The role of oxidative stress in diabetic cardiomyopathy: an experimental study

E. AKSAKAL¹, N. AKARAS², M. KURT³, I.H. TANBOGA³, Z. HALICI⁴, F. ODABASOGLU⁵, E.M. BAKIRCI¹, B. UNAL²

¹Department of Cardiology, School of Medicine, Atatürk University, Erzurum (Turkey)
²Department of Histology, School of Medicine, Atatürk University, Erzurum (Turkey)
³Department of Cardiology, Erzurum Education and Research Hospital, Erzurum (Turkey)
⁴Department of Pharmacology, School of Medicine, Atatürk University, Erzurum (Turkey)
⁵Department of Biochemistry, Faculty of Pharmacy, Atatürk University, Erzurum (Turkey)

Abstract. – Background: Diabetes mellitus (DM) has a negative effect on cardiovascular functions. Little, however, is known of the overall effect of DM on the cardiac histology or the pathophysiological basis of this.

Aim: We aimed to investigate the role of oxidative stress on the pathogenesis of diabetic cardiomyopathy in an experimental model.

Materials and Methods: 12 week-old female Sprague Dawley rats were randomly allocated into a healthy control group (n=6) and an DM group (n=6). After 12 weeks of alloxan induced DM, the groups’ cardiac tissues were histopathologically analyzed and examined for determination of oxidant and antioxidant enzymes [activities of catalase (CAT), superoxide dismutase (SOD), and myeloperoxidase (MPO) and amount of reduced glutathione (GSH) and lipid peroxidation (LPO)].

Results: When compared to the control group, the DM group showed cardiomyopathic changes. In the DM group, activities of CAT (144±0.9 vs 112±1.4, p < 0.05) and LPO amount (27.0±0.74 vs 14.4±0.20, p < 0.05) were significantly increased whereas activities of SOD (142±0.2 vs 146±0.7, p < 0.05) and amount of GSH (3.48±0.01 vs 3.73±0.01, p < 0.05) were significantly decreased when compared to the control group. Besides, activities of MPO (7.3±0.02 vs 8.6±0.11, p < 0.05) were comparable between groups.

Conclusions: Using the experimental animal model, we were able to demonstrate that DM causes cardiomyopathic changes, and we propose that these changes could be mediated by an oxidative stress.

Key Words: Experimental diabetes mellitus, Oxidative stress, Heart, Cardiomyopathy.

Introduction

Cardiovascular complications have become the major cause of morbidity and mortality for people suffering from diabetes mellitus (DM)¹. Apart from coronary artery disease and changes in blood pressure, DM causes the deterioration of the cardiac structure and function, a condition referred to as diabetic cardiomyopathy². Several studies have shown that hyperglycemia is a major risk factor directly associated with cardiac damage, such as myocardial fibrosis and collagen deposition, both of which are thought to be the cause of abnormal myocardial relaxation or diastolic dysfunction²,³. Although the underlying pathogenesis has not been exclusively studied, various factors, such as impaired calcium homeostasis⁴, activation of renin-angiotensin system⁵, altered substrate metabolism such as enhanced fatty acids metabolism⁶,⁷, activation of transcriptional pathways such as the peroxisome proliferator-activated receptor signaling network that regulates myocardial substrate use⁸, mitochondrial dysfunction and increase in uncoupling protein expression⁹,¹⁰, impaired leptin and endothelin metabolism¹¹ and increased oxidative stress¹², have been proposed to be involved in the pathogenesis of diabetic cardiomyopathy. In this study, therefore, we used an experimental model to evaluate, as other parameter, the role that oxidative stress has on the pathogenesis of diabetic cardiomyopathy.

Materials and Methods

Animals and Experimental Groups

The rats were housed in accredited facilities in accordance with international guidelines, and the studies were approved by and conducted in accordance with the Institutional Animal Care and Use Committee of Atatürk University. This study used 12 adult (12 week-old) female Sprague Dawley rats from the Atatürk University Experimental Animal Laboratory (ATADEM). The animals were

To prepare the tissue homogenates, the tissues were ground with liquid nitrogen in a mortar. The ground tissues (0.5 g each) were then treated with 4.5 mL of the appropriate buffer. The mixtures were homogenized on ice for 15 minutes using an Ultra-Turrax homogenizer. The homogenates were then filtered and centrifuged using a refrigerated centrifuge at 4°C. These supernatants were then used for the determination of enzymatic activities and amounts. All assays were performed at room temperature in triplicate.

