Erythrocyte Antioxidants and Hexokinase Activity Alterations in CCl4-Induced Cirrhotic Rats Through Naltrexone Treatment

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Abstract- Cirrhosis is the consequence of chronic liver injury Considering the crucial role of oxidative stress in the progression of liver cirrhosis, we aimed to investigate the ameliorative effect of NTX against oxidative stress in carbon tetrachloride (CCl_4)-induced cirrhotic rats. Eighty-four male Wistar rats were randomly assigned into 4 groups (21 rats /group I) receiving CCl_4; (II) NTX+CCl_4; (III) mineral oil (M) (as the control); (IV) NTX+M. The animals in each group were sacrificed in 3 different time-points 2 weeks, 6 weeks (early cirrhosis) and 8 weeks (advanced cirrhosis). Liver function tests, NO metabolites, GSH level, as well as the activity of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxides (GPX), and hexokinase (HK) were assessed. NTX was able to ameliorate liver injury, revealed by attenuation of ALT activity, which was significantly enhanced due to cirrhosis induction, as well as pathological evaluation. HK was also increased significantly after treatment with CCl_4 while NTX moderated this increase. Although CCl_4 treatment did not have a significant effect on GSH levels, NTX was able to considerably increase GSH in blood. The activity of CAT and SOD as well as NO levels were all augmented by NTX in CCl_4-treated rats. Naltrexone demonstrates antioxidative effects in liver cirrhosis and may confer a protective effect against hepatic cirrhosis through modulation of oxidative stress.

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Keywords: Liver cirrhosis; Naltrexone; Antioxidant; Oxidative stress; Carbon tetrachloride

Introduction

Hepatic fibrosis is a chronic condition which has high mortality rate (1,2) and is associated with increased risk of hepatocellular carcinoma (3,4). It is estimated that currently more than 800 million people are diagnosed with liver diseases, with a mortality rate of approximately 2 million deaths per year. Thus, there is an urgent need for improvement of prevention strategies as well as suitable management and treatment of these disorders (5).

Oxidative stress plays a crucial role in the pathogenesis of hepatic disorders (6,7), liver fibrosis and cirrhosis are associated with systemic oxidative stress (8). Some circulatory markers of oxidative stress such as SOD, CAT, GPX and HK could reflect the progression of disease, because these enzymes have an essential role in protecting cells from superoxide radicals and its consequent cascade of reactive oxygen species (ROS) (9,10). Studies have shown that the activity of antioxidant enzymes, GSH content, and plasma NO metabolite are altered in the liver tissue during progression of liver diseases (9-11). CCl_4-induced cirrhosis is caused by an imbalance between the production of free radicals and antioxidant defense, which results in pathological damage (6).

Naltrexone (NTX), an opioid receptor antagonist (12) blocks endogenous opioids, which are increased during liver injury (13). Studies on various models of cirrhosis

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have shown that NTX could prevent the progression of liver fibrosis (14-16). Endogenous opioids have been implicated in oxidative stress (17) and evidence suggests that NTX exerts anti-inflammatory and immune modulatory functions (18,19). NTX is able to normalize hepatic lipid peroxidation (LPO) and suppress apoptosis in a rat model of chronic cholestasis (17). We had previously shown that NTX could exert a protective effect on erythrocyte membrane in an animal model of liver cirrhosis (20). Therefore, in this study we aimed to evaluate the effect of NTX on the antioxidant status in CCl_4-induced cirrhosis in rats.

Materials and Methods

Animal model and sample collection

Male Albino-Wistar rats weighing 200-250 g, were used in this study. The rats were housed in an environment with a temperature of 23±2° C, 50±5% humidity, and a 12 h light/dark cycle. They had free access to tap water and standard chow. All procedures were performed according to the Animal Care Guidelines published by the National Institute of Health in USA and were approved by the ethics committee of Tehran University of Medical Sciences.

