

# The Possible Role of Nitric Oxide and Oxidative Stress in the Enhanced Apoptosis of Cardiac Cells in Cirrhotic Rats

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**Abstract-** Cirrhosis has been related with hyperdynamic circulation, manifesting as increased cardiac output and decreased systemic vascular resistance. In the present study we examined the cirrhosis outcome on apoptosis of rat hearts. We also tried to explore the role of nitric oxide (NO) and oxidative stress in the probable changed apoptosis of cirrhotic hearts. Twenty eight days after ligation of bile duct, heart tissues were tested for apoptosis. The extent of malondialdehyde (MDA), and the activities of catalase (CAT), glutathione peroxidase (GSHPx) and superoxide dismutase (SOD) have been calculated in heart tissues. The cirrhotic hearts exhibited structural defects and greater apoptosis. Chronic treatment of cirrhotic rats with L-NAME, a non-selective inhibitor of NO synthase, inhibited heart structural defects and reduced apoptosis of hearts. We also showed that cirrhotic rat hearts had an enhanced level of MDA and reduced activities of CAT, GSHPx and SOD. When the animals were treated by L-NAME chronically, the MDA level reduced and activities of CAT, GSHPx and SOD augmented in cirrhotic heart. In conclusion, increased apoptosis of cirrhotic hearts probably happen due to NO overproduction and increased oxidative stress in hearts of cirrhotic rats.

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## Introduction

Cirrhosis is accompanying with cardiovascular abnormalities. The circulation becomes hyperdynamic, well-defined as increased cardiac output and decreased peripheral vascular resistance and blood pressure. The peripheral vasodilatation has also been reported in local vascular beds for instance the mesenteric/splanchnic, pulmonary, renal, and skeletal muscle. In spite of the augmented baseline cardiac output, the ventricular contractile response to stimuli is reduced, this state called cirrhotic cardiomyopathy (1). Reduced systolic and diastolic responses to stress, electrophysiological irregularities, including prolongation of repolarization (increased electrocardiographic QT interval), and

cardiac chamber hypertrophy or enlargement have been observed in cirrhotic cardiomyopathy (1,2).

Nitric oxide (NO) was suggested as a mediator of splanchnic vasodilatation in portal hypertension in 1991 (3). Since then several evidences have been reported from human and experimental models of cirrhosis that alteration of NO activity influences diverse vascular beds in different methods. Cirrhotic patients have augmented plasma levels of nitrites and nitrates which are NO degradation products (4). In our previous paper, we showed that the basal abnormalities and the decreased chronotropic and inotropic responses to isoproterenol were improved by treatment with L-NAME, a non-selective inhibitor of NO synthase (NOS) in cirrhotic rats, suggesting the role of NOS in these

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events (5). Chronic L-NAME administration also corrected the QT prolongation in cirrhotic rats (6) and restored the susceptibility of cirrhotic and cholestatic rats to arrhythmias (6,7).

Oxidative stress has been known as essential factor in the promotion of chronic liver damage of various etiologies to liver cirrhosis. These etiologies include alcoholic liver disease, nonalcoholic steatohepatitis, chronic viral hepatitis and cholestatic diseases (8,9). Earlier studies have proved that the activity of liver antioxidant systems is decreased with disease severity (10,11). Gut-derived endotoxemia owing to gut barrier failure plays a serious role in the progression of the hepatic injury. The hepatic macrophage cells (Kupffer) exert essential role in this process that are stimulated by endotoxin to release several cytokines and proinflammatory mediators (12). There are several reports showed that treatment with antioxidants is helpful in preventing this process by direct antioxidant effects in liver tissue or through decrease of intestinal endotoxemia (12,13). Other reports showed that antioxidants similar L-NAME and N-acetylcysteine reduced cardiac nitrotyrosine levels leading to normalization of cardiac function in cirrhosis (14).