The activities of CAT, SOD, and MPO and the amount of GSH and LPO were analyzed as previously described13-16.

Dissection and Histological Examination in Paraffin Sections

After removing the hearts, they were fixed into a 10% buffered formalin for 24 to 48 hours in order to prepare them for histopathological examinations. After the fixation and routine preparation of the samples were carried out according to the conventional light microscopic technique, they were embedded in paraffin. 5-µm-thick sections were cut with a microtome (Leica RM2125RT) and stained with hematoxylin-eosin in order to conduct the routine histological examination. All sections were studied and photographed by a light photomicroscope (Olympus BH 40).

Statistical Analysis

Data are expressed as mean ± standard deviation. To compare continuous variables, Student’s-t test was used. The statistical evaluations were performed using the SPSS 13.0 (SPSS Inc., Chicago, IL, USA) for Windows. The significance level was set at p < 0.05.

Results

Histopathological Results: Conventional Light Microscopy by H&E

The endocardium, which is composed of three distinct segments (a single layer of squamous endothelial cells, a subendothelial layer of elastic and collagen fibers and smooth muscle cells, and a subendocardial layer of small blood vessels, nerves, and Purkinje fibers), had a normal appearance. The myocardium, which is the muscular wall of the heart attached to the fibrous cardiac skeleton, had no abnormalities (Figure 1 a-d). The cardiac muscle cells (Figure 1 d-f), with

Experimental Models

Alloxan-Induced Diabetes Procedure

Diabetes was induced in female Sprague Dawley rats by intraperitoneal administration of a single dose of 150 mg/kg of aqueous alloxan monohydrate (Sigma-Aldrich Co., St Louis, MO, USA). The aqueous alloxan monohydrate was dissolved in a freshly prepared solution of 0.9% NaCl and injected intraperitoneally to rats that had fasted for one night. After alloxan application, the pancreas secretes insulin at high levels. As a consequence, a fatal form of hypoglycemia can occur. To prevent this adverse effect, five doses of a 20% m/100 mL glucose solution were injected intraperitoneally 4-6 hours after the injection of the alloxan, and a 5% glucose solution was added to the drinking water of the animals for a 24 hour period. After 72 hours, blood samples were taken from the tail vein of the animals in order to determine the fasting blood glucose level in the plasma using the Accu-Check Active® blood glucose monitor one time in a 3 day period. At the end of this period, a rat was determined to be diabetic, if their plasma glucose level was 200 mg/dl or over. The diabetic rats were kept alive for 12 weeks.

Research Methods

Biochemical Investigation of Heart Tissues

The rats’ hearts were kept at −80°C for 3 days in order to conduct a biochemical investigation on them. After the 3 days, catalase (CAT), superoxide dismutase (SOD) and myeloperoxidase (MPO) activities were performed on the hearts, and the amount of reduced glutathione (GSH) and lipid peroxidation (LPO) present in the tissues of the rats’ hearts were determined.

To prepare the tissue homogenates, the tissues were ground with liquid nitrogen in a mortar. The ground tissues (0.5 g each) were then treated with 4.5 mL of the appropriate buffer. The mixtures were homogenized on ice for 15 minutes using an Ultra-Turrax homogenizer. The homogenates were then filtered and centrifuged using a refrigerated centrifuge at 4°C. These supernatants were then used for the determination of enzymatic activities and amounts. All assays were performed at room temperature in triplicate.

The activities of CAT, SOD, and MPO and the amount of GSH and LPO were analyzed as previously described13-16.

