

# Hepatic morphological changes and oxidative stress in chronic streptozotocin-diabetic rats

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## Abstract

**Oxidative stress (OS) is a biological entity quoted as responsible for several pathologies including diabetes. Diabetes mellitus (DM) has been also associated to human cirrhosis. The present work was designed to study the occurrence of OS as well as morphologic alterations in rat livers following induction of DM. Two groups of rats were used: Control and Diabetic. DM was induced in the second group by streptozotocin (STZ) in a single dose of 60 mg/kg, injected i.p. The occurrence of OS was determined in liver homogenates by measuring the hydroperoxide-initiated chemiluminescence and the activity of antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase). Liver sinusoids were morphometrically analyzed. In conclusion, livers from the diabetic group did not show evidence of the occurrence of OS, as it would be expected, but dilation of hepatic sinusoids was documented and it was significantly different from control group.**

**Key words: Diabetes, liver, chemiluminescence, free radicals, sinusoids.**

## Introduction

Several experimental models of type 1 and type 2 diabetes mellitus (DM) are available in rats.<sup>1</sup> Streptozotocin

(STZ) is a frequently used drug that exerts a diabetogenic effect through a specific damage of the pancreatic beta cells, mimicking type 1 DM. It also induces systemic microvascular alterations, as observed in retina and kidney.<sup>2,3</sup>

The liver constitutes an important parenchyma whose functions have been found altered in patients with DM.<sup>4</sup>

Oxidative stress (OS) can be associated to an increased rate of reactive oxygen species (ROS) generation, a decrease of antioxidant defences or a combination of both. ROS-mediated alterations include damage to cells, tissues or organs and are proposed as a major factor in the mechanism of several diseases including atherosclerosis,<sup>5</sup> DM<sup>6,7</sup> and chronic alcoholic intoxication.

An increased production of oxygen-derived free radicals as well as a decrease in the activity of free radical scavenger systems have been reported in DM.<sup>8</sup> It has also been proposed that an increase in OS could contribute to tissue damage in DM.<sup>9</sup> Moreover, OS is an important factor in the etiology and pathogenesis of DM.<sup>10</sup> Hepatic glutathione levels were found decreased in STZ-diabetic rats.<sup>11</sup> Recently, lipid peroxide levels were found increased and superoxide dismutase activity decreased in liver homogenates of STZ-diabetic rats<sup>12</sup> and the levels of the antioxidant enzymes catalase, glutathione peroxidase and superoxide dismutase are diminished in liver of experimental diabetic rats.<sup>13</sup>

The aim of the present work was to study the occurrence of OS and morphological alterations in rat livers following induction of chronic STZ DM.

## Methods

### Animals

Wistar male rats weighing 240-270 g were used. All animals were housed individually in an environmentally controlled room and allowed free access to food and water. This experiment was performed according to the Guidelines for Animal Experiment of the School of Pharmacy and Biochemistry, University of Bs. As.

### Experimental model

The animals were divided at random into 2 groups (n=5):

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Manuscript received: 21 February, 2005 and accepted: 11 April, 2005.

- Group I, control.
- Group II, diabetic

### Experimental diabetes

Experimental diabetes was induced in rats by the administration of a single injection of STZ (60 mg/ kg; ip). Before the assays were performed, rats of Group II were maintained during 3 months, recording body weight and glycemia weekly.

### Biochemical parameters

Blood samples were obtained before sacrifice and processed for determination of glycemia and tissue damage markers. Levels of glucose, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured in serum samples using conventional laboratory kits. Glycemia values are given in mg/dL of blood and the enzyme activities in U/L of serum.

### Tissue homogenates

Liver samples processed for the determination of hydroperoxide-initiated chemiluminescence (0.5-1.0 g of wet weight) were homogenized in 120 mM KCl, 30 mM phosphate buffer (pH 7.4) at 0-4 °C. The suspension was centrifuged at 600 x g for 10 min at 0-4 °C to remove nuclei and cell debris. The pellet was discarded and the supernatant was used as 'homogenate' (MM3)<sup>14</sup> and for the measure of antioxidant enzyme activities.

