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World J Gastroenterol 2006 November 14; 12(42): 6835-6841
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Antioxidants vitamin E and C attenuate hepatic fibrosis in biliary-obstructed rats

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Supported by Trakya University Research Fund. TUBAP No. 548
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Received: 2006-05-24 Accepted: 2006-09-10

Abstract

AIM: To investigate whether antioxidants vitamin E and C can retard development of hepatic fibrosis in the biliary-obstructed rats.

METHODS: Fifty Wistar albino rats were randomly assigned to 5 groups (10 rats in each). Bile duct was ligated in 40 rats and they were treated as follows: group vitC, vitamin C 10 mg/kg sc daily; group vitE, vitamin E 15 mg/kg sc daily; group vitEC, both of the vitamins; bile duct-ligated (BDL, control) group, physiological saline sc. The fifth group was assigned to sham operation. At the end of fourth week, the rats were decapitated, and hepatic tissue biochemical collagen content and collagen surface area were measured. Hepatic tissue specimens were histopathologically evaluated according to Scheuer system. Serum hyaluronate levels were measured by ELISA method.

RESULTS: Despite being higher than sham group, hepatic collagen level was significantly decreased in each of the vitC, vitE and vitEC groups (32.7 ± 1.2, 33.8 ± 2.9, 36.7 ± 0.5 μg collagen/mg protein, respectively) compared to BDL (48.3 ± 0.6 mg collagen/g protein) (P < 0.001 for each vitamin group). Each isolated vitamin C, isolated vitamin E and combined vitamin E/C supplementation prevented the increase in hepatic collagen surface density (7.0% ± 1.1%, 6.2% ± 1.7%, 12.3% ± 2.0%, respectively) compared to BDL (17.4% ± 5.6%) (P < 0.05 for each). The same beneficial effect of vitamin C, vitamin E and combined vitamin E/C treatment was also observed on the decrease of serum hyaluronate levels compared to BDL group (P < 0.001). The relative liver and spleen weights, serum transaminases, cholestatic enzymes, bilirubins and histopathological inflammation scores were not different between the antioxidant treatment groups and the control. However, fibrosis staging scores were obviously reduced only in the vitamin E/C combination group (vit EC: 2.4 ± 0.8 *vs* BDL: 3.1 ± 0.7; P < 0.05).

CONCLUSION: Each antioxidant vitamin E, vitamin C and their combination retard hepatic fibrosis in biliary-obstructed rats. Oxidative stress may play a role in the pathogenesis of hepatic fibrosis in secondary biliary cirrhosis.

Key words: Hepatic fibrosis; Biliary obstruction; Collagen; Vitamin E; Vitamin C

Soylu AR, Aydogdu N, Basaran UN, Altaner S, Tarcin O, Gedik N, Umit H, Tezel A, Dokmeci G, Baloglu H, Ture M, Kutlu K, Kaymak K. Antioxidants vitamin E and C attenuate hepatic fibrosis in biliary-obstructed rats. *World J Gastroenterol* 2006; 12(42): 6835-6841

http://www.wjgnet.com/1007-9327/12/6835.asp

INTRODUCTION

Hepatic fibrosis is a highly integrated cellular response to tissue injury[1]. It is essentially characterized by activation of hepatic stellate cells, secretion and accumulation of extracellular matrix proteins[2]. Various causes of cholestasis such as primary biliary cirrhosis, primary sclerosing cholangitis, biliary strictures, atresia, cysts or neoplasia may induce hepatic fibrosis that may potentially culminate in biliary cirrhosis. However, mechanisms underlying the development of hepatic fibrosis in cholestatic liver disease are not thoroughly elucidated.

