Effect of sılymarın on oxıdatıve stress and lıver hıstopathology ın experımental obstructıve jaundıce model[1]

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ABSTRACT

PURPOSE: To investigate the effect of silymarin on oxidative stress and hepatic injury induced by obstructive jaundice in an experimental model.

METHODS: Thirty Wistar-Albino type female rats were divided into 3 groups each including 10 rats. Only laparotomy was performed in group 1. Bile duct ligation was performed in group 2. In group 3, bile duct ligation was performed and orogastic silymarin 300 mg/kg/day dose was given for seven days. At the end of seven days, rats were sacrificed. The blood and liver tissue samples were taken to be examined biochemically and histopathologically.

RESULTS: The plasma and liver levels of malondialdehyde were significantly lower in silymarin group than in the bile duct ligated group. Although liver levels of GSH were significantly higher in silymarin group than in the bile duct ligated group, there was no significant difference between the plasma GSH levels of these groups. In silymarin group; the enlargement of hepatocytes, dilatation of canaliculi and the edema were regressed.

CONCLUSION: Silymarin diminished the harmful effects of obstructive jaundice on liver.

Key words: Jaundice, Obstructive. Silymarin. Oxidative Stress. Liver. Rats.

Introduction

Cholestatic liver disease, which may be due to primary bile duct disease or secondary causes such as stones or tumors, may involve both intrahepatic and extrahepatic bile ducts, or may be limited to one or the other. Management of the patient with cholestatic liver disease depends on defining the probable cause, initiating appropriate intervention or treatment, and the recognition and care of possible complications including cholangitis, coagulation defects, and liver damage progressing to biliary fibrosis and cirrhosis. Awareness of the complications that may be associated with cholestasis and providing the appropriate management are necessary[1]. The toxic bile products and neutrophil migration are known to induce oxidative stress and apoptosis that results in damage to the liver and, eventually, extrahepatic tissues and organs[2]. The bile duct ligation (BDL) model in rats has been used widely to investigate the cholestatic liver disease associated with oxidative stress and fibrogenesis. Experimental BDL model in rats stimulates the proliferation of hepatocyte progenitors and biliary epithelial cells, resulting in proliferating bile ductules with accompanying portal inflammation and fibrosis[3]. Although several drugs have been tested for the prevention of cholestasis-related tissue damage, no medication has been widely accepted in clinical practice.

Silybum marianum is the scientific name for Milk thistle or St. Mary's thistle that is a plant domestic to the Mediterranean region and belongs to the Asteraceae family. Silymarin is a natural compound that is present in species derived from *Silybum marianum*. The plant contains at least seven flavolignans including silybin, silydianin, silychristine, and the flavonoid taxifolin. Silymarin has been used worldwide for many years as a complementary alternative medicine because of the beneficial effects associated with the treatment of hepatic diseases. The hepatoprotective and antioxidant activities of silymarin are known to be caused by its control of free radicals which damage cellular membranes and cause lipid peroxidation. The cytoprotective effect on the liver is also caused by the inhibition of the cyclooxygenase cycle, leukotrienes, and the production of free radicals in Kupffer cells. All these effects reduce inflammation. Silymarin also protects the genomic injury, increases hepatocyte protein synthesis, decreases the activity of tumor promoters, stabilizes mast cells, chelates iron, affects the synthesis of RNA and DNA, and decelerates calcium metabolism. Furthermore, silymarin maintains the integrity of the hepatocyte membrane and impedes the entrance of toxic substances**.** It is capable of donating electrons to stabilize free radicals and reactive oxygen species (ROS) due to its phenolic nature. Silymarin also affects intracellular glutathione, which prevents lipoperoxidation of membranes. In general, silymarin has antioxidant, anti-fibrotic, anti-proliferative, immunomodulatory, and antiviral properties[4].

Based on the antioxidant, anti-inflammatory, anti-apoptotic and hepatoprotective properties of silymarin, the present study was conducted to investigate the effects of silymarin on oxidative stress paameters and hepatocyte apoptosis in experimental obstructive jaundice model. To the best of our knowledge, the effect of silymarin on obstructive jaundice has not been previously investigated in the literature.

Methods

The procedures in this experimental study were performed in accordance with the National Guidelines for The Use and Care of Laboratory Animals and approved by Animal Ethics Committee of Ankara Education and Research Hospital.

Thirty Wistar-Albino male rats, weighing 250± 25 g, were housed under constant temperature (21±2ºC) individually in wire cages with 12 hour light-dark cycle. Twelve hours before anesthesia, animals were deprived of food, but had free access to water two hours before anesthesia. No enteral or parenteral antibiotics were administered at any time. The rats that died during the experiment were excluded from the experiment and no new rat was included.

