



# Evaluating the Oxidative Stress Markers and Nitric Oxide Levels of Liver in the Experimental Model of Diabetes Mellitus

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## ABSTRACT

**Introduction:** Occurrence of oxidative stress and alterations of its related variables during diabetes mellitus is one of the main reasons of tissue damage and dysfunction. Therefore, we assessed the oxidative stress markers and nitric oxide levels of liver in the streptozotocin-induced diabetes mellitus in rats.

**Methods:** To perform the study, male Wistar rats were randomly divided into two groups (n=6): normal and diabetic groups. A single intravenous injection of streptozotocin (45 mg/kg) was used for inducing diabetes. At termination of the study, the oxidative stress markers were determined in the livers, including the contents of malondialdehyde (MDA) and NOx (nitrite/nitrate) as well as the activity of superoxide dismutase (SOD).

**Results:** Induction of diabetes increased blood glucose of the diabetic rats above 450 mg/dl. This value in diabetic rats did not change during the study. Diabetes increased the MDA levels in the livers of diabetic rats compared to normal group. The SOD activity of livers decreased in diabetic animals compared to normal rats. Also, NOx content of liver decreased in the livers of diabetic animals compared to normal group.

**Conclusion:** It is concluded that chronic hyperglycemia in diabetic states overproduces ROS and causes oxidative stress in liver by weakening the antioxidant capacity. Diabetes also alters the NO biosynthesis and bioavailability of liver, which can lead to liver dysfunction.

**Keywords:** Diabetes mellitus, Liver dysfunction, Antioxidant capacity, Oxidative stress.

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## INTRODUCTION

Diabetes is a common metabolic disease with the major complications, which is mainly characterized by hyperglycemia caused by impaired insulin secretion or dysfunction [1]. Worldwide, the prevalence of diabetes is projected to increase from 382 million (8.3%) in 2013 to 592 million (10.1%) in 2035 [2]. Diabetes is also associated with major defects in the metabolism of lipids and proteins, resulting in many severe and life-threatening complications,

including neuropathy, nephropathy, retinopathy, vasculopathy, and hepatopathy [3, 4]. Fibrosis, abnormal deposition of fat and glycogen, cirrhosis, and increased activity of liver enzymes are some of the liver abnormalities associated with diabetes. also, hepatocytes growth and cell number changes alter liver size during diabetes [5, 6]. In fact, Hyperglycemia, which is mainly caused by insulin resistance, affects the metabolism of lipids, carbohydrates, and proteins and can lead to nonalcoholic fatty liver disease (NAFLD), which can further progress to

nonalcoholic steatohepatitis, cirrhosis, and eventually hepatocellular carcinoma [7].

Increasing information shows that the production of free radicals due to the oxidation of glucose and glycosylation of proteins, as well as defects in antioxidant defense systems, play an important role in the pathogenesis of both types of diabetes [8, 9]. It has been suggested that the most important cause of liver damage in diabetic patients is oxidative stress caused by hyperglycemia and the subsequent disturbance in carbohydrate, protein, and lipid metabolism. These events lead to further oxidative stress and activation of inflammatory cascades [10, 11]. So, oxidative stress induced by diabetes and inflammation activates the transcription of pro-apoptotic genes in liver and leads to the death of liver cells [11]. According to the above introduction the current study aimed to investigate the effects of diabetes mellitus (DM), induced by intravenous injection of streptozotocin as a standard animal model for inducing diabetes in rat [12], on **the oxidative stress markers and nitric oxide levels** in the liver of diabetic rats.

## **MATERIALS AND METHODS**

### **Animals**

All procedures of the study were approved by the Institutional Care and Use of Animals Committee of the University of Baqiyatallah Medical Sciences, and were performed in accordance with accepted standards of animals use and care. The ethical code for present study was IR.BMSU.AEC.1402.034. We used male Wistar rats, weighing approximately 210-230 g, obtained from the animal house facility center of Baqiyatallah University of Medical Sciences. Before to procedures, we allowed the rats to acclimatize to the new situation with controlled temperature (22-24°C), humidity (40-60%), light period (07.00-19.00), and also free access to the rat chow and water.

### **Induction of Diabetes**

An intravenous injection of streptozotocin (STZ, Sigma Aldrich) was used for induction of type 1 of diabetes. In brief, under light anesthesia by using ethyl ether, diabetes was induced by an

intravenous injection of 45 mg streptozotocin per kg body weight of rats (45 mg/kg) in lateral tail vein. Five days after injection of streptozotocin, blood glucose levels were tested to confirm diabetes and the rats with blood glucose levels over 450 mg/dL were considered as the diabetic animals.

