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Original Research Article

Effect of vitamin C inhibiting liver fibrosis and lipid peroxidation in biliary obstruction of Wistar rats

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ABSTRACT

Background: The aim of this study was to evaluate the potential inhibiting effects of vitamin C as an antioxidant against liver fibrosis and lipid peroxidation in the bile duct ligation- induced biliary obstruction of Wistar rats.
Methods: A total of 25 male Wistar albino rats were divided into five groups: sham operated, control (bile duct ligation/BDL) without given vitamin C, BDL with vitamin C 75 mg, BDL with vitamin C 150 mg, and BDL with vitamin C 225 mg. Each group contained 5 animals. Vitamin C was given orally on day 3 after operation and after 14 days following vitamin C administration, all animals were performed laparotomy to obtain liver tissue samples for histopathological investigation of liver fibrosis and blood samples for malondialdehyde (MDA) as lipid peroxidation measurement.
Results: The changes demonstrating hepatic fibrosis including moderate to markedly thickened wall of central veins, localized to diffuse perisinusoidal fibrosis, enlarged portal track, increased number of septa, and thickened width of septa were observed in BDL groups. MDA measurement were also observed in all groups. Treatment of biliary obstruction in BDL groups with vitamin C given orally attenuated liver damage. Both the MDA measurement and histopathologic investigation of hepatic fibrosis were observed to be reduced with the vitamin C treatment.
Conclusions: Our data indicate that vitamin C inhibited liver fibrosis and lipid peroxidation in bile duct ligation-induced biliary obstruction of Wistar rats.

Keywords: Vitamin C, Biliary obstruction, Liver fibrosis, Lipid peroxidation, Bile duct ligation

INTRODUCTION

The evaluation and management of the patient with biliary obstruction is a common problem facing the general surgeon. Over the past 40 years, significant advances have been made in our understanding of the pathophysiology, diagnosis, and management of the jaundiced patient. Similarly, advances have been made in perioperative and operative management that have resulted in improved survival of the jaundiced patient.[1] Biliary obstruction produces local effects on the bile ducts, which lead to derangements of hepatic function and, ultimately, to widespread systemic effects. Jaundiced patients are at increased risk for hepatic dysfunction, renal failure, cardiovascular impairments, nutritional deficiencies, bleeding problems, infections, and wound complications, and their perioperative mortality and morbidity are increased. Obstructive jaundice affects multiple organ systems, including hepatic, renal, cardiovascular, hematologic, and immune systems.[1] Complete biliary obstruction causes cholestatic injury to the liver, including hepatocellular necrosis and apoptosis, bile duct epithelial cell proliferation, stellate cell activation, and, eventually, liver fibrosis.[2] Even though the exact mechanisms of biliary fibrosis are unknown, one of the implicated factors is the toxic effect of hydrophobic bile salts on the

hepatocytes and biliary epithelial cells by obstruction of the bile flow in the liver.[3] Immunohistochemically, it was found that the majority of cells observed in the fibrosis regions were positive cells (spindle cells) for alpha smooth muscle actin (α-SMA). It is suggested that the spindle cells, probably transforming from myofibroblasts, play an important role in the pathogenesis of hepatic fibrosis.[4]

Liver fibrosis is described as a process of scarring due to acute and chronic injury to the liver. Liver parenchymal cells carry out the regeneration process to maintain the hepatocellular mass and its function in an acute injury to the liver. This acute process is related to the inflammatory and fibrogenic response which is characterized by the process of deposition of the extracellular matrix. This seems contrary to research reveals that liver injury occurs in a long time will cause continued production of growth factors, proteolytic enzymes, angiogenic factors and fibrogenic cytokines.[1] Continuous liver injury in cases of biliary obstruction in addition to causing liver fibrosis, will also increase lipid peroxidation levels in plasma. An article entitled "Levels of plasma lipid peroxides before and after choledocholithotomy in patients with obstructive jaundice" by Tsai in 1992, states that there is involvement of lipid peroxidation in damage to liver cells due to biliary obstruction in patients with choledocolitiasis and high levels of plasma lipid peroxidation were related to the severity of jaundice.[5] Shimizu et al in 2012 reported the correlation between liver fibrosis and lipid peroxidation which stated that lipid peroxidation together with the formation of reactive oxygen species (ROS), tumor growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α) play a role in the process of liver fibrosis.[6]

Vitamin C (ascorbic acid) was discovered by Szent-Gyorgyi in 1928. Vitamin C is contained in fruits, including citrus, oranges or limes, and green leafy vegetables.[7] A journal entitled "Chemoprotective role of vitamin C in liver diseases by Marin et al in 2013 stated that the role of vitamin C in a healthy diet is well known. In developing countries, food and drinks that contain large amounts of vitamin C have become known as a healthy diet. Although vitamin C has been recognized as an antioxidant that has a protective effect, the role of ascorbic acid as an active form of vitamin C in various physiological processes of the human body, especially in the liver, is still need to be known and studied.[8]

Aim of this study is to evaluate the potential inhibiting effects of vitamin C as an antioxidant against liver fibrosis and lipid peroxidation in the biliary obstruction of Wistar rats.

