

Original Article

The effect of silymarin on hepatic regeneration after partial hepatectomy: is silymarin effective in hepatic regeneration?

Suleyman Cetinkunar¹, Serhat Tokgoz², Bulent Caglar Bilgin³, Hasan Erdem¹, Recep Aktimur⁴, Serpil Can⁵, Huseyin Serkan Erol⁶, Atilla Isgoren⁷, Selim Sozen⁸, Yilmaz Polat⁹

¹Adana Numune Egitim ve Arastirma Hastanesi, Genel Cerrahi Klinigi, Adana, Türkiye; ²Ankara Diskapi Yildirim Beyazit Egitim ve Arastirma Hastanesi, Genel Cerrahi Klinigi, Ankara, Türkiye; ³Kafkas Universitesi Tip Fakultesi, Genel Cerrahi Ana Bilim Dalı, Kars, Türkiye; ⁴Samsun Egitim ve Arastirma Hastanesi, Genel Cerrahi Klinigi, Samsun, Türkiye; ⁵Kafkas Universitesi Tip Fakultesi, Fizyoloji Ana Bilim Dalı, Kars, Türkiye; ⁶Ataturk Universitesi Veterinerlik Fakultesi, Biyokimya Ana Bilim Dalı, Erzurum, Türkiye; ⁷Ankara Universtesi, Veterinerlik Fakultesi, Ankara, Türkiye; ⁸Namik Kemal Universitesi Tip Fakultesi, Genel Cerrahi Ana Bilim Dalı, Tekirdag, Türkiye; ⁹Medikal Park Hastanesi, Genel Cerrahi Klinigi, Elazig, Turkiye

Received December 18, 2014; Accepted February 10, 2015; Epub February 15, 2015; Published February 28, 2015

Abstract: Aim: Silymarin from *Silybum marianum* was found to reduce liver injury. The aim of the present study was to investigate the effects of silymarin on hepatic regeneration in partially hepatectomized rats. Methods: Thirty Wistar-Albino rats were divided into 3 groups of 10 animals as sham, control and experimental groups. In the sham group (n=10) abdominal incision was closed after laparotomy. In the control group (n=10), the rats underwent 70% hepatectomy after laparotomy. In the experimental group (n=10) after partial 70% hepatectomy, silymarin (200 mg/kg/d) were given to rats for 10 days. Rats in three groups were sacrificed on 10 days. Aspartate (AST) and alanine transaminase (ALT), gamma glutamyl transferase (GGT), ALP, LDH and total bilirubin levels were measured using intracardiac blood samples. Tissue malondialdehyde (MDA) and tissue glutathion (GSH) and Superoxide dismutase (SOD) levels were measured. To reveal the increase in the mass of the remnant liver tissue in the control and experimental groups relative weight of the liver was calculated. Histopathological analysis of the liver was performed using a semi-quantitative scoring system. Results: A statistically significant difference among three groups was not shown for AST and ALT levels. A statistically significant difference was found between the groups as for total bilirubin and gamma glutamyl transferase levels. Increases in relative liver weights were seen with time in Groups 2 and 3. A statistically significant difference was not found for tissue malondialdehyde, Glutathion and Superoxide dismutase levels between hepatectomy and hepatectomy + silymarin groups. On liver tissue sections of the rats in the hepatectomy + silymarin group, increased regeneration and lipid peroxidation were observed accompanied by decreased antioxidant response. Conclusion: It has been observed that silymarin with many established functions such as antiproliferative, anti-inflammatory and energy antioxidant effects, does not contributed to proliferative regeneration of the liver-which has very important metabolic functions -after partial hepatectomy; instead it will decrease serum levels of transaminases.

Keywords: Silymarin, hepatic regeneration, hepatectomy.

Introduction

Mortality rates of hepatic resection, which was previously a dreaded surgical intervention, currently have dropped to less than 5% thanks to better comprehension of liver anatomy, and physiology and application of these operations in the light of these information [1]. Recovery of baseline liver mass and functions after loss of

hepatic tissue for any reason is called hepatic regeneration [1-5]. When the liver reaches the size where it can meet the functional requirements of the body and fulfil its metabolic functions, it stops growing further [2-7]. Interestingly, in case of transplantation of a liver larger than the recipient's naive liver, hepatic mass decreases till optimal liver/body ratio is achieved [8, 9].

