tests after 5-FU administration (El-Sayyad and Hassan 2013). The data obtained from our study were similar to the findings study and the expression of 8-OHdG increased in renal tissues of mice in the 5-FU group. On the other hand, some studies have shown that the application of various antioxidant agents inhibits oxidative stress-induced DNA damage (Ince et al. 2014; Sengul et al. 2021). This shows that SLY has an antioxidant effects and thus reduce ROS-mediated 8-OHdG expression, thus preventing oxidative DNA damage.

Inflammatory cytokines have been reported to contribute to the pathogenesis of tissue damage (Gelen and Şengül 2020; Lavery et al. 2010; Lacour et al. 2005). As a result of a study, it was reported that IL-6 level increased with 5-FU administration (Chang et al. 2017). The inhibitory effect of flavonoids on IL-6



release has also been reported (Peluso et al.2015). In previous studies, the inhibitory effect of SLY, which is a flavonoid, on IL-6 was reported (Shahidi et al.2017). The data we obtained in our study show that 5-FU treatment significantly increases IL-6 expression in liver and kidney tissues. However, we found that SLY treatment caused a significant decrease in the IL-6 increase caused by 5-FU in liver and kidney tissues. This result is likely due to the anti-inflammatory properties of SLY.

## Conclusion

According to our results, the mechanisms of 5-FU-induced hepatorenal toxicity are seen and confirm the potential antioxidant and anti-inflammatory of SLY. Other findings of this study support the role of oxidative stress, inflammation, DNA damage in the pathogenesis of 5-FU-induced liver and renal toxicity. As a result, SLY eliminates 5-FU induced liver and kidney toxicity in mice. The protective effects of SLY are likely due to its powerful antioxidant and anti-inflammatory properties.

## Declarations

**Contributions** E.S. V.G. S.Y. E.S. Y.D. G.E. M.G.: experiment design, experiment application, samples collection. E.Ş. V.G. E.S. Y.D. M.G.: serum markers and tissue antioxidant estimation, data curation and analysis, final reviewing. S.Y. G.E.: histopathological and immunohistochemical investigation. All authors contributed to the writing and editing, and they read and approved the final manuscript.

**Availability of data and materials** The authors confirm that the data and materials supporting the findings of this study are available within the article.

**Funding information** This work was not supported by any institution.

### Compliance with ethical standards

**Ethical approval** The study was designed and conducted according to ethical norms approved by the Atatürk University Rectorate Animal Experiments Local Ethics Committee (Erzurum, Turkey) (Protocol No. 2019/17).

