









# The effect of silymarin given before partial hepatectomy on liver regeneration in rats with ischemic preconditioning during liver resection

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## Abstract

**Background:** The regeneration capacity of liver tissue after partial hepatectomy closely affects morbidity and mortality. We aimed to investigate the effect of silymarin and ischemic preconditioning (IPC) before partial hepatectomy on liver regeneration.

**Methods:** Thirty rats were randomly divided into 5 groups (n=6). Serum AST, bilirubin, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6) values were studied 48 hours after hepatectomies. Mitotic count, congestion, necrosis, cytoplasmic vacuolization, and presence of neutrophils were evaluated histopathologically. Proliferating cell nuclear antigen (PCNA) antibody was studied immunohistochemically.

**Results:** The AST value (2071.5 $\pm$ 938.4) was the highest in the partial hepatectomy (PH) group. The Ischemic Preconditioning Partial Hepatectomy Group (IPC) (1535.5 $\pm$ 204.8) and the Silymarin+Partial Hepatectomy Group (Silymarin group) (1192.3 $\pm$ 526.3) had lower levels of AST values; however, the AST values were the lowest in the Silymarin+Ischemic Preconditioning+ Partial Hepatectomy Group (IPC+ Silymarin group) (1002.8 $\pm$ 348.9). Likewise, the highest improvement in bilirubin levels was observed in the IPC+ Silymarin group (0.33 $\pm$ 0.11). IL-6 (11.54 $\pm$ 2.89) and TNF- $\alpha$  (39.2 $\pm$ 22.73) values were the lowest in the IPC group, and interestingly, both were higher in the silymarin-treated groups. It was observed that these values increased in Silymarin group and IPC+ Silymarin group who received silymarin treatment compared to PH group and IPC group who did not receive silymarin. In histopathological analysis, it was found that the mitosis rate and PCNA percentage were higher in the Silymarin and IPC+ Silymarin groups.

**Conclusions:** The use of silymarin before hepatectomy and IPC during partial hepatectomy increase liver regeneration.

**Keywords:** Liver Resection, Silymarin, Ischemic Preconditioning, Liver Regeneration.

*Cite this article as: Yavuz A, Göbüt H, Büyükkasap Ç, Dikmen K, Bostancı H, Yalcinkaya A, Çingirt M, Gülbahar Ö, Taşkın Türkmenoğlu T, Dalgıç A. The effect of silymarin given before partial hepatectomy on liver regeneration in rats with ischemic preconditioning during liver resection. Arch Curr Med Res. 2022;3(3):181-186*

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## INTRODUCTION

Partial resection of the liver is a procedure performed for various reasons, including trauma-related injury, donor hepatectomy, and the treatment of cysts, abscesses, and primary or secondary liver tumors (1). The regeneration capacity of the remaining liver tissue after partial resection of the liver closely affects morbidity and mortality. Ischemia/reperfusion (I/R) injury is a primary factor associated with the occurrence of liver failure in the postoperative period following liver resection (2). Ischemic preconditioning (IPC) is a method that is applied prior to procedures likely to cause I/R injury and has been suggested to prevent or ameliorate I/R injury in various organs, including the liver (3).

Hepatic I/R injury and IPC have been associated with the levels of reactive oxygen species, nitric oxide, adenosine, TNF- $\alpha$  and, chemokines (4, 5). The administration of IPC has been demonstrated to protect sinusoidal endothelial cells and hepatocytes through modulation of the endogenous oxidant/antioxidant system (5). The protective effects conferred by IPC after hepatic surgery have been associated with improved survival and smaller liver necrosis after I/R injury (6). Silymarin is a compound extracted from the seeds of *Silybum marianum* (colloquially known as Milk thistle) that may provide hepatoprotective effects through the inhibition of free radicals formed by the metabolism of critical toxic substances, such as acetaminophen (7, 8). Silymarin is established to have anti-fibrotic, anti-inflammatory, anti-apoptotic, and anti-oxidant effects, and thus, may alleviate possible dysfunctions of the liver. Consistently, there are numerous studies which have shown the protective and regenerative effects of silymarin in liver diseases. Put together, these suggest a role for silymarin in preventing or reducing I/R injury, either as a stand-alone treatment or in combination with IPC (8).