Housed in groups of six per cage for at least 7 days under temperature/humidity controlled conditions and exposed to a 12 hour light/dark cycle. 12 week-old female Sprague Dawley rats were allocated randomly into i) non-diabetic healthy control group (Group I, n=6), and ii) diabetic group (Group II, n=6). In this study, the four basic evaluation criteria were used for analyzing the samples, including: (1) Plasma glucose concentration level during starvation; (2) Body weight measurement; (3) Detection of tissue biochemistry; (4) Histopathology by conventional light microscopy.
features such as cross-striated banding pattern (Figure 1b), centrally located pale-staining nuclei (Figure 1c, d and f), and endomysial connective tissue (Figure 1d and f), are the main components of the myocardium and are found in myocytes. The myocardium contains a rich capillary network and intercalated disks that are found at the interface between adjacent cardiac muscle cells. The epicardium, also referred to as the visceral layer of the pericardium, is the outermost layer of the heart wall and is composed of a simple squamous epithelium called mesothelium. The epicardium had normal appearances.

In the diabetic group (Figure 2a-i), the structural changes were able to be organized under three distinct categories: (1) myocyte, including abnormalities in general appearance (Figure 2a, c, d, f and g), necrosis (Figure 2f and g), an increase in necrotic cell density (Figure 2b, c and g), and loss of cells in parts of the area of section profiles (Figure 2f and g); (2) connective tissue component, including focal fibrous scarring of the myocardium (Figure 2b and e), inflammatory cell infiltration (Figure 2d and g), and edema (Figure 2a-i); and (3) blood vessels, including thickness in arteriole wall (Figure 2c), endothelial changes in capillary and perivascular fibrosis (Figure 2c).

**Tissue Biochemical Results**

The tissue biochemical values and the statistical analysis of the control group and the diabetic group are shown in Table I.

In the diabetic group, CAT activities (144±0.9 vs. 112±1.4 mmol/min/mg tissue, \( p < 0.05 \)) and LPO amounts (27±0.74 vs. 14.4±0.2 nmol/g tissue, \( p < 0.05 \)) were significantly increased, whereas SOD activities (142±0.2 vs. 146±0.7 mmol/min/mg tissue, \( p < 0.05 \)) and GSH amounts (3.48±0.01 vs. 3.73±0.01 mmol/mg tissue, \( p < 0.05 \)) were significantly decreased when compared to the control group. MPO activities (7.3±0.02 vs. 8.6±0.11 mmol/min/mg tissue, \( p < 0.05 \)), however, were similar between the two groups.

**Discussion**

This study was performed to investigate the impact of experimental DM on the histopathology of the heart and tissue oxidative status. In this experimental study we have shown, histopathologically, that diabetes causes various cardiomyopathic changes, such as myocyte necrosis and an increase in cell loss, myocardial inflammation, edema and

![Figure 1](image_url)

**Figure 1.** Light microscopic photomicrograph of non-diabetic healthy control groups. The section was stained with hematoxylin-eosin. CMs; Cardiac muscle cell, Cp; Capillary, Ct; Connective tissue, Ent; Endothelium, F; Fibroblast, NcCs; Nuclei of cardiac cell, SMs; Smooth muscle cell. (Magnification, 400 for a and b, 500 for c, d, e and f).
Figure 2. Light microscopic photomicrograph of diabetic groups. The section was stained with hematoxylin-eosin. Ed; Edema, En; Endocardium, Ent; Endothelium, F; Fibroblast, Fb; Fibrosis, LI; Lymphocytes Infiltrating, Nc; Necrotic Cell, SMs; Smooth muscle cell. (Magnification, 400 for a and b, 500 for c, d, e, f and g).
an increase in fibrosis, thickening in arteriole wall, capillary and perivascular fibrosis and endothelial changes in the heart tissue. Moreover, we have shown that these cardiomyopathic alterations were accompanied with changes in the tissue oxidant and antioxidant balance. Therefore, we proposed that experimental DM causes cardiomyopathic changes in the rat heart and that these changes are mediated by oxidative stress.

Diabetes mellitus is the cause of myocyte cell apoptosis and/or necrosis without ischemia in the heart tissues of the rats. In the present study, cardiomyopathic changes, such as abnormal myocardial architecture, inflammatory cell infiltration, edema, increase of the fibrosis and connective tissue component, enlarged interstitial space, and widely dispersed necrotic cells appeared to have increased in rats with DM. The cellular apoptosis and/or increase in necrotic cell density in cardiomyocytes and the loss of cells in parts of the area of section profiles are very important pathological mechanisms for causing cardiomyopathy in DM, rats’ heart tissues.

