EXPERIMENTAL STUDY

The effect of alpha-lipoic acid on oxidative parameters and liver injury in rats with obstructive jaundice

Atalay E[1], Ozdemir MT[1], Tur BK[1], Erdogdu HI[1], Sisman P[2]

Department of Internal Medicine, Kafkas University, Kars, Turkey. **erayatalay2067@hotmail.com**

ABSTRACT

INTRODUCTION: The aim of this study is to investigate the effects of obstructive jaundice on the liver and effectivity of alpha-lipoic acid on liver damage and oxidative stress.

MATERIALS AND METHODS: Thirty-six male Sprague-Dawley rats were divided into 3 groups per 12 animals, namely into Group I (control group): the bile duct was only mobilized by laparotomy, Group II (bile duct ligation group - BDL): the common bile duct was closed with clips and OJ was caused after laparotomy, and Group III (bile duct ligation and alpha-lipoic acid group – BDL+LA): after closing the common bile duct, LA was administered in an intramuscular dose of 50 mg/kg for 10 days. On the 10th day, malondialdehyde, glutathione and superoxide dismutase levels were measured in liver and histopathological evaluation was performed.

RESULTS: AST (U/L)/ALT(U/L) in groups I, II and III were 155.33/51.83, 445.28/165.89, 380.78/173.33, respectively ($p < 0.005$). Superoxide dismutase and glutathione levels were lower in patient groups than in the control group (0.31 µl/g vs 0.36 µl/g; $p < 0.05$). After the lipoic acid treatment, none of the biochemical markers of liver improved. Only the increase in superoxide dismutase (0.31 µl/g and 0.34 µl/g in groups II and III, respectively) and glutathione levels (0.16 µl/g and 0.22 µl/g in groups II and III, respectively) was statistically significant ($p < 0.05$).

CONCLUSIONS: Histopathological damage was statistically significantly decreased and antioxidant levels were statistically significantly increased after LA treatment *(Tab. 1, Fig. 6, Ref. 23)*. Text in PDF *www.elis.sk.*

KEY WORDS: obstructive jaundice, alpha lipoic acid, antioxidant, liver damage, biochemical markers.

Introduction

Obstructive jaundice (OJ) is a severe disease occurring due to different causes such as pancreatic cancer, gallstones or bile duct stricture. Despite improvements in the diagnosis and treatment of OJ, high mortality and morbidity rates have not been markedly reduced. Major complications such as pulmonary dysfunction, renal failure, sepsis, peripheral vasoconstriction, gastrointestinal hemorrhage, coagulopathy, impaired wound healing and cardiovascular problems are frequently encountered (1).

Hepatic inflammation is an important feature of cholestatic liver disease in both humans and experimental animals. The inflammatory features of obstructive cholestasis include portal tract edema, neutrophil infiltration in the portal tracts, proliferation of the biliary epithelial cells, portal tract fibrosis, and, at last, necrosis. OJ could be induced in rat by common bile duct ligation, which causes cell damage that probably leads to cirrhosis and portal hypertension (2). Besides, bacterial translocation, endotoxemia, defective host immune response, retention of hydrophobic bile acids, the increase in inflammation-related cytokines, lipid peroxidation and oxidative stress are also important problems in this disease (3–6).

In OJ, antioxidative activity is reduced and free radical production increases (7). In studies conducted on rats with bile duct ligation, the activity of antioxidants such as superoxide dismutase (SOD) and glutathione (GSH), which are the major compounds having roles in clearing the oxygen radicals from the milieu, and malondialdehyde (MDA) have been examined. MDA is a marker for oxidative stress with mutagenic and cancerogenic effects. The degree of lipid peroxidation can be estimated by the amount of MDA in tissues. GSH is an important antioxidant and it is capable of preventing damage incurred to important cellular compounds caused by reactive oxygen species. SOD is an enzyme that alternately catalyzes the dismutation of superoxide radicals into either ordinary oxygen molecule or hydrogen peroxide. Superoxide is produced as a byproduct of oxygen metabolism and causes cell damage if not regulated.