**Conflict of Interest** The authors declare that there are no conflicts of interest.

**Consent to participate** All authors voluntarily participated in this research study.

**Consent to publish** Not applicable

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## Figures

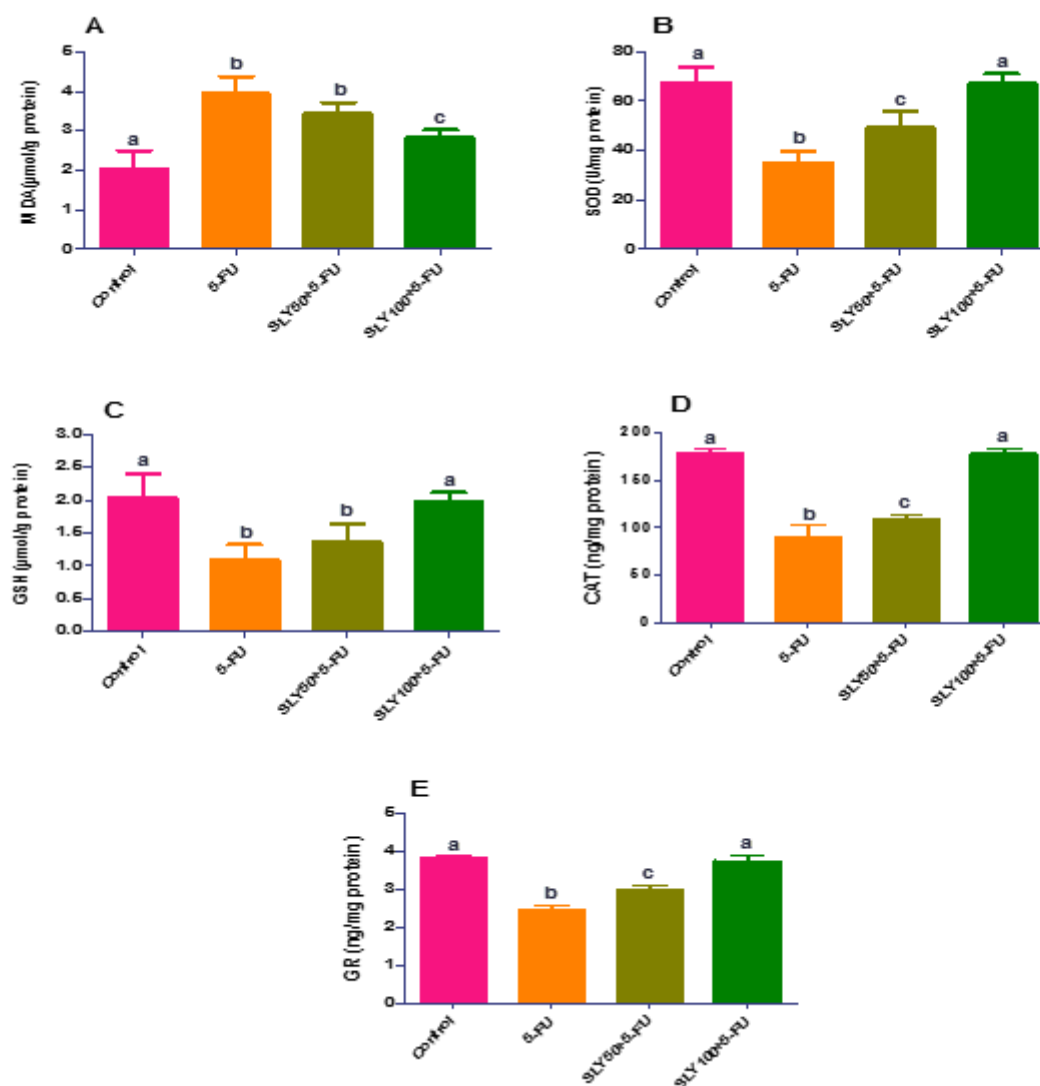


Figure 1

The MDA (A) and GSH (C) levels and SOD (B), CAT (D), and GR (E) activities in liver tissues, a,b:  $p < 0.001$ , a,c; b,c:  $p < 0.05$ ).

