Erythrocytes Membrane Alterations Reflecting Liver Damage in CCl_4-Induced Cirrhotic Rats: The Ameliorative Effect of Naltrexone

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Received: 25 Nov. 2015; Revised: 12 Jan. 2016, Accepted: 04 Feb. 2016

Abstract- Cirrhosis is the consequence of chronic liver disease. Deleterious effects of oxidative stress on hepatocytes may be reflected in the erythrocyte membrane. Naltrexone (NTX) has been shown to attenuate hepatocellular injury in fibrotic animal models. The aim of this study was to investigate the progressive effect of CCl_4 on the liver and whether the improvement of liver cirrhosis can be monitored through alterations in the erythrocyte membrane. In this study, 84 male Wistar rats were divided into 4 groups and received reagents (i.p.) as follows: 1- CCl_4, 2- NTX + CCl_4, 3- Mineral Oil (M), and 4- NTX + M. After 2, 6 and 8 weeks, the blood and liver tissue samples were collected. Plasma enzyme activities, the content of erythrocyte GSH and some membrane compositions, including protein carbonyl, protein sulfhydryl, and malondialdehyde were assessed. After 6 and 8 weeks, plasma enzyme activities and the content of protein carbonyl were higher in CCl_4 group significantly, as compared to other groups (P<0.001). NTX significantly diminished protein carbonyl and plasma enzyme activities (P<0.001). GSH did not change until the 6th week. However, CCl_4+NTX increased it significantly as compared to CCl_4 group (P<0.05). Protein sulfhydryl showed changes in NTX+CCl_4 group which indicated a significant increase in protein sulfhydryl content in a 6th week compared to CCl_4 group (P<0.05). MDA did not show any significant alteration. CCl_4-induced cirrhosis is accompanied by increased content of oxidative stress markers, especially protein carbonyl of RBC membrane and plasma enzyme activities. This study shows that the progression of liver cirrhosis and the ameliorative effect of NTX can be followed through alterations of these markers.

Acta Med Iran, 2016;54(10):631-639.

Keywords: Cirrhosis; Carbon tetrachloride; Naltrexone; Oxidative stress

Introduction

Liver cirrhosis is a major yet largely preventable and under-appreciated cause of global health loss and has emerged as a major cause of the global health burden (1). Liver disease is now the fifth most common cause of death (2). This liver injury is associated with abnormalities in the systemic circulation (3) and increased risk of hepatocellular carcinoma (4,5). Despite increasing knowledge about molecular and cellular mechanisms involved in liver carcinogenesis (6), the only hepatocellular carcinoma (HCC) curative treatment is transplantation (7). Liver biopsy is the best way that provides valuable information for making treatment decisions, but it is invasive, costly, difficult to repeat and has life-threatening complications (8,9). Accordingly, non-invasive tools to assess liver disease, especially its progression or remission, have been largely needed. Some procedures have been introduced as more safe ways for recognizing and following liver cirrhosis like FibroTest (FT); combination of serum markers based on five and six biochemical markers (10); or serum markers of fibrogenesis (8), but they have shown only a moderate correlation with histological changes in fibrosis and cirrhosis (8,11). Considering the important role of oxidative stress in the pathogenesis of cirrhosis (12,13), it seems that systemic markers of oxidative stress could reflect the intensity of disease

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progress in the liver (14-16).

The biological membrane which is composed of oriented proteins and lipids which have a dynamic and semi-fluid structure and even minor changes in its fluidity may cause abnormal function and pathological consequences (17). Reactive oxygen species (ROS)-mediated lipid peroxidation leads to accumulation of cytotoxic products such as MDA and 4-hydroxy alkenals such as 4-hydroxy-nonenal (4-HNE) (18) which impair various cellular functions (16,19). Proteins may also be damaged by ROS leading to structural changes and loss of function (20). Oxidation of proteins leads to carbonyl residue formation which is an irreversible reaction. So the presence of carbonyl groups in proteins is a marker of ROS-mediated protein oxidation (21). Changes in protein sulfhydryl oxidation are also suggested to play an important role in the pathogenesis of hepatotoxins (22). Erythrocyte membrane alterations have been shown to be of useful tools and peripheral biomarkers of some diseases (23). Studies also have shown that lipid and protein oxidation in erythrocyte membrane, as well as GSH content alteration, occurs in erythrocytes in liver fibrosis and cirrhosis, although controversial results have also been reported (24-26).