Rats were randomly divided into three groups each including 10 animals: group I, sham-operated; group II, ligation and division of the common bile duct (BDL); group III, BDL followed by oral supplementation of silymarin 300 mg/kg/day (Sigma, Istanbul, Turkey) with nasogastric tube that was inserted daily and taken off after silymarin supplementation. Animals were sacrificed on postoperative day 7 by high-dose ketamine hydrochloride. Blood and liver samples were taken for biochemical and histopathological evaluation.

Animals were anesthetized by intramuscular injection of 30 mg/kg ketamine hydrochloride (Ketalar®; Parke-Davis, Istanbul, Turkey) and 5 mg/kg xylasine (Rompun®, Bayer, Istanbul, Turkey). Midline laparotomy was performed under sterile conditions. In the sham-operated group (group I) the common bile duct (CBD) was freed from the surrounding soft tissue and was manipulated without ligation and transection. In group II and III, CBDs of the rats were identified, double ligated with 5-0 silk, and divided between the ligatures. The same surgeon performed all procedures. The abdominal incisions were closed in two layers with continuous 3-0 silk sutures. Animals were allowed to feed

after the operation.

After sacrifice, liver samples were taken and kept on an ice bath until homogenization. The tissues were homogenized in (20 w/v) serum physiologic solution, then centrifuged 4000x *g* for 15 min and upper clear supernatants were used in the assays. All the procedures were performed at +4°C throughout the experiments. Protein level of the clear supernatants was studied by Lowry's method[5]. Malondialdehyde (MDA) levels (nmol/mg) and GSH-peroxidase (Px) (mIU/mg) enzyme activities were measured in the supernatants. MDA levels were measured by thiobarbituric acid reactive substances method[6]. After the samples were preincubated with fish oil and xanthine-oxidase system at room temperature for 1 h, MDA level was determined. GSH-Px activity was measured by following changes in NADPH absorbance at 340 nm[7]. Plasma levels of MDA and GSH were determined by the same method.

The histopathological analyses of this study was carried out in Histology and Embriology Department of Ufuk University Faculty of Medicine. For light microscopic analyses, Liver tissues removed from each group of rats were fixed in 10% formaldehyde solution for 4 days, washed in flowing water and were dehydrated with rising concentrations of ethanol. Tissues were infiltrated with and embedded in paraffin after obtaining tranparency in xylene. Embedded tissues were cut into sections of 5 μm thickness by Leica RM 2245 and were mounted on glass slides. They were stained with stained with hematoxylin and eosin (H&E), Mallory-Azan dyes. The examinations were performed by the same researcher blinded to the treatment groups on Olympus BX51 and were photographed with Olympus DP71.

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 15.0 for Windows (SPSS Inc., Chicago, USA). According to the Shapiro-Wilk test, both MDA-plasma and GSH-Px-plasma values were normally distributed about the mean. All other variables were nonnormally distributed. Data were presented as means±standard deviation. Whether there were any differences among the groups was evaluated by one-way ANOVA or Kruskal-Wallis variance analysis, where appropriate. When the p values from the variance analysis were statistically significant, the Tukey HSD or Mann-Whitney U multiple comparison test was used to know which group differed from any of the others. All results were accepted as statistically significant when p<0.05).

Results

The levels of MDA and GSH are summarized in Table 1. Both plasma and liver levels of MDA were significantly lower

in silymarin group than in the bile duct ligated group (p=0.008 and 0.001, respectively). Although liver levels of GSH were significantly higher in silymarin group than in the bile duct ligated group (p=0.001), there was no significant difference between the plasma GSH levels of these groups (p > 0.05).

TABLE 1 - MDA and GSH levels of liver and plasma.

GROUPS	MDA-Liver (nmol/mg tissue)	GSH-Liver (nmol/mg tiss.)	MDA-Plasma (IU/mL)	GSH-Plasma (IU/mL)
Sham (I)	0.394±0.06[a]	0.058± 0.004[a]	5.871± 1.5752[a]	0.5081± 0.1119
BDL (II)	0.6298±0.159[b]	0.0389± 0.0051[b]	9.5902± 2.6321[b]	0.4296± 0.1098
BDL+ Silymarin (III)	0.4446±0.0764	0.0592± 0.0068	6.6791± 0.9191	0.6099± 0.0291

[a]Significantly different, I *vs* II
[b]Significantly different, II *vs* III

The micrographs of group I show the regular structure of the liver which is a solid organ composed of tightly packed plates of epithelial cells termed hepatocytes (H). The outer layer of surface of the liver is covered by a capsule composed of collagenous tissue called Glisson's capsule (c) over which is a layer of mesothelial cells (m) from the peritoneum (Figure 1, A1-A2). Mallory-Azan stained sections of group I show a typical portal tract (P) containing three main structures including terminal portal venule (PV), terminal branches of the hepatic artery (A) and bile ductules (B). Lymphatics are also present in the portal tracts, but their walls are delicate and often collapsed (Figure 2, A1-A3).

