

# Opioid Receptors Blockade Modulates Apoptosis in a Rat Model of Cirrhotic Cardiomyopathy

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## Abstract

**Background:** Cirrhosis is a common consequence of chronic liver inflammation is known to be associated with various manifestation of cardiovascular dysfunction, which has been introduced as a cirrhotic cardiomyopathy. Some possible pathogenic mechanisms has been reported and still more details should be explored. **Aim:** The present study is the first study to explore the contribution of endogenous opioids in the apoptosis process in a rat model of cirrhotic cardiomyopathy. **Materials and Methods:** Cirrhosis was induced in rats by bile duct ligation (BDL) and resection. Cardiomyopathy was confirmed using trichrome staining for fibrosis. Naltrexone, an opioid antagonist was administered for 29(1) days. Apoptosis was detected using terminal transferase deoxyuridine triphosphate nick end labeling assay with some modification. Statistical evaluation of data was performed using analysis of variance test.  $P < 0.05$  was considered to be statistically significant. **Results:** Left ventricular (LV) wall thickness was significantly ( $P < 0.001$ ) lower in the BDL group than the sham group, either receiving naltrexone or saline. No significant difference was seen in LV wall thickness or LV end diastolic diameter in BDL group receiving either saline or naltrexone. The apoptosis density of cardiac specimens of sham operated and BDL rats were dramatically different from each other. The cardiac specimens of BDL rats contained multiple apoptotic cells. In saline treated samples (BDL-saline vs. sham-saline), apoptosis density was significantly increased in BDL-saline group ( $P < 0.001$ ). Cardiomyocyte apoptosis was significantly decreased in the BDL-naltrexone group compared to BDL-saline group ( $P < 0.001$ ). There was no significant change in apoptosis density in sham groups receiving either naltrexone or saline. **Conclusion:** Apoptosis occurs during cirrhotic cardiomyopathy and endogenous opioid receptors blockade using naltrexone decreases its amount, but cardiac function may not be improved.

**Keywords:** Apoptosis, Cirrhotic cardiomyopathy, Naltrexone, Opioids

## Introduction

Cirrhosis is a common consequence of chronic liver inflammation characterized by replacement of liver tissue by fibrous scar and regenerative tissue, leading to a progressive loss of liver function.<sup>[1]</sup> Cirrhosis is known to be associated with various cardiovascular abnormalities, including increased cardiac output, decreased arterial pressure and total peripheral resistance, systolic incompetence, diastolic dysfunction and

electrophysiological abnormalities in the absence of any known cardiac disease. These abnormalities are named as a clinical entity of cirrhotic cardiomyopathy.<sup>[2-4]</sup>

Over-activation of endogenous opioids system contributes to impaired cardiac contractility in response to both  $\alpha$  and  $\beta$ -adrenoceptors stimulation and inhibiting opioid system pharmacologically correct bradycardia, reduced chronotropic and inotropic response in cholestatic rats.<sup>[5-7]</sup> Endogenous opioids can attenuate apoptosis of hepatocytes in a rat model of cholestasis and reducing liver antioxidant defense system was hypothesized as a mechanism through, which increased level of opioid system lead to hepatocytes damage and apoptosis.<sup>[8]</sup>

Recent studies have shown apoptosis has a causal role in various cardiac pathologies including acute myocardial infarction, ischemic-reperfusion damage and cardiomyopathies and several mediators such as endogenous cannabinoids, nitric

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oxide (NO) and carbon monoxide (CO) activate apoptosis pathway.<sup>[9,10]</sup>

Given the increasing data showing accumulation of endogenous opioids in cholestasis and their various roles, e.g., in angiogenesis pathway during cholestasis, this study was to explore the effect of endogenous opioids on apoptosis of cardiomyocytes in a rat model of cirrhotic cardiomyopathy.