Intraperitoneal (i.p.) injections of CCl_4 at the dosage of 750 mg/kg of CCl_4, dissolved in 1 ml mineral oil, every other day, were used for the induction of cirrhosis. Mineral oil (1 ml i.p. every other day) was used as the control. NTX was dissolved in normal saline and was administered subcutaneously at the daily dosage of 10 mg/kg.

Animals were randomly assigned to 4 main groups: each group containing 21 rats. Treatment for each group was as follows: (I) CCl_4 (test); (II) NTX+CCl_4; (III) mineral oil (M) (control); (IV) NTX+M. The CCl4-hepatotoxicity have been reported to follow a three-phased course; early necrosis, characterized by the rising enzymes levels, and an early damage of the liver, early cirrhosis characterized by extensive fatty infiltration and an increasing necrosis, and advance liver damage demonstrated by diminished synthesis ability and liver atrophy (21). Therefore, blood and liver tissue samples were collected at three separate time points after the beginning of injections including 2 weeks early damage, 6 weeks (early cirrhosis) and 8 weeks (advanced cirrhosis), to consider both early and late phases of liver injury.

Histological evaluation

Liver tissue samples were taken immediately after blood sample collection, and through surgical operation of rats under general anesthesia by ether. Tissue samples were fixed in 10% formaldehyde solution, followed by fixation in 70% ethanol. After embedding in paraffin, the samples were sectioned and stained with heamatoxylin-eosin (H and E) and Masson's trichrome, following standard procedures to investigate liver histological and fibrotic alterations. The sections were studied under light microscope.

Plasma biochemical measurements

Blood was collected in heparin-containing tubes and centrifuged at 3000 rpm for 10 min at 4° C. Plasma was separated and kept frozen at −80° C for future analyses. The activity of alanine aminotransferase (ALT) was measured using a commercially available kit (Pars Azemoon, Tehran, Iran). Plasma Nitrate and Nitrite levels were determined as an index of NO production, according to Griess method (22).

Enzyme activity assay

Erythrocytes were washed and the hemolysate was prepared using ice-cold phosphate-buffered saline (155 mM, pH 7.4). The concentration of hemoglobin in the hemolysate was measured by Drabkin's method (23).

All the activity assay kits were purchased from BioVision (Mountain View, CA, USA).

HK was measured according to the produced glucose-6-phosphate, which gave rise to NADH by glucose-6-phosphate dehydrogenase. The resulting NADH caused the production of a colored compound with strong absorbance at 450 nm.

CAT activity was assessed by first reacting with H_2O_2 to yield water and oxygen. The unconverted H_2O_2 reacted with OxiRed™ probe to produce a colored compound with absorption at 570 nm which was conversely proportional to CAT activity.

GPX activity was quantified through a coupled reaction with glutathione reductase (GR), using cumene hydroperoxide as the substrate, with detection sensitivity of ~ 0.5 mU/ml. Consumption of NADPH by GR, which was proportional to GPX activity, was measured as the reduction in the absorbance at 340 nm.

SOD activity was examined based on the inhibition of the reaction of superoxide with WST-1. Absorbance was measured at 450 nm.

Glutathione measurement

The concentration of GSH in hemolysate was assayed by a colorimetric method using the GSH assay kit from BioVision (Mountain View, CA, USA). First, 5-

sulfosalicylic acid (SSA) was used for the removal of proteins from samples and for the protection of GSH oxidation and γ-glutamyl transpeptidase reaction. The levels of GSH was measured by glutathione recycling system using 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and GR. DTNB and GSH reacted to generate 2-nitro-5-thiobenzoic acid with absorbance at 412 nm. Concentration of GSH was expressed as µmol/g Hb.

Statistical analysis

Results are presented as mean±SEM for at least duplicate experiments. Statistical significance was assessed by Analysis of variance (ANOVA), followed by Tukey's post-hoc test. A *P* of 0.05 or less was considered statistically significant.