### Hydroperoxide-initiated chemiluminescence

Hydroperoxide-initiated chemiluminescence of rat homogenates was measured in a liquid scintillation counter. Homogenates were placed in 25 mm diameter and 50 mm height low potassium glass vials. Reaction medium contained 120 mM KCl, 30 mM phosphate buffer (pH adjusted to 7.5). Protein content was adjusted at 1.0 mg/mL of protein homogenate. Chemiluminescence measurements were made after the addition of 3 mM *tert*-butyl hydroperoxide to the reaction medium.<sup>15</sup> Measurements were carried out at 30 °C. Results are expressed as counts per minute (cpm)/mg of protein.

### Superoxide dismutase

Superoxide dismutase activity was determined spectrophotometrically in plasma samples by measuring the inhibition of the rate of autocatalytic adrenochrome formation at 480 nm in a reaction medium containing 1 mM epinephrine and 50 mM glycine - buffer (pH adjusted to 10.2 with NaOH 1M). The enzymatic activity is expressed as SOD units (U) per mg of protein. One SOD

unit is defined as the amount of enzyme that inhibits the rate of adrenochrome formation in 50%.<sup>16</sup>

### Catalase

Catalase activity was measured in the homogenates treated with Triton X-100 by following the decrease of absorption at 240 nm. The reaction medium consisted of 50 mM phosphate buffer (pH adjusted to 7.2) and 10 mM H<sub>2</sub>O<sub>2</sub>. The results are expressed as pmol catalase/ mg of protein.<sup>17</sup>

### Glutathione peroxidase

Glutathione peroxidase activity was measured in the homogenates by following NADPH oxidation at 340 nm as described by Flohé and Gunzler.<sup>18</sup> The reaction medium consisted of 30 mM phosphate buffer (pH adjusted to 7.0), 0.17 mM GSH, 0.2 U/ mL glutathione reductase and 0.5 mM *tert*-butyl hydroperoxide. The glutathione peroxidase activity is expressed as mU/mg of protein. One unit corresponds to 1 umol NADPH per min per mg of protein.

### Protein determination

Protein concentration in homogenates was measured by the method of Lowry et al<sup>19</sup> using bovine serum albumin as standard.

### Microscopy and image analysis

After sacrifice, livers were removed, weighed and fixed in buffered formalin, included in Paraplast and stained with hematoxylin-eosin, PAS and Masson's trichromic.

The hepatic sinusoidal area was measured by an automatic computerized semiquantitative method using the Scion Image Beta 0.4 software, coupled to an optical microscope equipped with a VCC video camera. Results were expressed as the inverse log of intensity per sinusoidal area (ILGV/area). These soft images are properly standardized for background lighting ILGV/area results proportional to the unweighted average optical density, which is then used to determine optical density and sinusoidal area. At least 9 sections per rat were analysed at the level of the 3 zones of the liver lobular histology.<sup>20</sup> Selected areas for quantification were measured as pixels per area (square inches). Standard referenced area utilized was 8.33 square inches.<sup>21</sup>

For High resolution Optic Microscopy (HROM) tissue was fixed in 3% glutaraldehyde buffered with sodium cacodylate, embedded in epoxy and stained with toluidine blue.

### Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM). Each value represents one animal,

data were statistically analyzed by factorial analysis of variance (ANOVA) followed by the Neumann-Keuls test for comparison of means. Differences were considered significant at  $p < 0.05$  in the bilateral situation.

## Results

The general conditions of the animals were assessed by the evaluation of clinical parameters. As shown in Figure 1A, fasting glycemia values in the diabetic animals were significantly higher when compared to controls. Serum levels of tissue damage marker enzymes AST (Figure 1B) and ALT (Figure 1C) were significantly increased in the diabetic rats as compared to the controls. No change was found for serum creatinine levels (data not shown).

The presence of oxidative damage can be evaluated by the determination of tert-butyl hydroperoxide-initiated chemiluminescence in liver homogenates (Figure 2). Hydroperoxide-initiated chemiluminescence showed no modifications in diabetic rats.

The antioxidant system in diabetic rats liver homogenates showed an increased SOD activity as compared to the control ( $3.4 \pm 0.2$  USOD/mg prot, Figure 3A). When catalase activity (CA) in liver homogenates was measured, it was found to be significantly decreased in diabetics as compared to the controls (Figure 3B). Also glutathione peroxidase activity in diabetic liver homogenates decreased significantly ( $p < 0.001$ ) when compared to controls ( $42 \pm 2$  mU/mg prot.). Enzyme activity decreased 38 % in the diabetic liver, as shown in Figure 3C.