Carbon tetrachloride (CCl_4); a well-known hepatotoxin (27), which is widely used to induce hepatic cirrhosis in experimental models (28-30), was utilized in the current study. Cytochrome P450 system in liver microsomes converts CCl_4 to free radicals which are highly reactive and cause lipid peroxidation and toxicity (31).

It has been shown that endogenous opioids may modulate oxidative stress and therefore affect cell proliferation and survival. Naltrexone (NTX) is a μ-opioid receptor antagonist and blocks endogenous opioids receptors which are increased during a liver injury (32). Also, it has been shown that NTX ameliorates liver injury in many animal models of cirrhosis (33-35). There is evidence that NTX normalizes hepatic reduced glutathione (GSH) and lipid peroxidation (LPO) levels and meanwhile, suppresses hepatic cell apoptosis in chronic cholestasis (36). Although increased oxidative stress in liver cirrhosis has been shown in previous studies (37), it is not yet clear whether amelioration of liver cirrhosis in different stages of the disease is accompanied by an improvement in oxidative stress status and if NTX is beneficial in this regard. Thus, in the current study, we examined the changes in oxidative stress markers including GSH, MDA, and protein carbonyl and sulfhydryl content in erythrocyte membrane during progression of liver cirrhosis and the consequence of blocking opioid receptors by NTX in CCl_4-induced cirrhotic rats to clarify if progression of liver cirrhosis by CCl4 and especially its amelioration by NTX can be monitored via these oxidative stress markers.

Materials and Methods

Chemicals

All chemicals were obtained from Sigma-Aldrich (unless specified), at the appropriate grades.

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 Institutes of Health in the USA and were approved by the ethics committee of Tehran University of Medical Sciences. Treated animals were randomly assigned to 4 groups (21 Rat/group) and these 21 Rats divided into 3 subgroups (7 Rat/subgroup) to be sacrificed in 3 different times after beginning of the study. The animal receives substrates as follows: (I) CCl_4 (test; treated with CCl_4 750 mg/kg, dissolved in 1 ml mineral oil i.p. every other day), (II) NTX+CCl_4; (treated with CCl_4 750 mg/kg, dissolved in 1 ml mineral oil i.p. every other day and NTX 10 mg/kg dissolved in normal saline daily), (III) mineral oil (control; treated with 1 ml mineral oil i.p. every other day), (IV) NTX + mineral oil (treated with 1 ml mineral oil *i.p.* every other day and NTX 10 mg/kg dissolved in normal saline daily). Blood and liver tissue samples were collected at 2, 6 and 8 weeks after the beginning of injections.

Histological evaluation

Liver tissue samples were taken immediately after blood collecting and surgical operation of rats under general anesthesia by ether and were fixed in 10% formaldehyde solution, followed by fixing in 70% alcohol. After embedding in paraffin, the samples were sectioned and stained with haematoxylin-eosin (H & E) reagent. The sections were studied under a light microscope.

Plasma biochemical measurements

Blood was collected in heparin-containing tubes,

centrifuged at 3000 rpm for 10 min at 4° C, plasma, and buffy coat were removed, and plasma was kept frozen at −80°C for analysis. The activity of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and gamma-glutamyltransferase (GGT) were measured using commercially available kits (Pars Azemoon).