In the current study we are trying to find if cirrhosis have any effect on cardiac apoptosis and whether L-NAME, a non-selective inhibitor of NOS, could change apoptosis in cirrhotic rat hearts.

## Materials and Methods

### Animal

Male Sprague-Dawley rats weighing 200-250 g were used in this study. The rats were handled in agreement with the standards defined in the "Guide for the Care and Use of Laboratory Animals" (NIH US publication 86-23 revised 1985 and accepted by Shiraz University of Medical Sciences). The animals were kept in standard polycarbonate cages in a temperature-controlled room ( $24\pm 2^\circ$  C) with 12 h light/12 h dark cycle with free access to food and water.

### Animal model of cirrhosis

The animals were randomly divided into 4 groups; each group consisted of ten rats. (1) sham-operated controls treated with saline, (b) sham-operated controls treated with L-NAME (10 mg/kg), (c) cirrhotic animals treated with saline, (d) cirrhotic animals treated with L-NAME (10 mg/kg). In brief, bile duct ligation was done as defined before (15). Laparotomy was done under anesthesia (ketamine HCl, 50 mg/kg, i.p. and xylazine

10 mg/kg, i.p.). The bile duct was recognized, manipulated and one untied loose tie was left in place in the sham rats, however, in cirrhotic rats, the bile duct was doubly ligated. Then the abdominal wall was closed in two layers. Four weeks after the surgery, cirrhotic rats showed jaundice, muscle wasting, ascites and liver failure. This is a well-known rat model of biliary cirrhosis, and the liver histology and serum biochemistry characteristic of cirrhosis and liver failure have formerly been recognized (16,17). The groups received intraperitoneal injections of L-NAME (10 mg/kg) or saline once daily for 7 days during the fourth week after the surgery.

### Heart haematoxylin and eosin staining

For histological staining, formalin-fixed slices of the cardiac lobes were cut into 3 mm sections. Haematoxylin-eosin (H and E) was used for staining. A pathologist who was blinded to the information of groups and laboratory data evaluated the histology.

### Apoptosis of heart cells (Terminal deoxynucleotidyl transferase dUTP nick end labeling assay)

Twenty eight days after bile duct ligation or sham surgery, rats' cardiac tissues were fixed in 10% formalin, and then tissues were embedded in paraffin and sectioned at 5 mm. The slides were processed for a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay. An ApopTag in situ detection kit (Roche Diagnostics, Mannheim, Germany) was used according to the producer's guidelines. The slides were treated with hydrogen peroxide ( $H_2O_2$ ) and incubated with the reaction mixture containing TdT and digoxigenin-conjugated dUTP for 1 h at  $37^\circ$  C. Labelled DNA was visualized with peroxidase-conjugated anti-digoxigenin antibody using 3, 3'-diaminobenzidine (DAB) as the chromogen. Rat testicular tissue was used as positive control (18). For negative control, TdT was omitted from the reaction mixture. At least 10 random sections of each heart were quantified. Values were expressed as the number of TdT-labelled nuclei per 106 nuclei.

### Malondialdehyde determination

Tissue MDA was dignified using the thiobarbituric acid-reactive substance assay, as defined by Ohkawa *et al.*, (19). As an external standard, 1,1,3,3-tetramethoxypropane was used and the levels of lipid peroxides are expressed as nanomoles of MDA per gram wet tissue.

### Catalase, glutathione peroxidase and total superoxide dismutase determination

Tissue sections were homogenized in triton X-100 (1%), and the homogenates were diluted with potassium phosphate buffer. The reaction was initiated by adding  $H_2O_2$  to the reaction mixture, and the extent of enzyme activity was quantitated based on the ability of tissue CAT to decompose  $H_2O_2$  by monitoring the reduction in absorbance at 240 nm. By measuring the absorbance changes after 1 min, CAT activity was calculated and expressed as nmol per mg protein.

