

## The Effect of Dexamethasone on Expression of Inducible Nitric Oxide Synthase Gene During Liver Warm Ischemia-reperfusion in Rat

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### Abstract

**Background:** Liver ischemia / reperfusion Injury (IRI) is one of the major causes of liver failure during various types of liver surgery, trauma and infections. The present study investigates the effect of dexamethasone on the liver injury and inducible nitric oxide synthase gene expression during hepatic warm ischemia/reperfusion in rats.

**Materials and Methods:** 24 male Wistar rats (200-250 g) were randomly divided into 3 groups of 8 rats each: 1) saline - administered group (Control), 2) saline - administered ischemia/reperfusion insulted group (IR), and 3) dexamethasone - administered IR group (DEX + IR). Dexamethasone was injected twice at a dose of 8 mg/kg intraperitoneally (60 min before ischemia and immediately after reperfusion). After 1 h of ischemia and 3 hours of subsequent reperfusion, blood and liver samples were collected.

**Results:** Ischemia significantly increased serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the IR group while these parameters were significantly reduced by dexamethasone in DEX + IR group ( $P < 0.05$ ). In parallel with this finding, according to histopathological imaging, dexamethasone reduced hepatic tissue damages. In addition elevated gene expression of inducible nitric oxide synthase (iNOS) in IR was significantly decreased in DEX + IR group ( $P < 0.05$ ).

**Conclusion:** Dexamethasone, as an anti-inflammatory drug, can decline hepatic IR stimulated damages through inhibition of immune mediated reactions and inhibition of iNOS gene expression.

**Keywords:** Liver; Dexamethasone; Ischemia; Reperfusion

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### Introduction

Liver ischemia – reperfusion injury (IRI) as a result of cut off oxygen and food supplies occurs during surgery, infections, trauma and liver transplantation which can cause liver dysfunction and rejection of transplanted tissue (1). There are various mechanisms and factors involved in expansion of IRI, but in general, the direct attack of reactive oxygen species (ROS) to the cell components and disruption of liver microcirculation during reperfusion are considered the main causes of liver damages. Nitric oxide (NO),

one of these active radicals, plays a major role in preventing vasoconstriction in health status. The exact balance between the vasodilator (NO) and vasoconstrictor (endothelin-1) regulates the hepatic perfusion. In the liver, NO is synthesized by endothelial (eNOS) and inducible nitric oxide synthases (iNOS). Under normal conditions, NO is produced by eNOS, while in pathological conditions, iNOS produces NO in excess amount of the value needed for natural vasodilation. In addition to

reaction with O<sub>2</sub> and formation of harmful molecule proxynitrite (ONOO<sup>-</sup>) radicals, NO can cause systemic hypotension and reduction in blood flow to the liver during reperfusion (2, 3). In addition, numerous studies have shown that although up-regulation of iNOS gene expression occurs very slowly and lately, it continues several hours or days after reperfusion. On the other hand, unlike eNOS derived NO, NO produced by iNOS exerts harmful effects on the liver tissue (3). Therefore, the up-regulation of iNOS gene and an excessive NO generation during IR can probably cause hepatic tissue injury. Dexamethasone, an anti-inflammatory drug, can also suppress iNOS gene expression in hepatocytes, epithelial and vascular smooth muscle cells (4). Therefore, in this study the effects of dexamethasone on the expression of iNOS and damage of liver tissue during IR were examined.

### Materials and Methods

Twenty four male Wistar rats weighing 200-250 g were obtained from the animal center in Mazandaran University of Medical Sciences. The animals were housed under standard laboratory conditions (12-hour of light and dark cycles, moisture 55±5% and temperature 23 ± 1 °C). All methods were performed in accordance with the Institutional Animal Ethics Committee. The rats were randomly assigned into three groups, each consisting of eight rats:

(1) Control group, were only injected saline 0.9% (Control); (2) ischemia-reperfusion group which received saline (IR) and (3) those with IR, received dexamethasone (IR + DEX). In control group surgery was performed similar to other two groups.

The rats were kept fasting for 18 hours prior to surgery but drinking water was provided. They were anesthetized with Ketamine (60 mg/kg, IP) and Xylazine (10 mg/kg, IP). After creating a longitudinal incision on the ventral midline and bringing out the liver, in groups IR and IR + DEX, the left branches of portal vein and hepatic artery were clamped down for 60 minutes to persuade complete ischemia of hepatic middle and left lobes. After 3 hours of reperfusion, the rats were sacrificed, and their blood and liver samples were collected for further tests. Liver tissues were stored in RNA-later solution at -70 °C. Serum from the blood samples was stored at -20 °C (5).