Preparation of erythrocyte membrane

Erythrocyte membrane ghosts were prepared according to Dodge *et al*., with a little modification (38). Briefly, erythrocytes were washed four times with cold (4°C) phosphate-buffered saline (155 mM, *p*H 7.4). Hypotonic lysis of cells was performed in cold 5 mM sodium phosphate buffer (*p*H 8.0) followed by centrifugation (30 min, 4°C, 20000×g). The supernatant (hemolysate) was decanted carefully, and the ghosts were washed four times subsequent to hemolysis. Biuret method was used for estimating total protein content of the erythrocyte membrane with bovine serum albumin (BSA) as a standard (39).

Protein carbonyl content

Protein carbonyl contents in erythrocyte membrane were evaluated using 2,4-dinitrophenylhydrazine (DNPH) assay with slight modifications (40). The erythrocyte ghosts containing 0.5 mg protein were precipitated with 10% trichloroacetic acid, (4:1, w/v) and centrifuged at 4 °C for 5 min at 11000×g. Supernatant was discarded, and the pellets were resuspended in 500 µl of 10 mM DNPH in 2 M HCl and allowed to rest at room temperature for 60 min, vortexing every 10-15 min to facilitate the reaction with proteins. Proteins were precipitated with 50% trichloroacetic acid and centrifuged at 4° C for 5 min at 11000×g. After washing the pellet with 500 µl ethanol:ethyl acetate (1:1, v/v) three times, the insoluble materials were removed by centrifugation and dissolved in 0.6 ml of 6 M guanidine hydrochloride at 37° C. The absorbance of the samples was measured at 370 nm. Carbonyl group content was calculated using a molar absorption coefficient of $2.2\times104\ M^{-1}cm^{-1}$ and expressed as µmol/mg protein.

Protein sulfhydryl measurement

Erythrocyte membrane protein sulfhydryl content was determined using the Ellman reagent containing 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (41). Briefly, erythrocyte membrane ghost samples containing 0.5 mg protein were denatured with sodium dodecyl sulfate and reacted with Ellman reagent. The absorbance of the resulting thiophenylate anions was measured at 412 nm. The sulfhydryl content was calculated using molar absorption coefficient of $1.36\times104\ M^{-1}cm^{-1}$ and expressed as µmol/mg protein.

Reduced glutathione (GSH) assay

The concentration of GSH was assayed using GSH Assay Kit (BioVision, Mountain View, CA, USA). The concentration of GSH was expressed as µmol/gHb.

The concentrations of hemoglobin in the hemolysate samples were measured according to Drabkin's method (42).

Lipid peroxidation measurement

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) formation (43). Briefly, erythrocyte membrane ghosts containing 0.5 mg protein, were suspended in a reagent with 15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid and 0.25 N HCl. The solution was heated in a boiling water bath for 15 min. After cooling, the precipitate was removed by centrifugation. Absorbance was measured at 535 nm. MDA concentration was calculated using its molar absorption coefficient of $1.56\times105\ M^{-1}cm^{-1}$ (44) and expressed as µmol/mg protein.

Statistical analysis

Results are presented as mean±SEM for at least two repeats of the experiment. Statistical significance was assessed by ANOVA, followed by Tukey's post-hoc test. A *P*.value of 0.05 or less was considered statistically significant.

Results

Biochemical parameters

As shown in Table 1, the plasma activity of ALT, ALP and GGT were significantly higher in the CCl_4 group as compared to those in other groups ($P<0.05$) indicating the progression of liver injury. The results showed that NTX was able to partially reverse the increased GGT activity and could significantly reverse the increased ALP and ALT activities in NTX+CCl_4 group ($P<0.001$); revealing protective and to some extent, curative effect of NTX against the stress caused by CCl_4.