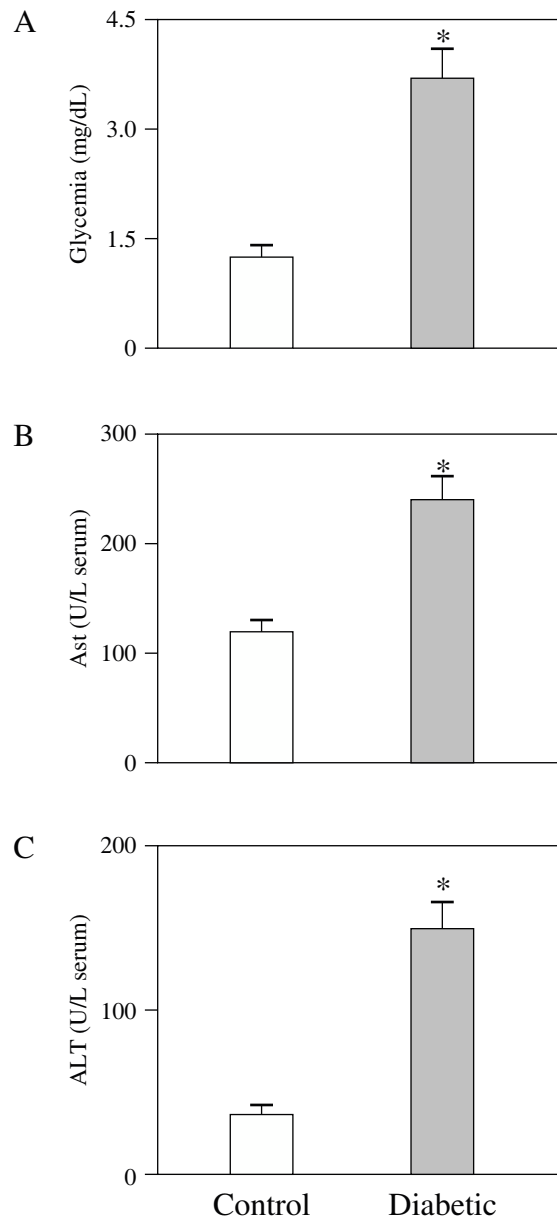
As can be seen in Table I, significant differences were found in the sinusoidal area of diabetic animals when compared with controls. These results, recorded on a frame work area of 8.33 inches,<sup>22,23</sup> show that sinusoidal area determined at three zones expressed the same phenomena (no significant differences were registered in the different zones).

Light microscopy showed normal features in group I (Figure 4), while group II showed sinusoidal dilation in the pericentral vein zone as the major changes (Figure 5).

## Discussion

Liver was selected as a target for oxidative stress evaluation because its important metabolic functions and role in DM. The occurrence of OS was evaluated by measurement of tert-butyl hydroperoxide-initiated chemiluminescence and the antioxidant enzymes activities.

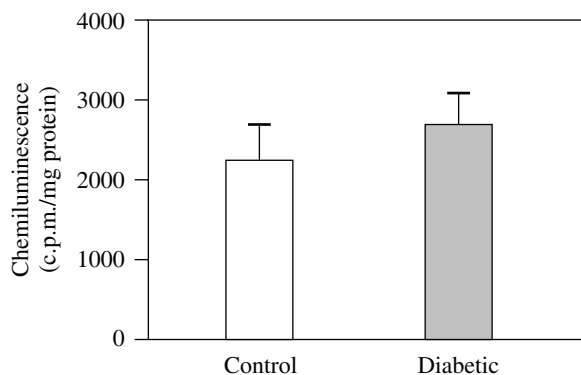
The determination of tert-butyl hydroperoxide initiated chemiluminescence is a sensitive assay that has been applied to detect OS associated to experimental pathological situations such as ethanol treatment in rats,<sup>24</sup> barbital treatment in mice,<sup>25</sup> tumor-bearing in mice,<sup>26</sup> and adriamycin cardiotoxicity in rats, mice and rabbits.<sup>27,28</sup> The rationale of the assay is that two samples of tissue with sim-



**Figure 1.** Biochemical parameters. Glycemia, as mg/dL of blood (A), aspartate aminotransferase (AST, B) and alanine aminotransferase, as U/L of serum (ALT, C) in control (open bars, n=5) and diabetic (filled bars, n=5) rats. Data are the mean  $\pm$  SEM. \* $P < 0.01$  compared with the control group.

ilar characteristics will yield higher or lower chemiluminescence depending on the levels of endogenous antioxidants. A lower level of antioxidants as a consequence of a previous situation of oxidative stress will correspond to a higher chemiluminescence and this will indicate the occurrence of oxidative stress.

