Involvement of Nitrergic and Opioidergic Systems in the Oxidative Stress Induced by BDL Rats

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Authors’ contributions

This work was carried out in collaboration between all authors. Authors ARD and AG designed the study, managed the literature searches and supervised the study. Author AHD wrote the protocol, carried out the procedure, identified the clinical sign of the BDL model in rats and performed the experimental process. All authors read and approved the final manuscript.

ABSTRACT

Aims: Liver cirrhosis is the irreversible end result of fibrous scarring and associated with prominent morbidity and mortality. We aimed to investigate the involvement of nitrergic and opioidergic systems in the oxidative stress induced by bile duct ligation (BDL) rats by evaluating the oxidative stress markers such as malondialdehyde (MDA), protein carbonyl content and total thiol in the plasma.

Methodology: Rats received injections of 3 mg/kg Nω-Nitro-L-arginine methyl-ester-hydrochloride (L-NAME: a NO-synthase inhibitor), 10 mg/kg opioid-receptors antagonist naltrexone (NTX), L-NAME (3 mg/kg)+NTX (10 mg/kg) or saline once daily for 28 days after BDL or sham surgery. Oxidative stress markers and biochemical indexes were measured in plasma samples.

Results: Results showed that serum levels of total bilirubin (TB) and alkaline phosphatase (ALP) to be significantly increased in BDL rats as compared with the Sham-operated (SO) group.
Liver cirrhosis is associated with outstanding morbidity and mortality which is described by diffused disorganization of the normal hepatic structure of regenerative nodules and fibrotic tissue [1]. Oxidants include superoxide (O_2^-·), hydroxyl radical (OH·), hydrogen peroxide (H_2O_2), and other reactive oxygen species (ROS) which is believed to have some role in this process [2]. Cells have developed antioxidant systems which restore oxidants into non-toxic molecules, protecting the organism from lethal effects of oxidative stress. Oxidative stress is an interruption in the oxidant-antioxidant balance, resulting in tissue damage and has an important role in the pathogenesis of cirrhosis [3,4]. Jaeschke and et al. suggested that oxidant stress-induced liver injury is resulted from lipid peroxidation mediated by ROS, mitochondrial dysfunction and apoptotic cell death [5]. Even though ROS are vital in cell signaling pathways, excessive ROS production leads to damage of the cell membrane, lipids and proteins [6,7] and creating malondialdehyde (MDA), protein SH (P-SH) groups and carbonylated proteins. Carbonylated proteins are a established marker of severe oxidative stress that often leads to loss of protein function and inducing liver cirrhosis [8,9].

Elevated plasma levels of endogenous opioid peptides, mostly methionine enkephalin, have been demonstrated in cholestatic patients and rats [10,11], furthermore, both acute and chronic activation of opioid receptors in animals have been reported to cause liver damage through increased oxidative stress and enhanced plasma liver enzyme activities [12,13]. Previous studies have reported that NTX returns hepatic reduced glutathione (GSH) and lipid peroxidation (LPO) levels in chronic cholestasis to normal, also, ameliorates liver injury and adjusts oxidative stress in many animal models of cirrhosis [14-16].

We have previously reported evidence for nitric oxide (NO) overproduction in cholestasis [17,18]. NO plays an important role in increased oxidative stress in cirrhotic rat livers by a rapid reaction with superoxide anion (O_2^-·) [19].

The rodent bile duct ligation (BDL) model has been widely used to evaluate the consequences of cholestasis (20). Considering the crucial role of nitricergic and opioid systems in the cholestatic/cirrhotic rats and based on disturbances in the oxidant-antioxidant balance in this disease, we aimed to investigate the involvement of opioid and NO systems in oxidative stress after bile-duct ligation in rats by evaluating the oxidative stress markers such as MDA, protein carbonyl content, total thiol and FRAP in plasma.