Dexamethasone was injected twice at a dose of 8 mg/kg intraperitoneally (60 min before ischemia and immediately after reperfusion) (6).

#### Measurement of serum alanine and aspartate aminotransferases

To evaluate the severity of liver damage, serum ALT and AST levels were measured using Pars Azmoon test kits applying standard spectrophotometric methods.

#### Total RNA extraction and Real time PCR

Liver cells total RNA was extracted by RNeasy plus mini kit according to the manufacturer's instructions (Qiagen, Germany). RNA purity and concentration was determined by measuring the absorbance ratio at 260/280 nm using UV spectrophotometer (Thermo scientific, USA). In order to assess the integrity of extracted RNA, 1% agarose gel electrophoresis with DNA Green Viewer<sup>TM</sup> (pars tousbiotechnology) was used. In next step, reverse transcription was performed to synthesize cDNA using 1 µg of RNA (EURx, Poland). Then iNOS mRNA expression analysis was done by reverse transcription - real time PCR. Briefly, about 50 ng of cDNA and specific primers (10µM) were subjected to PCR using SYBR Green PCR Master Mix reagent (EURx, Poland) for a total volume of 20 µL. The PCR cycles were performed as follows: UNG pre-treatment at 50 °C for 2 min, initial denaturation at 95 °C for 10 min and 40 cycles, each consisting of denaturation at 94 °C for 15 seconds, annealing at 60 °C for 30 seconds, the extension at 72 °C for 30 seconds and final extension at 72 °C for 5 minutes. Dissociation curves were analyzed to ensure the specificity of PCR products and lack of primer dimers. The efficiencies of all reactions were analyzed by standard curve and the obtained threshold cycles were normalized to β-actin gene. For standardization the β-actin gene was used as an internal control. The sequences of the primers are listed in Table 1.

**Table 1.** Primer sequences used in real time PCR

Gene	Primer sequence 5'→3'	Product length (bp)
iNOS	Sense: 5'-TGGTGAGGGGACTGGACTTT-3' Antisense: 5'-CCAACCTCTGCTGTCTCCGT-3'	207
β-actin	Sense: 5'-CCCATCTATGAGGGTTACGC-3' Antisense: 5'-TTAATGTACGCACGATTTC-3'	145

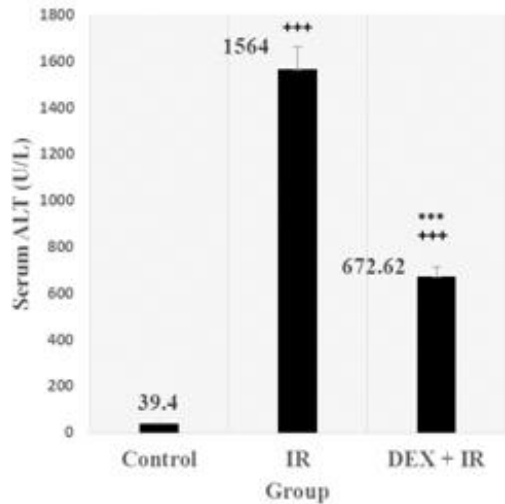
#### Histopathological examination of liver tissue

For histopathologic examination, liver tissues were fixed in 10% formalin and the prepared slides were stained with Hematoxylin and Eosin (H & E), and finally were observed by microscope (7).

#### Statistics

For data analysis, SPSS software version 13 was used. All results have been reported as mean ± SEM. Changes in ALT, AST were analyzed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test. Real time PCR data were

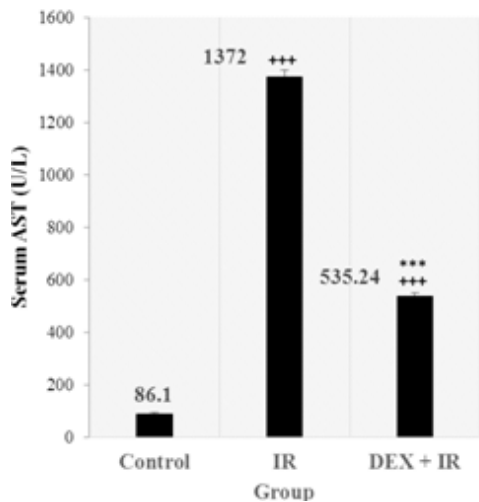
analyzed by REST-RG software. In all tests the level of significance was considered as less than 0.05 ( $P < 0.05$ ).