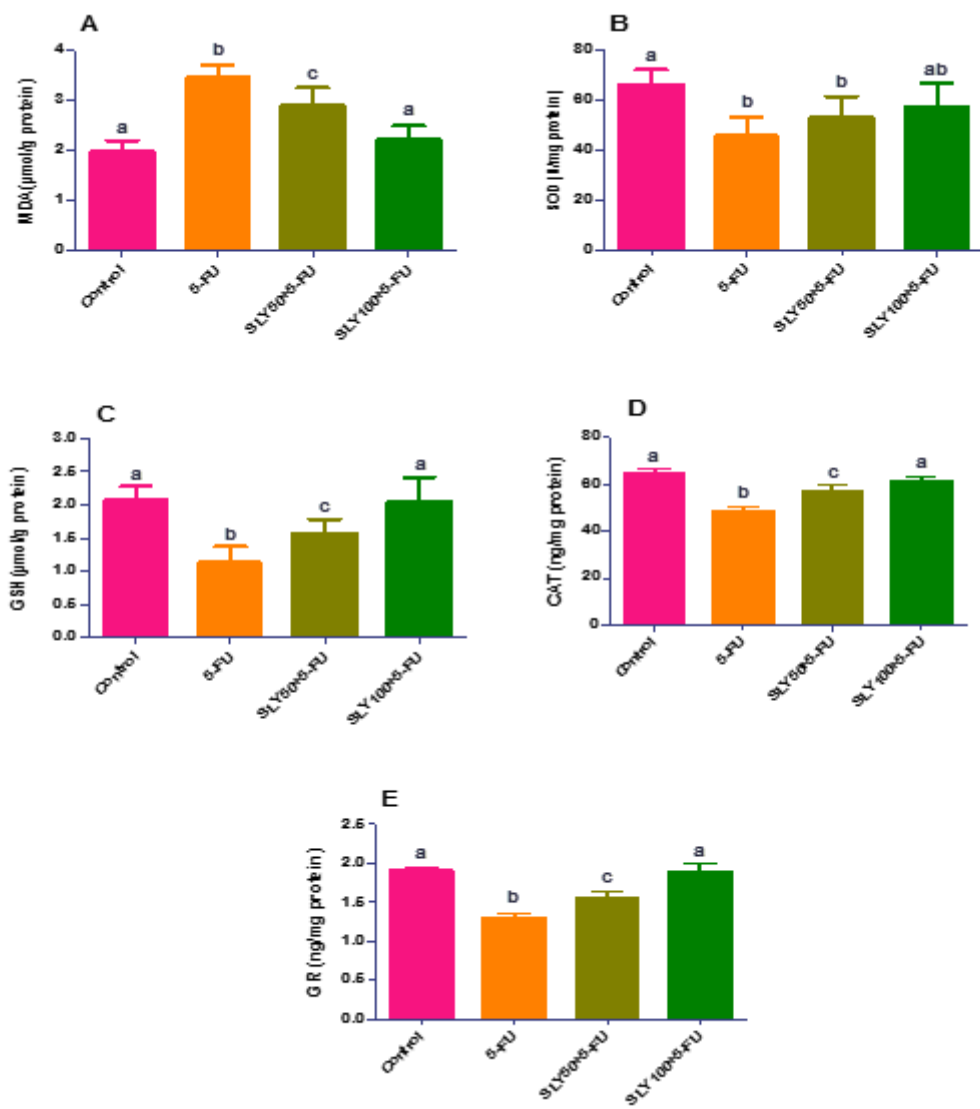
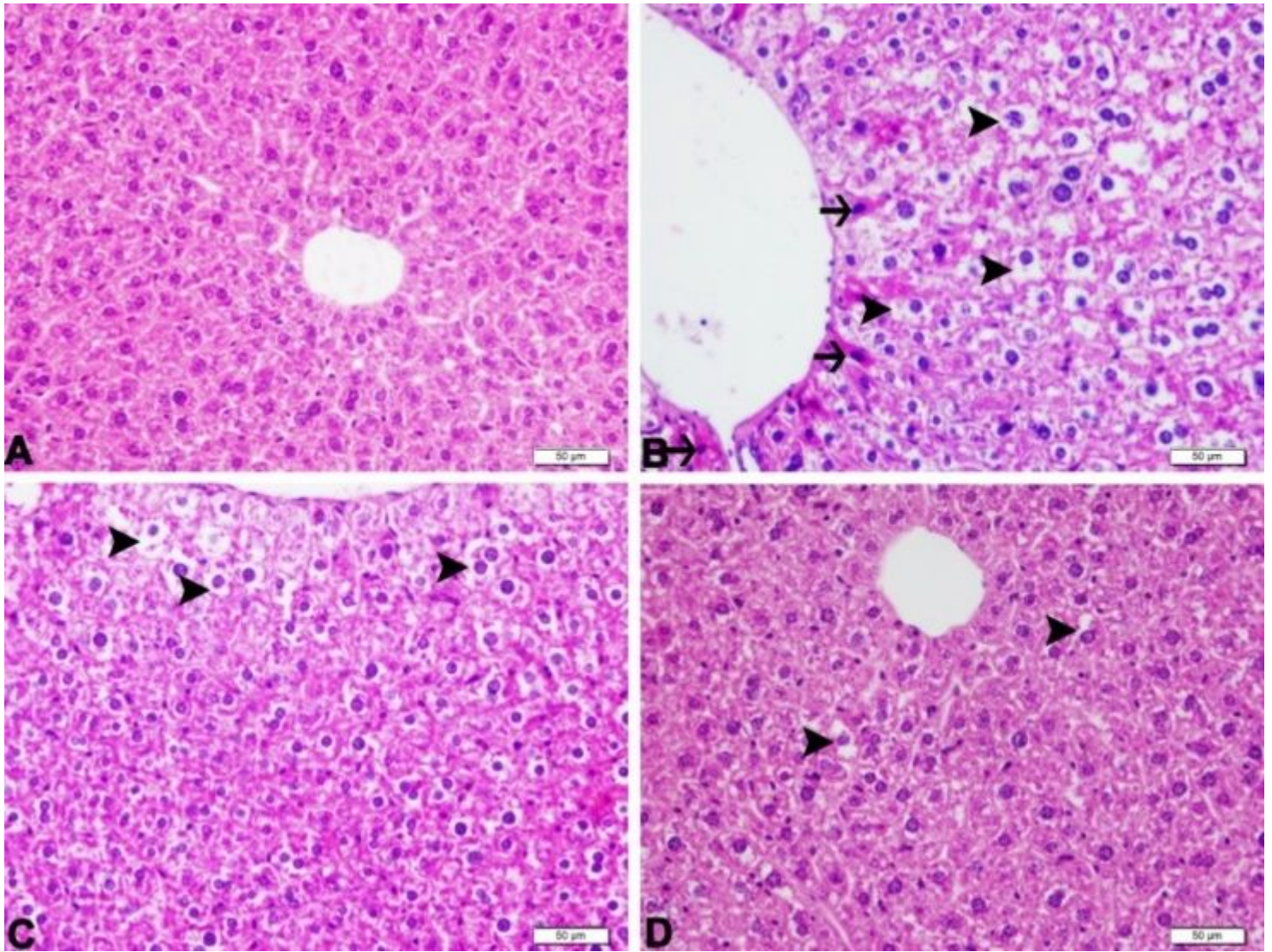