## Materials and Methods

### Animals and procedures

Adult male Sprague–Dawley rats (230–250 g, Pasteur Institute of Iran, Tehran, Iran) were used in this study. Rats were housed in a temperature controlled vivarium with a 12:12 h light–dark cycle and had free access to rat chow and water. All animal procedures were in accordance with Guide for the Care and Use of Laboratory Animals (National Institutes of Health US publication no. 85-23 revised 1985). All experiments were performed in agreement with the ethical considerations, recommended by the Pasteur Institute of Iran. The 24 rats were randomly divided in two groups: Sham group and bile duct ligation (BDL) group. Each group was also divided in two subgroups in order to treat with either naltrexone (kindly provided by Francopia-Sanofi Chimie, Aramon, France) or saline. Each of the four groups contained six rats. BDL was performed as described, previously.<sup>[9]</sup> Briefly, under general anesthesia, the common bile duct was exposed through a midline abdominal incision under sterile condition, ligated in two places with a silk thread and sectioned between the ligatures. In the sham-operated rats, the bile duct was manipulated and no ligation was performed. Finally, the abdominal wall was closed in two layers. BDL and resection is a standard animal model of cholestasis and the liver histology and serum biochemistry typical of liver injury have been previously documented in detail.<sup>[11]</sup> The naltrexone (1 mg/kg/day) or normal saline was administrated from the day of surgery for 29(1). As cirrhosis occurs 4 weeks after BDL in comparison with the normal liver in this model, the rats were sacrificed 29(1) day after operation [Figure 1].

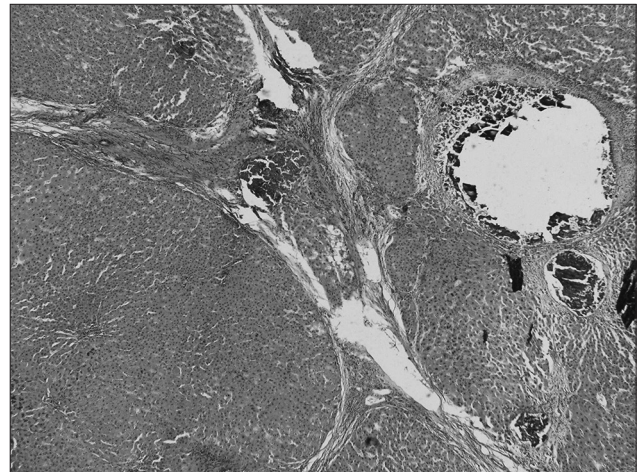
### Drug administration and sample collection

In the BDL-naltrexone and sham-naltrexone groups, naltrexone (20 mg/kg/day; s.c.) was injected and the BDL-saline and sham-saline groups received a daily injection of sterile saline solution for 29(1) days following sham and BDL procedures. The rats were killed with an overdose of sodium pentobarbital and cardiac samples were collected and immersed in phosphate buffered formalin as a fixative and then embedded in paraffin. Then, 5 µm sections were obtained for specific staining.

### Cardiomyopathy determination

#### *Ventricular wall thickness*

After 4 weeks, the rats were sacrificed and sampling was performed. Left ventricular (LV) wall thickness was measured at the base of papillary muscle level in all samples and compared



**Figure 1:** Histopathologic evaluation of liver sample from bile duct ligation group, showing fibrotic bundles and also bile plugs, confirming cirrhosis development

with each other. Significantly decreased LV wall thickness was considered as determinants of cardiomyopathy development.<sup>[12]</sup>

### Pathologic evaluation

Hematoxylin and eosin (H and E) and Masson's trichrome staining methods were performed on the prepared slides for cardiomyopathy determination. Attenuated, stretched and irregular cardiomyocytes in H and E staining and presence of focal fibrosis in Masson's trichrome staining were considered as evidences of cardiomyopathy.<sup>[13]</sup>

### Analysis of apoptosis and specific staining

Staining was performed using APO-bromodeoxyuridine (BRDU) terminal transferase deoxyuridine triphosphate nick end labeling (TUNEL) assay kit (Invitrogen Corporation, CA, USA) with some modifications. Briefly, after deparaffinization, slides were treated with proteinase K for 15 min and rinsed in rinsing buffer (included in the APO-BRDU kit) shortly. Then treated with deoxyribonucleic acid labeling solution containing bromodeoxyuridine triphosphate and terminal deoxynucleotidyl transferase enzyme (prepared according to manufacturer's protocol) and then incubated with anti BRDU mAb (included in the APO-BRDU kit) for about 45 min in 37°C. For visualizing the attached antibodies, EnVision solution (DAKO Corporation, Glostrup, Denmark) and diaminobenzidine solution (DAKO Corporation, Glostrup, Denmark) were used. Non-specific binding was inhibited using non-immune serum. After extensive washing, the slides were mounted and evaluated under the light microscope. The number of apoptotic cells in 10 high power fields of hot spot areas in light microscopy was calculated in each specimen. Human reactive lymph node tissue was used as a positive control for apoptosis.