Alpha-lipoic acid (LA) is a naturally occurring compound in both prokaryotic and eukaryotic cells. For many years, it has been used for diabetic polyneuropathy without serious side effects. It is also used in liver diseases, including alcohol-induced conditions, mushroom poisoning, metal intoxication and biliary cirrhosis (8). It reduces free radicals, including cellular membrane lipid peroxides, and scavenges free radicals at their mitochondrial source. In

[1]Department of Internal Medicine, Kafkas University, Kars, Turkey, and [2]Endocrinology and Metabolism Clinic, Medicana Hospital, Bursa, Turkey

Address for correspondence: E. Atalay, Department of Internal Medicine, Kafkas University, Kars, Turkey.
Phone: +05306959045

cells, LA is reduced to its dihydro form, which is a more potent antioxidant. In the literature, there were studies investigating the positive effect of LA on the liver (9–11) and intestine (12, 13) in rats with OJ. In this study, we aimed to determine whether lipoic acid has a protective effect in obstructive jaundice. While evaluating the effect of lipoic acid we also determined the changes in oxidative stress.

As a result, we planned to assess the effect of bile duct ligation on oxidative stress and morphology of the liver. We also planned to evaluate the potential protective role of LA in hepatic damage in rats with OJ.

Methods

Animals

We performed this study in the Experimental Animals Unit of Kafkas University. The protocol was approved by local ethics committee (affiliated to Turkish Ministry of Environment) for animal experiments of the Medical Faculty of The University of Kafkas on their meeting on 18.02.2015 under number 09. Animals were obtained from our Experimental Animals Unit. Thirty-six male Sprague Dawley male rats weighing 200 ± 5–240 ± 5 g were used. The mean age of the rats was 120 ± 20 days. They were housed under the conditions of room temperature, 40–50 % humidity and 12-h dark/light cycles. During the experiment, they were fed with standard food and tap water.

Study design

The rats were randomly divided into 3 groups, with 12 animals in each group.

Group I   (n = 12; control group): the bile duct was only mobilized by laparotomy.

Group II (n = 12; bile duct ligation group – BDL): The common bile duct was closed with clips and OJ was caused after laparotomy.

Group III (n = 12; bile duct ligation and alpha-lipoic acid group – BDL+LA): after closing the common bile duct and causing OJ, alpha-lipoic acid was administered intramuscularly for 10 days.

The medical procedures were applied to the animals in groups as 1, 2, and 3. respectively.

Surgical procedure

Each animal was fixed in a supine position on the operating table for the surgical procedure. All rats were anesthetized with an intramuscular dose of ketamin and xylazine. Just under the xyphoid, a 3-cm midline incision was performed. In Group I, the common bile duct was found and mobilized. Then the abdomen was closed. In groups II and III, the common bile duct was separated from the surrounding tissues by vessel clips and ligated. In Group III, after inducing obstructive jaundice, LA (Thioctacid 600T sol 15x24 ml/ 600 mg Meda Pharma GmbH & Co. KG, 61352 Bad Hamburg) was administered intramuscularly in a dose of 50 mg/kg/day for 10 days.

On days 3, 7 and 10 after the operation, a 2-cc volume of blood was extracted from the heart. Plasma was separated by centrifugation and serum was kept at –20 °C before carrying out the measurement for determining the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), γ-glutamyl transpeptidase (GGT), and concentrations of total bilirubin (TB) direct bilirubin (DB), urea, and creatinine.

For evaluating the liver toxicity, ALT (U/L), AST (U/L), GGT (U/L) and bilirubin (mg/dl) levels were assessed. The analysis of liver toxicity was based on the examination of bile canalicular amount and damage, inflammatory reaction and cell count in the portal space, hydropic degeneration of hepatocytes, necrosis and fibrosis. We also tried to determine the activity of antioxidants such as superoxide dismutase (SOD) and glutathione (GSH) as well as malondialdehyde which is the last product of lipid peroxidation. We finally investigated the effect of lipoic acid on liver toxicity. No animals died during the study and all animals were analyzed. On day10 after the operation, all rats underwent laparotomy under anesthesia. In groups II and III, the bile canals were found to be dilated.