### **Experimental protocols**

To perform the study, the rats were randomly divided into two groups of equal numbers (six rats in each group) as follows: normal group (normal healthy rats that used as normal control) and diabetic group (diabetic rats that used as diabetic control). The rats of normal and diabetic groups were kept in the standard situation with free access to the rat chow and water during study. Blood glucose levels at the beginning (day 5) and termination (day 60) of the experiment were measured using a commercial kit (Pars Azmoon Company, Tehran, Iran) by an enzymatic colorimetric method according to the manufacture protocol.

### **Tissue preparation**

At termination of the study (day 60), under deep anesthesia using ethyl ether, the liver tissues were rapidly removed, washed in an ice-cold phosphate buffer saline (PBS), immersed in liquid nitrogen and finally kept on -80 °C until biochemical analysis. Then, the tissues were weighed and homogenized 1:10 in ice-cold PBS. The homogenates were centrifuged at 14000×g for 15 minutes at 4 °C. After centrifugation, the supernatants were removed and used for measurement of malondialdehyde (MDA) and NOx contents (nitrite/nitrate levels) as well as superoxide dismutase (SOD) activity.

### **Determination of protein levels**

The method of Bradford was used to quantify the protein levels of brains and livers for data calculation. To calculate the protein concentration, bovine serum albumin (BSA; Sigma, Germany) was used as a standard.

### **Determination of malondialdehyde (MDA)**

The method of Satoh (Satoh, 1978) was used to measure the content of malondialdehyde (MDA), as an index of oxidative damage [13]. "0.5 mL of tissue homogenate was added to 1.5 mL of 10%

trichloroacetic acid (TCA), vortexed and incubated for 10 min at room temperature. 1.5 mL of supernatant and 2 mL of thiobarbituric acid (0.67%) were added and placed in a boiling water bath in sealed tubes for 30 min. The samples were allowed to cool to room temperature. 1.25 mL of n-butanol was added, vortexed and centrifuged at 2000 g for 5 min. The resulting supernatant was removed and measured at 532 nm on a spectrophotometer. MDA concentrations were determined by using 1,1,3,3-tetraethoxypropane as standard. The content of MDA was ultimately represented as nmol/mg protein [14].

#### Assessment of superoxide dismutase (SOD) activity

The activity of superoxide dismutase (SOD) was determined using the method designated by Winterbourn (Winterbourn et al., 1975) based on the ability of this enzyme to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide [15]. "For the assay, 0.067 M potassium phosphate buffer, pH 7.8 was added to 0.1 M EDTA containing 0.3 mM sodium cyanide, 1.5 mM NBT and 0.1 mL of sample. Then, 0.12 mM riboflavin was added to each sample to initiate the reaction and was incubated for 12 min. The absorbance of samples was read on a Genesys 10 UV spectrophotometer at 560 nm for 5 minutes. The amount of enzyme required to produce 50% inhibition was taken as 1 U and results were expressed as U/mg protein [16].

#### Determination of nitrate/nitrite (NO<sub>x</sub>) content

The content of nitrite/nitrate (NO<sub>x</sub>), as an index of nitrosative damage, was assessed by the colorimetric reaction (Griess reagent). "0.1 mL of homogenate solution was deproteinized by adding 0.2 mL of zinc sulfate solution and then centrifuged for 20 min at 4000 g and 4 °C to separate supernatant for nitrate assay. 0.1 mL of supernatant (as a sample) or pure water (as blank) or sodium nitrite (as standard) was mixed with 0.1 mL vanadium III chloride to reduce nitrite to nitrate. 0.05 mL sulfanilamide (0.01 %) and 0.05 mL N-[1-naphthyl] ethylenediamine dihydrochloride (NED, 0.01 %) were incubated for 30 min in dark place at 37 °C. After that, the absorbance of the solution was determined at the wavelength of 540 nm. Nitrite/nitrate concentration was estimated from a standard

curve generated from the absorbance of each sodium nitrate solution. The content of NO<sub>x</sub> was ultimately represented as nmol/mg protein [17].

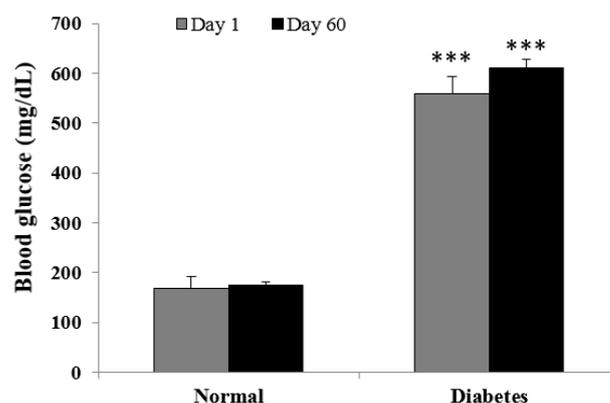
#### Statistical analysis

To perform the statistical analysis for data of the current study, the SPSS (v.21, Chicago, IL, USA) was used. The t-test was used to analyze the data between two groups (normal and diabetic groups). All data were expressed as mean±SEM. A p<0.05 was considered statistically significant.