### Statistical analysis

The results are expressed as mean (standard error of the mean). Statistical analysis was performed using SPSS

version 16.0 (SPSS Inc., Chicago, IL, USA). Normality of data was evaluated with the Kolmogorov-Smirnov test and statistical evaluation of data was performed using analysis of variance, followed by the Tukey's *post hoc* test.  $P < 0.05$  was considered to be statistically significant.

## Results

### Histologic evaluation of cardiomyopathy

All BDL rats and none of rats in sham operating group (either receiving naltrexone or saline) revealed changes compatible with cardiomyopathy in histologic examinations. The amount of fibrosis in cardiac biopsies of BDL rats was obvious in Masson trichrome staining and naltrexone injection decreased the amount of fibrotic bundles [Figure 2a-c].

### LV wall thickness

LV wall thickness was measured in all groups as an indicator of cardiac LV function. LV thickness in BDL rats and sham operated rats were 2.54 (0.16) and 3.75 (0.1) mm, respectively and this difference was statistically significant ( $P < 0.001$ ). There was no significant difference between LV thickness in sham operated rats receiving either saline or naltrexone (3.8 [0.2] vs. 3.6 [0.1] mm). Within BDL group, no significant difference was identified between LV wall thickness in saline and naltrexone receiving rats (2.26 [0.26] vs. 2.75 [0.14] mm), although the wall thickness was lower in BDL-saline group than BDL-naltrexone group ( $P = 0.1$ ).

### Evaluation of apoptosis density

The apoptosis density of cardiac specimens of sham operated and BDL rats were dramatically different from each other [Figure 3]. The cardiac specimens of BDL rats contained multiple apoptotic cells. On the other hand, cardiac muscle specimens of sham-operated rats largely consisted of very few apoptotic cells. Indeed, in saline treated samples (BDL-saline

vs. sham-saline), apoptosis density was significantly increased in BDL-saline samples ( $P < 0.001$ ).

There was no significant change in apoptosis density in sham groups receiving either naltrexone or saline ( $P = 0.15$ ). As identified by specific staining (TUNEL assay), cardiac myocyte apoptosis density was significantly decreased in BDL-naltrexone group compared with BDL-saline group ( $P < 0.001$ ). Figure 4a-c shows a representative picture of apoptotic cardiac myocytes in sham and BDL groups receiving naltrexone and saline after specific staining for apoptosis.

## Discussion

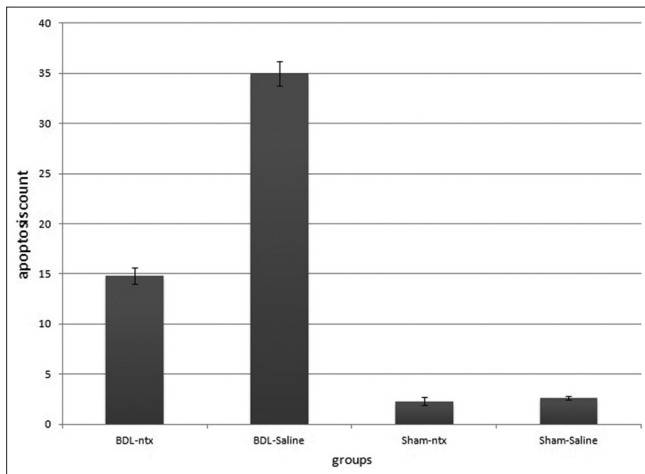
Here, we demonstrated the presence of apoptosis in a rat model of cirrhotic cardiomyopathy and for the 1<sup>st</sup> time we revealed preventive effect of endogenous opioid blockade on cardiac myocytes apoptosis. Our data revealed increased number of apoptotic cells in cardiac samples of cirrhotic rats and showed that blocking the endogenous opioids using naltrexone as an opioid antagonist decreased the amount of apoptotic cells. Our findings are to some extent in line with some other studies focusing on the current topic. According to the literature, apoptosis plays a pivotal role in cardiomyopathy and related studies have tried to explain the underlying mechanisms and pathways. Some studies have shown the increased expression of Mitogen-activated protein kinases (MAPKs) -proteins involved with growth, proliferation, differentiation and apoptosis- in cirrhosis. They have introduced an increased expression level of specific isoforms of MAPKs in cirrhosis, which contributes to cell death and apoptosis.<sup>[14]</sup> Overproduction of NO is another theory for development of cirrhotic cardiomyopathy. NO is produced in cardiac microvascular endothelial cells from either constitutive or inducible NO synthase (iNOS). It is mentioned that NO produced by iNOS has cardiotoxic effect, which could be due to its direct adverse effect on cardiac myocytes or apoptosis induction.<sup>[15-17]</sup>