Membrane protein modification

Erythrocyte membrane protein carbonyl content was dramatically increased in the CCl_4 group with a peak level at the 6th week. This value was significantly diminished in the CCl_4+NTX group as compared to CCl_4 group

(P<0.001), however, it was still higher than the control group (P<0.01). Protein carbonyl levels were decreased especially at week 8 (P<0.001) and returned to that of control levels in CCl_4+NTX group (Figure 1a).

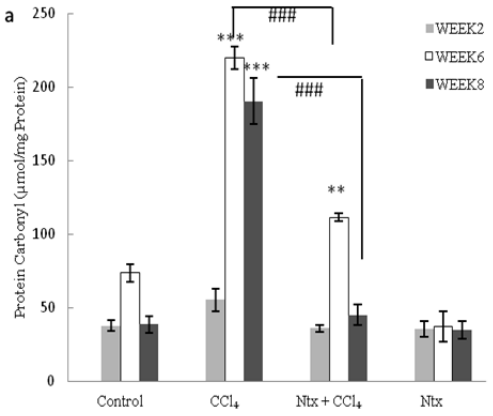

Figure 1a. Protein carbonyl level of erythrocyte membrane

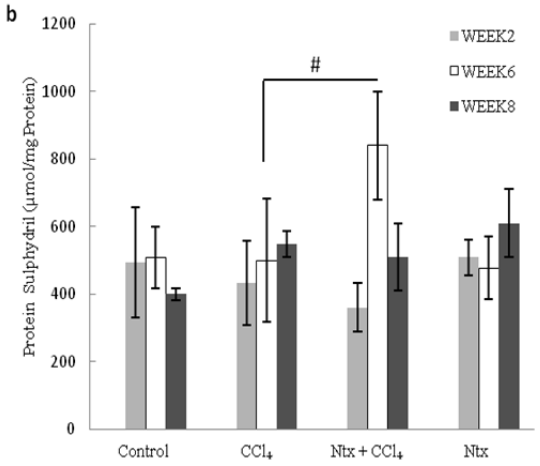

Figure 1b. Protein sulfhydryl level of erythrocyte membrane

Erythrocyte membrane protein carbonyl (a) and protein sulfhydryl (b) in NTX treated CCl_4-induced cirrhotic rats. CCl_4 and CCl_4+NTX are test groups. *, Comparison of the groups with control with P<0.05, #, comparison of two test groups with P<0.05 at the same week after injection. Repetition of * and # signs two times means P<0.01 and three times means P<0.001. Abbreviations are as in Table 1.

There were no significant changes in protein sulfhydryl level of erythrocyte membrane samples in CCl_4 treated rats in comparison with control group. However, there was a significant increase in protein sulfhydryl content of the NTX+CCl_4 group at week 6 compared to CCl_4 group (P<0.05) (Figure 1b).

Membrane lipid peroxidation

NTX slightly decreased CCl_4-induced MDA production in CCl_4+NTX group but this effect was not significant as compared to both control and CCl_4 groups (Figure 2) and no increase was observed in any groups in comparison to that of the control (P<0.05).

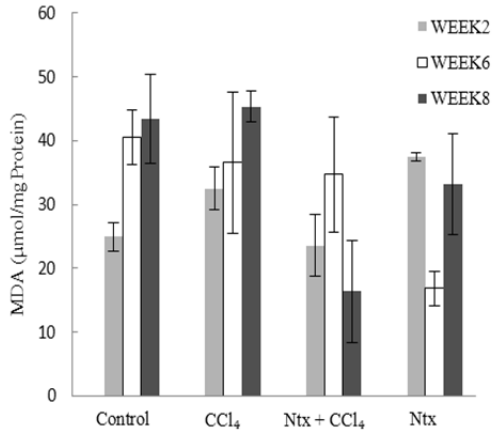

Figure 2. MDA level of erythrocyte membrane

Malondialdehyde (MDA) content as an indication of lipid peroxidation of erythrocyte membrane content in NTX treated CCl-induced cirrhotic rats. CCl and CCl+NTX are test groups. Abbreviations are as in Table 1.