The micrographs of group II showed some histopathological findings such as swelling of the hepatocytes surrounding the portal venule and most importantly enlargement of the hepatic sinusoids (Figure 1, B1-B2). Mallory-azan stained sections assesed the collagen augmentation in the portal tract of group II. Bile ductules were merkedy increased in number because of looping and reduplication (Figure 2, B1-B3).

In group III, sinusoidal dilatation was decreased and the paranchymal liver tissue with hepatic sinusoids lying between the hepatocyte plates was in regular appearance (Figure 1, C1-C2). The histopathological evidence of the bile ductules' proliferation was markedly reduced presumably because of removal of the bile duct epithelial cells via apoptosis. The collagen deposition was markedly regressed (Figure 2, C1-C3).
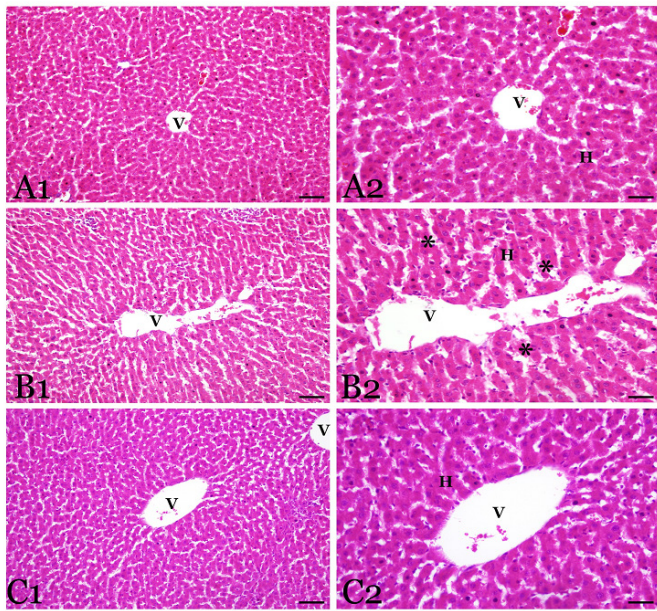
FİGURE 1 - The histopathological view of hepatic venule and hepatocyte plates by sinusoids lined by endothelial cells. **A**: Group I (sham-operated), **B**: Group II (ligation and division of the common bile duct), **C**: Group III (silymarin supplemented after bile duct ligation). V: hepatic venule, H: hepatocyte plate, *: dilated hepatic sinusoids. The scale bar is 20 μm in A1, B1, C1 and 10 μm in A2, B2, C2 micrographs.
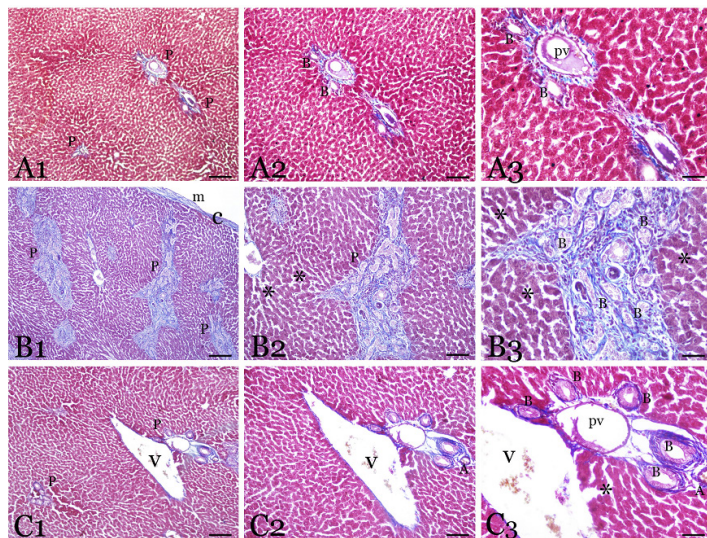

FİGURE 2 - The histopathological view of portal tract. **A**: Group I (sham-operated), **B**: Group II (ligation and division of the common bile duct), **C**: Group III (silymarin supplemented after bile duct ligation). P: portal tract, V: hepatic venule, H: hepatocyte plate, *: dilated hepatic sinusoids, m: mesothelial cells, c: capsule, PV: portal venule, A: hepatic artery branch, B: bile duct branch. The scale bar is 40 μm in A1, B1, C1; 20 μm in A2, B2, C2 and 10 μm in A3, B3, C3 micrographs.