Figure 2

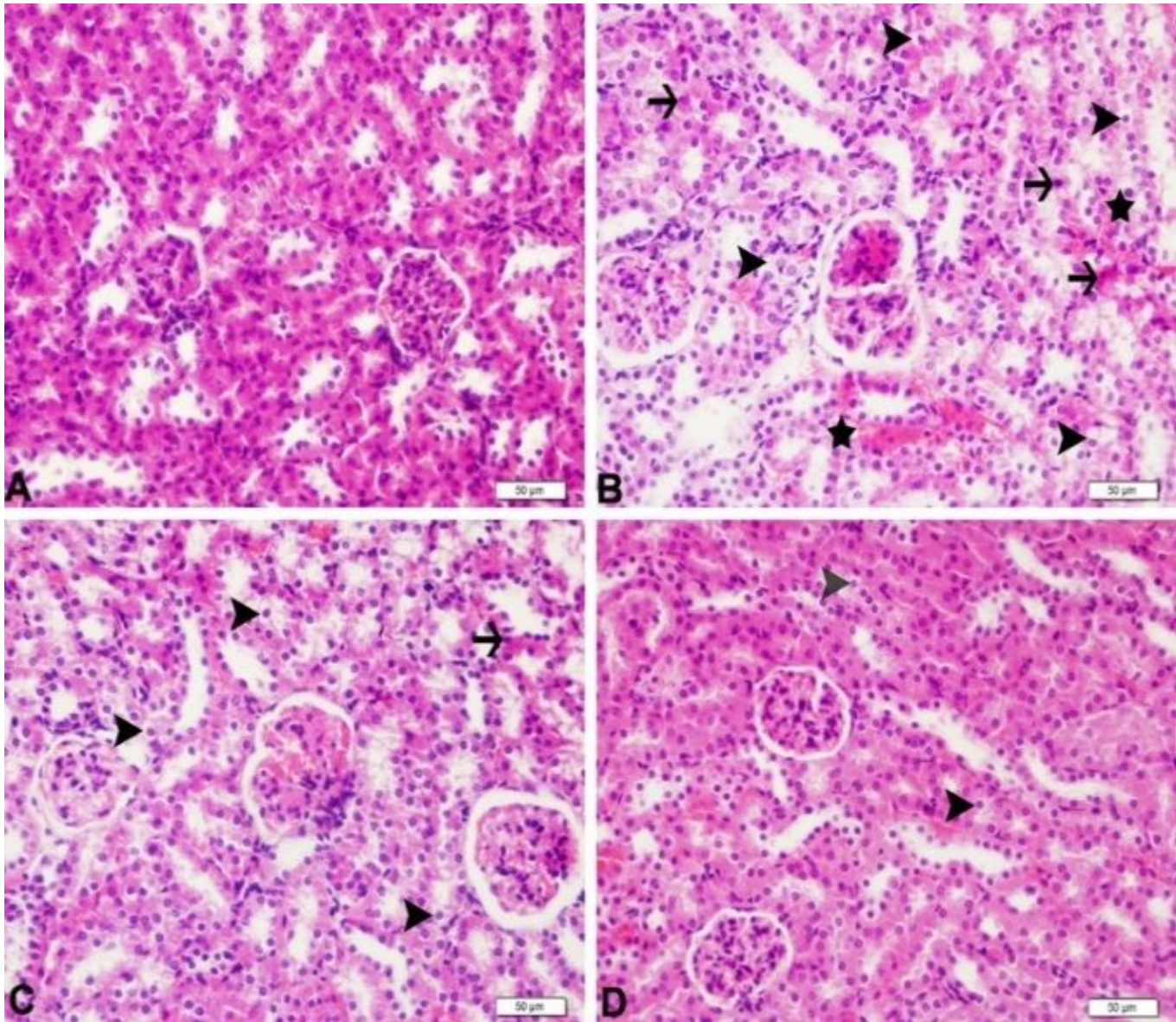
The MDA (A) and GSH (C) levels and SOD (B), CAT (D), and GR (E) activities in renal tissues ( a,b:  $p < 0.001$ , a,c; b,c:  $p < 0.05$ ).