**Figure 2:** Histopathological evaluation of cardiac tissue stained with Masson trichrome method showing fibrotic bundles in cardiac samples of rats. Fibrotic bundles get blue color in trichrome staining method. Presence of fibrosis in cardiac tissue is a histologic clue for cardiomyopathy. (a) Cardiac sections from control group with normal histologic features and no evidence of fibrosis. As the results of sham-saline and sham naltrexone groups were the same just one picture provided to show normal tissue ( $\times 100$ ). (b) Marked fibrosis in cardiac tissue of bile duct ligation (BDL)-saline group (blue colored bundles showed by arrows) ( $\times 100$ ). (c) Cardiac sections from BDL-naltrexone group with mild interstitial fibrosis (blue colored bundles showed by arrows) ( $\times 100$ ). The amount of fibrosis in this group is not as severe as the BDL-saline group. The pictures are captured with low magnification to better exhibit the amount of fibrosis



We found no significant change in LV wall thickness after naltrexone injection, which demonstrate limited protective role of opioid system blockade on cardiac remodeling. To the best of our knowledge, in spite of the increasing data showing accumulation of endogenous opioids in cholestasis<sup>[18]</sup> and their various roles, e.g., in angiogenesis pathway during the cholestasis<sup>[11,19]</sup> there are just few studies exploring their effect in cirrhotic cardiomyopathy. We examined the role of endogenous opioids in cirrhotic cardiomyopathy using naltrexone. Naltrexone is a non-specific opioid receptor antagonist with a strong affinity to both mu and kappa receptor subtypes and is proposed as a treatment for pruritus in cirrhosis.<sup>[20,21]</sup> Our data revealed that naltrexone injection decreased apoptosis in BDL-naltrexone group compared with BDL-saline group. There are some studies showing the effects of opioids on apoptosis in various organs, for example Svensson *et al.* showed that opiate can induce apoptosis in neural tissue.<sup>[22]</sup> Some other studies have examined different pathways through which opioids can lead to apoptosis such as toll like receptors activation.<sup>[8,23]</sup>



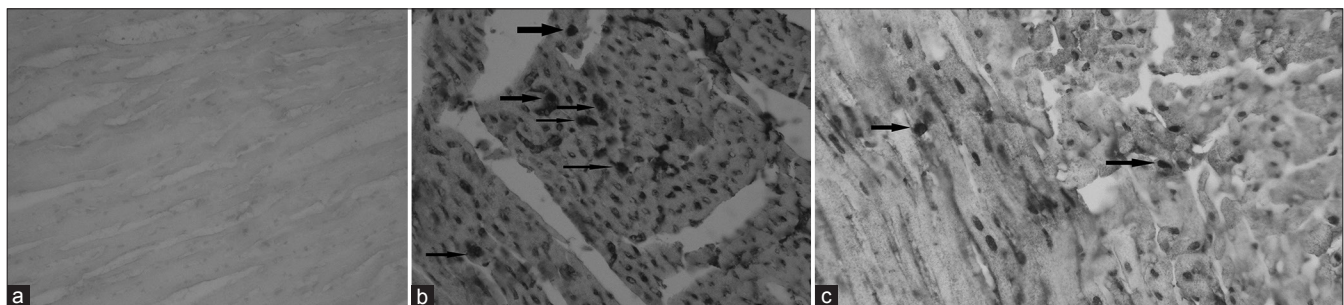
**Figure 3:** Mean number of apoptotic cells in different groups. Specifically stained slides for apoptosis were analyzed by counting apoptotic cells in ten representative high power fields. There were a markedly increased number of apoptotic cells after bile duct ligation (BDL). The amount of apoptotic cells are decreased after naltrexone injection in BDL rats

Data from this study and our previous studies<sup>[6-8]</sup> provide consistent evidence linking excess opioids and apoptosis in various organs in cirrhotic experimental models and warranted further studies evaluating the possibility of improving clinical outcome. We revealed the protective effect of naltrexone against the cardiac myocytes apoptosis, but the relation between apoptosis and cardiac function is not fully understood.