It is suggested that free radicals might contribute to the development of hepatic fibrosis in biliary obstruction[3-5]. Cholestasis *per se* reduces antioxidative capacities in liver

mitochondria in bile duct-ligated rats[6,7]. Accumulation of hydrophobic bile acids and inflammatory cells in the liver tissue may cause increased production of free radicals in biliary obstruction[8-10]. Bile acids especially, enhance reactive oxygen species released by polymorphonuclear leukocytes[11]. In addition, intraluminal bile salt deficiency in extrahepatic biliary obstruction results in vitamin E malabsorption[12]. Serum and hepatic tissue levels of vitamin E are reduced threefold in biliary-obstructed rats[13]. Accordingly, hepatic tissue levels of lipid peroxides were increased in bile duct-ligated rats[13]. Although the presence of oxidative stress in the course of biliary cirrhosis has been reported, it is still unclear whether it is the cause or consequence of tissue injury or contributes any to hepatic fibrosis. This may partly stem from inadequate number of studies performed to test the efficiency of antioxidants in the prevention of hepatic fibrosis in chronic cholestatic liver diseases.

Type I collagen is the major extracellular matrix protein deposited in the liver during hepatic fibrosis[1,2]. It was reported that lipid peroxides stimulated hepatic deposition of type I collagen in cultured human hepatic stellate cells[14,15]. This suggestion established a possible link between the lipid peroxidation (i.e. oxidative stress) and hepatic fibrosis. Studies demonstrating the beneficial effect of vitamin E supplementation in prevention of both enhancement of lipid peroxidation and synthesis of type I collagen might support this suggestion[16-18]. However, it has been reported that short-term treatment (seven days) of bile duct-ligated rats with vitamin E completely prevented the increase in lipid peroxidation in liver and plasma, but failed to prevent tissue injury histologically[19]. Thus, the current controversies between the few studies performed to evaluate the role of oxidative stress in tissue injury induced by bile duct ligation make newer studies to be performed necessary.

In the liver, vitamin E is the primary fat-soluble chain-breaking antioxidant protecting lipid bilayers whereas hydrophilic vitamin C is the scavenger particularly in plasma, cytosol and other aqueous compartments[20]. In the present study, we aimed to investigate the effect of antioxidants vitamin E and C on hepatic fibrosis in bile duct-ligated rats. Vitamin C was combined to obtain the benefit from synergism of these drugs as the vitamin C reacts with oxidised vitamin E, thereby regenerating vitamin E[21]. Serum hyaluronate levels were used as a marker of liver fibrosis and to predict the response to therapy.

MATERIALS AND METHODS

Weanling 50 female Wistar albino rats weighing 198 ± 15 g were housed in cages with stainless steel wire tops and with 12-h light-dark cycles under standard animal laboratory conditions at room temperature. The rats had free access to standard rat chow and water. Under aseptic conditions, extrahepatic cholestasis was induced by double ligation with 5/0 polipropilene sutures and transection of the common bile duct in between. In sham-operated rats, the operation was performed in the same way but without bile duct ligation or transection.

The rats were randomly assigned to 5 groups, 10 in each. The 40 bile duct-ligated rats were treated as follows: vitC group, given subcutaneous vitamin C at a daily dose of 10 mg/kg (ascorbic acid; Redoxon®; Roche, Istanbul, Turkey); vitE group, given subcutaneous vitamin E at a daily dose of 15 mg/kg (D-α-tocopherol acetate; Evigen®; Aksu Farma, Istanbul, Turkey) as previously described[22]; vitEC group, given both vitamins by the same route and dose; BDL (bile duct-ligated) (control) group, given subcutaneous physiologic saline. The sham group rats were sham-operated. Administration of vitamin C and E was started immediately after bile duct ligation. At the end of the study period (fourth week), the rats were weighed and decapitated and trunk blood was obtained. Serum samples were collected for liver biochemical tests and serum hyaluronate determinations and then stored at -86℃. The total body weights, liver and spleen weights of each rat were also recorded. Hepatomegaly was defined as liver wet weight of more than 3 g per 100 g rat body weight[23].

This study was approved by the Local Ethical Committee, School of Medicine, Trakya University.

Liver tissue sampling

The left, middle and right lobes of each liver were explored. By cutting six different 5 mm × 5 mm × 5 mm slices, each liver was randomly sampled in 0.75 cm^3 tissue. The slices sampled were fixed in 10% buffered formalin, routinely processed and blocked into paraffin for detecting collagen content by biochemical methods and image analysis.