GSHPx enzymes catalyse the reduction of  $H_2O_2$  to water using glutathione (GSH) as a reducing reagent, the. In the assay, oxidized GSH is reduced to GSH by the enzyme GSH reductase, which oxidizes NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) to NADP in the catalytic cycle. The alteration in absorbance at 340 nm subsequent the oxidation of NADPH is the base of quantitating tissue GSHPx activity. GSHPx activity was calculated and stated as nmol per mg protein.

Superoxide anions were produced from manganese (II) chloride and mercapthoethanol in the existence of acid-EDTA. The extent of SOD was calculated according to its capability to inhibit NADH oxidation in the reaction mixture after the adding tissue homogenate. NADH oxidation was calculated by checking the diminution in absorbance at 340 nm during the reaction. Total SOD activity was stated as units per mg protein.

### Statistical analysis

Data were presented as mean $\pm$ SEM. One-way ANOVA followed by Tukey multiple comparisons were used to analyse the data. Differences were considered to be significant at  $P<0.05$ .

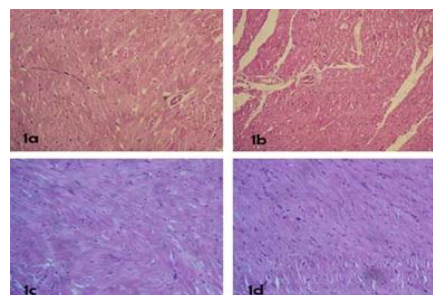
## Results

### Haematoxylin and eosin staining of the heart tissues and cardiac cell apoptosis

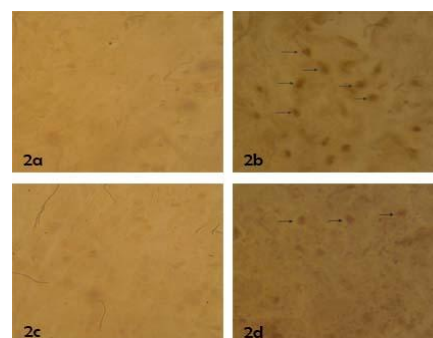
Cirrhotic hearts presented essential abnormalities when compared with sham hearts (Figures 1a,1b). Moreover, chronic administration of L-NAME did not induce any structural changes in the hearts of sham animals (Figure 1c) while structural abnormalities in the heart of cirrhotic rats were prevented by chronic L-NAME administration (Figure 1d).

TUNEL positive cells were rarely recognized in sham rat hearts. Apoptosis of cardiac cells were greater in cirrhotic rats compared with sham ones ( $P<0.01$ ); but L-NAME administration decreased apoptosis in cirrhotic

rat hearts ( $P<0.01$ ). Chronic treatment with L-NAME did not alter the extent of apoptosis in cardiac sham animals (Figures 2,3).

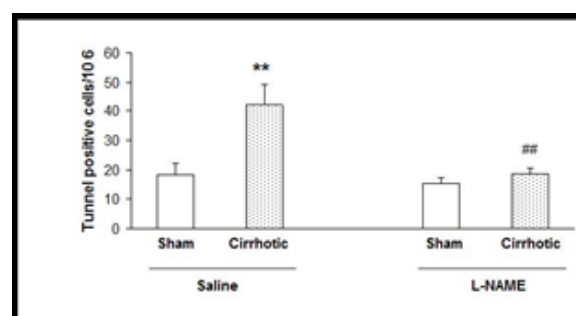


**Figure 1.** Gross morphology in rat hearts. The pictures presenting haematoxylin and Eosin (H and E) staining of heart tissues on day 28 in (a) sham group, (b) cirrhotic group, (c) L-NAME-treated sham group and (d) L-NAME-treated cirrhotic group. The rats received L-NAME (10 mg/kg) or saline once daily for 7 days during the fourth week after the bile duct ligation surgery.