2. MATERIALS AND METHODS

2.1 Reagents

The opioid receptor antagonist naltrexone (NTX HCl), a NOS inhibitor Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME HCl), Thiobarbituric acid (TBA) and (5,5′-dithiols-(2-nitrobenzoic acid)) (DTNB) were obtained from Sigma Chemical Co (St Louis, MO, USA). FeSO_4·7H_2O was purchased from Riedel-de Haen, Germany. Tripyridyltriazine (TPTZ), trichloroacetic acid (TCA), 2, 4-dinitrophenylhydrazine (DNPH), and formaldehyde were obtained from Merck (Germany).

2.2 Animal Manipulation

Male Sprague–Dawley rats weighing 200–250 g 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 in accordance with the Animal Care Guidelines published by the National Institutes of Health in USA and were approved by the ethics committee of Tehran University of Medical Sciences.

2.2.1 Animal model of cholestasis

Animals were divided into four groups (n=10 for each group): 1) BDL animals treated with saline, 2) BDL animals treated with L-NAME (3 mg/kg), 3) BDL animals treated with NTX (10 mg/kg), 4) BDL animals treated with NTX (10 mg/kg)+L-NAME (3 mg/kg). For each group equal number of sham-operated (SO) animals were prepared. BDL was performed in rats as described previously [20]. In brief, rats were laparotomized under general anesthesia induced by ketamine HCl (50 mg kg\(^{-1}\)) and xylazine HCl (10 mg kg\(^{-1}\)). The general bile duct was exposed through a midline abdominal incision. It was then ligated in two loci with a silk thread and sectioned between the ligatures. SO age-matched rats provided as the control. Sham operation consisted of laparotomy and bile duct recognition and handling without ligation. The animals received intraperitoneal injections of L-NAME (3 mg/kg), NTX (10 mg/kg) or saline once daily for 28 days after BDL or sham surgery. On day 28, animals were sacrificed by cardiac puncture under general anesthesia and blood and liver tissue samples were collected.

2.3 Plasma Biochemical Measurements

Blood was collected in heparin-containing tubes, centrifuged at 3000 rpm for 10 min at 4°C, plasma were removed and kept frozen at −80°C for analysis. Plasma TB, ALP, albumin (Alb), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using commercially available kits (Pars Azemoon, Iran). Bradford method was used for estimating total protein content of the plasma with bovine serum albumin (BSA) as a standard [21].

2.4 Histological Evaluation

Liver tissue samples were taken immediately under general anesthesia after blood collecting and were fixed in 10% formalin solution, followed by fixing in 70% alcohol. After embedding in paraffin, the samples were sectioned and stained with hematoxylin-eosin (H&E) reagent. The liver sections were studied under light microscope in double-blind examinations.

2.5 FRAP Assay

Plasma total antioxidant capacity was determined according to Benzie and Strain [22]. This method is based on the ferric reducing ability of plasma (FRAP) which is estimated from the reduction of a ferric tripyridyltriazine complex in the ferrous form at low pH. Solutions to make up the FRAP reagent were prepared: 300 mmol/L acetate buffer, 10 mmol/L TPTZ/HCl solution, and 20 mmol/L ferrocene chloride. Standard solutions of FeSO\(_4\).7H\(_2\)O ranging in concentration from 1000 to 31.125 µmol were made. The absorbance of the resulting blue color was measured at 593 nm, and the total antioxidant capacity of plasma was determined using a standard curve.

2.6 Protein Carbonyl Content

Protein carbonyl contents in plasma were evaluated using DNPH assay with slight modifications [23]. The plasma containing 1 mg protein was precipitated with 10% TCA, (4:1, w/v) and centrifuged at 4°C for 5 min at 11000×g. Supematant 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 make easy the reaction with proteins. Proteins were precipitated with 200 µl of 50% TCA 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), the insoluble materials were removed by centrifugation and dissolved in 0.6 ml of 6 M guanidine hydrochloride at 37°C for 15 min. After centrifugation at 4°C for 5 min at 11000×g, the absorbance of the supernatant was measured at 370 nm. Carbonyl group content was calculated using a molar absorption coefficient of 2.2×10\(^4\) M\(^{-1}\)cm\(^{-1}\) and expressed as µmol/mg protein.