Tert-butyl hydroperoxide-initiated chemiluminescence, recorded in the livers of diabetic rats showed the same values observed in controls. These results are not in accordance with other authors that stated that OS was present as a contributory factor to diabetic pathology.<sup>29</sup> It must be



**Figure 2.** Chemiluminescence. Chemiluminescence as cpm/mg of protein in liver homogenates from control (open bars, n=5) and diabetic (filled bars, n=5) rats. Data are the mean  $\pm$  SEM.

recalled that the decreased activity of catalase and glutathione peroxidase found in the diabetic group could possibly mean an enzyme consuming effect. On the other hand, other authors reported that OS is not overtly or systematically increased in DM, except in final stages of its evolution when complications appear.<sup>30</sup> Another possible explanation for these results could be that chronic diabetic rat livers could produce some unknown antioxidant agent.

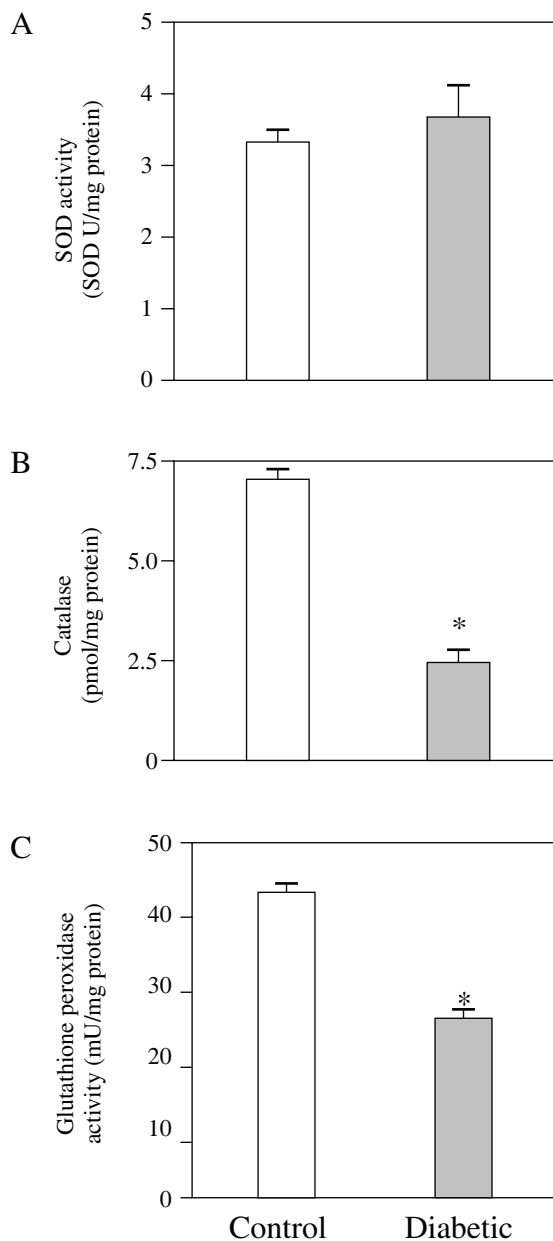
It can also be suggested that STZ-induced diabetes produced a sort of antioxidant effect through an unknown metabolite, glucose or advanced glycation end products.<sup>21</sup>

Liver antioxidant enzymatic defences were evaluated to determine parenchyma responses to the possible presence of OS in these pathologies. The results obtained by measuring antioxidant enzymes, were also intriguing. Diabetic animals showed that liver catalase and glutathione peroxidase activity decreased significantly.

It is difficult to assess in what extent liver microcirculation, including its endothelial cells, participate in the vascular damage produced by chronic diabetes as occurred in other vascular beds.<sup>31-34</sup> Besides, it is not clear if liver microvascular system is even more modified. The endothelial damage described by other authors<sup>1,35</sup> could be the first event in vascular injury, producing cellular alterations. Accordingly to Huszka et al,<sup>36</sup> NO production is reduced in diabetes in correlation with endothelial damage.