For biochemical and histopathological analysis, the liver was extracted up to seven minutes after laparotomy. For biochemical examination, the tissues were placed into phosphate tampons and for histopathological analysis, those tissues were fixed. Liver extracts were examined as for the presence of the products of lipid peroxidation, namely MDA levels and tissue key antioxidants, namely GSH and SOD.
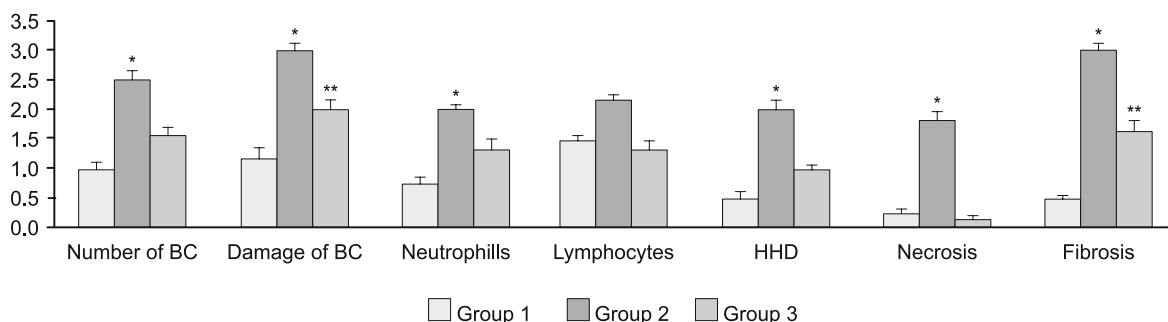
Fig. 1. Histopathological evaluation of the liver between groups. Scores of fibrosis, hydropic degeneration, necrosis, number and degeneration of bile canaliculi, number of cells for inflammatory response and number of cells in portal space are evaluated.

Biochemical analysis

The liver tissues of the rats, which were sacrificed secondary to hypovolemia following the blood drawing, were placed into the glass tubes filled with the phosphate buffer (pH = 7.4). The levels of glutathione (GSH), which is the key antioxidant, superoxide dismutase (SOD), and MDA, which is a product of lipid peroxidation, were measured in the liver tissue.

SOD measurement

The SOD activity was measured according to the method described by Sun et al (62).

The calculation of SOD activity

The amount of formed formazan was read at 560 nm in 3-ml quartz cuvettes. The activity values (EU) were obtained with the help of the following formula while taking the dilution coefficients into consideration and giving SOD activity in mmol/minutes/mg tissue. The effect of each factor was given after 6 repetitions.

EU/mg tissue = ΔAblind – ΔAsample / ΔAblind

The measurement of the amount of total glutathione (GSH)

The measurement was performed according to the method developed by Sedlak and Lindsay (63). For this purpose, homogenization was carried out with the addition of 4.5 ml 50 mM Tris-HCl (pH 7.4) to a 0.5 g tissue sample.

The calculation of the GSH amount

The GSH amounts in the samples were given in nmol/mg tissue. The effect of each factor was determined after six repetitions.

LPO measurement

It was done according to the method described by Ohkawa et al., which is based on the principle of measurement of the color that is formed by MDA with TBA in the acidic environment, at 532 nm (61).

The calculation of LPO amount

The LPO amounts in samples were described in nmol/MDA/g of tissue. The effect of each factor was given after 6 repetitions.

Histopathological examination

Liver tissues were immediately placed into formaldehyde solution, embedded in paraffin, cut into sections and stained with hematoxylin-eosin. Histopathological evaluation of fibrosis was made under light microscope with x400 magnification and x200 magnification. The histopathological evaluations were made by a pathologist not aware of which group the specimens belonged to.

Bile canalicular amount and damage, inflammatory reaction and cell count in the portal space (PNL/LEN), hydropic degeneration of hepatocytes, necrosis, and fibrosis were examined according to Ishak (12). Scores for fibrosis and hydropic degeneration, number and degeneration score of bile canaliculi, and number of cells associated with inflammatory response are given in Figure 1.