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Silymarin from seed of milk thistle or *Silybum marianum* was traditionally used as raw extract and composed of silibinin, silychristin, isosilybin, silydianin, dehydrosilybin, and taxifolin [10]. It was found to reduce liver injury caused by acetaminophen, carbon tetrachloride, radiation, iron overload, phenylhydrazine, alcohol, cold ischemia and *Amanita phalloides* [11]. Silymarin prevents liver damage by maintaining the integrity of the plasma membrane, thereby suppressing the leakage of enzymes [12].

The aim of the present study was to investigate the effects of silymarin on hepatic regeneration in partially hepatectomized rats.

Materials and methods

In our study 30 Wistar-Albino strain female rats weighing 180-260 gr were used. The rats were divided into 3 groups of 10 animals as sham, control and experimental groups.

In the sham group (n=10) abdominal incision was closed after laparotomy. In the control group (n=10), the rats underwent 70% hepatectomy after laparotomy. In the experimental group (n=10) after partial 70% hepatectomy, silymarin (200 mg/kg/d) were given to rats for 10 days.

Preoperatively the rats were deprived of food and water for 6 hours. To prevent the effect of diurnal variations in regenerative response, operations were performed during the first half of the day. In all operations, anaesthesia was achieved with 50 mg/kg ketamine HCl (Ketalar) and 10 mg/kg xylazine (Alfazyne). Preoperatively all rats were weighed with precision scale and their body weights were recorded. After operation site cleaning with povidine iodine, through a 2.5 cm-long midline incision, laparotomy was performed. In compliance with the method described by Higgins et al. middle and left lateral lobes were ligated at their attachment to the vena cava with 4/0 silk sutures and 70% hepatectomy was realized [13]. Then the abdomen was closed in 2 layers. Six hours after the surgical intervention, the rats were fed through oral route. Rats in the sham and control groups were fed with standard laboratory pellets and tap water. However rats in the experimental group received silymarin through orogastric feeding tubes at once daily morning doses of 200 mg for 10 days. Rats in three groups were

sacrificed on 10 days after ketamine HCl anaesthesia (50 mg/kg). For biochemical analysis 3 ml intracardiac blood samples were drawn. In the sham group, liver, in the control and experimental groups remnant liver tissue were excised.

Biochemical evaluation

Aspartate (AST) and alanine transaminase (ALT), gamma glutamyl transferase (GGT), ALP, LDH and total bilirubin levels were measured using intracardiac blood samples.

Malondialdehyde measurement method: As an indicator of lipid peroxidation, tissue malondialdehyde (MDA) levels were measured based on the method described by Ohkawa et al. [14]. MDA was reacted with 0.67 thiobarbutiric acid (2%-thiobarbutiric acid; TBA) solution and the product was extracted with N-butanol. As a standard malondialdehyde solution malondialdehyde bis-(dimethyl acetal) was used to prepare standard solutions at 1-40 nM. Absorbances of the samples were read in spectrophotometre at 540 nm using a microplate reader and the calculations were made based on automatically drawn standard curves and the results were expressed as nmol/g protein.

Glutathion and Superoxide dismutase measurement method: Tissue GSH was measured according to the method described by Beutler et al. [15] Precipitating solution was prepared using metaphosphoric acid, disodium EDTA and NaCl. Disodium phosphate solution was prepared using disodium hydrogen phosphate (Na_2HPO_4). DTNB solution was prepared using [5,5'-Dithio-bis (2-nitrobenzoic acid)] and sodium citrate. Glutathion standard (reduced glutathion) was used to prepare 1-60 mg/dl standard solutions. Absorbances of standard and sample solutions were read in spectrophotometre at 412 nm.) SOD activity was measured by the method described by Fridovich (16). Results were presented as U/mg protein.

Besides, instead of sample solution, the same amount of pure water (blind sample) was used to record absorbances. The result was subtracted from the estimates of sample and standard solutions and the difference between two values were used to calculate the amount of GSH. The results were expressed in $\mu\text{g}/\text{mg}$ protein.