Biochemical collagen content determination

The collagen content of the liver was assayed by the colorimetric method described by Lopez de Leon and Rojkind[24]. The principle is the coloring of collagenous protein by Sirius red (36554-8, 2610-10-8; Aldrich Chemical, Deisenhofen, Germany) and non-collagenous proteins by fast green (14280; MERCK KGaA, Darmstadt, Germany). Fifteen micrometer-thick liver slices taken from each paraffin block were layered on glass slides. Slices were deparaffinized and assayed as originally described. Collagen content was calculated using the formula described by the authors[24] as microgram collagen per milligram protein.

Slices at 5-, 10- and 15- μm thickness were tested beforehand and the optimum depth was determined to be 15 μm, which gave higher absorbency.

Histological image analysis of liver fibrosis

Five-micrometer liver sections of each paraffin block were stained with trichrome for collagen. SAMBA 4000-image analysis system (Unilog, France) was used to determine liver collagen content. Only lobular areas of the liver sections were randomly sampled with × 20 objective magnification. Portal areas were excluded from the analysis by a meticulous area selection. Each representative image frame was 1.278 mm × 0.958 mm (= 1.224 324 mm^2). One hundred frames were selected for analysis to reach about 1 cm^2 of liver tissue sample. A homemade algorithm generated with SAMBA-IPS software was run. The program automatically outlined and measured the total liver area and the blue-stained collagen area within each frame. Me-

Table 1 Liver biochemical tests and relative liver and spleen weights of rats at the end of 4th wk of bile duct obstruction (mean ± SE)

parameter	Vit C	Vit E	Vit EC	Sham	Control
AST (IU/L)	548 ± 82	446 ± 75[b]	505 ± 58[a]	223 ± 26	473 ± 64[a]
ALT (IU/L)	156 ± 34	148 ± 16	156 ± 23	98 ± 9	165 ± 29
ALP (IU/L)	446 ± 95	403 ± 41	396 ± 27	296 ± 35	477 ± 59
GGT (IU/L)	64 ± 13	86 ± 5	74 ± 8	64 ± 4	101 ± 17
Total bilirubin (mg/dL)	5.9 ± 1.2[d]	7.2 ± 0.9[d]	7.1 ± 1.3[d]	0.5 ± 0.2	8.4 ± 1.4[d]
Conjugated bilirubin (mg/dL)	3.9 ± 0.6[d]	3.7 ± 1.0[d]	3.5 ± 0.6[d]	0.2 ± 0.1	4.2 ± 0.7[d]
Albumin (g/dL)	3.2 ± 0.1[b]	3.0 ± 0.1[a]	3.3 ± 0.2	3.8 ± 0.0	2.9 ± 0.1[d]
Relative liver weight (%)	5.9 ± 0.4[a]	5.6 ± 0.6[a]	5.9 ± 0.5[b]	3.4 ± 0.6	5.9 ± 0.1[d]
Relative spleen weight (%)	0.40 ± 0.07	0.39 ± 0.08	0.42 ± 0.08	0.34 ± 0.04	0.44 ± 0.05

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; T. bilirubin: Total bilirubin; C. Bilirubin: Conjugated bilirubin. [a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$ *vs* sham.

Table 2 Serum hyaluronate levels, liver collagen content by biochemical method and collagen surface density by image analysis of groups at the end of 4th wk of study (mean ± SE)

parameter	Vit C	Vit E	Vit EC	Sham	Control
Liver collagen content microgram collagen/mg protein	32.7 ± 1.2[b,d]	33.8 ± 2.9[b,d]	36.7 ± 0.5[a,d]	23.8 ± 1.3[d]	48.3 ± 0.6[a]
Collagen surface density (%)	7.0 ± 1.1[c]	6.2 ± 1.7[c]	12.3 ± 2.0[c]	2.6 ± 0.7[c]	17.4 ± 5.6
Hyaluronate (ng/mL)	63 ± 25[d]	87 ± 28[d]	47 ± 25[d]	56 ± 16[d]	783 ± 111

[a]$P < 0.001$, [b]$P < 0.01$ *vs* sham; [c]$P < 0.05$, [d]$P < 0.001$ *vs* control.

asurements of each frame were added to each other consecutively. The total liver lobular area, total blue spectrum-stained collagen area, and the ratio of the collagen within the liver tissue were summed. Histological image analysis was performed at the Pathology Department of Haydarpasa Hospital.