**Figure 1.** The effect of dexamethasone on serum ALT activity in the studied groups. The values are expressed by mean  $\pm$  SEM. The level of significance was considered as less than 0.05 ( $P < 0.05$ ). \*\*\*Significantly different from the control group ( $P < 0.001$ ). \*\*\*Significantly different from the IR group ( $P < 0.001$ ) Control: the group receiving saline, IR: Ischemia-reperfusion Injury affected group, DEX + IR: IR group receiving dexamethasone.

**Ethics Statement**

All methods were performed in accordance with the Institutional Animal Ethics Committee.

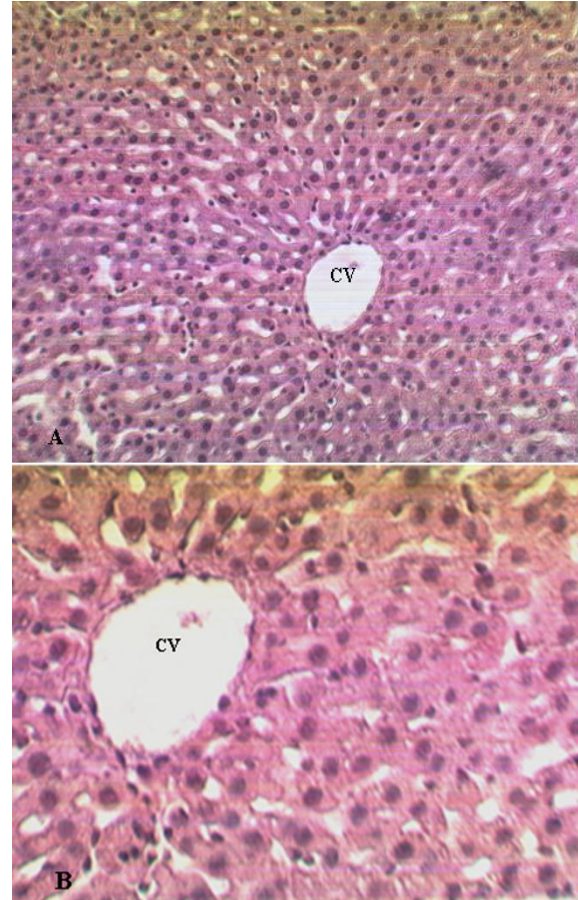


**Figure 2.** The effect of dexamethasone on serum AST activity in the studied groups. The values are expressed by mean  $\pm$  SEM. The level of significance was considered as less than 0.05 ( $P < 0.05$ ). \*\*\*Significantly different from the control group ( $P < 0.001$ ). \*\*\*Significantly different from the IR group ( $P < 0.001$ ). Control: the group receiving saline, IR: Ischemia-reperfusion Injury affected group, DEX + IR: IR group receiving dexamethasone.

**Results**

*Serum ALT and AST levels*

Serum ALT level in control group was  $39.4 \pm 2.1$  U/L which significantly increased to  $1564 \pm 99.8$  in the IR group receiving saline ( $P < 0.001$ ). Dexamethasone injection caused a significant reduction of ALT to  $672.62 \pm 42.5$  U/L in DEX + IR group ( $P < 0.001$ ) (Figure 1).



**Figure 3.** Liver histological sections in Control group (H & E, A, B, magnification 200X magnification 400X, respectively). CV: Central Venule.