In addition to the surgical techniques applied to minimize the likelihood of hepatic dysfunction following partial liver resection, a number of chemical and herbal drugs have been used to increase regeneration and prevent potential adverse outcomes (9). In this experimental study, we aimed to investigate and compare the effects of silymarin and IPC in the prevention / alleviation of hepatic dysfunctions following partial hepatectomy in rats by measuring parameters associated with inflammation and liver functions.

## MATERIALS AND METHODS

### Animal care and study design

This study was carried out in accordance with the Declaration of Helsinki, after obtaining permission from Gazi University Animal Experiments Local Ethics Committee with code number G.U.E.T.-15.047.

Thirty Wistar-Albino male rats weighing between 200-300 grams were used in the study. Rats were randomly divided into 5 groups, each consisting of 6 rats. Rats were received by the Experimental Research Center and were acclimatized for 1 week before the study. All rats were kept in sufficiently large cages with standard environmental housing (21 $\pm$ 2 °C, standard rodent chow, tap water, 45-65% humidity, 12-hour day/night cycles with automatized lighting).

Except for the sham group, all rats underwent partial hepatectomy. The treatments were performed based on the relevant groups and included silymarin administration and IPC. Silymarin (Carsil® 90 mg tablet, Sopharma PLC, Bulgaria) was administered at a dose of 200 mg/kg/day via orogastric tube. IPC was performed by subjecting rats to three cycles of 5-minute ischemia followed by 10-minute reperfusion (total duration 45 minutes).

### Experimental groups

**Sham group:** The sham group underwent laparotomy and only hepatic manipulation was performed. **Partial hepatectomy group (non-treatment):** 70% liver resection was performed after laparotomy. **IPC Group:** The rats in this group underwent IPC before 70% liver resection. **Silymarin group:** These rats received silymarin for 6 weeks before the procedure. After 6 weeks, 70% liver resection was performed. **IPC + silymarin group:** These rats received silymarin for 6 weeks before the procedure. After 6 weeks, IPC was performed before 70% liver resection.

### Surgical procedure

Surgeries were applied under intraperitoneally-administered general anesthesia using 50 mg/kg ketamine (Ketalar®, Parke Davis and Co. Inc., 40mg/kg) and 10 mg/kg xylazine (Rompun®, Bayer Ag, Leverkusen, Germany; 5mg/kg). Briefly, the abdomen was entered with a midline abdominal incision in the supine position, and for IPC, using a microvascular clip distal to the portal vein and hepatic artery, 5 minutes of ischemia followed by 15 minutes of reperfusion followed by 45 minutes of total hepatic ischemia was performed. In accordance with

the method described by Higgins et al., the left lateral and median lobes of the liver were tied with 4/0 silk sutures at the junction with the vena cava, and 70% liver resection was performed.(10). At the end of the experiment, after the samples were taken, the rat's heart was removed and euthanized.

### Sample acquisition

Each rat was euthanized 48 hours after their respective procedure and tissue and blood samples were obtained. Total working time was kept equal in all groups. Tissue specimens were fixed with 10% neutral buffered formaldehyde. Blood samples were collected via cardiac puncture and were centrifuged at 1500×g for 10 minutes to obtain sera, which were stored at -20 °C until analysis.

### Measurement of biochemical parameters

As inflammatory parameters, we measured serum TNF- $\alpha$  levels and IL-6 which were quantified with enzyme-linked immunosorbent assays (ELISA) (SunRed Rat TNF- $\alpha$  ELISA Kit and SunRed Rat IL-6 ELISA Kit). Serum AST and total bilirubin levels were studied in a Roche Cobas 8000 Autoanalyzer equipped with the necessary commercial kits at the Medical Biochemistry Laboratory of Gazi University Hospital.