2.7 Total Thiols in Plasma

This procedure assays total thiol (P-SH and GSH) in plasma using spectrophotometric method with slight modifications [9]. Briefly, an
aliquot of plasma (50 µl) is mixed in a 1.5-ml micro tube with 150 µl of the Tris-EDTA buffer followed by the addition of 10 µl of 10 mM DTNB and 790 µl of absolute methanol. The test tube is capped, and the color was extended for 15-20 min, followed by centrifugation at 3000×g for 10 min at room temperature. The absorbance of the supernatant is measured at 412 nm (A) and subtracted from a DTNB blank (B) and a blank containing the sample without DTNB (C). Total SH groups are conveniently calculated using a molar absorption of 13,600 M⁻¹ cm⁻¹ as follows:

\[(A - B - C) \times \frac{1.0/0.05}{13.6} = (A - B - C) \times 1.47 \text{ mM}\]

2.8 Lipid Peroxidation Measurement

The TBA assay was taken from Ohkawa [24] and Buege [25] and adapted for our purpose. Briefly, 200 µl of plasma was suspended in 800 µl of reagent with 15% w/v TCA, 0.375% w/v TBA and 0.25 N HCl. The solution was heated in a boiling water bath for 30 min. After cooling, the precipitate was removed by centrifugation for 10 min at 3500×g. Absorbance was measured at 535 nm. Tetramethoxypropane was used as an external standard, and the level of TBA reactions was expressed as µmol of malondialdehyde.

2.9 Statistical Analysis

Results are presented as mean±SEM for at least two repeats of the experiment. Statistical significance was assessed by one way ANOVA, followed by LSD and Tukey’s post-hoc test. A P value of <0.05 was considered as statistically significant.

3. RESULTS AND DISCUSSION

3.1 Biochemical Parameters

As shown in Table 1, serum levels of TB and ALP were markedly increased in BDL rats as compared to that of the SO group, indicating the presence of biliary cirrhosis as a result of BDL, whereas serum Alb and ALT levels did not show any difference between the groups. The results showed that NTX could significantly reverse the increased ALP activity (258.16±23.38 vs 396.93±41.29; P<0.05) and the TB content (0.93±0.06 vs 5.73±0.571; P<0.05) in BDL+NTX group; revealing protective and to some extent, the curative effect of NTX against the damage caused by BDL. Treatment with L-NAME+NTX caused a significant increase in the Alb, but had no effect on the AST and ALT levels compared to BDL group. It can be seen from the data in Table 1 that BDL rats showed a significant increase in FRAP level as compared with a SO control group (P<0.05). Treatment with NTX caused a significant decrease in the FRAP level as compared to BDL group (P<0.05). Nevertheless, at the end of the experiment, FRAP level was significantly higher in the BDL+L-NAME group as compared to the related SO, BDL and BDL+NTX groups (P<0.05). In addition, there was no evidence of mortality attributable to the doses of NTX, L-NAME and NTX+L-NAME used in this study.

3.2 Histological Study

The liver of SO rats showed normal histology changes. In BDL groups, however, portal inflammation, moderate fibrosis and bile duct proliferation were seen. Meanwhile, portal inflammation and bile duct proliferation were diminished in the L-NAME and NTX-treated groups as compared with the BDL group (Fig. 1).

3.3 Plasma Protein Carbonyl

Fig. 2 shows that plasma protein carbonyl content was slightly increased in the BDL group as compared with SO control. After 4 weeks of intervention, protein carbonyl content was significantly lower in BDL+L-NAME+NTX group as compared to the related SO, BDL and BDL+L-NAME groups (P<0.05).