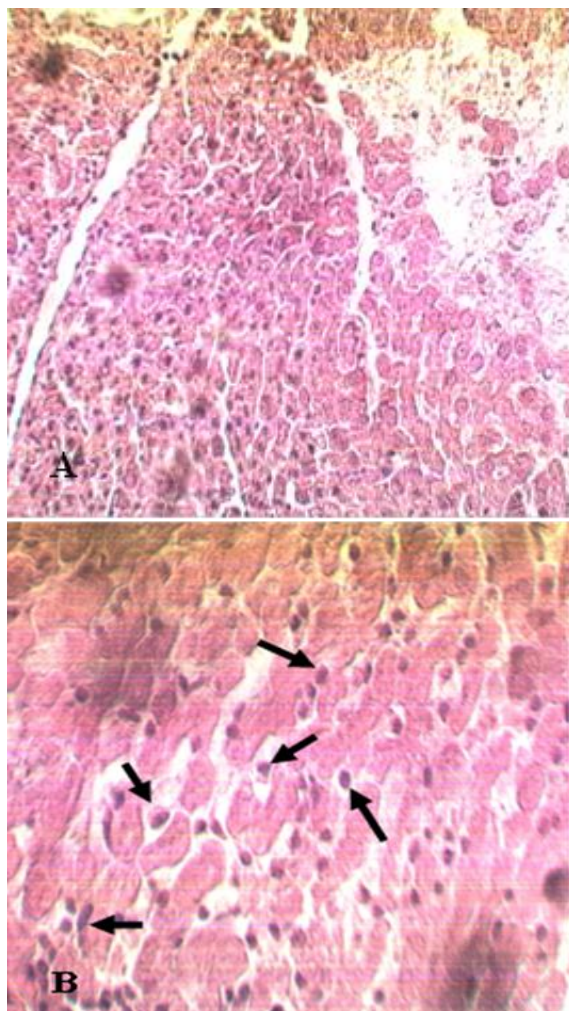
Serum AST level increased in IR group to  $1372 \pm 26$  U/L, which significantly differed from that of the control group ( $86.1 \pm 7.1$  U/L) ( $P < 0.001$ ); dexamethasone in DEX + IR group significantly decreased AST to  $672.62 \pm 14.3$  U/L ( $P < 0.001$ ). Figure 2 illustrates the mean values of AST among the three groups.

*Liver histopathology*

In control group a few number of Kupffer and also polymorphonuclear cells were observed in liver sinusoids. The overall order of liver lobules was natural (Figure 3).

In IR group which received saline during IR, lobules

structure has been destroyed and the number of Kupffer cells significantly increased within the sinusoid. In these samples hepatocytes with pyknotic nucleus were also observed (Figure 4).



**Figure 4.** Liver histological sections in IR group (H & E, A, B, magnification 200X magnification 400X, respectively). CV: Central Venule.

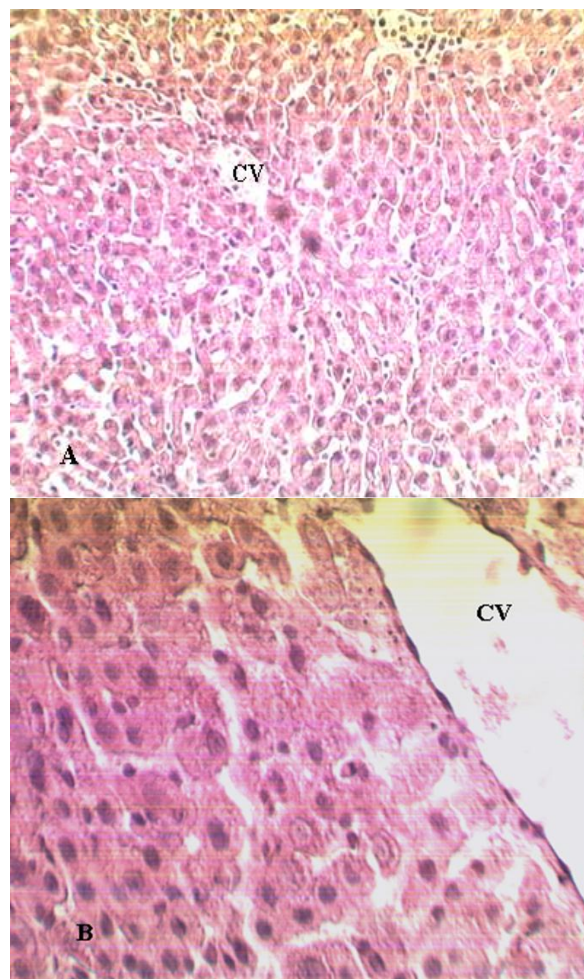
In the third group that received dexamethasone during IR (DEX + IR), sinusoids have normal appearance and a number of hepatocytes with pyknotic nucleus were observed but their number was less than the IR group. There were a number of damaged hepatocytes around the lobular central vein. In this group overall form of the lobules was found to be normal (Figure 5).

#### *iNOS genes expression*

As can be seen in Figure 6, IR significantly increased iNOS gene expression in comparison to the control group ( $P < 0.001$ ). Comparison of IR and DEX + IR groups showed that dexamethasone significantly reduced the expression of iNOS ( $P < 0.01$ ).