METHODS

Study design

The design of this study was experimental study with parallel design.

Study place and period

The study was conducted at the animal laboratory, University of Padjadjaran, Bandung, West Java, Indonesia. The period of the study was in March 2020.

Animal

In this study, the inclusion criteria of the samples were healthy male Wistar albino rats (agile, calm breathing, thick and shiny furry), weighing 300 grams and averaging 16 weeks of age. The exclusion criteria were rats that are ill or unable to adapt before the research begins. Food and tap water were available ad libitum. In the animal laboratory of medical faculty, Padjadjaran University, room temperature range was 21^0-22^0 C, lighting control was 12 hour/light and 12 hour/dark cycle, and humidity ranged from 55 to 60%. All animals received human care according to the criteria outlined as 3R and 5F principles.

Sampling technique

The sampling technique used in this study based on calculations according to Federer, and with anticipation of dropped out animals during the study, the total number of experimental animals was 25 rats.

Experimental groups

A total of 25 male Wistar albino rats were divided into 5 groups with 5 animals in each: sham operated group (laparotomy only, without BDL), control group (BDL) without vitamin C administered, BDL group with vitamin C 75 mg given orally, BDL group with vitamin C 150 mg given orally, and BDL group with vitamin C 225 mg given orally.

Experimental procedures

The rats were anesthetized with ketamine 0.5 cc/kg body weight and the bile duct exposed through a midline abdominal incision. The bile duct was located and obstructive jaundice induced by a double ligation at proximal and distal of bile duct with a 4-0 silk. The rats in the vitamin C treated group were given vitamin C in a dose of 75 mg, 150 mg, and 225 mg, once a day orally by a metal orogastric tube for 14 days, starting 3 days after the BDL procedure. The sham operated group and BDL untreated rats were also intubated with the same volume of clear water as the BDL groups treated with vitamin C. After 14 days of treatment, all animals were performed re-laparotomy to obtain liver tissue samples for histopathological investigation of liver fibrosis and blood collection to measurement of malondialdehyde (MDA) as lipid peroxidation indicator.

Histopathologic evaluation

At the end of the surgical procedure, the liver specimens were immersed in a buffer formalin solution and sent to

laboratory of anatomical pathology of Hasan Sadikin hospital to investigate the fibrosis process. Fibrosis was assessed in sections stained with Masson's trichrome. The histopathological fibrosis was evaluated based on semiquantitave severity score as: no fibrosis (scored as 0), mild fibrosis (scored as 1), moderate fibrosis (scored as 2), severe (scored as 3), and cirrhosis (scored as 4). Histopathological examination was carried out by a pathologist who had no prior knowledge of the animal groups.

Malondialdehyde measurement

Lipid peroxidation was evaluated by measurement of plasma malondialdehyde (MDA) which was obtained from blood. The MDA measurement was based on 2-thiobarbituric acid reactive substances (TBARS) with spectrophotometer to read the wavelength. TBARS are low-molecular-weight end products (mainly MDA) that are formed during the decomposition of lipid peroxidation products. Increased levels of TBARS usually demonstrated in liver disorders disease. The procedure involved the following steps: blood from rats was collected into a ethylenediaminetetraacetic acid (EDTA)-contained blood container then sent to biochemistry laboratory, blood collected in centrifugation tube was centrifuged at a speed of 3000 rpm for 10 minutes, the blood plasma located on the surface is separated and taken to be analyzed, and MDA concentration was measured with Quantichrom™ (TBARS) assay kit (DTBA-100).

Statistical analysis

All statistical analysis was carried out using SPSS statistical software (SPSS for Windows, version 23).

Data from histopathologic investigation were presented in crosstabulation for each parameter. Differences in measured parameters among the five groups were analyzed with a non-parametric test (Kruskal-Wallis) and dual comparisons between groups exhibiting significant values were evaluated with a Mann-Whitney U-test. These differences were considered significant when the probability value was less than 0.05. Data from MDA measurement presented in mean standard deviation (SD) and significant value between groups was tested with Tukey test.