3.4 Plasma Total Thiol Content

As can be seen in Fig. 3, plasma total thiol content was slightly decreased in the BDL group as compared with relevant SO control. However, administration of NTX caused a significant increase in total thiol content as compared to BDL+L-NAME+NTX group (P<0.05).
| Parameter | Saline | L-NAME | NTX | L-NAME+NTX |
|-----------|--------|--------|-----|------------|
| ALP (IU/L)| 215.58±15.25 | 396.93±41.29<sup>ab2,3</sup> | 237.83±34.39 | 214.77±18.17 |
| AST (IU/L)| 100.02±10.83 | 61.25±9.99<sup>ab2</sup> | 62.65±3.02 | 104.07±8.46<sup>ab1,3,4</sup> |
| ALT (IU/L)| 44.82±3.42 | 40.74±3.76<sup>b2</sup> | 30.56±5.10 | 23.13±1.01 |
| TB (mg/dL)| 1.17±0.133 | 5.73±0.571<sup>ab2,3</sup> | 1.97±0.062 | 0.45±0.076 |
| Alb (g/dL)| 4.18±0.137 | 3.95±0.114<sup>b2,4</sup> | 4.01±0.119 | 3.95±0.084 |
| FRAP (µmol/L)| 276.06±22.1 | 468.9±19.4<sup>ab2,3,4</sup> | 286.5±15.1 | 255.2±10.7 |

Table 1. Biochemical and FRAP tests at the end of the experiment

Table 1. Biochemical tests and the ferric reducing ability of plasma (FRAP) assay after the completion of the study using sham-operated (SO), Naltrexone (NTX), and Nω-Nitro-L-arginine methyl ester (L-NAME) treated BDL cirrhotic rats (Test), each value represents mean±SEM (n=7) and experiments performed in duplicate.

<sup>a</sup>Significantly different from SO, P-value<.05.  <sup>b</sup>Significantly different from BDL cirrhotic rats (Test) group, P-value<.05
Fig. 1. Representative hematoxylin/eosin-stained sections of rat liver specimens in (a) sham-operated (SO) (b) BDL (c) BDL+NTX (d) BDL+L-NAME and (e) BDL+NTX+L-NAME groups (scale bar, 0.1 mm)

Fig. 2. Protein carbonyl content after the completion of the study using sham-operated (SO), Naltrexone (NTX), and Nω-Nitro-L-arginine methyl ester (L-NAME) treated BDL cirrhotic rats (Test)

*Significantly different from SO, P-value<0.05.
^Significantly different from BDL cirrhotic rats (Test) group, P-value<0.05

3.5 Plasma Lipid Peroxidation

As shown in Fig. 4, the results demonstrated a significant increase in MDA level in the BDL group as compared to SO rats (P<0.05). Treatment of NTX in BDL rats during the study could significantly decrease the MDA production (P<0.05) as compared to BDL group. MDA production significantly increased in BDL+L-NAME group as compared to related SO, BDL+NTX and BDL+L-NAME+NTX, also the results indicate a significant increase in MDA level in the BDL+NTX and BDL+L-NAME+NTX as compared to related SO rats (P<0.05).

Cholestasis can be described as an impairment of the bile flow. As a result, the bile acids, bilirubin and other cholephils release into the liver and blood, elevated levels of bile acids in the liver can cause apoptosis or necrosis of...
hepatocytes and ultimately lead to cirrhosis [26]. In this study, NTX (opioid receptors blocker) and L-NAME (a non-specific inhibitor of NOS isoenzymes) were used to manipulate nitrergic and opioidergic systems in BDL cirrhotic rats. The main test of cholestasis is the elevation of total bilirubin in the serum. Although its mechanism is not perfectly known, this increase may be due to the weakened tight junctions between hepatocytes in the BDL rats that diminish excretion of total bilirubin, resulting in further efflux back into the serum [27]. These results are in accord with the histological documents provided by H&E staining of the liver tissues from the experimental and control groups which showed the formation of fibrotic scars and inflammation; the signs which are characteristic for liver cirrhosis. Administration of opioid receptor blocking agent in BDL group significantly decreased ALP activity and total bilirubin level in the plasma, which confirms the antioxidative effects of NTX and its role in protecting cellular membranes. The serum albumin level is generally reduced in liver cirrhosis [28] for the reason that, albumin not only is a regulator of plasma oncotic pressure, but also prevents the development of edema by exerting balance between hydrostatic and colloid osmotic pressure within blood vessels [29].