### Histopathological evaluation

After the macroscopic examination of tissues fixed with 10% neutral buffered formaldehyde, the tissue specimens were embedded in paraffin and cut at 5 $\mu$ m-thickness. Examination was carried out with H&E staining under a light microscope. Mitosis, congestion, necrosis, cytoplasmic vacuolization, and neutrophil presence were scored semi-quantitatively. The mitosis count was calculated from 30 consecutive high-magnification fields. Congestion was classified into three groups (mild, moderate, significant); presence of necrosis into four groups (absent, mild,

moderate, severe); cytoplasmic vacuolization into four groups (absent, mild, moderate, severe); neutrophil inflammation into two groups (present, absent). Also, proliferating cell nuclear antigen (PCNA) was studied with immunohistochemical antibodies with the Ventana Benchmark XT immunohistochemistry automatic staining device.

### Statistical analysis

Descriptive statistics are given as the median of the frequency percentage distribution. Nonparametric tests were used as it was an animal study. The Kruskal-Wallis test was used to compare three groups of quantitative data, and benforonia correction was made to find out which group the difference originated from. Mann-Whitney U test was used for comparison of 2 groups. P<0.05 was considered statistically significant.

## RESULTS

### Effects on serum TNF- $\alpha$ and IL-6 levels

When the groups were compared, it was found that PH increased IL-6 (20.2 $\pm$ 14.42) and TNF- $\alpha$  (66.36 $\pm$ 39.48) values; however, IL-6 (11.54 $\pm$ 2.89) and TNF- $\alpha$  (39.2 $\pm$ 22.73) values were the lowest in the IPC group; rivetingly, both values were higher in the groups that were given silymarin.

### Effects on serum AST and Total Bilirubin levels

It was evaluated that (Table 1) the AST value (2071.5 $\pm$ 938.4) was the highest in the PH group, but the AST value was lower in the IPC (1535.5 $\pm$ 204.8) and Silymarin (1192.3 $\pm$ 526.3) groups. It was observed that the AST value was the lowest in the IPC + silymarin group (1002.8 $\pm$ 348.9) and significantly improved the AST level with a synergistic effect. Likewise, the highest improvement in bilirubin levels was observed in the IPC + silymarin group (0.33 $\pm$ 0.11).

**Table 1. IL-6, TNF- $\alpha$ , AST and Total Bilirubin values of the study groups**

	IL-6 (Mean $\pm$ SD)	TNF- $\alpha$ (Mean $\pm$ SD)	AST (Mean $\pm$ SD)	Total Bilirubin (Mean $\pm$ SD)
<b>Grup S</b>	10.99 $\pm$ 1.92	22.75 $\pm$ 10.16	393.5 $\pm$ 82.3	0.05 $\pm$ 0.01
<b>Grup PH</b>	20.2 $\pm$ 14.42	66.36 $\pm$ 39.48	2071.5 $\pm$ 938.4	0.38 $\pm$ 0.20
<b>Grup IPPH</b>	11.54 $\pm$ 2.89	39.2 $\pm$ 22.73	1535.5 $\pm$ 204.8	0.42 $\pm$ 0.13
<b>Grup SPH</b>	38.78 $\pm$ 26.27	97.40 $\pm$ 59.11	1192.3 $\pm$ 526.3	0.40 $\pm$ 0.12
<b>Grup SIPPH</b>	26.74 $\pm$ 10.59	77.39 $\pm$ 20.20	1002.8 $\pm$ 348.9	0.33 $\pm$ 0.11

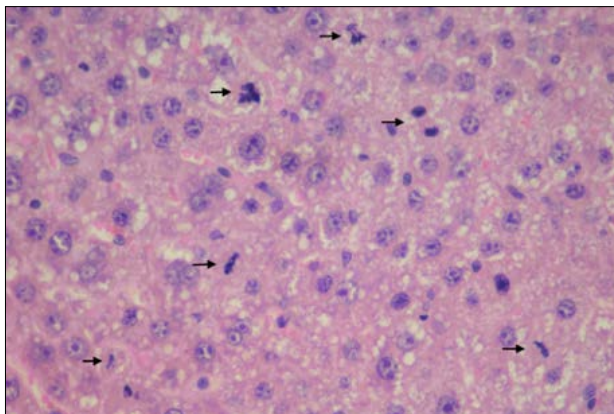
**Histopathological Evaluation**

When the histopathological scores were examined (Table 2), it was found that the mitosis rate and PCNA percentage were higher in the groups given silymarin (Silymarin group and IPC + silymarin group) compared to