Discussion

Obstructive jaundice occurs from a variety of disorders resulting from the mechanical stenosis or occlusion of the biliary ducts followed by the failure of bile flow into the duodenum.

This blockage results in the elevation of potentially toxic bile acids in the liver and blood. Obstruction of the extrahepatic bile duct from the hepatic hilum to the duodenal papilla is considered extrahepatic obstructive jaundice which is mainly characterized by bilirubinemia derived from elevated direct bilirubin. This is not an independent disease but a symptom of various diseases. Regardless of the cause, cholestasis often results in inflammation, hepatocellular injury, bile duct proliferation, and fibrosis. If the cause is not corrected and cholestasis persists, fibrosis progresses to cirrhosis and ultimately liver failure occurs. Bile duct ligation (BDL), the most extensively studied model in animals, represents a severe form of extrahepatic obstructive jaundice. In addition to the increase in oxidative stress markers in bile duct ligated animals, the levels of antioxidant defenses decrease. This could contribute to the increased levels of oxidative stress. Since there has been great interest in understanding the mechanism by which cholestasis produces hepatic oxidative stress, several studies have focused on the role of oxidative stress in the development of hepatocellular injury during cholestasis[8,9].

Several studies have indicated that bile acids may be important mediators of oxidative stress in hepatocytes. Bile acid concentrations increase in both animal models of cholestasis and in humans with cholestasis. Because of this, hepatocytes are exposed to very high concentrations of potentially toxic bile acids which stimulate production of reactive oxygen species (ROS) by hepatocytes and this is a very important for bile acid toxicity. Another potential source of ROS in the liver during cholestasis is inflammatory cells, which generate superoxide through NOX2. In both rat and mouse models of BDL, an early neutrophilic inflammatory infiltrate was observed. Although activation of Kupffer cells can also be detected during this time, an increase in the number of liver macrophages is generally not seen before 1 to 2 weeks after BDL. Despite the capacity of Kupffer cells to generate ROS and cause liver injury under certain inflammatory conditions, after BDL Kupffer cells are a more important source of cytokines, which initially mediate the inflammatory response and later also promote fibrosis rather than cause direct cell injury by oxidant stress. Thus, the main effect of Kupffer cell activation in obstructive cholestasis is to further promote an inflammatory and later a fibrotic response, but not to cause direct cell damage by oxidant stress. In contrast to Kupffer cells, neutrophils are considerably more cytotoxic. First, neutrophils are mobile and are able to accumulate in high numbers at a focus of inflammation. Second, neutrophils do not just generate superoxide and hydrogen peroxide through NOX2, but also release myeloperoxidase, which forms hypochlorous acid, a very aggressive oxidant and

chlorinating species, as well as additional secondary oxidants such as various chloramines. ROS generated by activated neutrophils, including the neutrophil-specific oxidant hypochlorous acid, can diffuse into the target cells and induce an intracellular oxidative stress. If neutrophil accumulation at the site of inflammation is prevented or NOX2 is inhibited, liver injury is substantially reduced. Ample evidence indicates that oxidative stress occurs in the liver during cholestasis. The source of oxidative stress appears to be primarily inflammatory cells that accumulate in the liver at early stages of disease. ROS produced by these cells not only damage hepatocytes, but may be an important stimulus for fibrosis by stimulating formation of lipid peroxidation products that activate hepatic stellate cells. Therapeutics designed to abrogate oxidative stress during cholestasis may be effective to treat this disease, however, the specific ROS generated and the cellular source and location of the oxidant stress need to be considered when choosing proper antioxidants for therapy[8].

Silybum marianum, commonly known as 'milk thistle' is one of the oldest and thoroughly researched plants in the treatment of liver diseases. The extracts of milk thistle is being used as a general medicinal herb from as early as 4th century B.C. The active constituents of the plant are obtained from the dried seeds where it is present in higher concentrations than in other parts of the plant and consist of four flavonolignans which are collectively known as silymarin. Silymarin is a complex mixture of four flavonolignan isomers, namely silybin, isosilybin, silydianin and silychristin. The cytoprotective effects of silymarin are mainly attributable to its antioxidant and free radical scavenging properties. Silymarin can also interact directly with cell membrane components to prevent any abnormalities in the content of lipid fraction responsible for maintaining normal fluidity. The inhibitory effect on 5-lipoxygenase pathway resulting in inhibition of leukotriene synthesis is a pivotal pharmacological property of silymarin. Silymarin has a regulatory action on cellular and mitochondrial membrane permeability in association with an increase in membrane stability. It has established efficacy in the restoration of liver function and regeneration of liver cells[10].