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Table 1. Tissue levels of MDA, GSH, and SOD activities in the groups

Groups	MDA (nmol/g tissue)	GSH (nmol/g tissue)	SOD (U/mg tissue)	Relative weight (%)
Sham (n=10) (Group I)	1.25 ± 0.33	8.34 ± 0.8	8.25 ± 0.21	
Hepatectomy (n=10) (Group II)	38.16 ± 6.27	6.13 ± 1.03	6.94 ± 1.24	22.10 ± 2.34
Hepatectomy + Silymarin (n=10) (Group III)	40.25 ± 1.73	6.34 ± 0.72	7.34 ± 2.22	28.20 ± 2.75
P Value	I versus II	0.001	0.01	0.05
	II versus III	0.68	0.91	0.55

MDA=malondialdehyde; GSH=reduced glutathione; SOD=Superoxide Dismutase; vs=versus; *P<0.05 was considered to be statistically significant.

Table 2. Clinical parameters in Sham, Hepatectomy, and "Hepatectomy + Silymarin" rats

	Sham (n=10) (Group I)	Hepatectomy (n=10) (Group II)	Hepatectomy + Silymarin (n=10) (Group III)	P Value I vs II	P Value II vs III
AST (U/L)	174.2 ± 22.4	198.4 ± 159	173.1 ± 31.7	0.24	0.37
ALT (U/L)	54.8 ± 5.9	51.14 ± 17.3	45.9 ± 10.5	0.16	0.33
LDH (U/L)	1845.1 ± 823.5	1867.1 ± 595	1852 ± 755	0.44	0.28
Total bilirubin (mg/dL)	0.1 ± 0.06	3.6 ± 2.6	0.26 ± 0.2	0.001	0.002
ALP (U/L)	118.2 ± 46.7	181.1 ± 56.2	188.1 ± 49.4	0.001	0.18
GGT (U/L)	1.8 ± 0.61	3.57 ± 2.8	1 ± 0.8	0.001	0.001

AST=Aspartate aminotransferase; ALT=Alanine aminotransferase; LDH=Lactate dehydrogenase; ALP=Alkaline phosphatase; GGT=Gamma glutamyl transferase; vs=versus; *P<0.05 was considered to be statistically significant.

To reveal the increase in the mass of the remnant liver tissue in the control and experimental groups relative weight of the liver was calculated. From the weight of the liver extracted during laparotomy the weight of the 70% hepatectomized liver was subtracted and the ratio between this difference and the total weight of the liver was calculated. The value obtained was multiplied by 100 to find the rate of regeneration. Body weight of the rat was multiplied by 0.034 to estimate the total weight of the liver. Results were expressed as percentages (%).

Histopathological analyses

Preparation of the liver tissues: Liver tissues harvested from the rats at the end of the experiment were fixated for 48 hours in buffered neutral formaldehyde solution. After fixation, liver tissues were stepwise subjected to routine histopathological procedures (dehydration, clearing and embedding) and 5 µm-thick cuts were obtained. All sections were stained with hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS).

Histopathological analysis of the liver: Semi-quantitative scoring was used based on the light microscopic evaluation in consideration of the following criteria: Degenerative cellular changes, death of a necrotic cell, adhesive leu-

kocyte, inflammatory cell infiltration and alterations in the sinusoidal area. Each parameter was scored between 0 (none) and 4 (severe) points.

Statistical analyses: Data were analyzed using the Statistical Package for Social Sciences (SPSS) software version 13.0 for Windows (SPSS Inc., Chicago, IL). Parametric tests were applied to data of normal distribution and non-parametric tests were applied to data of questionably normal distribution. The Kruskal-Wallis test and Mann-Whiney U-test were used to compare independent groups. Data are expressed as mean ± SD or median (interquartile range), as appropriate. Statistical significance was assumed for P<0.05.

Results

The experiment started with 30 rats and a total of 6 death events occurred in the control (n=2) and the experimental group (n=4) within the first 24 hours due to bleeding, while in the sham group any deaths were not observed. To complete total number of the study subjects to 10, 2 rats were included in the control and 4 in the experimental group, therefore the study was completed with 30 rats.