Statistical analysis

Calculations were performed using the program of SPSS (Statistical Package for Social Sciences) 16.2. ANOVA test was used for the analysis of laboratory examinations and Duncan test was used for the differences in groups. We used Bonferroni test for comparing independent two groups. The value of $p < 0.05$ was considered statistically significant.

Results

There was no difference in ALT, AST, AP, GGT, TB and DB levels measured on days 3, 7 and 10 ($p > 0.05$). The determinants measured on day 10, and their comparison according to their groups are given in Figure 2. Biochemical liver damage markers AST (U/L)/ALT(U/L) in groups I, II and III were 155.33/51.83, 445.28/165.89, 380.78/173.33, respectively ($p < 0.005$). Changes in liver enzymes in the course of experiment per group are listed in Table 1. Superoxide dismutase and glutathione levels were lower in patient groups than in the control group (0.31 µl/g vs 0.36 µl/g;
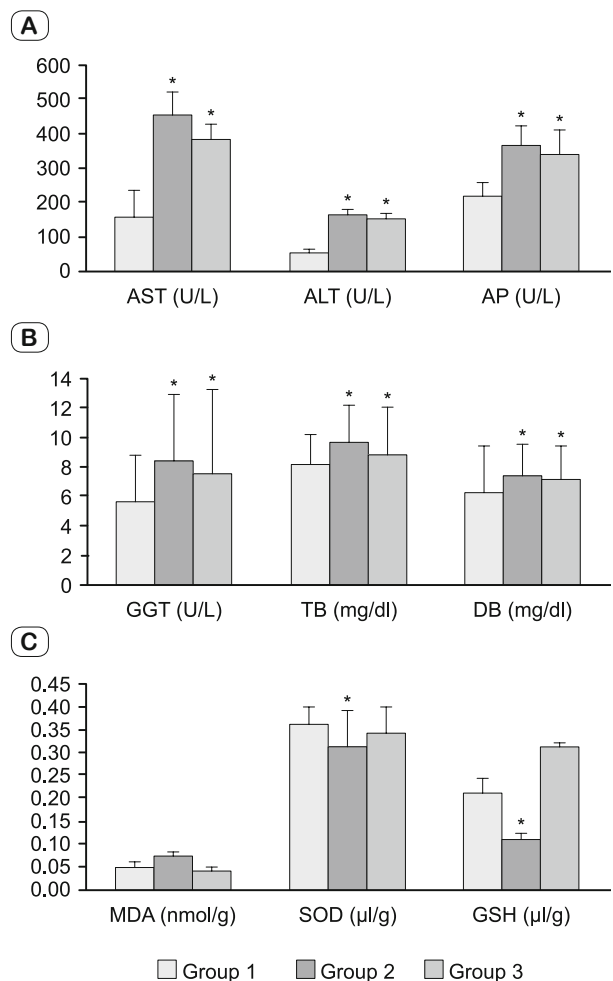