Nitric oxide (NO), heme oxygenase 1 (HO1) and carbon monoxide (CO) are endogenous compounds that activate guanylate cyclase,<sup>35</sup> leading to the generation of cyclic guanosine monophosphate, which in turn mediates various physiological functions. Moreover, an excessive production of CO, as a consequence of HO-1 overexpression, could play an important role in the modulation of vascular tone under different pathological situations.<sup>1,2</sup>

Beside this, cytokines play also an important role and endothelin 3 is the major factor involved in the regulation of vascular resistance in the liver. All the former path-



**Figure 3.** Oxidative stress parameters. A: Superoxide dismutase (SOD) activity as U/mg of protein in liver homogenates from control (open bars, n=5) and diabetic (filled bars, n=5) rats. Data are the mean  $\pm$  SEM. B: Catalase activity as pmol/mg of protein in liver homogenates from control (open bars, n=5) and diabetic (filled bars, n=5) rats. Data are the mean  $\pm$  SEM. \* $P < 0.01$  compared with the control group. C: Glutathione peroxidase activity as mU/mg of protein in liver homogenates from control (open bars, n=5) and diabetic (filled bars, n=5) rats. Data are the mean  $\pm$  SEM. \* $P < 0.01$  compared with the control group.

ways should be explored to achieve a comprehensive state of the sinusoidal modulation under the present experimental conditions.

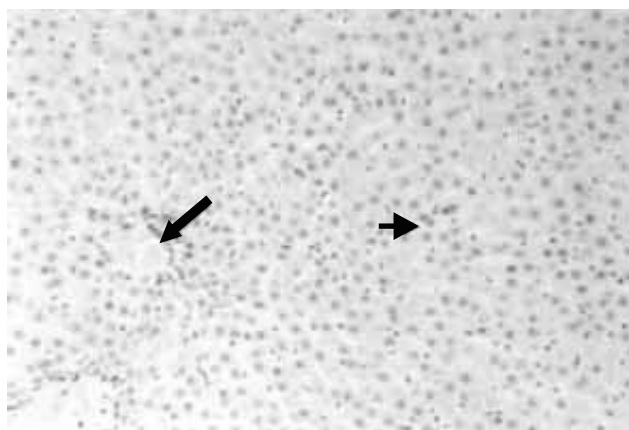
It can be concluded that under these experimental conditions chronic diabetic rats do not suffer liver OS, as could be expected.

**Table I.** Liver sinusoidal area quantification.

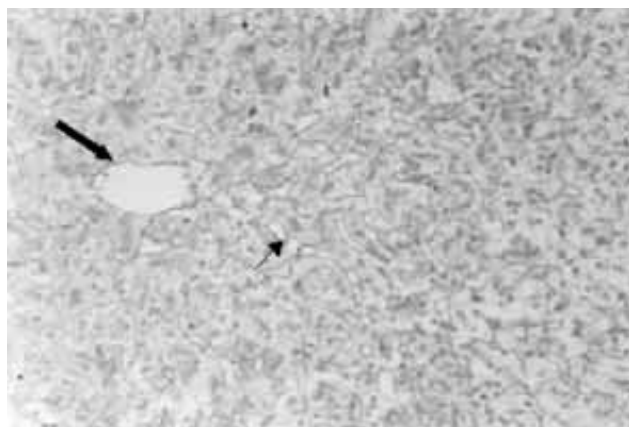
Group	Sinusoidal area/field
Control (n = 87)	27,263.37 ± 698.64
Diabetic (n = 63)	35,364.43 ± 867.20 <sup>a</sup>
t & p test t	T = 7.32 - 0.0001

The selected areas for quantification were measured as pixels per area. The standard area used was 8.33 square inch.

<sup>a</sup>, indicate significant difference ( $P < 0.0001$ ) according to student t test, two-sided  $P$  value.  $n$ : number of fields measured.



**Figure 4.** Light micrograph from a control rat liver. Large arrow shows a central vein and small arrow shows normal parenchymal cells (Magnification 600 X).



**Figure 5.** Light micrograph from a diabetic rat liver. Large arrow shows a central vein and small arrow shows a dilated sinusoid. (Magnification 600 X).

## Acknowledgements

This work was supported by grants from University of Buenos Aires (FA 056) and Fiorini Foundation.

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