Glutathione content

GSH content did not show any significant changes in CCl_4 group as compared to the control group, but the results indicate that NTX significantly increased it when administrated simultaneously with CCl_4 significantly, especially 8 weeks after injection as compared to control (P<0.05) and CCl_4 groups (P<0.01) (Figure 3).

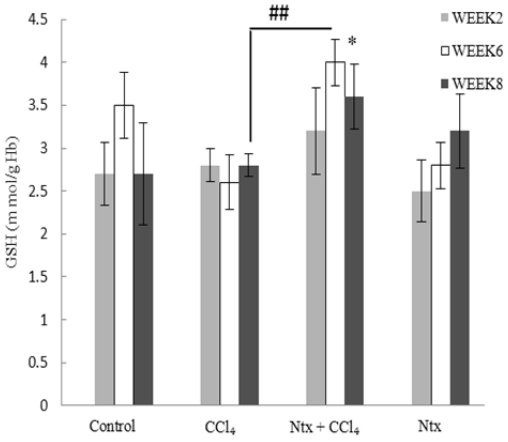

Figure 3. GSH level of erythrocyte membrane

Reduced glutathione (GSH) content in NTX treated CCl_4-induced cirrhotic rats. CCl_4 and CCl_4+NTX are test groups. *, comparison of the groups with control with P<0.05, #, comparison of two test groups with P<0.01 at the same week after injection. Abbreviations are as in Table 1.

Histological study

Photohistographs of hematoxylin-Eosin stained sections of the liver tissues are shown in Figure 4. In control animals, the architecture of the hepatic parenchyma tissue was normal. Slight necrosis and granules of fat were observed in CCl_4 treated rats after 2 weeks. At the same week, some areas of necrosis and hyperemia were noticed in NTX+CCl_4 group. In the 6th week, infiltration of inflammatory cells was evident, and the cells were surrounded by blood. Comparison of the tissue samples from the CCl_4 and NTX+CCl_4 groups during the 6th week showed less fibrosis in the latter, which confirms a protective role for NTX. After 8 weeks, extensive bridging fibrosis (portal to portal and portal to central linkage with fibrotic bands), necrosis and inflammation were observed, and many inflammatory cells were seen. These histopathological events are in line with the biochemical changes, and prove cirrhosis progression in CCl_4 treated animals after eight weeks. At the 8thweek, severe hyperemia and increased kupffer cells are visible in the NTX+CCl_4 group.

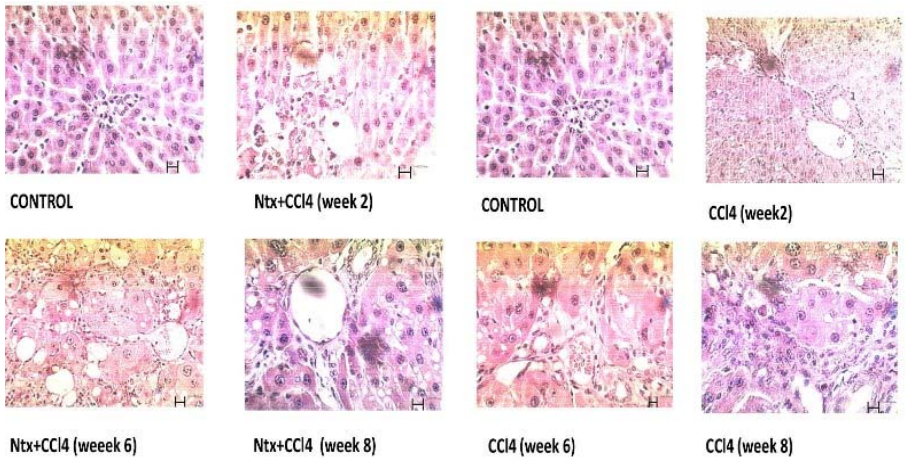

Figure 4. Hematoxylin-Eosin staining of liver tissue from the control group, CCl_4-treated and NTX+CCl_4 treated groups after 2, 6, and 8 weeks of injection. Abbreviations are as in Table 1.