Fig. 2. The characteristics and comparison of the groups. Asterics (*) means the differences between groups ($p < 0.05$).
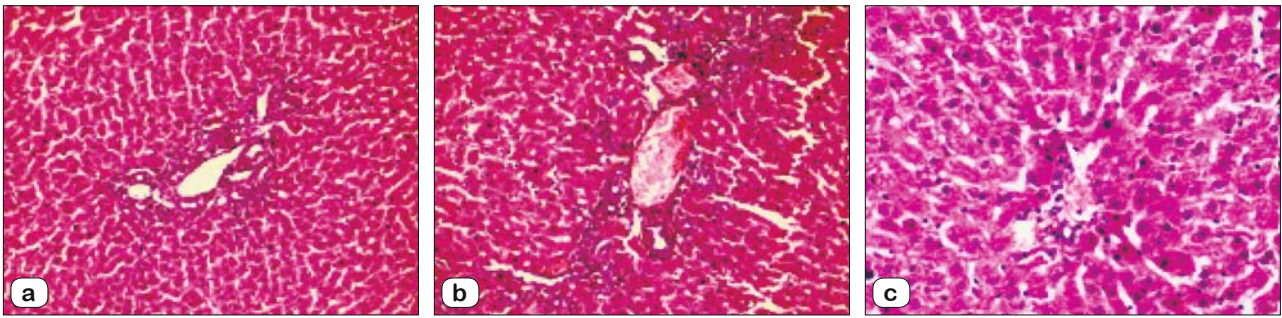
Fig. 3. The number of bile ductules: (a) The decline in the number of bile ductules in the BDL+LA group, (b) the increased number of ductules in the BDL group, (c) the normal number of ductules in the sham group (a and b: x200 magnification, c: x400 magnification, HE).
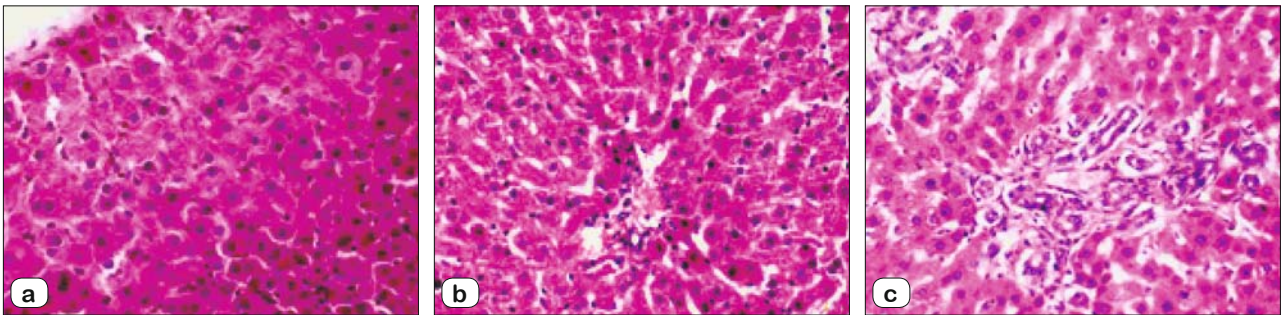

Fig. 4. Hydropic degeneration in the hepatocytes: (a) hydropic degeneration field in the hepatocytes belonging to BDL group, (b) normal liver tissue belonging to the sham group, (c) liver tissue belonging to BDL+LA group (x400 magnification, HE).
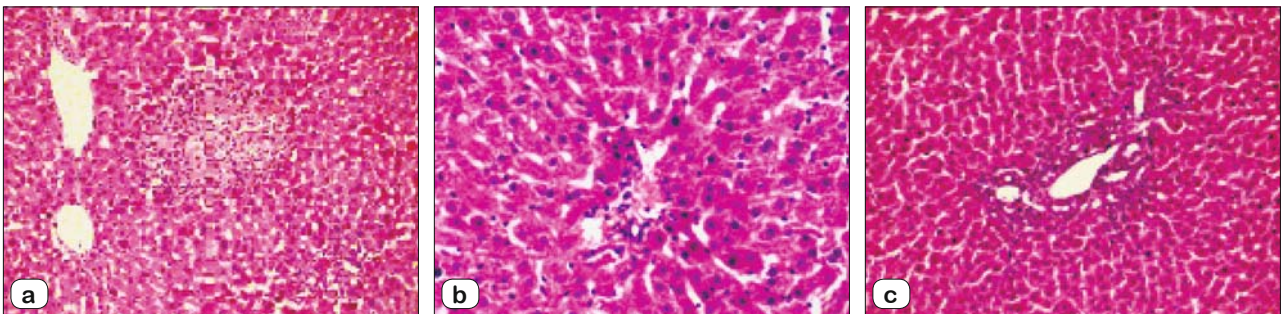