Results

Here we developed an animal model of liver cirrhosis by injection of CCl_4 and followed the course of the disease progression for 8 weeks. We first measured the levels of ALT to evaluate the liver dysfunction in response to CCl_4. As shown in figure 1, induction of cirrhosis was accompanied by a significant elevation in the level of ALT in CCl_4-induced cirrhotic rats which was significantly higher than control group, 2 weeks after injection of CCl_4 and rose to its maximum levels in the 6th week and remained high in the 8th week. Although NTX administration to CCl4-treated rats caused a more prominent increase in the ALT activity at the beginning of the experiment, it gradually decreased ALT activity which became significant compared to CCl_4 group in the 8th week (Figure 1). NTX alone did not change the ALT activity compared to control rats.

These alterations were correlated with the histological images provided by H and E and Masson's trichrome staining of the liver tissues from the experimental and control groups. The pathological results showed the presence of collagen and the formation of fibrotic scars and inflammation in both CCl4 and CCl4+NTX groups, which are characteristics of liver cirrhosis (24,25). A mild congestion was seen in the group that received NTX+M.

We also interrogated the levels of NO metabolites in plasma in cirrhotic rats and in response to NTX. Plasma NO metabolites were significantly decreased in cirrhotic rats 6 weeks after treatment with CCl4, while NTX dramatically increased NO metabolites in all of the studied time points compared to the CCl4-induced cirrhotic rats in their corresponding times. NTX per se did not influence NO metabolite levels.

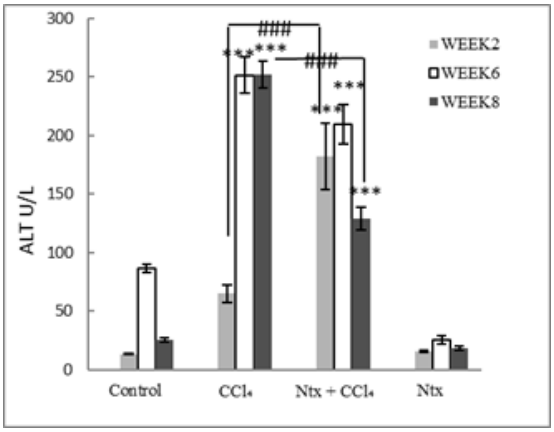

Figure 1. The activity of alanine aminotransferase (ALT) in different groups of rats including control (treated with mineral oil); CCl_4 group (treated with carbon tetrachloride); Ntx+CCl4 (treated by both CCl4 and Naltroxene); Ntx (treated with Naltrexone and mineral oil). Each value represents the mean ± SEM. *Significantly different from control group, * *P*<0.05, ** *P*<0.01, *** *P*<0.001. #Significant difference between Ntx+CCl_4 and CCl_4 groups, compared at the same time point. # *P*<0.05, ## *P*<0.01, ### *P*<0.001