RESULTS

Histopathologic findings

The architecture of hepatic lobules of the sham operated rats' shows normal (0) in central vein, perisinusoidal fibrosis, portal tract, number of septa, and width of septa. In control group of rats with BDL, there was 60% moderate liver fibrosis and 40% severe liver fibrosis. In 75 mg of vitamin C treated group, shows 20% mild liver fibrosis, 40% moderate, and 40% severe liver fibrosis. In 150 mg of vitamin C treated group, there was 40% mild, 40% moderate, and 20% severe liver fibrosis. In 225 mg of vitamin C treated group, we found 60% mild and 40% moderate liver fibrosis. (Table 1 and 2) (Figure 1 and 2).

Using light microscopy, Masson's trichome staining shows: mild fibrosis appears near the triangular structure not involving the veins, moderate fibrosis shows that fibrosis forms hexagonal form but still not predominant to veins and severe fibrosis include collapsed of veins (narrowing) and fibrosis seems to be predominant (Figure 1).

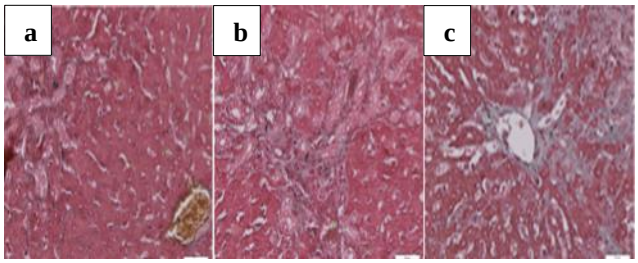

Figure 1: Light microscopy showed fibrosis of the liver (a) mild fibrosis (b) moderate fibrosis (c) severe fibrosis.

Table 1: Summary results from histopathological investigations of liver fibrosis.

Group	Histopathological result				Total	P
	F0	F1	F2	F3		
A	5 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	5 (100.0%)	0.004[a]
B	0 (0.0%)	0 (0.0%)	3 (60.0%)	2 (40.0%)	5 (100.0%)	
C	0 (0.0%)	1 (20.0%)	2 (40.0%)	2 (40.0%)	5 (100.0%)	
D	0 (0.0%)	2 (40.0%)	2 (40.0%)	1 (20.0%)	5 (100.0%)	
E	0 (0.0%)	3 (60.0%)	2 (40.0%)	0 (0.0%)	5 (100.0%)	
Total	5 (20.0%)	6 (24.0%)	9 (36.0%)	5 (20.0%)	25 (100.0%)	

[a]Significant difference between groups ($p<0.05$), Kruskal-Wallis test. Group A: Wistar rats with sham operation (laparotomy only, without bile duct ligation), group B: Wistar rats with bile duct ligation, without vitamin C administration, group C: Wistar rats with bile duct ligation and given vitamin C 75 mg orally, group D : Wistar rats with bile duct ligation and given vitamin C 150 mg orally, group E : Wistar rats with bile duct ligation and given vitamin C 225 mg orally, F0: normal liver histology (no signs of fibrosis), F1: mild liver fibrosis, F2: moderate liver fibrosis, F3: severe liver fibrosis and F4: cirrhosis liver.