**Figure 2.** Apoptosis of heart cells

Pictures showing TUNEL-positive nuclei on day 28 in (a) sham group, (b) cirrhotic group, (c) L-NAME-treated sham group and (d) L-NAME-treated cirrhotic group. Arrows show TUNEL-positive nuclei. The groups received L-NAME (10 mg/kg) or saline once daily for 7 days during the fourth week after the bile duct ligation surgery.

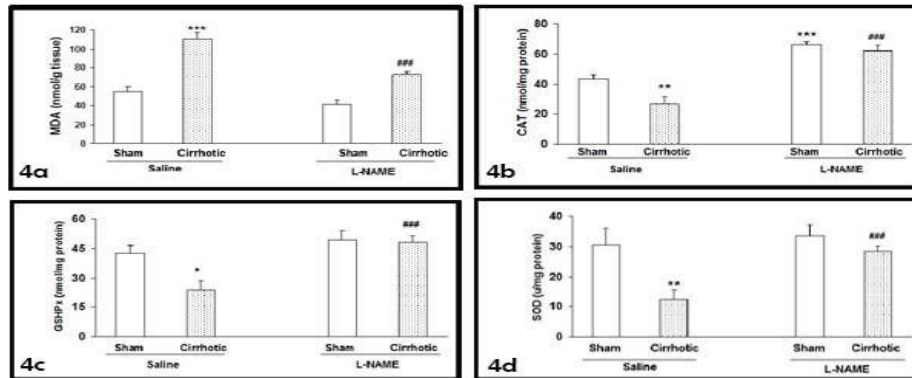


**Figure 3.** Cardiac cell apoptosis using TUNEL assay in sham and cirrhotic animals after treatment with saline or L-NAME. \*\* $P<0.01$  compared with corresponding sham group and ## $P<0.01$  compared with saline-treated cirrhotic group.

**Lipid peroxidation and antioxidant enzyme activities**

Cirrhotic rats showed higher level of malondialdehyde in their hearts ( $P<0.001$ ). Administration of L-NAME decreased MDA levels in cirrhotic group ( $P<0.001$ ) (Fig. 4a). The activities of CAT, GSHPx and SOD in cirrhotic

hearts reduced compared with sham ones. Treatment with L-NAME increased the activities of CAT in sham hearts ( $P<0.001$ ). Besides that L-NAME treatment enhanced CAT, SOD and GSHPx activities in cirrhotic rat hearts significantly ( $P<0.001$ ) (Figures 4b,4c,4d).



**Figure 4.** a) Malondialdehyde levels (MDA) and antioxidant enzyme activities: Catalase (CAT) (b), Glutathione Peroxidase (GSHPx) (c) and Superoxide Dismutase (SOD) (d) in rat cirrhotic and sham hearts. Data are expressed as mean±SEM.\*  $P$ .value<0.05, \*\*  $P$ .value<0.01 and \*\*\*  $P$ .value< 0.001 compared to saline-treated sham group and ###  $P$ .value< 0.001 compared to saline-treated cirrhotic group.

**Discussion**

In the present study we demonstrated that cirrhotic hearts presented structural abnormalities and increased apoptosis compared with sham hearts. Chronic administrations of L-NAME in cirrhotic rats not only improved heart structural abnormalities but also ameliorated enhanced-apoptosis of cirrhotic hearts. Cirrhotic rat hearts also indicated an increased level of MDA and decreased activities of CAT, GSHPx and SOD. In chronic L-NAME administration, the extent of MDA reduced and activities of CAT, GSHPx and SOD augmented in cirrhotic heart rats .

In cirrhosis, cardiac output increases while systemic vascular resistance and arterial pressure drop (20,21). Although there is the enhanced basal cardiac output, cardiac response to physiologic or pharmacologic stimuli is defined to be subnormal (1,22), a phenomenon called "cirrhotic cardiomyopathy" (23). Systolic and diastolic dysfunction, electrophysiological changes, and macroscopic and microscopic structural alterations have been defined in cirrhotic cardiomyopathy. The results of our study are in line with previous reports. The results of H&E staining confirmed structural abnormalities in cirrhotic hearts. Moreover cardiac cell apoptosis was significantly higher in cirrhotic rats compared with sham ones.