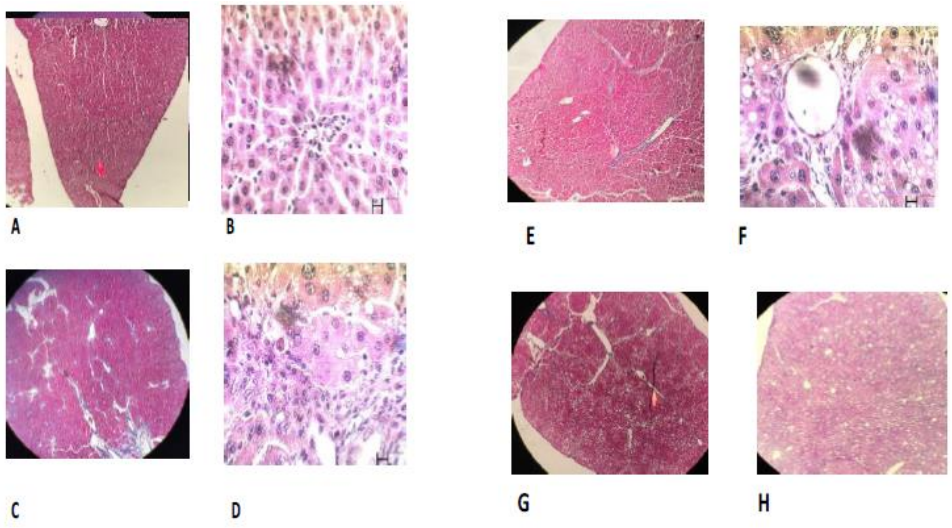

Figure 2. Masson's trichrome and H&E staining of liver sections 8 weeks after treatment of different groups of rats.A is Masson's trichrome and B is H&E staining for baseline group. C isMasson's trichrome and D is H & E staining for cirrhotic group. E isMasson's trichrome and F is H & E staining for cirrhotic group, treated Ntx. G isMasson's trichrome and H is H&E staining for baselinegroup, treatedNtx

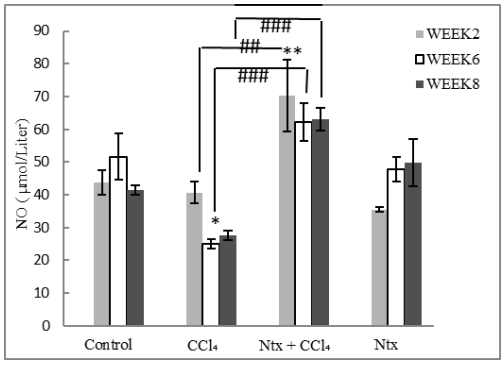

Figure 3. Nitric oxide (NO) metabolite in plasma of rats treated with carbon tetrachloride (CCl_4), naltrexone (Ntx) or their combination (Ntx + CCl4) compared to control rats. Each value represents the mean ± SEM. *Significantly different from control group, * P<0.05, ** P<0.01, *** P<0.001. #Significant difference between Ntx+CCl_4 and CCl_4 groups compared in the same time point # P<0.05, ## P<0.01, ### P<0.001.

The results of GSH assay showed that CCl_4 did not cause any significant change in the GSH levels (Figure 4). NTX alone gradually increased GSH, but this alteration did not reach significant levels. Nevertheless, treatment of cirrhotic rats with NTX was accompanied by a significant increase in the GSH levels compared to cirrhotic rats. Eight weeks after treatment of cirrhotic rats with NTX, the GSH level was even significantly higher than control group.

Hexokinase activity was remarkably increased in the cirrhotic group, especially after 8 weeks of treatment; however, NTX could significantly reduce it in CCl_4+NTX group (Figure 5) such that the activity of HK in cirrhotic rats that received NTX was similar to control group. NTX by itself did not affect the HK activity.

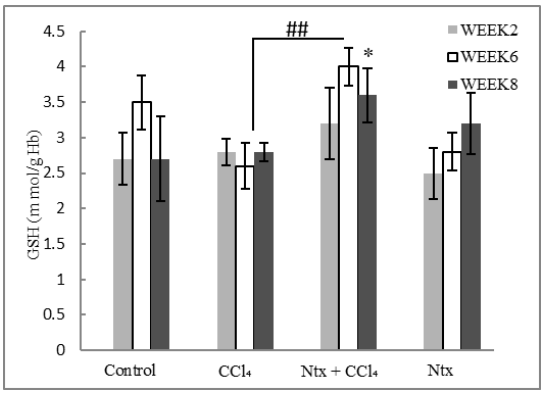

Figure 4. Reduced glutathione (GSH) content in different groups of rats treated with carbon tetrachloride (CCl_4), naltrexone (Ntx), or their combination (Ntx+CCl4), compared to control rats. Each value represents the mean±SEM. *Significantly different from control group, * P<0.05; #Significant difference between Ntx+CCl_4 and CCl_4 groups at the same week, ## P<0.01