Table 1. Biochemical parameters

	Time (week 2)			
Enzymes	**Control**	**CCl_4**	**CCl_4+NTX**	**NTX**
ALT (U/l)	25.47±2.01	64.84 ±8***	181.93±8***	18.35±1.63
ALP (U/l)	159.35±6.94	237±21	312±14**	125.44±6.89
GGT (U/l)	3.58±0.36	7.7±091	8.56±0.28	7.1±0.84
	Time (week 6)			
Enzymes	**Control**	**CCl_4**	**CCl_4+NTX**	**NTX**
ALT (U/l)	86.36±3.39	251.28±15***	209.42±17***	25.46±3.57
ALP (U/l)	118.55±9.25	409.3±42***	489.08±34***	147.77±17.57
GGT (U/l)	5.63±0.46	12.55±1.03***	10.51±0.85***	4.06±0.67
	Time (week 8)			
Enzymes	**Control**	**CCl_4**	**CCl_4+NTX**	**NTX**
ALT (U/l)	13.36±29	251.87±12***	129.02±10***###	15.46±0.92
ALP (U/l)	144.05±13.47	582.8±47***	351.53±41***###	143.36±10.05
GGT (U/l)	7.38±1.7	11.16±0.76	10.72±1.36***	1.9±0.41

Table 1 shows biochemical parameters of control and test groups. Data are presented as mean±SEM. Asterisks denote significant differences from the control group, * and # denote significantly different between NTX+CCl_4 and CCl_4 groups as the same week. $P<0.05$, ** $P<0.01$, *** $P<0.001$, # $P<0.05$, ## $P<0.01$, ### $P<0.001$. GGT: gamma glutamyl transferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; CCl_4: carbon tetrachloride; NTX: Naltrexone; M: mineral oil.

All enzymes activities were significantly higher in CCl_4 groups indicating the progression of liver injury. The results showed that NTX was able to partially reverse the increased GGT activity and can significantly reverse the increased ALP and ALT activities in NTX+CCl_4 group.

Discussion

The imbalance between the production of free radicals and antioxidants results in pathological damages (12). In the current study, we aimed to induce cirrhosis with CCl_4 which is widely used to compel hepatic fibrosis and cirrhosis in experimental models (29). In CCl_4-induced cirrhotic rats, hepatic responses to CCl_4 administration are similar to those of human hepatic cirrhosis (30). Liver injury induced by CCl_4 is the best characterized system of xenobiotic-induced hepatotoxicity in living systems (7). Since the development of liver cirrhosis is a progressive procedure, cross-sectional assessment of different markers may not give enough insight for evaluation of disease progression. Thus we used animal models of liver damage from early to advanced cirrhosis to give a better perspective of biochemical alterations during stages of the disease progression and amelioration. We found significant increases in the activity of enzymes ALT, ALP, and GGT in this model, indicating severe impairment of the liver function (45). These alterations were correlated with the histological documents provided by H and E staining of the liver tissues from the experimental and control groups which showed the presence of collagen and formation of fibrotic scars and inflammation; the signs which are characterized for liver cirrhosis.

We found that CCl_4 caused systemic oxidative stress along with liver damage, reverberating on erythrocytes. This effect was evident by increased protein carbonyl, which is a stable marker of oxidative stress. Since CCl_4 is a hepatotoxin, it seems that this oxidative stress is secondary to liver injury. Protein carbonylation is one of the major physical changes which occurs in the early stages of oxidative stress and has more stability as compared to other parameters of oxidative stress such as glutathione disulfide and MDA. Thus, Protein Carbonyl (CO) groups have been suggested as biomarkers of oxidative stress (21). Lipid peroxidation which produces MDA is one of the basic reactions involved in the oxidative deterioration of polyunsaturated lipids (46), and MDA is also a marker of oxidative damage to membrane lipids. Systemic oxidative stress has been shown to be associated with liver cirrhosis (47) and the increased oxidative markers in different stages of liver damage has been previously reported by this group (48) However, whether liver amelioration is accompanied by a gradual improvement in these markers has not been investigated before.