Tissue levels of MDA, GSH and SOD levels and Relative weights of the liver in three groups

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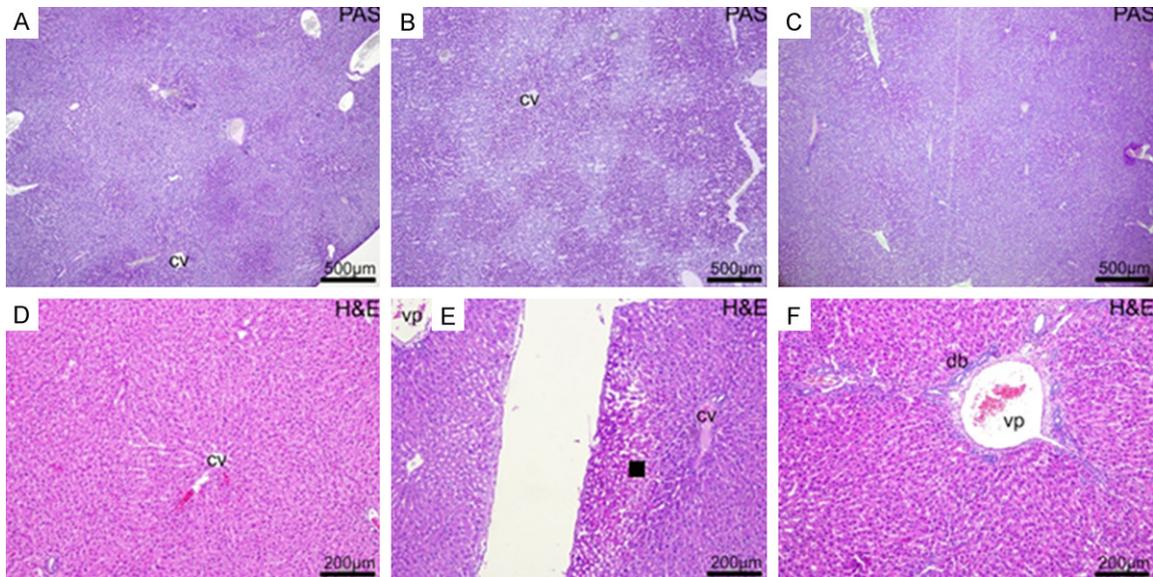


Figure 1. Appearance of liver tissue under low magnification in the Control (A-D), Hepatectomy (B-E) and Hepatectomy + Silymarin groups: (C-F) Appearance of necrotic foci in the hepatectomy group (H) and increased number of bile ducts in the hepatectomy + Silymarin group (B). (central vein=vc; portal vein=vp; bile duct=db; necrotic area=black square; Hematoxylin-Eosin stain=H&E; Periodic Acid- Schiff stain=PAS).