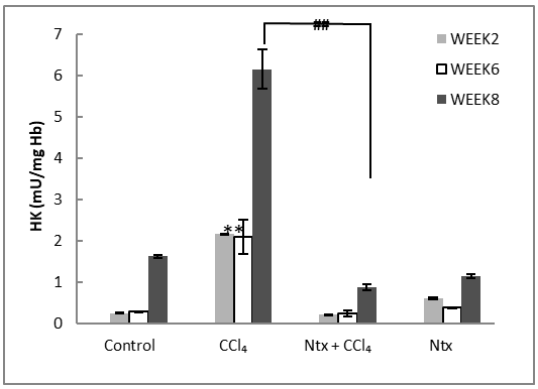

Figure 5. Hexokinase activity (HK) in different groups of rats treated with Carbon tetrachloride (CCl_4), Naltrexone (Ntx), or their combination (Ntx+CCl4) compared to control rats. Each bar represents the mean±SEM. *Significantly different from control group at P<0.05; ##Significant difference between Ntx+CCl_4 and CCl_4 groups at P<0.01.

The activity of GPX was increased in cirrhotic rats, 2 weeks after induction of cirrhosis and remained significantly elevated. NTX, when combined with CCL_4, was not effective on reducing GPX activity until 8 weeks after beginning of administration; however, it was still higher than control group (Figure 6). Treatment with NTX alone also caused a substantial increase in GPX activity which was higher than that in control group at all of the treatment time points.

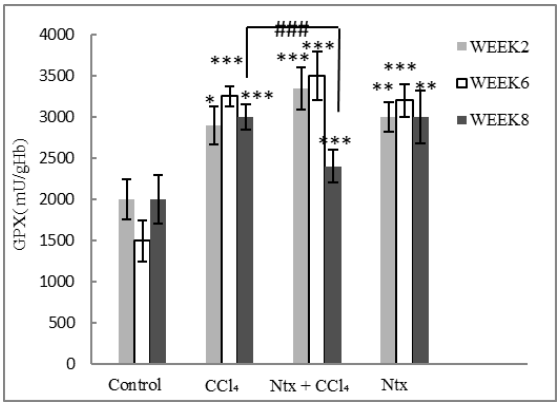

Figure 6. Glutathione peroxidase (GPX) activity in different groups of rats treated with carbon tetrachloride (CCl_4), Naltrexone (Ntx), or their combination (Ntx+CCl4) compared to control rats. Each bar represents the mean ± SEM. *Significantly different from control group. * P<0.05, ** P<0.01, *** P<0.001. #Significant difference between Ntx+CCl_4 and CCl_4 groups. # P<0.05, ## P<0.01, ### P<0.001

Induction of cirrhosis did not result in any change in CAT activity (Figure 7). Nevertheless, NTX increased CAT activity both in cirrhotic rats and those that only received NTX, suggesting that the effect of NTX on CAT was independent from cirrhotic status.

SOD activity was also measured as another important antioxidant enzyme. The results showed that SOD was not altered in cirrhotic rats (Figure 8). Interestingly, NTX caused a dramatic rise in SOD activity only in cirrhotic rats and NTX alone did not significantly influence the activity of SOD.

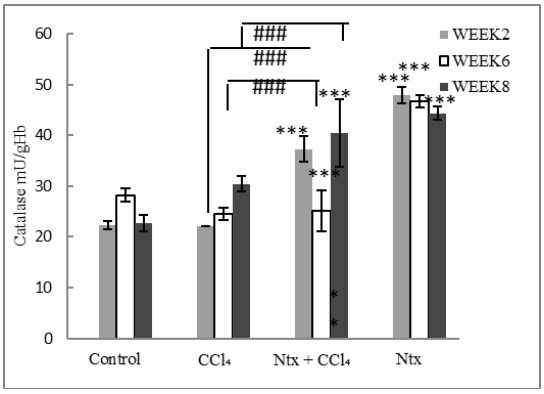

Figure 7. Catalase (CAT) activity in different groups of rats treated with carbon tetrachloride (CCl_4), Naltrexone (Ntx), or their combination (Ntx+CCl4) compared to control rats. Each value represents the mean±SEM. *Significantly different from control group; *** P<0.001. #Significant difference between Ntx+CCl_4 and CCl_4 groups ### P<0.001