The role of nitric oxide in the pathophysiology of cardiac dysfunction in cirrhosis was first reported in

1996 (24). Following reports by Lee *et al.*, (25) have demonstrated that serum cytokine levels are enhanced in cirrhotic rats, and that the negative inotropic effect of interleukin-1β could be reversed by pre-incubation of isolated papillary muscle with an NO synthase (NOS) inhibitor. Similarly, we have reported that elevated NO production in bile duct-ligated (BDL) animals induces bradycardia (26,27). Another study indicated that abnormal cardiac chronotropic function in cirrhosis is associated with increased nitration of cardiac proteins. In this study we indicated that structural abnormalities in the heart of cirrhotic rats were prevented by L-NAME administration. In addition, L-NAME treatment reduced apoptosis in cirrhotic rat hearts. These results approve the role of nitric oxide in structural abnormalities of cirrhotic hearts.

Patients with cirrhosis have increased plasma levels of nitrites and nitrates which are the NO degradation products (4). There is also evidence of increased eNOS (28,29), iNOS (30) or nNOS (31) upregulation in the systemic circulation of cirrhotic subjects. It has been demonstrated that exhaled air from cirrhotic patients contains elevated levels of NO compared with controls. The augmented content of NO associates with the severity of disease and degree of hyperdynamic circulation (32). One of essential results of the increased levels of NO is its cellular toxicity. The direct toxicity of NO per se is modest, but is greatly increased on reaction with superoxide anions ( $O_2^-$ ) to form peroxynitrite

(ONOO<sup>-</sup>). Although this reaction was viewed initially as a route for NO inactivation, the formation of peroxynitrite transforms two relatively unreactive free radicals, 'NO and O<sub>2</sub><sup>-</sup>', into much more bioreactive species. The formation of peroxynitrite depends on the concentration of both 'NO and O<sub>2</sub><sup>-</sup>' and, therefore, on the activities of both NOS and superoxide dismutase (33).

Oxygen-derived free radicals (among the more important of these is O<sub>2</sub><sup>-</sup>), and consequently lipid peroxidation, are clearly enhanced in liver diseases. Under these conditions, peroxynitrite is readily formed from the reaction between O<sub>2</sub><sup>-</sup> and 'NO. This reaction is at least three times faster than dismutation by superoxide dismutase (34).

In the current study we indicated that cirrhotic rat hearts presented increased level of MDA and decreased activities of CAT, GSHPx and SOD. These data are similar with several previous studies which have been demonstrated the role of oxidative stress in liver injury (10,11,35). In chronic administration of L-NAME, the level of MDA decreased and activities of CAT, GSHPx and SOD increased in cirrhotic heart rats which support the role of nitric oxide in these abnormalities.

Nitric oxide induces apoptosis by modulating multiple sites along the signaling pathways. In the death receptor pathway, NO increases the Fas expression and the ceramide production, which leads to caspase activation. In the mitochondrial pathway, NO increases the Bax/Bcl-2 ratio, resulting in an increased mitochondrial permeability and cytochrome c release. NO also inhibits the electron transport chain complex I–III to reduce energy production and increase p53, both of which contribute to the pro-apoptotic effects of NO. In addition, NO increases caspase activity through downregulation of IAP (36). In the present research we demonstrated that L-NAME treatment could reverse cirrhosis-induced heart apoptosis in rats which confirm the role of nitric oxide in cirrhotic hearts apoptosis.

In conclusion, cirrhotic hearts demonstrated structural abnormalities and apoptosis, which are improved by L-NAME treatment, suggesting the role of increased NO overproduction in this effect. Besides that increased levels of MDA and decreased levels of CAT, GSHPx and SOD have been observed in cirrhotic hearts which are reversed by L-NAME treatment, indicating the possible role of NO.

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