**Figure 3**

Liver tissue, control group (A), normal histological view; 5-FU group (B), severe hydropic degeneration (arrowheads) in the central region, severe necrosis (arrows); SLY50+5-FU groups (C), medium level hydropic degeneration (arrowheads); SLY100+5-FU groups (D), mild hydropic degeneration (arrowheads), H&E, Bar: 50 µm.

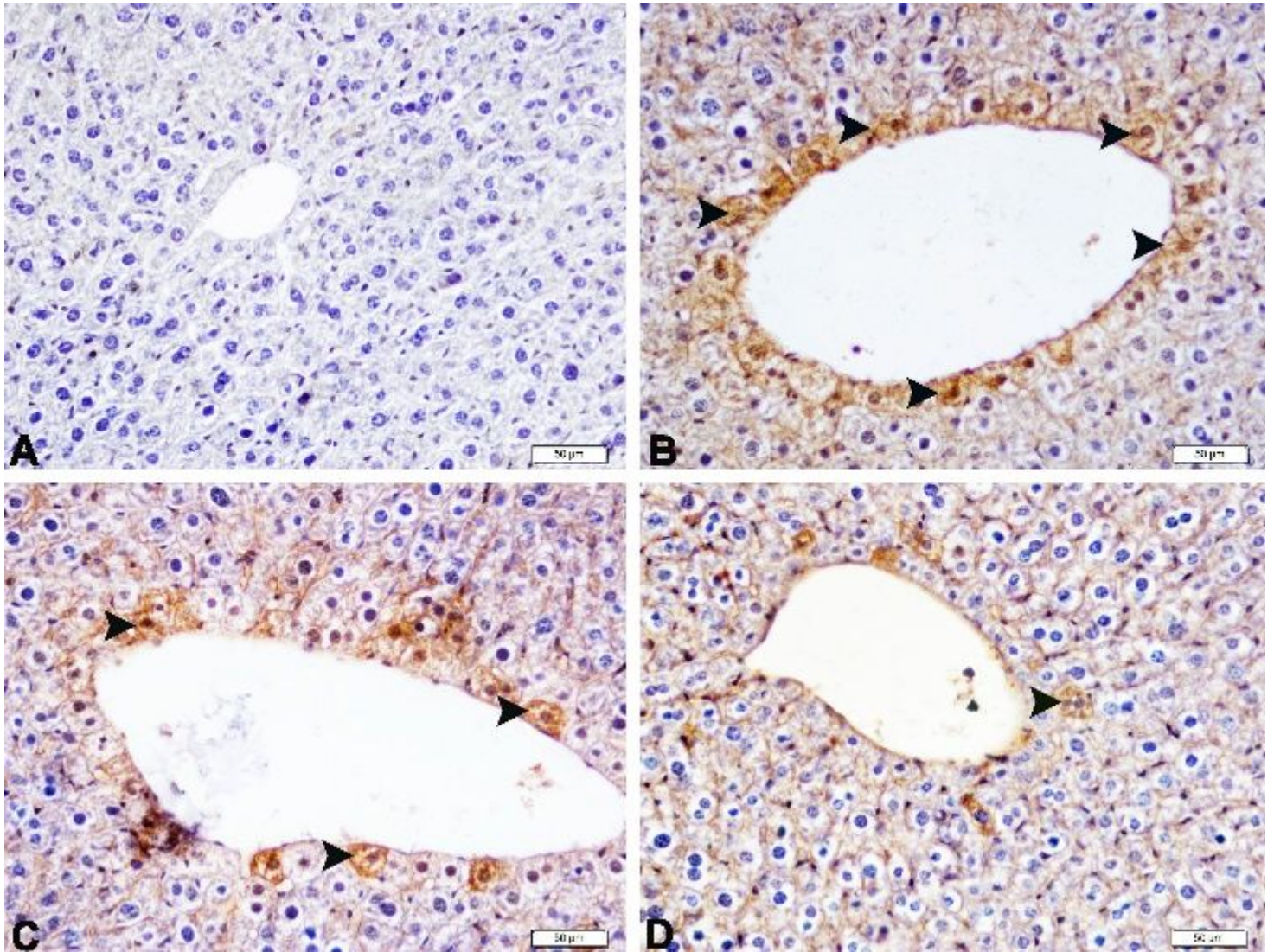




**Figure 4**

Kidney tissue, control group (A), normal histological view; 5-FU groups (B), severe necrosis (arrows) in the tubular epithelium, severe degeneration (arrowheads), severe hyperemia in the vessels (stars); SLY50+5-FU groups (C), mild necrosis in the tubular epithelium (arrow), moderate degeneration (arrowheads); SLY100+5-FU groups (D), mild degeneration in the tubular epithelium (arrowheads), H&E, Bar: 50 µm.