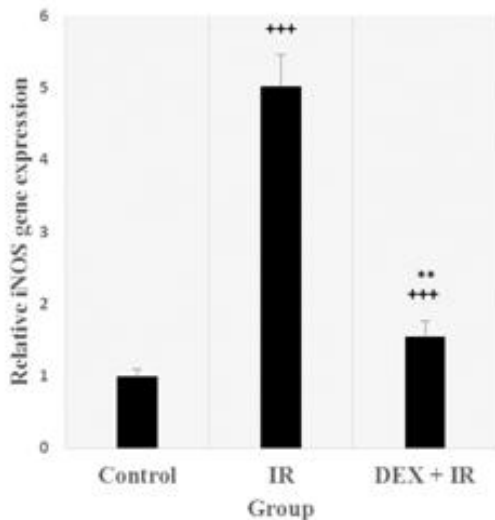
#### **Discussion**

Ischemia-reperfusion includes a complex series of molecular reactions which occurs during reperfusion after a period of ischemia and induce liver damages. Reduction of oxygen, nutrients, and as a result, ATP, activates parenchymal, endothelial and Kupffer cells which initiate a series of destructive processes through production of proinflammatory cytokines and could end to IRI (8, 9).



**Figure 5.** Liver histological sections in DEX + IR group (H & E, A, B, magnification 200X magnification 400X, respectively). CV: Central Venule.

In present study the effect of dexamethasone on serum indicators of liver tissue injury and iNOS gene expression during hepatic warm IR was investigated. The main advantage of this study was that the effect of dexamethasone on the iNOS gene expression was done for the first time. Our findings revealed, dexamethasone significantly reduced elevated level of serum ALT and AST following IR. Several studies have shown that reperfusion after a period of ischemia elevated serum ALT and AST levels due to damages to hepatic parenchymal cells (5, 9-11).



**Figure 6.** Elevated level of *iNOS* gene expression after hepatic IR was diminished by dexamethasone. Results are expressed as a mean  $\pm$  S.E.M. of 8 rats per group. \*\*\*Significantly different from the Control group ( $P < 0.001$ ). \*\*Significantly different from the IR group ( $P < 0.01$ ). Control: the group receiving saline, IR: ischemia-reperfusion affected group, DEX + IR: IR group receiving dexamethasone.

Our results are consistent with those findings. In addition, histological study of the liver tissue suggested that dexamethasone reduced liver damage. Liang et al. demonstrated increase in serum ALT and AST levels due to ischemia and confirmed liver tissue damages through histopathological imaging (10). One possible mechanism to explain this effect is that the glucocorticoid dexamethasone suppressed the production of NF- $\kappa$ B, TNF- $\alpha$ , IL-1, prostaglandins and other destructive factors (12-14). In addition to the deleterious effects of inflammatory cytokines and activation of the immune system, impaired liver microcirculation is another cause of tissue damages during reperfusion (3). One of the major causes of this phenomenon is damage to vascular endothelial cells which in normal conditions produce an appropriate amount of NO through eNOS enzyme and prevent vasoconstriction. On the other hand, NO can be produced by the iNOS enzyme which its gene is induced in a variety of hepatic cells in response to inflammation and produces large amounts of NO in a long time (15-17). The surplus NO has two effects: 1- NO by the reaction with superoxide anion produces proxynitrite (ONOO<sup>-</sup>), an active radicals which can directly react with and destroy biomolecules like proteins, lipids, etc; 2- the excess NO can also cause systemic hypotension and circulatory shock which reduces hepatic perfusion (18).

Therefore, if the expression of iNOS gene is reduced or inhibited, the liver will remain safe from the harmful effects of excess NO during IR (19). This

study shows that dexamethasone reduced iNOS gene expression. The possible mechanism of this effect is the suppression of NF- $\kappa$ B which is a transcription factor influencing the expression of many genes involved in inflammatory processes (4, 16).

In conclusion, our findings unraveled the protective effect of dexamethasone on ischemia/reperfusion liver injury of rats through inhibiting the expression of iNOS and NO overproduction.

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#### Authors' Contribution

GhM, GhK, VA performed sampling and experiments, and assembled input data. GGh designed primers. RB analysed the data. KhA designed the study, interpreted the data and wrote the paper.

#### Conflict of Interest

The authors declare no conflict of interest.

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