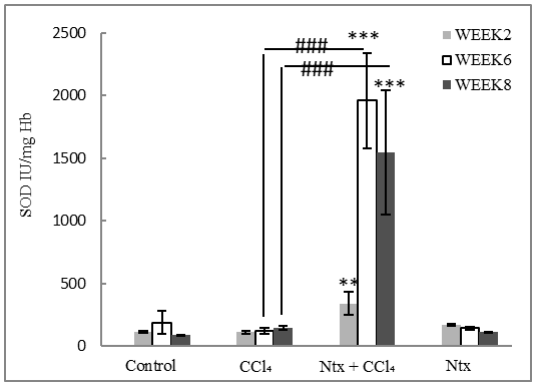

Figure 8. Superoxide (SOD) activity in different groups of rats treated with Carbon tetrachloride (CCl_4), Naltrexone (Ntx), or their combination (Ntx+CCl4) compared to control rats. Each value represents the mean±SEM. *Significantly different from control group, ** P<0.01, *** P<0.001. #Significant difference between Ntx+CCl_4 and CCl_4 groups,### P<0.001

Discussion

Oxidative stress has been considered as an important pathological mechanism in initiation and advancement of liver diseases. Various factors including drugs, alcohol, and toxins may cause oxidative stress in liver, which leads to severe liver diseases such as cirrhosis (26). In the present study we investigated alterations in erythrocytes antioxidant system after induction of cirrhosis and during progression of the disease. Additionally, we evaluated the effect of NTX on cirrhosis and its associated oxidative disturbance.

Studies have shown that NTX blocks endogenous opioid receptors, which are increased during liver injury and could ameliorate liver injury in different animal models of cirrhosis. For example Javadi-Paydar *et al*., showed that memory impairment in rats due to cholestasis was improved by receiving NTX for 28 days (13). Additionally, Kiani *et al*., showed that NTX could prevent liver fibrosis in biliary duct ligated (BDL) rats after 28 days of intervention (14-16).

In this study, we showed that long-term treatment of cirrhotic rats with NTX could modulate ALT activity and partially reduce its level in CCl4-induced cirrhotic

rats. Consistently, recent studies have shown that NTX could amend ALT in liver disorders. For example, Wang *et al*., reported decreased activity of ALT in animal models of hepatitis after 12 weeks of treatment with NTX (27). In the beginning of administration of NTX to CCl4-treated rats, we observed a remarkable rise in ALT activity which declined in the following weeks with continuous intervention with NTX. Some other hepatoprotective compounds have also shown to exert their dramatic ameliorative effect on ALT after long-term treatment (28).

The results of our study showed that the induction of cirrhosis was accompanied by decreased NO production. In line with our findings, Shah *et al*., investigated nitric oxide synthase (NOS) and NO levels in animal models of cirrhosis and reported that the cirrhotic animals produced significantly less NO compared to control animals. They showed that NOS activity was considerably decreased in liver tissue from cirrhotic animals, although the NOS protein levels were unchanged (29) present investigation shows that NO metabolites are diminished in CCl4-induced cirrhosis that may be due to decreased hepatic eNOS activity. The results about NO metabolites in this study are similar to those reported recently in the bile duct ligation model of liver injury, (30) NO is one of the main endogenous vasodilators; thus, its reduced levels would be harmful to liver perfusion portal hypertension (31). NO synthesis requires an intricate regulation and alters because of NOS modulation. Researche in the fibrotic livers of bile duct-ligated rats have shown that eNOS activity is uniformly decreased in cirrhotic liver. Therefore decreased NO levels in cirrhotic models might be a direct consequence of reduced eNOS activity in hepatocytes (21)

Here we showed that NTX significantly increased NO metabolites in CCl4-induced cirrhotic rats and by this mechanism might be effective in opposing the detrimental effects of cirrhosis on NO production. Beneficial effect of NTX on NO producing system has been previously shown in cholestasis-induced memory impairment and induction of NOS has been suggested as a mechanism involved in the improvement of spatial recognition memory by NTX (13).