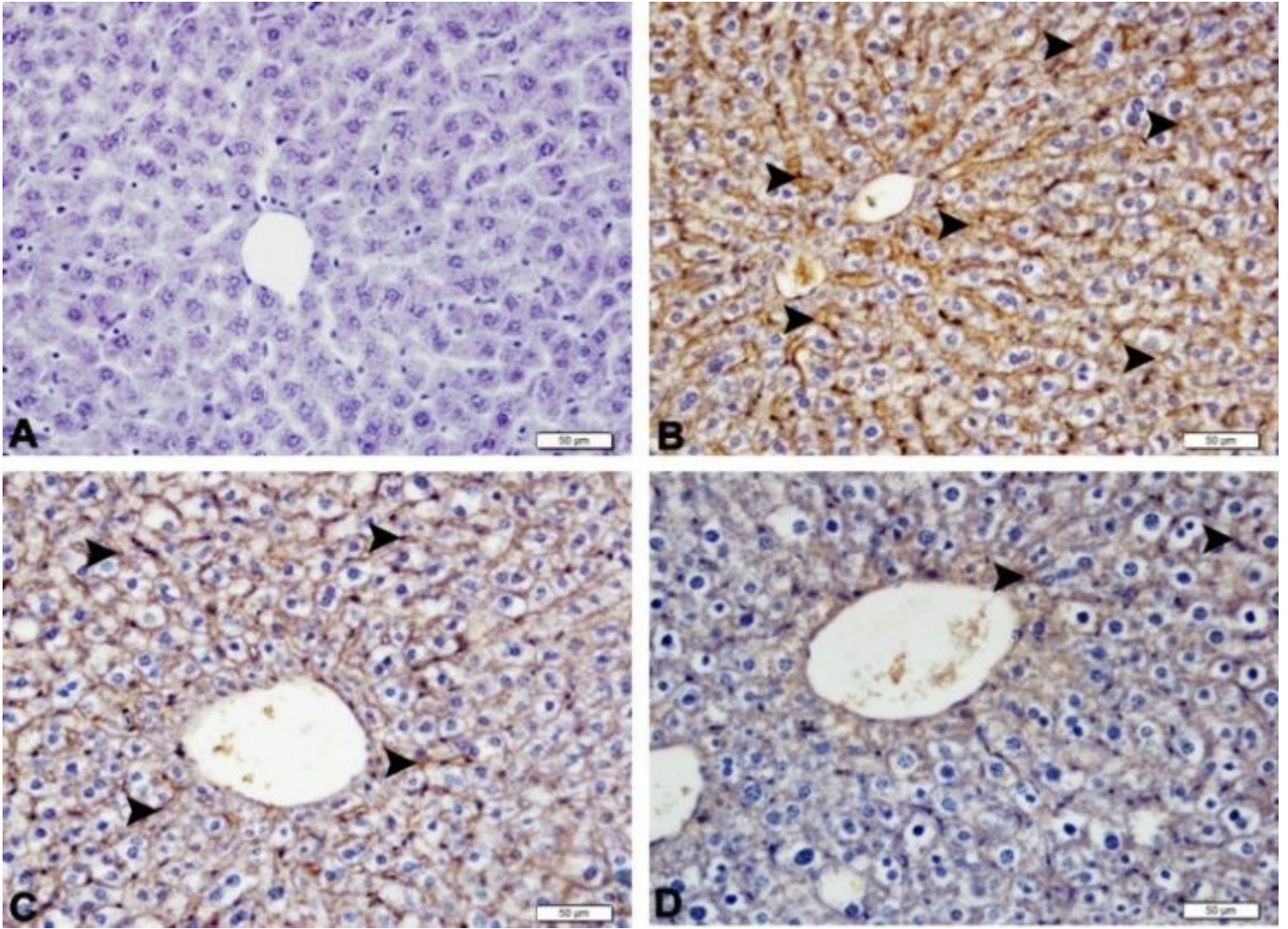




**Figure 5**

Liver tissue, control group (A), negative 8-OHdG expression; 5-FU group (B), severe 8-OHdG expression in hepatocyte (arrowheads); SLY50+5-FU groups (C), moderate 8-OHdG expression (arrowheads); SLY100+5-FU groups (D), mild 8-OHdG expression (arrowheads), IHC-P, Bar: 50 µm.