Histopathological investigations

Five-micrometer liver sections were stained by HE and trichrome. Necroinflammatory activity grading and fibrosis staging were performed with Scheuer system[25].

Biochemical assays and serum hyaluronate

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), bilirubins (total and conjugated) and albumin were assayed in an Olympus AU 800 biochemical automated analyzer.

Hyaluronate quantitative test kit was used to measure serum hyaluronate levels (Corgenix Westminster, CO, USA) by ELISA method.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SE). The Shapiro-Wilks test was used to assess the normality of continuous data. For all variables, the statistical differences between groups were tested using one-way analysis of variance for normally distributed data. The nonparametric Kruskal-Wallis test was used for nonnormally distributed data. Multiple comparisons were made using the Tukey post hoc multiple comparisons test for normally distributed data, and the non parametric Tukey post hoc multiple comparisons test for nonnormally distributed data. $P < 0.05$ was considered statistically significant. Correlation was calculated using Pearson's test. SPSS/PC+ version 11.0 statistical analysis program (SPSS Inc., Chicago, Illionis) was used for the statistical analysis.

RESULTS

Forty percent of rats in each vitC, vitE, vitEC and control groups were died, whereas there was no mortality in the sham group. The causes of deaths were biliary peritonitis and various infectious complications. There were no statistically significant differences among the animals in vitamin treated groups and controls in terms of mortality rates and causes of deaths. The numbers of rats that were alive at the end of study period and taken into account were 6, 6, 7, 6 and 10 for vitC, vitE, vitEC, BDL and sham groups, respectively. Body weights of the rats included in the study were not significantly changed at the end of the study period in any of the groups. However, relative liver weights, as expressed in grams per 100 g of body weight, were significantly increased in all BDL groups compared to sham ($P < 0.05$ for both vitC and vitE groups; $P < 0.01$ for vitEC; $P < 0.001$ for control group) (Table 1). No significant difference was observed among the alternative antioxidant regimens or controls. The relative spleen weights were the same among the groups including the sham-operated (Table 1).

Biochemical collagen content measurement and histological image analysis for fibrosis

The hepatic collagen contents in control group were significantly increased compared with the sham-operated group by biochemical method ($P < 0.001$) (Table 2). However, the antioxidant vitamin groups had significantly less total liver collagen compared to control (physiologic

Table 3 Histologic fibrosis staging and grading of necroinflammatory activity scores according to Scheuer system at the end of the study (mean ± SE)

parameter	Vit C	Vit E	Vit EC	Sham	Control
Stage	3.2 ± 0.4[b]	2.5 ± 0.5[b]	2.4 ± 0.8[a,b]	0.5 ± 0.5	3.1 ± 0.7[b]
Grade	1.5 ± 0.5[d]	1.5 ± 0.5[d]	1.4 ± 0.5[d]	0.2 ± 0.4	1.9 ± 0.7[f]

[a]$P < 0.05$ *vs* control; [b]$P < 0.001$, [d]$P < 0.01$, [f]$P < 0.001$ *vs* sham.