Alterations of redox status plays both direct and indirect roles in the pathogenesis of cirrhosis (32), and GSH depletion is often associated with cell and tissue injury (33). One of the results of the current research was the increased GSH levels by NTX in cirrhotic rats. Several studies suggest that NTX modulates oxidative stress by increasing GSH level, while GSH is suppressed by increased endogenous opioids during liver disease (14). Treatment of bile duct cirrhotic rats by NTX could also increase GSH synthesis (34). In this study no alteration was seen when normal rats were treated with NTX suggesting that NTX exerts its positive effect on GSH only when a liver damage and pre-existing oxidative stress is present.

A cellular mechanism to prevent oxidative damage and combat GSH depletion is to increase HK activity and produce more NADPH through the pentose phosphate pathway (35). The resulting NADPH helps reduce GSSG to GSH and stabilize redox status inside cells (35). Here we showed that induction of cirrhosis was accompanied by a remarkable elevation in HK activity, indicative of oxidative stress and the need for up-regulation of antioxidant defense. HK activity was especially increased with the development of the disease demonstrating that the rise in HK can reflect the severity of cirrhosis. NTX opposed the changes in HK activity and balanced it to its normal activity.

In our study CAT activity was not changed in cirrhotic rats but was increased after receiving NTX. In the research conducted by Szuster *et al*., it was shown that CAT activity was increased in the sera of patients with liver cirrhosis or pancreatitis but not in the patients harboring both diseases (36). Another study showed that CAT activity was increased in endothelial cells of the cirrhotic patients (37). However, Goth *et al*., showed normal levels of serum CAT activity in the liver cirrhosis (38). Based on the current and previous findings it seems that there is a controversy between results which may be due to the multifactorial regulation of CAT activity (39). Opioid use has been shown to be associated with decline in CAT activity in liver (40,41); thus, elevation of CAT by NTX, which is an opioid antagonist, might be attributed to its opioid-antagonistic effects (42). Another study has also suggested the involvement of opioid peptides in the regulation of CAT activity (43).

Here we showed a significant increase in GPX activity in cirrhotic rats. Consistently, some other investigations showed increased GPX activity in erythrocytes during hepatitis (44). GPX uses GSH and performs its antioxidant activity by producing its oxidized form (GSSG) (45). It seems possible that elevation of GPX activity in erythrocytes might be an adaption mechanism to the oxidative stress. NTX alone increased GPX activity compared to control rats. Consistently, in the research performed by Almansa *et al*., the augmentation of GPX by NTX has been reported (19). However, its administration in CCl4-treated rats

did not exert any added benefit and the GPX activity was not significantly different from cirrhotic rats.

In this study we found that SOD activity was not affected by induction of cirrhosis. Consistently, Ozenirler *et al*., showed that erythrocyte SOD activity in patients with active liver cirrhosis was not significantly different from that of the controls (46). However, some previous studies have described controversial results regarding SOD activity in cirrhosis. For example, Togashi *et al*., reported that the levels of Cu, Zn-SOD was significantly lower while Mn-SOD was not significantly different in non-alcoholic cirrhotic patients (47). Clemente *et al*., showed that serum Mn-SOD was significantly higher in cirrhotic patients compared to healthy subjects (48). On the contrary, Koruk *et al*., and Hadi *et al*., reported lower SOD activity in cirrhotic patients (49,50). In our studied animals, the progression of disease did not lead to any changes in SOD activity. In line with our findings, it has been shown that severity of the cirrhosis does not influence the SOD activity (48). Here we showed for the first time that NTX causes a rapid and consistent rise in the SOD activity in the cirrhotic animals. Interestingly, the same effect was not observed in normal animals receiving NTX suggesting that the effect of NTX differs in normal and disease state and that it only induces SOD in the background of a liver injury.

The results of this study demonstrate that the induction of cirrhosis is accompanied by alterations in oxidative status especially the activities of HK and GPX. NTX exerts ameliorative effects in cirrhosis by modulation of most of the oxidative stress components and its effect in cirrhotic state was reflected on the alteration of NO and GSH levels as well as CAT activity. Thus, NTX might be considered an efficient therapy to combat oxidative stress in liver diseases.

Acknowledgements

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