## RESULTS

#### Effect of STZ-induced diabetes on blood glucose

Figure 1 illustrates the representative changes of blood glucose during the study in normal and diabetic rats. Blood glucose of the normal animals was 168±24 mg/dL at beginning of the test. This value did not significantly change during the study at day 60 (175±6 mg/dL). Also, blood glucose of the diabetic rats was 559±35 mg/dL at beginning of the test (after diabetes induction). This value did not change in diabetic rats during the test at days 60 (610±17 mg/dL). Finally, there were no significant differences in the blood glucose levels of two groups at the different mentioned times (days 1 and 60) during the study.



**FIGURE 1.** Blood glucose of diabetic rats (mg/dL) at the beginning (day one) and termination (day 60) of the study. All values are presented as mean ± SEM (n= 6). \*\*\* (p<0.001) as significant difference compared with normal group

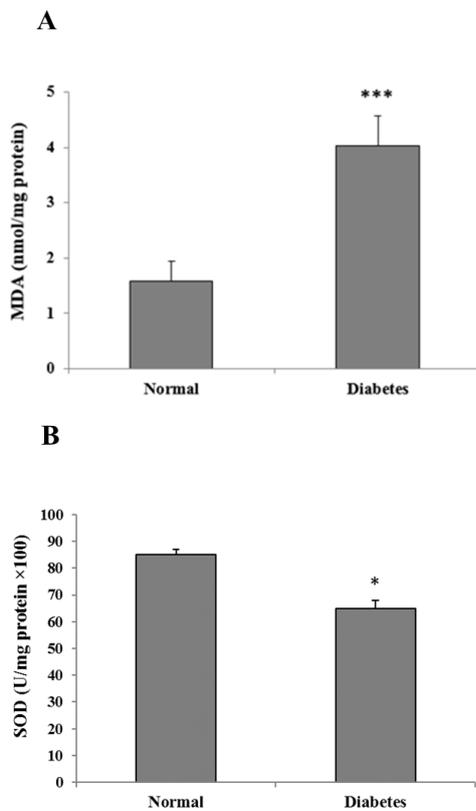
#### Effect of diabetes on the MDA levels of liver

As shown in Figure 2-A, the mean value of MDA levels in the livers of normal rats was 1.58±0.37

nmol/mg protein at termination of the study. The MDA levels significantly increased in the livers of diabetic rats ( $4.03 \pm 0.54$  nmol/mg protein) compared to normal animals ( $p < 0.001$ ), at termination of the study.

**Effect of diabetes on the SOD activity of liver**

Figure 2-B illustrates the SOD activity of livers at termination of the study. The mean value of the SOD activity in the livers of normal rats was  $85 \pm 2$  U/mg protein. Chronic hyperglycemia significantly decreased the SOD activity of livers in diabetic rats ( $65 \pm 3$  U/mg protein) compared to normal animals ( $p < 0.05$ ).

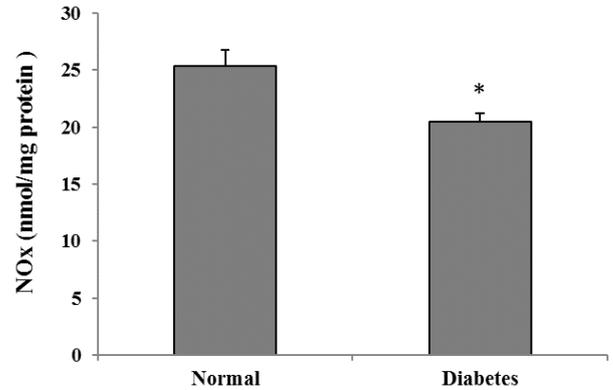


**FIGURE 2.** The effect of chronic hyperglycemia on the oxidative stress markers of livers, A; MDA levels (nmol/mg protein) and B; SOD activity (U/mg protein × 100), in diabetic rats at termination of the study. All values are presented as mean ± SEM (n= 6). \* ( $p < 0.05$ ) and \*\*\* ( $p < 0.001$ ) as significant difference compared with normal group

**Effect of diabetes on the NOx content of liver**

As shown in Figure 3, the mean value of NOx content in the livers of normal rats was  $25.37 \pm 1.36$  nmol/mg protein at termination of the

study. This value significantly decreased in the livers of diabetic rats ( $20.47 \pm 0.77$  nmol/mg protein) compared to normal animals ( $p < 0.05$ ), at termination of the study.