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/silibinin to treat a range of liver and gallbladder disorders such as acute and chronic hepatitis, cirrhosis and toxin-induced hepatitis[11].

The use of the silymarin extract, silybum marianum, in 72 patients with non-alcoholic fatty liver disease on a restricted diet significantly reduced the levels of alanine aminotransferase (ALT) and aspartame aminotransferase (AST). In this study,

silymarin was also reduced the high serum levels of gamma-glutamyl transpeptidase (GGT), probably for stabilization of the hepatocyte membrane structure, thereby preventing toxins from entering the cells. The levels of TNF-α was also decreased after silymarin therapy, as a consequence of the anti-inflammatory action of the drug. This clearly demonstrated a reduction of the hepatic inflammation. The results indicated that silymarin was effective to reduce the biochemical, inflammatory and ultrasonic indices of hepatic steatosis[12].

Ethanol metabolism induces generation of excessive amount of ROS which results in immune dysfunction. Das and Mukherjee[13] examined the effects of silymarin on ethanol-induced oxidative stress and immunomodulatory activity in mice. The efficacy of silymarin was also compared with ascorbic acid, a potent antioxidant. Both silybin and ascorbic acid appeared to be equally good candidate in the prevention of ethanol-induced oxidative stress and normalized the cytokine metabolism. They concluded that these effects of silymarin were due to its antioxidant, antiinflammatory, antifibrotic, and metabolic activities.

In a randomized controlled double-blind pilot study Ladas *et al.*[14] investigated milk thistle on children with acute lymphoblastic leukemia (ALL) with chemotherapy associated hepatic toxicity in both the laboratory and clinical setting. Liver function tests were evaluated during the study period. At day 56, the milk thistle group had a significantly lower AST and ALT levels when compared with placebo group. In children with ALL, milk thistle reduced the chemotherapy-associated liver toxicity and did not antagonize the effects of chemotherapy agents used for the treatment of ALL. No significant difference was found in the frequency of side effects, incidence and severity of toxicities, or infections between the groups.

Kwon *et al.*[15] investigated the underlying mechanism of beneficial action of silymarin and found that silymarin enhanced hepatic glutathione generation and this effect might subsequently contribute to the antioxidant defense of liver. Pramyothin *et al.*[16] also confirmed the beneficial roles of silymarin against ethanol-induced liver injury in rats and concluded that possible mechanism might involve its antioxidant activity.

In another study, Polyak *et al.*[17] suggested that silymarin exerted anti-inflammatory and antiviral effects, and it might assist in the management of patients with chronic hepatitis C.

As mentioned before, obstructive jaundice causes significant oxidative stress and inflammatory reaction in liver. Thus, in the present study we investigated the effects of silymarin, which has anti-infammatory and antioxidant properties, on oxidative stress and the morphology of liver tissue in the

obstructive jaundice model in rats.

In histopathological examination, specimens from the sham group presented no morphological alteration. However, tissues of the obstructive jaundice group (Group II) displayed many histopathological changes such as the hepatocyte enlargement, hepatocyte degeneration, sinusoidal dilatation, edema around vena centralis, and marked bile pigment granule accumulation. In group III (obstructive jaundice+silymarin group), the enlargement of hepatocytes, dilatation of canaliculi and the edema were regressed. The regeneration of hepatocytes were obtained. The debris of apoptotic hepatocytes were phagocyted by the activated Kupffer cells but the reduced amount of bile pigments also existed. Consequently, silymarin ameliorated the harmful effects of obstructive jaundice on liver tissue, morphologically.

To investigate the mechanism of the therapeutic effect of silymarin, oxidative stress parameters for each group were measured. Both plasma and tissue levels of MDA were significantly lower in silymarin group than in the bile duct ligated group ($p=0.008$ and 0.001, respectively). Although liver levels of GSH were significantly higher in silymarin group than in the bile duct ligated group ($p=0.001$), no significant difference was found between the plasma GSH levels of these groups ($p > 0.01$). These results indicated that the protective effects of silymarin might be related at least partly to its antioxidant activity.

Conclusion

Silymarin diminished the harmful effects of obstructive jaundice on liver.

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