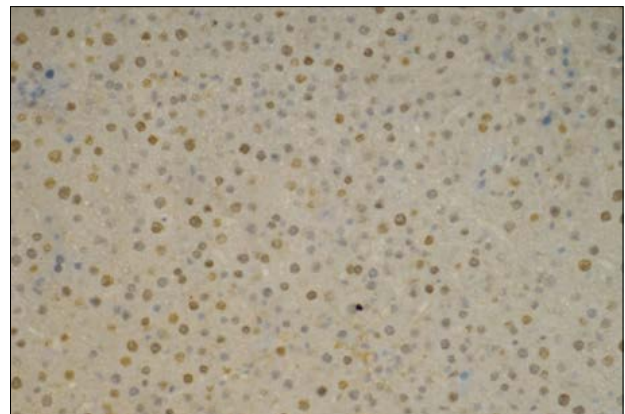
the PH group. Although the rates of congestion, necrosis, vacuolization, and neutrophils were worse in Silymarin and IPC + silymarin groups; it was thought that its hepatoprotective effect was due to preserving the mitosis rate and keeping the PCNA percentage high (Figure 1,2).

**Table 2. Comparison of the histopathological features of the remaining liver tissue, 48 hours after partial hepatectomy according to the groups**

	Grup S	Grup PH	Grup IPPH	Grup SPH	Grup SIPPH	P
Mitosis rate	0	42.0±21.24 37.0 (18-80)	23.0±25.76 14.0(1-60)	99.5±96.47 62.0 (13-236)	75.66±98.16 24.0(1-206)	0.27
Congestion (%)						0.001*
Mild	100	100	-	-	-	
Moderate	-	-	66.7	66.7	83.3	
Significant	-	-	33.3	33.3	16.7	
Necrosis (%)						0.006*
Absent	100	50	16.7	66.7	-	
Mild	-	50	50	16.7	-	
Moderate	-	-	33.3	16.7	100	
Severe	-	-	-	-	-	
Vacuolization (%)						0.39
Absent		-	-	-	-	
Mild	100	-	-	-	-	
Moderate	-	50	66.7	50	16.7	
Severe	-	50	33.3	50	83.3	
-	-	-	-	-	-	
Neutrophil (%)						0.44
Absent	100	100	66.7	83.3	66.7	
Present	-	-	33.3	16.7	33.3	
PCNA (%)	0	13.3±8.57 11.5 (6-30)	30.83±5.84 32.5 (20-35)	48.33±17.1 47.5 (30-70)	74.16±3.76 75.0 (70-80)	0.001*



**Figure 1. Diffuse cytoplasmic vacuolization in hepatocytes and marked increase in mitosis as indicated by arrows in the group that underwent Silymarin + ischemic preconditioning followed by partial hepatectomy (HEX40)**



**Figure 2. Widespread PCNA immunostaining in hepatocytes in the group that underwent Silymarin + ischemic preconditioning followed by partial hepatectomy (X20)**

## DISCUSSION

The ability of an organ to regain its mass as a result of tissue loss or loss of a part is defined as regeneration. Post-resection liver regeneration is a complicated process in which proinflammatory cytokines, hormones, and transcription factors play a role (11, 12).

Anaerobic metabolism in the ischemic process and post-reperfusion injury, which is mainly caused by free oxygen radicals, are closely related to dysfunction, morbidity, and mortality after hepatectomy.

Studies focused on IPC and intermittent clamping, and in a limited number of studies, it was shown that IPC was particularly effective in alleviating IRI (13).