We demonstrated the cardiomyocytes loss beyond the cardiodepressant effect of various mediators. The previous investigations proposed roles for NO, endocannabinoids, prostaglandins, CO, endogenous opioids and adrenergic receptor changes as major pathogenesis underling cirrhotic cardiomyopathy. Down-regulation of  $\beta$ -adrenergic receptors and blunted post-receptor signaling pathway at different levels in the cardiac tissue of cirrhotic rats has been demonstrated and other studies considering the increased level of tumor necrosis factor- $\alpha$  and cyclic guanosine monophosphate (cGMP) in cardiac homogenates proposed cytokine-iNOS-cGMP mediated pathway of action for NO in the pathogenesis of cirrhotic cardiomyopathy. An increased expression of inducible heme oxygenase (HO) and cGMP levels and reversal of blunted contractility of isolated papillary muscles with Zn-protoporphyrine IX (an HO inhibitor) in cirrhotic rats propose the cardiodepressant effects of CO occurring via activation of the HO-CO pathway and the stimulation of cGMP.<sup>[3,10,15]</sup>

In the previous experiments, the endogenous opioid peptides role in bradycardia and hyporesponsiveness of the cardiovascular system to exogenous stimulation in cholestatic rats was demonstrated and the incubation of the cirrhotic papillary muscles with naltrexone acutely corrected the basal contractile forces, the chronotropic and inotropic hyporesponsiveness of cirrhotic rats to isoproterenol stimulation.<sup>[5-7]</sup> However, we demonstrated that chronic opioid system blockage can reduce apoptosis and cardiomyocyte loss in cirrhotic rats.

Since there are no published data regarding the correlation between apoptosis, endogenous opioids and cirrhotic



**Figure 4:** Histopathological evaluation of cardiac tissues stained with terminal transferase deoxyuridine triphosphate nick end labeling (TUNEL) method to show apoptotic cells in cardiac samples of rats. Apoptotic cells are stained dark with TUNEL method. (a) shows cardiac sections from control group with normal histologic features and no evidence of apoptotic cells. As the results of sham-saline and sham-naltrexone groups were the same just one picture provided to show normal tissue (x400). (b) Multiple apoptotic cells (darkly stained nuclei which some of them are indicated by arrows) in cardiac tissue of bile duct ligation (BDL)-saline group, (x400). (c) Cardiac sections from BDL-naltrexone group with few apoptotic cells (some are indicated by arrows) (x400). The number of apoptotic cells was less than BDL-saline group

cardiomyopathy, our finding may provide an indication of a relationship between them. Unfortunately, we were unable to determine which apoptosis pathway (intrinsic vs. extrinsic) or opioid receptor is involved. Further research would define the responsible pathways.

In summary, our data revealed that apoptosis is one of the mechanisms, which develop in cirrhotic cardiomyopathy and beside the other discussed factors endogenous opioids can be responsible for at least a proportion of cirrhosis-induced cardiac dysfunction acutely by reducing chronotropic and ionotropic response and chronically by inducing apoptosis. However, considering no improvement in LV wall thickness as an indicator of LV function, the effect of apoptosis reduction by naltrexone in cardiac function cannot be concluded. We suggest further studies measuring cardiac ejection fraction or contractility in order to evaluate naltrexone role on cardiac function more precisely.

Because of compensatory mechanisms, severe manifestations of heart failure are usually obscured and certain interventions, which increase the effective blood volume and cardiac pre-load can make it clinically evident, thus prevention and management of cirrhotic cardiomyopathy is a matter of paramount importance in this special group of patients. Considering the other effects of opioids blockage in cirrhosis perhaps further research along this line will better describe the underlying mechanisms of cardiomyopathy.

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