Table 4 Correlation of serum hyaluronate with histologic fibrosis staging, collagen surface density and biochemical collagen content

		Fibrosis stage	Collagen density	Collagen content
Serum	*r*	+ 0.38	+ 0.56	+ 0.71
Hyaluronate	*P* <	0.025	0.005	0.0001

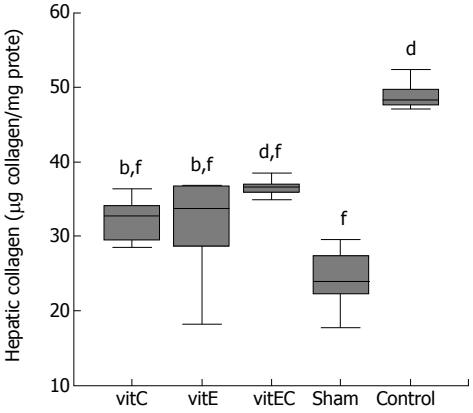

Figure 1 Effect of treatment on liver collagen content by biochemical method. [b]$P < 0.01$, [d]$P < 0.001$ *vs* sham; [f]$P < 0.001$ *vs* control.

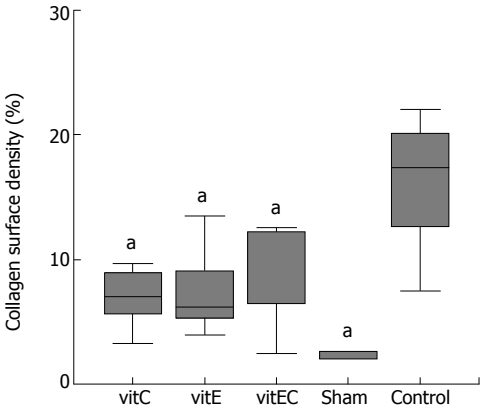

Figure 2 Effects of vitamin E, C and combination treatment on hepatic fibrosis as determined by computerized image analysis. [a]$P < 0.05$ *vs* control.

saline) group ($P < 0.001$ for all vitamin groups *vs* control group) (Figure 1). The total hepatic collagen contents in antioxidant groups were about 23% less than the BDL (control) group by biochemical method at the end of fourth week. Collagen surface areas were also found to be significantly better in rats treated with antioxidants by computerized image analysis compared to controls ($P < 0.05$) (Table 2, Figure 2). There were no statistically significant differences among the antioxidant treatment groups in regard to the total collagen contents biochemically or collagen surface densities by image analysis (Table 2). Although combination therapy was effective in reducing hepatic fibrosis, it was not statistically more effective than either drugs used alone ($P > 0.05$).

Biochemical findings

The biochemical hepatic profile, including AST, ALT, ALP, GGT, total and conjugated bilirubin measurements, showed no statistically significant difference among the treatment groups and controls ($P > 0.05$) (Table 1). In contrast, serum concentrations of the AST, albumin and bilirubins among the aforementioned biochemical parameters were significantly changed in all of the BDL groups compared to sham-operated group (at least, $P < 0.05$), and without difference in any of the antioxidant regimens that could suggest an improvement in hepatic functions ($P > 0.05$).

Histopathological findings and serum hyaluronate levels

Examination of the liver sections demonstrated inflammation (Figure 3A), bile duct proliferation (Figure 3B) around the portal tracts and fibrosis (Figures 3C and D) in all the BDL groups. Using Scheuer system, the histological necroinflammatory activity observed in the BDL groups were not different compared with any of the treatment regimens (Table 3). However, fibrosis staging was statistically significantly better in the vitamin E and C combination group ($P < 0.05$). The serum hyaluronate levels were significantly elevated in the control group compared to vitamin and sham groups ($P < 0.001$) (Table 2, Figure 4). There was significant correlation between the serum hyaluronate levels and fibrosis staging ($r = 0.38$, $P < 0.05$), biochemical collagen content ($r = 0.71$, $P < 0.0001$) and collagen surface density by image analysis ($r = 0.56$, $P < 0.005$) (Table 4).

DISCUSSION

Our study showed that antioxidants vitamin E and C retarded the development of hepatic fibrosis without effecting necroinflammation in biliary-obstructed rats. Antioxidants vitamin E and C individually had anti-fibrotic properties. They were equally effective.