Silymarin is a compound that has been used in the treatment of various diseases, especially liver diseases, for more than 2000 years (14). Zholobenko et al. showed that preconditioning of silymarin and its components affects signaling pathways (15). We thought that silymarin could increase hepatoprotection by increasing the effectiveness of IPC, via reducing the adverse effects of ischemia-reperfusion injury.

IRI induces cholestasis and temporarily reduces bile secretion. Changes in bile flow result in an increase in AST levels and plasma bilirubin levels and return to normal within 1-3 days (16). Therefore, we evaluated AST, bilirubin, and histopathological results to detect IRD.

AST levels were found to be statistically significantly lower in the Silymarin and IPC + silymarin groups compared to the other groups, but higher than the control group. Bilirubin values were found to be lower in the IPC + silymarin group. From these results, we thought that silymarin and ischemic preconditioning together reduced hepatocyte damage and increased their hepatoprotective activities.

In 2012, Wang et al. performed left lateral lobe and caudate lobe transplantation following IPC in rats in their study. They reported that histopathological damage was significantly lower in the preconditioning group, and TNF- $\alpha$  was higher in the preconditioning group in the early period and lower in the following periods. However, they reported that IL-6 expression increased significantly both in the early and late periods in the

preconditioning group. Accordingly, they suggested that IL-6 regulates hepatocyte proliferation (17). In our study, TNF- $\alpha$  and IL-6 levels were statistically significantly higher in the other groups compared to the control group, and the values were lower in the IPC group compared to the other groups; on the contrary, it was found that both values increased significantly in the group that underwent silymarin+partial hepatectomy. Based on these results, it can be thought that silymarin increases IL-6 levels and ischemic preconditioning decreases TNF- $\alpha$  and hepatocyte damage. Cescon et al. showed that direct IPC did not change the histopathological findings (18). Esin H et al., in a study investigating liver regeneration with dipyrindamole administration in rats that underwent partial hepatectomy, showed that mitotic index, PCNA, and relative liver weight (RA) were significantly higher in parallel with regeneration (19). In this study, we considered mitosis rate and PCNA percentages to evaluate liver regeneration in rats who underwent partial hepatectomy. When combined with other findings, we found that these values were significantly higher in the groups treated with silymarin and IPC and were an indicator of hepatoprotective effect.

Mitotic index and PCNA index were used frequently in studies on liver regeneration and played an important role in the interpretation of regeneration (20, 21). By Hou et al. mitotic index and PCNA index data were also used to determine the effect of an organic compound named FR167653 on liver regeneration in rats that underwent partial hepatectomy (22). In their study, it was found that the mitotic index and PCNA index, which were determined simultaneously from the liver sections of the control group rats who had undergone partial hepatectomy, were higher than the other groups. In our study, it was observed that the mitosis rate was higher in the silymarin group compared to the other groups, but there was no statistically significant difference compared to the other groups. It was found that the PCNA index was statistically significantly higher in the group that underwent Ischemic Preconditioning + Silymarin + Partial hepatectomy. With these results, it is thought that the application of IPC and Silymarin together is more effective on regeneration in groups that underwent partial hepatectomy.

In this experimental study, we compared the results of rats who underwent partial hepatectomy with those who underwent partial hepatectomy after using silymarin for

6 weeks before resection. We found that the regeneration ability of the remaining liver tissue was higher and the liver functions were less affected in the group given silymarin compared to the group that was not given. In addition, although it has been shown that IPC increases the regeneration of the remaining liver tissue and liver functions are less affected during partial hepatectomy, we found that the use of silymarin before hepatectomy increases regeneration more and affects liver functions less. With these results, it can be said that the use of silymarin and IPC before hepatectomy will increase liver regeneration. However, similar clinical studies are needed in order to apply these results in clinical practice.

### Declarations

The authors received no financial support for the research and/or authorship of this article. There is no conflict of interest.

This study was approved by the Ethics Committee of the Gazi University (Date: 26.06.2015, Ref No: 2017/15.047).

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