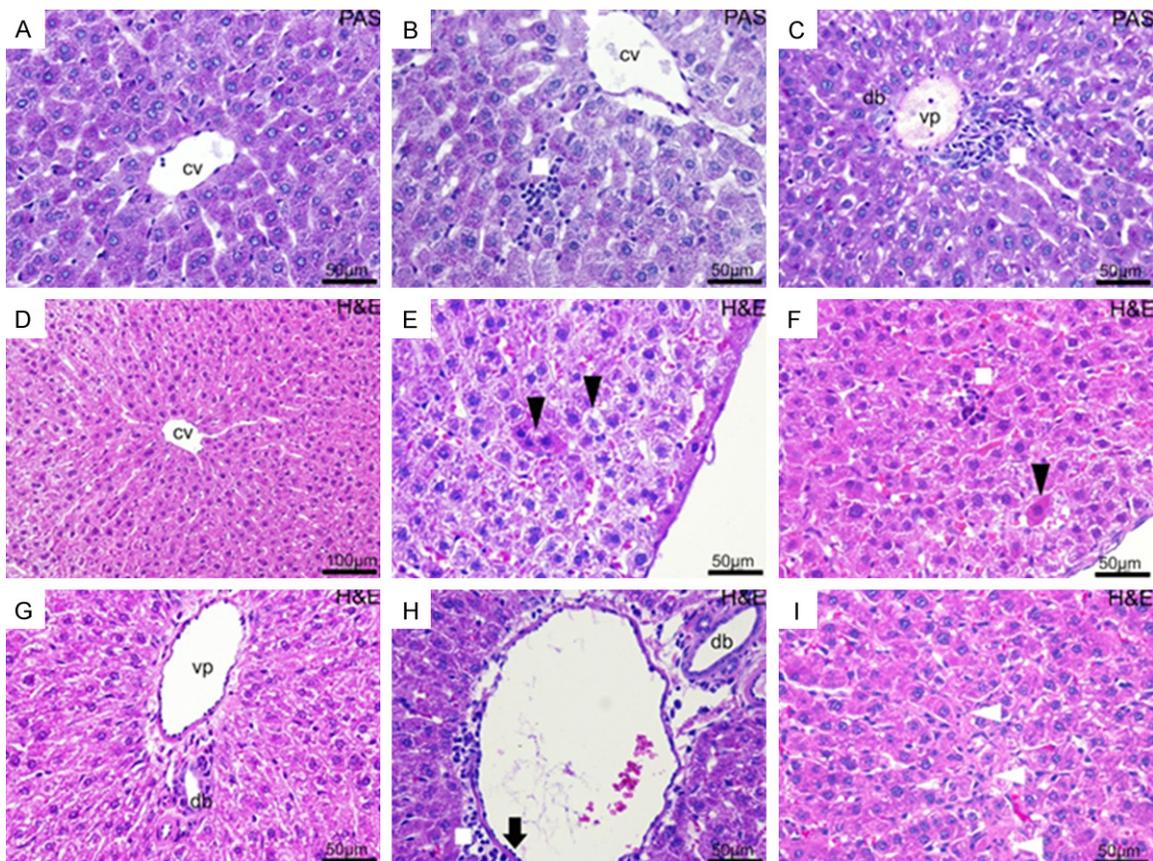


Figure 2. Appearance of liver tissue under high magnification in the Control (A, D, G), Hepatectomy (B, E, H) and Hepatectomy + Silymarin groups (C, F, I). Appearance of adhesive leukocytes (H) in portal vein, infiltration of inflammatory cells (B) apoptotic hepatocytes (E) in the hepatectomy group (E). (central vein=vc; portal vein=vp; bile duct=db; leukocytic infiltration=white square; adhesive leukocyte=black arrow; cellular degeneration=black triangle; apoptotic cell=white triangle; Hematoxylin-Eosin stain=H&E; Periodic- Acid Schiff stain=PAS).

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Table 3. Histopathological evaluation for rat hepatic tissues

	Sham (n=10) (Group I)	Hepatectomy (n=10) (Group II)	Hepatectomy + Silymarin (n=10) (Group III)
Increased number of biliary ducts (biliary duct proliferation)	0	1	1
Adhesive leukocyte		3	2
Inflammatory cell infiltration	0	3	2
Necrotic cell	0	3	3
Degenerative cellular changes	1	3	3
Focal hepatocyte necrosis	0	3	2
Alterations in the sinusoidal area	0	1	2
Total	1	17	15

0=None; 1=minimal; 2=Mild; 3=Moderate; 4=Severe.

mean \pm SD values of AST, ALT, ALP, GGT and their statistical analysis are shown in **Tables 1** and **2**. A statistically significant difference among 3 groups was not seen as for AST and ALT levels. A statistically significant difference was found between the groups as for total bilirubin and GGT levels (**Table 2**). Increases in relative liver weights were seen with time in Groups II and III. In intragroup comparisons, a statistically significant difference was obtained ($P < 0.05$).

Histopathological results

General appearances of the cut sections (small magnification) of all groups are seen in **Figure 1**, while histopathological changes are (higher magnification) mainly emphasized in **Figure 2**. Histopathological evaluation was made semi-quantitatively based on inflammatory cell adhesion and/or migration, necrotic or apoptotic hepatocytes and presence and intensity of cellular degenerations (**Table 3**). When liver tissue sections were analysed, typical liver histological structure was observed (**Figure 1A, 1D**). Hepatocytes and sinusoidal areas in centrilobular and midzonal regions had normal appearances (**Figure 2A, 2D**) while Remark cords had a regular structure. Portal vein, hepatic artery and choleduct observed in the portal area had a healthy appearance (**Figure 2G**). Then control and experimental groups were compared and predominant degenerative changes were observed both in the hepatectomy and hepatectomy + silymarin groups. Although the abovementioned histopathological changes were seen both in the hepatectomy and hepatectomy + silymarin groups, degeneration was more severe in the hepatectomy group. Priorly, necrosis and necrotic cells were evaluated and in the hepatectomy group intense focal necrotic foci