Fig. 5. Necrosis: (a) necrosis field belonging to BDL group (x200 magnification, HE), (b) normal liver tissue belonging to the sham group (x400 magnification, HE), (c) liver tissue belonging to BDL+LA group (x200 magnification, HE).
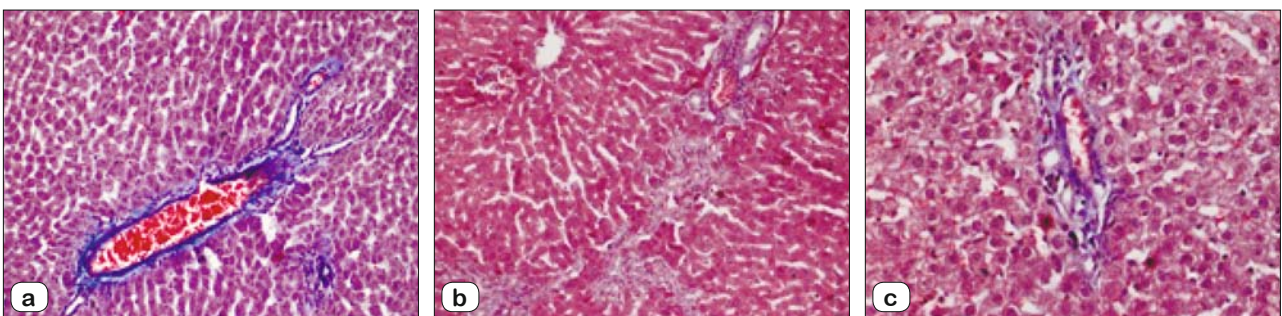

Fig. 6. Fibrosis: (a) image of the fibrotic expansion in some portal areas belonging to the sham group ± short fibrotic septa, (b) expansion in the portal areas with diffuse fibrosis + portoportal bridging in the BDL group, (c) fibrotic expansion in some portal areas belonging to the BDL+LA group (a and c: x400 magnification, b: x200 magnification, Mason trichrome).

Tab. 1. Changes in liver enzymes during days and between groups.

	Group I	Group II	Group III
AST(U/l) (Mean±SD)			
3rd day	178.65±65	524.83±148	424.86±206
7th day	140.75±37	479.83±203	394.33±130
10th day	148.5±60	361.16±87	323.33±75
ALT(U/l) (Mean±SD)			
3rd day	54.75±13	328.33±131	278.83±241
7th day	47.5±5	104.66±25	138.33±42
10th day	53.5±3	64.6±12.14	104.89±23
AP(U/L) (Mean±SD)			
3rd day	218.5±94	266±113	378±181
7th day	208±64	286.33±48	324.16±72
10th day	224±71	302.66±76	369.16±87
GGT(U/L) (Mean±SD)			
3rd day	9	10	10
7th day	3±0.81	6.8±4.9	7.1±7
10th day	3.25±0.5	8.5±9.3	10.83±10

$p < 0.05$). After lipoic acid treatment, none of the biochemical markers of liver improved. Only the increase in superoxide dismutase (0.31 µl/g and 0.34 µl/g in groups II and III, respectively) and glutathione levels (0.16 µl/g and 0.22 µl/g in groups II and III, respectively) was statistically significant ($p < 0.05$). MDA levels did not differ among groups. SOD and GSH levels in Group II were statistically lower than in groups I and III (Fig. 2).

The number and damage score of bile canaliculi, number of neutrophils as inflammatory cells in the portal space, hepatic hydropic degeneration, necrosis and fibrosis in Group II and also damage score of bile canaliculi and fibrosis in Group III were higher than those of Group I. In Group III, the number and damage score of canaliculi, number of neutrophils as inflammatory cells in the portal space, and values of hepatic hydropic degeneration, necrosis and fibrosis were lower than in Group II. The number of lymphocytes as inflammatory cells in the portal space did not differ among groups. Illustrations of pathological findings are represented in Figures 3–6.

Discussion

OJ results in the dilatation of bile ducts and accumulation of hydrophobic bile acids in liver cells. Neutrophil migration and toxic biliary products start the generation of oxygen free radicals, thus unbalancing the antioxidant status in the liver. Antioxidant defenses, as demonstrated by GSH, catalase and SOD, are found to be decreased while lipid peroxidation, as demonstrated by MDA levels, is found to be increased in rat models with OJ (7, 18, 19). There have been studies suggesting that LA reduced MDA levels and increased antioxidant levels such as GSH and SOD in hepatic pathologies (20–23).