In order to demonstrate the efficiency of vitamin E and C in preventing hepatic fibrosis, we started vitamin treatments on the first day of the experiment. As a limitation to our study, we could not measure and compare the serum or hepatic tissue vitamin E levels. However, it has recently been shown that subcutaneous administration of emulsified vitamin E to rats results in substantially elevated serum and liver concentrations of vitamin E comparable with levels achievable by dietary supplementation for conditions in which hepatic oxidative stress is present[26]. Therefore, we preferred to give our

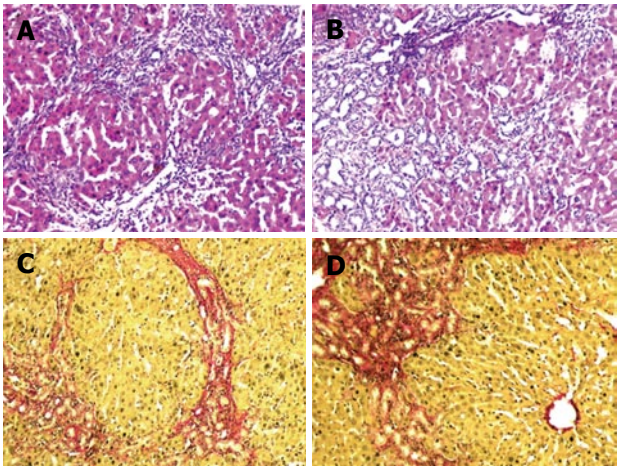

Figure 3 **A**: Necroinflammation disperses the lobular parenchyma from the portal areas (HE x 100); **B**: Prominent bile duct proliferation; **C**, **D**: Severe portal fibrosis with porto-portal bridging by sirius red collagen stain (x 100).

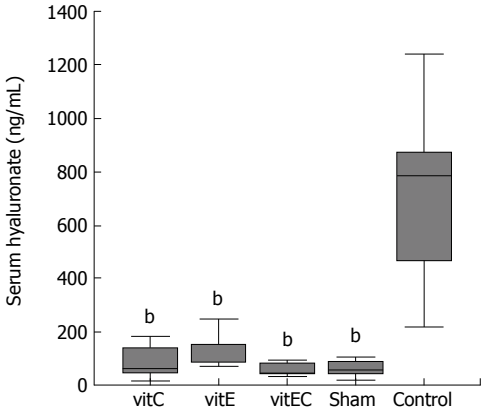

Figure 4 Effects of vitamin E, C and combination treatment on serum hyaluronate levels. $^{b}P < 0.001$ *vs* control.

rats the vitamins by parenteral route. In contrast, it was reported that oral supplementation with vitamin E and C fails to show any benefit in rats with biliary obstruction[27].

Mild to moderate degrees of hepatic inflammation is usually present especially early in biliary obstruction[28]. Neutrophils infiltrate periductal areas. In our study, we observed this low-grade inflammatory activity in all bile duct-ligated rats. However, antioxidant treatments did not improve the histologic necroinflammation and its surrogate markers serum transaminases and cholestatic enzymes. The reduction in hepatic fibrosis without improvement in necroinflammation needs to be clarified. One possible explanation may be the effect of vitamin E on rat hepatic stellate cell in inducing apoptosis and thus reducing extracellular collagen accumulation[29]. Therefore, in our study, the decrease in hepatic fibrosis in rats taking vitamin E may result from reduced hepatic stellate cell numbers. As another explanation, it is suggested that free radicals may induce apoptosis of hepatocytes in low dose but necrosis in high dose[3]. In biliary obstruction such as in our study, the levels of hepatic tissue free radicals may not be high enough to contribute to the hepatic necroinflammation and disturb liver biochemical enzymes. It would be better if we had preferred to use higher doses of vitamin E or C in order to overcome the necroinflammation and to improve the liver biochemical tests. In our recently published study, the combination of vitamins E and C was associated with decreased ethanol-induced hepatic glutathione peroxidase activity and hepatic fibrosis in protein-deficient rats fed with a high-fat liquid diet[30]. The improvement in hepatic fibrosis was accompanied by reduced necroinflammation and hepatomegaly. However, not all studies indicate that the reduction in fibrosis was due to the reduction in necroinflammation. Similar to the findings of our current study, it has been reported that vitamin E and C combination therapy, although retarded hepatic fibrosis, did not improve hepatic inflammation in patients with nonalcoholic steatohepatitis (NASH) where oxidative stress has a prime role in tissue injury[31]. Antifibrogenic effect of antioxidants vitamin E and C probably interfered with fibrogenesis by mechanisms other than prevention of inflammation or necrosis in both patients with NASH and in our biliary-obstructed rats. In concert with these two studies, the reduction of hepatic collagen by antioxidant sylimarin treatment in biliary-obliterated rats[32] was not accompanied by improvement in clinical findings or biochemical liver enzymes. Meanwhile, antifibrotic effect of sylimarin was mediated by downregulation of type I collagen synthesis[33]. In fact, quiescent stellate cells in tissue culture have the ability to respond to free radicals to express collagen independent of necrosis, inflammation or any other confounding variables[18]. Hepatic stellate cells have been shown to respond to antioxidant vitamin E by downregulating collagen secretion[16-18]. This response was supposed to occur by reduced transforming growth factor-β secretion from hepatic stellate cells in the liver[34].