**Tissue Oxidant/Antioxidant Balance**

In this investigation, we have demonstrated that as a result of experimental DM, CAT activities and LPO amounts were significantly increased, while SOD activities and GSH amounts were significantly decreased. MPO activities were, however, unchanged.

The pathogenesis of diabetic cardiomyopathy is multifactorial. For reduced myocardial contractility, many hypotheses, such as autonomic dysfunction, metabolic derangement, abnormalities in ion metabolism, alteration in structural protein, and increased oxidative stress, have been proposed. Oxidative stress represents the imbalance between the production and manifestation of the reactive oxygen species (ROS), as well as their detoxification. Many markers are used in the quantification of the oxidative stress. CAT, GSH and SOD compose the antioxidant defense system and LPO and MPO are components of the oxidative system.

Oxidative stress could possibly play an important pathophysiological role in DM complications. DM can alter the activities of oxidant/antioxidant enzymes in the heart, a matter of which is a well-known implication of increased ROS generation or impaired antioxidant defenses. In both type 1 and type 2 DM, increased ROS production was seen, and this situation was associated with increased apoptosis in the hearts of the rats. Therefore, increased ROS generation or impaired antioxidant defenses could be a trigger for diabetic cardiomyopathy.

SOD, CAT and GSH are important components of the antioxidant system, in that the SOD converts superoxides into oxygen and hydrogen peroxide and CAT converts hydrogen peroxide into non-toxic water. GSH plays an important role as an electron donor at the cellular level by reducing peroxide and free radicals and turning them into an oxidized form of glutathione disulfide. In our study, we found that CAT enzyme levels increased while SOD and GSH levels decreased. Conflicting results were obtained in the earlier studies of diabetic rat hearts. Udayakumar et al. found that there was a decrease in SOD and GSH, Kaul et al. found that there was a decrease in SOD, Kakker et al. found that there was an increase in SOD and CAT, and Wohaib et al. found that there was an increase in CAT.

The MPO enzyme catalyzes production of hypochlorous acid and is found mainly in leukocytes. MPO is a marker of neutrophil accumulation, which is an indicator of inflammatory activity. LPOs are further degraded to reactive aldehyde products, such as malondialdehyde, which can covalently bind to proteins and change their structure and function and, therefore, the very physiology of the cell. LPO levels constitute a good marker of peroxidative damage to cell mem-

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<th>Table I. Tissue biochemistry results of all experimental groups.</th>
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<tr>
<td><strong>Control (n = 6)</strong></td>
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<tr>
<td>CAT (mmol/min/mg tissue)</td>
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<tr>
<td>SOD (mmol/min/mg tissue)</td>
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<td>MPO (µmol/min/mg tissue)</td>
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<td>LPO (nmol/g tissue)</td>
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<td>GSH (nmol/mg tissue)</td>
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CAT: Catalase; SOD: Superoxide dismutase; MPO: Myeloperoxidase; LPO: Lipid peroxidation; GSH: Reduced Glutathione.
In our study we were unable to find any change in the levels of the MPO, but we did, however, observe an increase in the amount of LPO. Udayakumar et al found that the amount of LPO increased, which may be due to the depletion of antioxidant scavenger systems such as SOD. Furthermore, the authors concluded that GSH also acts as a free radical scavenger and participates in the elimination of reactive intermediates by reduction of hydrogen peroxide and that its high level may induce the activation of glutathione peroxidase, which in turn reduces lipid peroxidation in diabetic rats. Sun et al found increased MPO levels in diabetic rats; we, however, did not observe any change in the levels of the MPO.

In conclusion, we propose that diabetes mellitus causes serious damage to the structure and function of the heart and to the underlying mechanism partly associated with the oxidative status.

The summary of this study has been presented at 27th National Congress of Cardiology (October, 27-30, 2011, in Istanbul, Turkey).

References