In our study MDA levels increased in patients with OJ in the liver and decreased after LA administration, but those results were not statistically significant. However, antioxidant levels, GSH and SOD decreased after ligation of bile duct and increased with LA, while these results were statistically significant. A significant increase in antioxidant enzymes with LA treatment and

a significant decrease in liver damage indicators as a function of the protective effect in the liver are promising for us. As there was no statistical difference in GSH and SOD levels in groups I and III, we thought that the LA treatment was so effective that those antioxidant levels had been reached before the procedure levels. This result was more prominent in GSH. We wonder if we could speculate whether GSH regenerates more quickly than SOD, or LA improves the GSH levels more effectively. The questions as to why MDA liver level did not increase statistically significantly and why its level did not decrease with LA, remain unanswered. The reason why markers of oxidation did not change while antioxidant levels decreased may be explained by notion that cellular and blood MDA may differ and if we had the chance of examining MDA in blood we could have got different results.

It has been stated that a 10-day period is sufficient for the development of OJ-related factors increasing the mortality and morbidity rates, such as intestine barrier dysfunctions and systemic endotoxemia (17). Therefore, we examined the levels of ALT, AST, AP, GGT, TB, DB levels on day 10, but also on days 3 and 7 after the operation. No difference was found between these values of the latter three measurements. On day 10, the ligation of common bile duct resulted in elevated ALT, AST, AP, GGT, TB, DB levels. Those levels in animals with OJ and in those with OJ and LA treatment were statistically higher than in the sham group. Those levels decreased non-statistically after LA. In the literature, it was shown that these markers of liver damage were elevated after OJ and decreased after LA treatment (13-16). We think that if we repeat the study with larger sized groups, we may get satisfactory results.

We demonstrated an increase in the number and score of damage in bile canaliculi, number of neutrophils as inflammatory cells in the portal space, hepatic hydropic degeneration, fibrosis and necrosis after OJ. In accord with literature, the liver damage scores decreased after LA medication (14–16). There was no change in the number of lymphocytes as inflammatory cells in the portal space. Our data suggest that during the early stages of OJ, it is neutrophils rather than lymphocytes that are potentially active, and they react to the treatment earlier and become prominent among the inflammatory cells that invade the obstructed livers. As shown, they infiltrate the liver within three hours of BDL and remain there for days to weeks.

We have some limitations to our study. Firstly, we caused an experimental blockade in biliary tract leading to OJ in our animals, which in reality was an irreversible obstruction. This actually does not exactly match the progression of the disease in humans. However, as shown in numerous publications in the literature, this method has been used in animals to clarify human disease and evaluate the physiology and the treatment of experimental obstructive jaundice. This was the reason why we performed this model in our study. Nevertheless, this might be regarded as a limitation. Secondly, it has been stated that a 10-day period is sufficient for the development of OJ-related factors increasing the mortality and morbidity rates and is also sufficient for oxidative stress markers to emerge in animals. We have doubts as to whether this time is sufficient to see the changes in the tissue. For example, we wonder

if MDA changes in the tissue could have been more prominent if we had examined them later. Thirdly, our sample size may be a limitation. Fourthly, we determined the MDA, GSH and SOD levels in the liver. Serum values of these oxidative stress determinants may also be valuable.

Conclusion

Our study demonstrated that OJ has deleterious effects on the liver. It was also shown that histopathological damage was statistically significantly decreased and antioxidant levels were statistically significantly increased after LA treatment. The biochemical damage was lessened but it was not statistically significant. The importance of oxidative stress during OJ should be considered. LA has anti-inflammatory and antioxidant effects on the liver.

Our study results suggest that LA treatment decreases liver damage by increasing antioxidant enzyme levels in OJ. We believe that these results can be used to understand OJ-related consequences, as well as diagnose and treat OJ disease in humans.

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