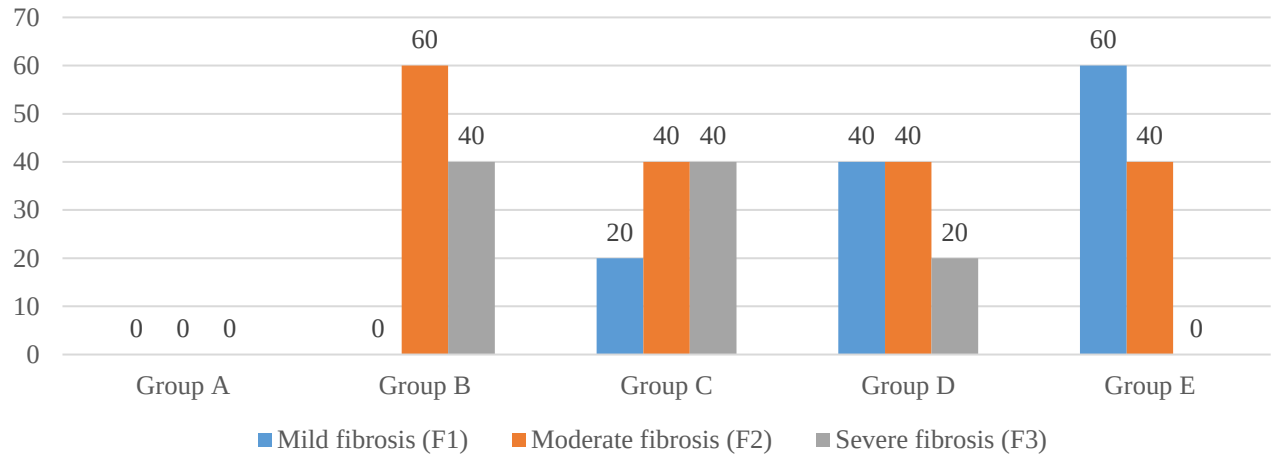

Figure 2: Liver fibrosis in 5 groups of Wistar rats (in %).

Table 2. Mann Whitney test for histopathological results.

Group		P value*	Significant difference
A	B	0.005	Present
	C	0.008	Present
	D	0.005	Present
	E	0.005	Present
B	C	0.729	Not present
	D	0.212	Not present
	E	0.031	Present
C	D	0.439	Not present
	E	0.118	Not present
D	E	0.419	Not present

*Mann Whitney test.

Table 3. Malondialdehyde (MDA) values of all groups (in µM), one-way Anova test.

	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	$\bar{X}$±SD	P value
Group A	10.249	11.155	10.651	10.545	10.679	10.65±0.33	0.01[a]
Group B	11.469	13.114	12.008	12.732	12.478	12.36±0.64	
Group C	10.007	11.221	10.529	10.223	11.33	10.66±0.59	
Group D	11.557	10.518	9.712	9.473	9.889	10.23±0.84	
Group E	9.78	8.825	9.112	9.527	8.59	9.17±0.49	

[a]Significant difference between groups ($p<0.05$), one way-Anova test.

Table 4. Tukey test for MDA results.

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% confidence interval	
					Lower bound	Upper bound
A	B	-1.70440*	0.38011	0.002	-2.8418	-0.5670
	C	-0.00620	0.38011	1.000	-1.1436	1.1312
	D	0.42600	0.38011	0.794	-0.7114	1.5634
	E	1.48900*	0.38011	0.007	0.3516	2.6264
B	A	1.70440*	0.38011	0.002	0.5670	2.8418
	C	1.69820*	0.38011	0.002	0.5608	2.8356
	D	2.13040*	0.38011	0.000	0.9930	3.2678
	E	3.19340*	0.38011	0.000	2.0560	4.3308

Continued.

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% confidence interval	
					Lower bound	Upper bound
C	A	0.00620	0.38011	1.000	-1.1312	1.1436
	B	-1.69820*	0.38011	0.002	-2.8356	-0.5608
	D	0.43220	0.38011	0.785	-0.7052	1.5696
	E	1.49520*	0.38011	0.007	0.3578	2.6326
D	A	-0.42600	0.38011	0.794	-1.5634	0.7114
	B	-2.13040*	0.38011	0.000	-3.2678	-0.9930
	C	-0.43220	0.38011	0.785	-1.5696	0.7052
	E	1.06300	0.38011	0.074	-0.0744	2.2004
E	A	-1.48900*	0.38011	0.007	-2.6264	-0.3516
	B	-3.19340*	0.38011	0.000	-4.3308	-2.0560
	C	-1.49520*	0.38011	0.007	-2.6326	-0.3578
	D	-1.06300	0.38011	0.074	-2.2004	0.0744

*The mean difference is significant at the 0.05 level

Evaluation of malondialdehyde

The results of malondialdehyde (MDA) in sham operated rats was 10.65±0.33 and in control group with BDL was 12.36±0.64. In 75 mg of vitamin C group, MDA value was 10.66±0.59 followed by 10.23±0.84 in 150 mg of vitamin C group. In 225 mg of vitamin C group, MDA was 9.17±0.49 (Table 3 and 4) (Figure 3).

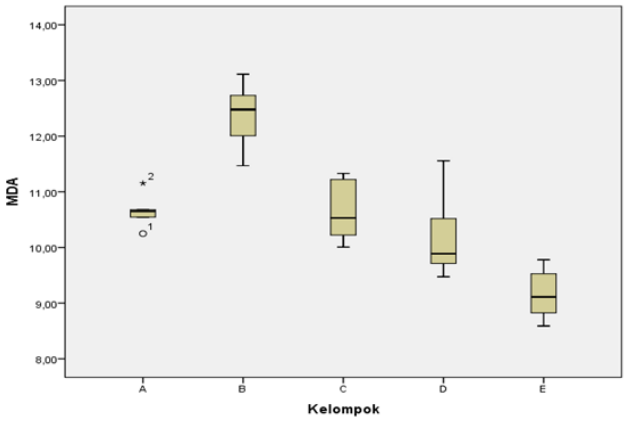

Figure 3: MDA values boxplot.