(**Figure 1E**) and degradation of hepatocytes in the liver tissue specimens were observed (**Figure 1E**). Secondly, histological findings detected in both hepatectomy and hepatectomy + silymarin groups included apoptotic hepatocytes with dense eosinophilic cytoplasm and heterochromatic nuclei (**Figure 2E**). However, presence of apoptotic hepatocytes and fibrotic connective tissue (**Figure 2I**) were less dense in the hepatectomy + silymarin group. Thirdly, in the hepatectomy group, in addition to degeneration of the portal vein endothelium, presence of intensely accumulated adhesive leukocytes and infiltration of inflammatory cells (**Figure 2B**) were detected (**Figure 2H**). Finally, on liver tissue sections of the rats in the hepatectomy + silymarin group, greatly increased number of biliary ducts in the portal area were observed (**Figure 1F**). In both groups, irregular and enlarged sinusoids were discerned (**Figure 1C**).

Discussion

Hepatic resection or partial hepatectomy decreases liver mass and though rarely it leaves damaged cells behind. In the 2/3 partial nephrectomy model, left and medial lobes are ligated and excised. Thus, 65-70% of the liver is excised [17]. Although the remaining hepatic segments are exposed to the effects of increased portal blood flow and pressure following partial hepatectomy, *in vivo* regenerative response studies partial hepatectomy has been demonstrated as the best method to provide pure hepatic regeneration not associated with cellular damage. Twenty-four hours after partial hepatectomy, active cellular replication process starts and continues till liver reaches its baseline weight. Within the first 10 days major regenerative changes occur and this process is completed within 4 or 5 weeks. Excised lobes do not assume their previous configura-

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tion. Regeneration more frequently proceeds as formation of new lobules and enlargement of the remaining lobules. Stimulants required for hepatic regeneration are humoral factors coming from pancreas, other extrahepatic organs and regenerated liver itself [18].

All liver cells divide and involve in the regeneration process [19]. Hepatocytes which constitute 80% of the liver mass and 60% of the number of hepatic cells, most rapidly induce cellular regeneration cycle. These cellular changes occur within minutes [20, 21]. Maximum DNA synthesis is seen in hepatocytes within 24 hours. Hepatocytes are followed by ductular epithelial cells, Kupffer cells, stellate cells and sinusoidal endothelial cells in order of decreasing rates of regeneration [22]. Silymarin, is a flavanoid extracted from silybum marianum fruit with hepatoprotective effects [23, 24]. Silymarin has also many potentially therapeutic effects and it is a very potent antioxidant which inhibits lipid peroxidation in hepatocytes [25]. In addition, it has anti-inflammatory activities mediating modification of the functions of hepatic Kupffer cells [26]. Moreover, the antioxidative, anti-lipid peroxidative, antifibrotic, anti-inflammatory, membrane stabilizing, immunomodulatory, liver regenerating, anti-tumour, anti-atherosclerotic, and anti-diabetic activities of silymarin were also reported [27]. Silymarin prevents liver damage by maintaining the integrity of the plasma membrane, thereby suppressing the leakage of enzymes [12].

As a cytoplasmic and mitochondrial enzyme, AST is found in many organs apart from liver including heart, skeletal muscle, kidney and brain tissues, as a cytoplasmic enzyme ALT is mainly found in liver and it is more specific than AST [1, 28]. It is recognized that serum transaminases are very sensitive in the demonstration of hepatocyte damage and independent from etiological factors, their values remain at high levels as far as persistence of liver damage [29]. Assessment of liver function can be made by the estimation of serum levels of metabolic enzymes like ALT, AST and ALP which are leaked out into systemic circulation during necrotic cell damage and hence are referred as sensitive indicators of liver injury [30, 31]. Increase in serum level of ALP is due to increased synthesis in presence of increasing biliary pressure [32]. Effective control of bilirubin level and alkaline phosphatase activity points towards an early improvement in the secretory mechanism