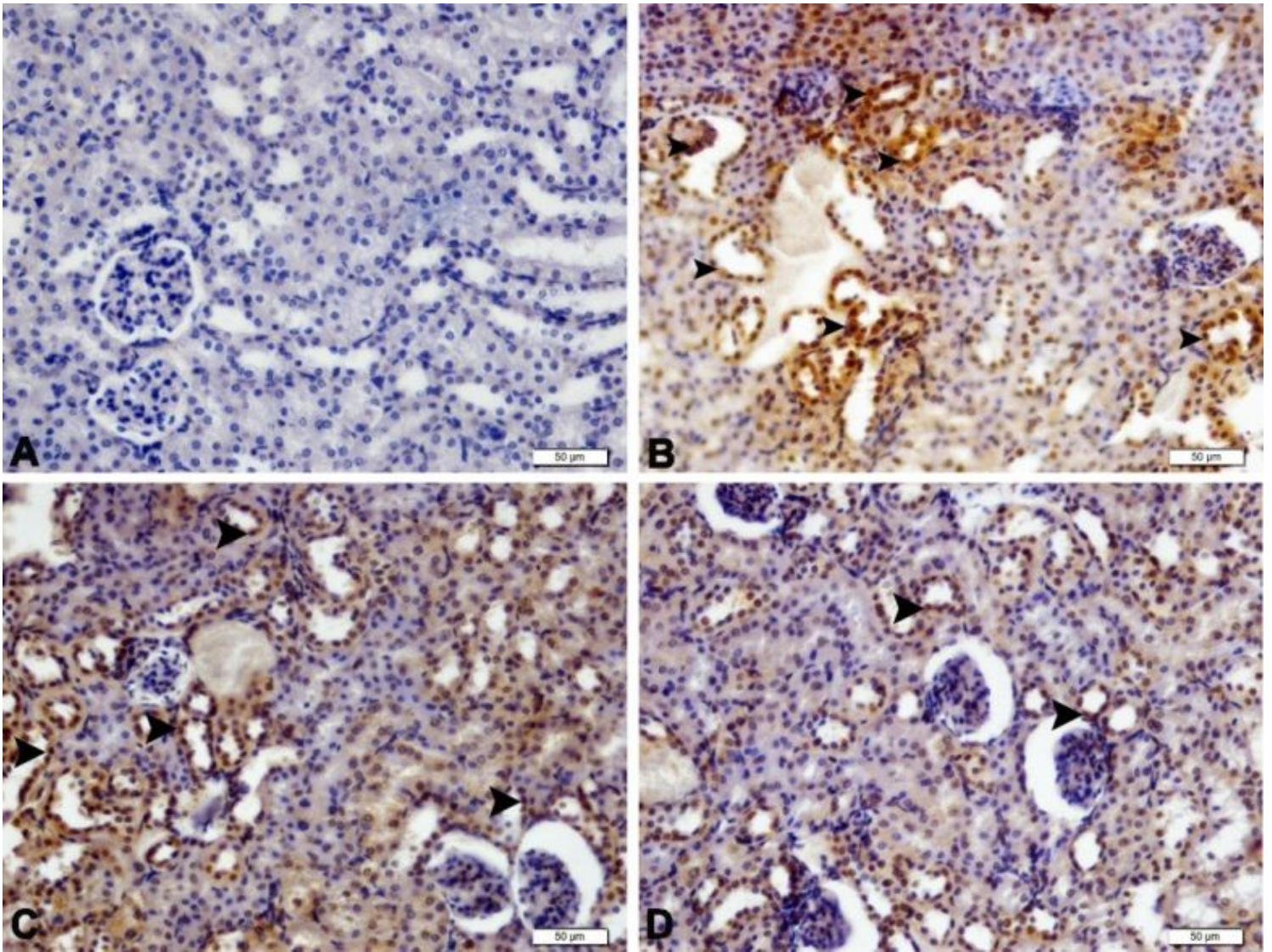




**Figure 6**

Liver tissue, control group (A), negative IL-6 expression; 5-FU group (B), severe IL-6 expression (arrowheads); SLY50+5-FU groups (C), moderate IL-6 expression (arrowheads); SLY100+5-FU (D), groups, mild IL-6 expression (arrowheads), IHC-P, Bar: 50 µm.