Assay of total anti-oxidant capacity measures the endogenous antioxidants such as bilirubin, reduced GSH and uric acid. It also measures several nonenzymatic antioxidants present in blood; for instance polyphenols, ascorbic acid, α-tocopherol and β-carotene [8]. One unanticipated finding was that BDL rats showed a significant increase in FRAP level as compared with SO control. The higher levels of FRAP in rats with liver cirrhosis may be due to increase in the level of plasma bilirubin and other endogenous antioxidants.

![Fig. 3. Total thiol after the completion of the study using sham-operated (SO), Naltrexone (NTX), and Nω-Nitro-L-arginine methyl ester (L-NAME) treated BDL cirrhotic rats (test)](image)

*Significantly different from SO, P-value<0.05.
*Significantly different from BDL cirrhotic rats (Test) group, P-value<0.05.

In our study bile duct ligated rats showed a slight increase in plasma protein carbonyl content as compared with SO control. These results seem to be consistent with other researches which not found a significant difference in serum protein carbonyl content between control and cirrhosis patients [30]. However, our previous studies indicated that protein carbonyl content was increased in RBCs membrane of cirrhotic rats [31,32]. Nonetheless, the exact situation in RBC membrane does not necessarily reflect the situation in the plasma. After 4 weeks of intervention with opioid receptor blocker and NOS inhibition in BDL group, protein carbonyl content was significantly lower as compared to the related SO, BDL and BDL+L-NAME groups (P<0.05). The presence of carbonyl groups in proteins is a marker of ROS mediated protein oxidation. Carbonylated proteins may interfere with the normal homeostasis of cell growth inducing liver cirrhosis [8].

Lipid peroxidation which produces MDA, is one of the critical reactions involved in the oxidative worsening of poly-unsaturated lipids [33]. In this study, MDA levels were significantly increased in cirrhotic rats as compared to control. MDA of BDL groups was increased 1.66 and 1.52-folds compared to related SO and BDL+NTX groups.
and its production significantly decreased in BDL+L-NAME+NTX and BDL+NTX group as compared to BDL+L-NAME ($P<0.05$), which confirms the involvement of the opioid system in oxidative effect during cirrhosis.

All of the plasma sulfhydryl (SH) groups are related to proteins. Plasma SH groups are susceptible to oxidative damage and are frequently low in patients suffering from diseases for instance rheumatoid arthritis and coronary artery disease. Plasma contains protein SH (P-SH) groups and small amounts of glutathione (GSH) [9,34]. The current study found that plasma total thiol content was slightly decreased in the BDL group as compared with SO control. Treatment with NTX caused a slight increase in the total thiol content compared to BDL group. Some studies found a significant universal protein sulfhydryl depletion during hepatotoxicity [35] or no significant change in protein sulfhydryl status [36]. NTX could transiently increase total thiol in plasma. Previous studies have proven that chronic administration of NTX in cholestatic rats recovered the amount of liver GSH significantly, and has protective effects against oxidative damage by blocking the opioid receptors [14,20]. Another study showed that cerebroventricular injection of an opioid agonist decreased hepatic GSH synthesis [12], suggesting the participation of endogenous opioids in regulation of redox state of hepatocytes [20]. Modifications 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 [37]. Opioid receptor blocker has beneficial effects on hepatic injury by ameliorating the oxidative stress and consequently pathologic alterations.

**4. CONCLUSION**

The results of the present study showed that nitrergic and opioidergic systems have various impacts in the oxidative stress induced by BDL rats. Opioid receptor blockage could significantly decrease the MDA production and protein carbonyl content, and also could slightly increase total thiol as compared to BDL group by decreasing endogenous opioids and moderately prevent the severe liver injury. NO inhibition had no effect on the protein carbonyl content, total thiol and MDA level, so we can conclude that nitrergic systems have not significant impact on these markers in BDL rats.

**CONSENT**

It is not applicable.

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**COMPETING INTERESTS**

Authors have declared that no competing interests exist.

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