**FIGURE 3.** The effect of chronic hyperglycemia on the NOx levels of liver (nmol/mg protein) in diabetic rats at termination of the study. All values are presented as mean ± SEM (n= 6). \* ( $p < 0.05$ ) as significant difference compared with normal group

**DISCUSSION**

The findings of the present study indicated that induction of chronic hyperglycemia mediated by DM in rats considerably increased the MDA levels of liver, as a main determinant of oxidative stress. Chronic hyperglycemia also weakened the antioxidant defense system of liver in diabetic animals. In this regard, the activity of SOD decreased in the liver of diabetic rats. The NOx content of liver also decreased following diabetes induction as a main marker or metabolite of nitric oxide molecule.

Overproduction of free radicals such as reactive oxygen radicals (ROS) increases widely in the different tissues of body particularly in liver during DM [18]. Accordingly, the activated kupffer cells (hepatic macrophages) in liver are the main cells that produces ROS through NADPH-oxidase or inducible nitric oxide (NO)-synthase activities [19]. In this regard, dysfunction of Kupffer cells is central to hepatic injuries and contributes to the pathogenesis of NAFLD in DM [20, 21]. Our result indicated that induction of diabetes for sixty days considerably increased the MDA levels of liver, as a main

maker of oxidative stress. These findings have been confirmed in most tissues and organs, which were affected by diabetes [18]. Hence, measurement of the oxidative stress indicators in diabetic patients showed that oxidative stress and the resulting damages occur in most tissues of the human body [22]. Induction of diabetes experimentally triggers oxidative stress in the liver of diabetic animals that is considered as a mechanism for diabetic chronic liver disease [23]. Overproduction of mitochondrial ROS is one of the underlying common pathogenic mechanism of glucotoxicity for diabetic complications [18]. Several ROS-producing sites have been identified in mammalian mitochondria in different tissues of the body [24]. Also, activation of pro-oxidant enzymes during diabetes such as NADPH-oxidase is one of the most important regulators of ROS production in liver [25]. Moreover, weakening of the antioxidant defense system of liver during hyperglycemia can be as another mechanism for induction of oxidative stress [26]. According to our findings, diabetes decreased the activity of SOD in the livers of diabetic rats [27]. The liver has the intricate and potent antioxidant systems, including SOD, catalase, and glutathione-related enzymes, such as glutathione S-transferase (GST) and glutathione peroxidase (GPX) [27]. This potent antioxidant defense system protects the liver cells from oxidative damage by scavenging free radicals and hydrolyzing hydrogen peroxides. In this regard, several studies have demonstrated that decreased SOD and catalase activities has increased oxidative stress and eventually liver damage during hyperglycemic situations [28]. In addition to these enzymes, GSH is a prominent factor in the homeostasis of GSH-related enzymes family. Reduction of its levels in the liver of diabetic animals correlate with decreased the activities of GST, GPX, and glutathione reductase [29].

Nitric oxide (NO) is biosynthesized from L-arginine amino acid in the endothelial cells (ECs) by endothelial NO-synthase (eNOS) and is known as the endothelium-derived relaxing factor [30]. According to evidence, synthesis of this vasodilator molecule is decreased during diabetes mellitus [24]. In this regard, the results of present study indicated that the levels of NO metabolites, NOx, decreased in the livers of diabetic rats. NO

production in the normal ECs has many important function, including preventing ECs apoptosis as well as neutrophil and platelet adhesion to the vessel wall, regulation of vascular tone and proliferation, and mediating flow-induced adaptive vascular modeling [31]. Hence, the amount of vasodilation response through NO is affected in people with uncontrolled diabetes [24]. Several reasons can cause a decrease in NO production and NO bioavailability during diabetes mellitus [32, 33]. One reason is oxidative stress that reduces NO bioavailability [24]. Vessels under prolonged exposure to hyperglycemia generates superoxide anion, which can act on NO to form peroxynitrite (ONOO<sup>-</sup>) [34]. This compound with reduced NO availability can contribute to vascular dysfunction in diabetes [24]. Additionally, activation of eNOS by phosphorylation in Ser1177 residue can increase NO production in response to vascular stimuli [35]. Based on the reports, hyperglycemia dampens phosphorylation of eNOS at Ser1177 residue, leading to enhance eNOS uncoupling and diminish the activity of eNOS [35].

## CONCLUSIONS

It is concluded that chronic hyperglycemia in diabetic states overproduces ROS and causes oxidative stress in liver. It seems that weakening the antioxidant defense system of liver by diabetes as well as reducing the SOD activity plays a crucial role in this phenomenon. Diabetes also changes the NO biosynthesis and bioavailability of liver, which can lead to liver dysfunction.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## FUNDING RESOURCES

There are no financial conflicts of interest to disclose.

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