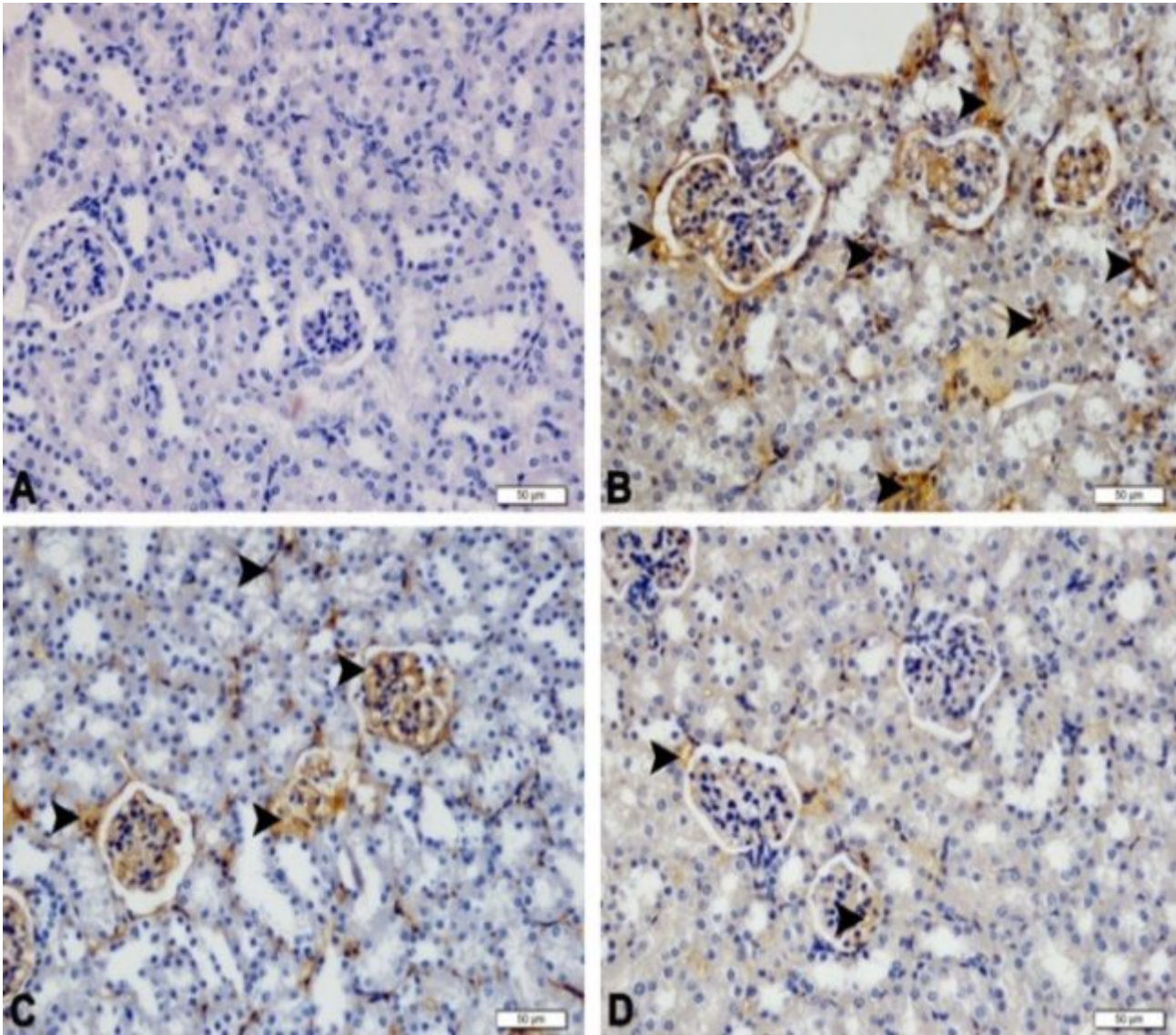




**Figure 7**

Kidney tissue, control group (A), negative 8-OHdG expression; 5-FU group (B), severe level of cytoplasmic 8-OHdG expression (arrowheads); SLY50+5-FU group (C), moderate 8-OHdG expression (arrowheads); SLY100+5-FU groups (D), 8-OHdG expression at mild level (arrowheads), IHC-P, Bar: 50 µm.





**Figure 8**

Kidney tissue, control group (A), negative IL-6 expression; 5-FU group (B), severe IL-6 expression (archery); SLY50+5-FU groups (C), moderate IL-6 expression (arrowheads); SLY100+5-FU groups (D), mild IL-6 expression (arrowheads), IHC-P, Bar: 50 µm.