DISCUSSION

Obstruction occurred in bile duct causes injury to hepatic cells followed by progressive fibrogenesis process. This fibrogenesis process include proliferation of collagen synthesis in hepatocyte which was exposed by free radicals.[3] Lipid peroxidation process is one of the markers of oxidative stress which includes the conversion of ROS to TGF-β and TNF-α which are thought to cause liver fibrosis.[5] This process occurs in cell membranes composed of PUFA and includes the separation of hydrogen from carbon, then will be replaced by the entry of oxygen to produce radicals in the form of lipid peroxyl and hydroperoxide.[9] Vitamin C is an organic compound found in many plants and animals and is known to have antioxidant properties. The antioxidant effect of vitamin C is the ability of vitamin C to donate hydrogen atoms and form ascorbil free radicals that are relatively stable and can reduce damage caused by oxidation processes.[10] Inhibition the process of formation of liver fibrosis and reduction of lipid peroxidation by the administration of vitamin C occurs due to neutralization of ROS by vitamin C and conversion of lipid peroxidation products to non-reactive lipid vitamin C-peroxidation products. [11,12] Following a decrease in the lipid peroxidation process, the formation of fibrosis the liver will also be inhibited through the mechanism of decreasing changes in ROS to TGF-β and TNF-α.[5]

Data from histopathological examination of liver tissue biopsy of Wistar rats showed that in the state of biliary obstruction without oral vitamin C there was a moderate to severe liver fibrosis process and this fibrosis process decreased significantly along with oral vitamin C administration at larger doses.

MDA plasma data results showed that the average value of plasma MDA levels in the Wistar rat groups with biliary obstruction treated with oral vitamin C was lower and near to the average value in the control group that performed sham operation. Vitamin C administration with the largest dose of 225 mg in the condition of biliary obstruction for 14 days in our study revealed to have an effect in inhibiting the occurrence of liver fibrosis and this also showed a low plasma MDA level when compared to the control group, the 150 mg group and 225 mg group. This was further supported statistically with Mann Whitney test for the assessment of liver fibrosis and the Tukey test for the assessment of MDA levels. Both of these results also show conformity with research conducted by Adikwu and Deo in 2013 which concluded that vitamin C has a hepatoprotective effect.[13] Our results shows consistent with the results of several previous studies of the effects of hepatoprotector and the effect of reducing the lipid peroxidation of vitamin C in rats which are treated with exposure to various chemical compounds, such as the study by Bashandy and Alwasel in 2011, Mossa in 2011, and also Krisnamoorthy and Sangeetha in 2013.[14-16] The ability of vitamin C in preventing liver fibrosis in this study is parallel with research conducted by Su et al in

2015 which stated that when vitamin C was given to Gulo-type mice treated with cholestatic liver injury, the liver fibrosis formed would be significantly reduced and this hepatoprotective effect was thought to be related to decreased of cell apoptosis and liver cell necrosis.[17]

The limitation of our study is that in executions, oral administration of vitamin C by using a metal gastric tube sometimes has difficulty in giving the right dose due to the effects of vomiting or regurgitation after animals have been given vitamin C in certain doses. In addition, to assess the occurrence of fibrosis of the liver, it would be better if performed in a span of time longer more than 14 days after BDL surgery. It aims to make bile acid exposure long enough in the hepatobiliary system of the rats, also by considering the possibility of dropping out during the treatment period. These limitations can be observed in the results of histopathological examination of liver tissue biopsy that does not show the existence of cirrhosis of liver tissue both in the control treatment group without administration of vitamin C or groups with oral administration of vitamin C at various dosage levels. Another limitation in this study is that the value of MDA does not yet have a standard. The value displayed depends on the range of measurement values owned by the examining reagent (kit) and MDA examination will provide more informative value if examined serially during the treatment period. Another important thing to note, recently there is still very small amount of literature which examines the effect of oral administration of vitamin C as an antioxidant specifically in providing hepatoprotective effects in rats and also in human studies with jaundice caused by biliary obstruction due to various causes.

CONCLUSION

Our data indicate that vitamin C inhibited liver fibrosis and lipid peroxidation in bile duct ligation-induced biliary obstruction of Wistar rats.

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