Bile duct ligation model of rats mimics best the clinical and histopathological aspects of hepatic fibrosis secondary to extrahepatic biliary obstruction in humans[35]. This model allows researchers to study in detail all the developmental stages of hepatic fibrosis, ultimately, terminating in cirrhosis within about 4 wk. In our study the beneficial effect of antioxidants did not reach the levels of improving clinical findings. Neither the liver sizes nor the spleen weights were affected by the antioxidant treatment. Mortality was not improved, either. Maybe, more prolonged study such as 5-6 wk' period would be necessary to observe the expected difference in liver and spleen weights and to judge the effect of treatment on the outcome. The high mortality in our rats was related to postoperative complications. However, these increased complications might also result from the liver damage or from dosage of vitamins which was borderline high for such a long-term treatment. Therefore, by using lower vitamin dosages further studies may also be designed for future experiments. In our study, although the number of rats taken into consideration was not too much, we used four different methods to quantify the extent of hepatic fibrosis. All of the results were in very good correlation and in concordance with each other.

Serum hyaluronate has been suggested to be used in prediction of hepatic fibrosis and the evaluation of treatment response of various antifibrotic drugs in human

studies[36] and in rat[37,38] models of hepatic fibrosis. Several influencing factors such as hepatic inflammatory activity and the indicators of this activity (i.e. liver chemical tests) may reduce diagnostic value of hyaluronic acid and other fibrosis indexes[39]. In our study the presence of low inflammatory activity in the livers of the rats and the absence of difference in serum biochemistry test results between the groups probably attributed to the good correlation between the hyaluronic acid serum levels and the hepatic tissue fibrosis measurements with all methods used. The serum levels of hyaluronic acid in our rats treated with antioxidants were as low as in sham group and correlated well with hepatic collagen quantity. Antioxidant treatment was very effective in preventing the elevation of serum hyaluronic acid levels. However, we could not explain such a significant reduction of the hyaluronic acid level in the treated rats, compared to the negative control with only slight changes in hepatic inflammation and liver biochemistry. It may be appropriate to investigate, if the presumed mechanism, how the vitamins may protect the liver, takes place, or if the hyaluronate serum levels are reduced by another pathway. Nevertheless, our study supported that serum hyaluronic acid levels may be used as an index of liver fibrosis and to evaluate the antifibrotic effects of antioxidant vitamins in biliary-obstructed rats.

In conclusion, oxidative stress might contribute to the pathogenesis of secondary biliary cirrhosis. Antioxidant vitamins E and C or combination of both attenuate the development of hepatic fibrosis without improving necroinflammatory activity in bile duct-ligated rats. Long-term, prospective studies in humans with chronic cholestatic liver diseases may be helpful to evaluate the beneficial effects of these vitamins.

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