of the hepatic cell. In our study, a statistically significant difference was found between groups regarding total bilirubin and gamma-glutamyl transferase levels. Especially increase in ALT is one of the reliable parameters indicating degradation of hepatocytes. However in our study a significant difference was not detected between groups as for AST and ALT levels. As a matter of fact, liver damage resolves at 72 hours after resection [33]. Since we made our measurements on the 10 day, we couldn't get adequate data concerning the effects of silymarin on hepatic functions following hepatic resection. Normally, released toxic oxygen metabolites and antioxidant defence systems are in equilibrium. However in cases where oxidative metabolism significantly gain momentum or blood supply of the tissues decrease, production of free radicals accelerates and antioxidant defence systems become inadequate. Increased oxidative stress during the early phase of liver regeneration had been observed as a cause of surgery and a reactive response of the reduced organ to compensate for the extra functional load [34-36]. We observed significantly increased oxidative stress and liver function tests levels and significantly decreased liver tissue GSH levels after partial hepatectomy. Increased oxidative stress could diminish the regeneration process. Measurement of MDA levels is very important in the determination of the levels of lipid peroxidation products. We analyzed MDA levels in liver tissue specimens. We couldn't find a statistically significant difference between hepatectomy and silymarin + hepatectomy groups as for liver MDA levels ($P=0.68$). Superoxide dismutase enzyme catalyzes dismutation of superoxide (SOD) radical into hydrogen peroxide. SOD enzyme fights against free radicals so as to obviate harmful effects of I/R injury. In the silymarin treated group hepatic SOD (superoxide dismutase) levels were markedly higher relative to hepatectomy group, but without any statistically significant intergroup difference ($P=0.910$). We observed increased lipid peroxidation and diminished antioxidant response in the silymarin-treated hepatectomized group. Its anti-oxidant property was demonstrated in previous studies [37]. The most extensively studied and disseminated property of silymarin is its hepatoprotective activity. Several clinical studies have been performed to evaluate the efficacy of silymarin to treat a range of liver and gallbladder disorders such as acute and chron-

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ic hepatitis, cirrhosis and toxin-induced hepatitis [38]. These effects were associated with decreased membrane lipid peroxidation, reduced free-radical release and restoration in the GSH levels [38]. Silymarin is known to reduce the rise in intracellular Ca^{2+} levels induced by tertbutyl hydroperoxide in rat hepatocytes, suggesting that the hepatoprotective effect of silymarin is not only due to the inhibition of lipid peroxidation but also modulation of intracellular calcium levels [39]. The ability to maintain calcium flux may be due to either silymarin's effect as an antioxidant by reducing intracellular free radical levels and/or some direct effect on mitochondria through modulation of mitochondrial calcium ion channels [40].

A massive centrilobular necrosis, central vein dilation, ballooning degeneration and inflammatory cellular infiltration of liver are associated with liver damage as evidenced with histological findings in present study [41]. However, silymarin was effective in prevention of these toxic histological changes associated with liver damage. Hua-Sheng et al. analysed the correlation between bile secretion and hepatic regeneration and they expressed regeneration rate as the ratio between total weight of the intact liver and residual weight of the liver following partial hepatectomy [42]. Therefore their regeneration rate was underestimated. However in our study regeneration rate was estimated by comparisons between total liver weights measured before and after hepatectomy. In our study an increase in the regeneration rate was observed in the silymarin- hepatectomy group

In conclusion, it has been observed that silymarin with many established functions such as antiproliferative, anti-inflammatory and energy antioxidant effects, does not contributed to proliferative regeneration of the liver -which has very important metabolic functions-after partial hepatectomy; instead it will decrease serum levels of transaminases. However, we think that its clinical use requires more comprehensive and numerous experimental and clinical studies.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Süleyman Çetinkünar, Adana Numune Training and Research Hospital, Clinic of General Surgery, Adana TURKEY, Serin evler Mh Ege Bagatur Bulv. 01200 Yuregir, Adana

Turkey. Tel: 00905054133397; Fax: 00903223-550144; E-mail: slmcetin@gmail.com

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