NTX is an endogenous opioids receptor blocker (49), and is available as a safe generic formulation already used as an adjunct in counseling treatment of alcohol abuse (50,51), although with FDA's black box label warning of hepatotoxicity (52). Co-treatment of NTX with CCl_4 during the time course of the study could moderately prevent the severe liver injury and significantly decrease the liver enzyme activities of plasma as compared to CCl_4 group. Here we showed for the first time that NTX was able to dramatically diminish protein carbonyl content in erythrocyte membrane during liver injury progression and bring it back to normal levels after 8 weeks.

In this study, MDA levels were not significantly increased in cirrhotic rats compared to control. However, NTX could partially decrease MDA levels as compared to CCl_4 group apparently by protecting the liver from oxidative stress caused by CCl_4.

Different mechanisms have been proposed for the anti-oxidative function of NTX. Several studies suggest that NTX modulates oxidative stress by increasing GSH level which is suppressed by increased endogenous opioids during liver disease through an unknown mechanism (33). Evidence supporting this concept comes from the studies in which intracerebroventricular injection of an opioid agonist decreased hepatic GSH synthesis (53), suggesting the involvement of endogenous opioids in the regulation of redox state of hepatocytes (54).

In order to investigate whether the beneficial effects of NTX is mediated by GSH, we measured GSH levels in erythrocytes during cirrhosis progression and treatment with NTX. NTX increased erythrocyte GSH levels significantly, especially after 8 weeks of treatment. It could also transiently increase protein sulfhydryl content in the erythrocyte membrane. Cells must be maintained at a high level of reduced GSH. GSH plays a key role in detoxification of xenobiotics in cells, maintenance of oxidative balance and keeping proteins in the reduced state. Alterations of redox status play both direct and indirect roles in the pathogenesis of cirrhosis. GSH depletion has been shown to be associated with loss of protein sulfhydryls (22). There are conflicting reports about the extent of protein sulfhydryl depletion during CCl_4-induced cirrhosis. One report found significant global protein sulfhydryl depletion during hepatotoxicity (55), whereas others identified only selective protein sulfhydryl alterations (56) or no significant change in protein sulfhydryl status (57). In this study, it was shown that although protein sulfhydryl levels were not significantly different between cirrhotic and normal rats, NTX could increase

protein sulfhydryl levels in cirrhotic rats concomitantly with GSH levels surge in the 6th week. Thus it seems that protein sulfhydryl content is related to GSH levels. The data suggests that during hepatic injury, NTX has beneficial effects in ameliorating the oxidative stress and subsequent pathologic alterations. However, further studies are needed to confirm the effectiveness of NTX in the management of hepatic cirrhosis.

The results of the present study showed that the oxidative stress was higher in CCl_4-induced cirrhotic rats, and this situation is reflected in altered oxidative stress markers in erythrocytes. NTX had a protective effect on liver damage as well as systemic oxidative status evident by reduced protein carbonyl formation in erythrocyte membrane, slightly decreased MDA production, and increased GSH and protein sulfhydryl levels in erythrocytes. Our findings confirm an anti-oxidative role for NTX and suggest that amelioration of liver cirrhosis can be followed by evaluation of oxidative stress markers, especially protein carbonyl content of erythrocytes.

Acknowledgment

Authors are grateful to the Research Council of Tehran University of Medical Sciences (Grant No. 19392). We also appreciate Professor Gholamreza Hasanzadeh for his valuable assistance in providing the histological data and the photographs.

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