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Histological Examination of Rat Heart Tissue with Chronic Diabetes

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Abstract

Diabetes mellitus causes structural and functional impairment of the system in the organism by affecting various organs and structures. In this study, we aimed to examine the changes in female rat heart tissue histologically by creating experimental diabetes. 16 female adult rats were used in the study. Rats were randomly divided into two groups as control and 3–month diabetes. The diabetes group was formed from subjects with blood glucose levels above 250mg/dl 72 hours after 40 mg / kg streptozotocin administration. At the end of the experiment, the heart tissues of the subjects were removed and taken into formaldehyde solution. To examine the histological structure, haematoxylin–eosin, and immunohistochemically neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) were stained. Heart tissue sections belonging to the control group had histologically normal appearance. In the diabetes group heart tissue sections, vacuolization in some cells and eosinophilic increase in the cytoplasm of some cells were observed. The nNOS and iNOS immunoreactivity was observed to be decreased in the diabetes group compared to the control group, but the decrease was not statistically significant. As a chronic disease, DM causes histological damage to the heart tissue. The resulting damage causes a decrease in nNOS and iNOS expression. It is important to maintain NOS enzyme levels to protect tissue from the harmful effects of diabetes and ensure normal physiological conditions.

Keywords: *Diabetes mellitus, Heart, nNOS, iNOS.*

Introduction

Diabetes mellitus is a serious disease that affects large masses worldwide. Risk factors such as hypertension, coronary artery diseases, hypercholesterolemia increase the rate of heart failure in diabetic

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patients (1). Also, regardless of coronary artery disease or hypertension, DM can cause cardiomyopathy by causing ventricular dysfunction (2).

DM triggers oxidative stress by causing prolonged hypoglycaemia and causes disruption of protein, lipid and carbohydrate metabolism in the cell and production of reactive oxygen species (3). The biosynthesis of NO, a free radical, is provided by nitric oxide synthase (NOS) (4). NOS has two basic isoforms: constitutive NOS (cNOS) and inducible NOS (iNOS). The cNOS enzyme consists of two basic isoforms. These are neuronal NOS / NOS1 and endothelial NOS / NOS3. iNOS is also known as NOS2 (5, 6). NOS enzymes are found in three isoforms: NOS1 / nNOS, NOS2 / iNOS and NOS3 / eNOS (7). It has been reported that all three NOS isoforms are found in mammalian cardiac tissue, and they modulate oxygen consumption, substrate use, hypertrophy, apoptosis and regenerative potential in cardiac cell biology (8).

DM shows its effect on cardiovascular diseases due to irregular free radical production in the cell. In this study, it was aimed to evaluate the changes in the heart tissue of female rats with experimental chronic diabetes on nNOS and iNOS immunoreactivity.

Methods

Animals

Sexually mature 12-weeks-old male Wistar rats, obtained from the Hakan Çetinsaya Experimental and Clinic Research Centre, Erciyes University, Kayseri, Turkey, were used for this study. They were housed in plastic cages in a well-ventilated rat house and allowed ad libitum access to food and water and kept at a 12-h light: dark cycle. All the animals received humane care according to the standard guidelines. The study protocol was accepted by the Erciyes University Experimental Animal and Local Ethics Committee (decision no: 12/105/2012). The rats were randomly assigned to two groups. This Control group (n=8) and Diabetes group (n=8).

Diabetes was induced in 12-week-old female Wistar rats by intraperitoneal injection of STZ (40 mg/kg) (Sc-200719, Santa Cruz Biotechnology, CA, USA) (9). Hyperglycaemia was confirmed 72 h after streptozotocin injection by measuring glucose levels in the blood obtained from the tail vein, using a glucometer. Animals with mean plasma glucose levels higher than 250 mg/dL were considered diabetic. Diabetes group at sacrificed 12 weeks after streptozotocin injection (10). At the end of the experimental period, the animals were killed by decapitation under intraperitoneal ketamine (75 mg/kg) + xylazine (10 mg/kg) anaesthesia. After decapitation, the heart tissues were quickly removed and were fixed. To evaluate the normal histological structure, haematoxylin-eosin (H-E) staining was performed.

Immunohistochemistry

To determine the differences in expression of nNOS and iNOS in heart tissue, the avidin-biotin-peroxidase method was used for marking. Paraffin sections (5 µm) were deparaffinized in xylene. The sections were rehydrated, rinsed in deionized water and antigen retrieval was carried out by microwave treatment in 0.01 M sodium citrate buffer (pH 6.0) at 95°C for 5 min. The slides were then cooled rapidly at room temperature for 20 min. The sections were washed with phosphate-buffered saline (PBS) and endogenous peroxidase activity was inhibited by 3% H_2O_2 in methanol for 10 min. For the next stages the ABC staining system using colouring kit was

used. All cross sections were washed with PBS and then to make sure to block outside the antigenic fields, block serum was applied for 20 minutes at room temperature. The histological sections were then incubated with nNOS (Pierce antibody product, PA3-032A1/200 dilution) and iNOS (Pierce antibody product, PA3-030A, 1/200 dilution) primary antibodies overnight at 4 ˚C. After washing with PBS, the sections were incubated with biotinylated secondary antibodies. The immunoreaction was amplified with the streptavidin–avidin–peroxidase complex, and the sections were visualized using 3,3P-diaminobenzidine tetrahydrochloride (DAB) and lightly counterstained with haematoxylin (11). Ten different areas were evaluated in terms of expression using the image J program.

Statistical analysis

All statistical analyses were carried out by using GraphPad Prism version 7.00 for Mac, GraphPad Software, La Jolla, California, USA. D'Agostino Pearson omnibus test was used to identify the normal distribution of the data. In the situation of quantitative variables with abnormal distribution, variables were compared using independent two-sample t-test with Mann-Whitney test. Quantitative variables with normal distribution were compared by using independent two-sample t-test with Welch's test. The data were expressed as 'median (min-max)'. $p<0.05$ was considered as statistically significant.

Results

Histological results

The control group heart tissue sections had a normal histological appearance. Myocardial muscle fibers were smooth, H-E staining was normal. In the diabetes group heart tissue sections, vacuolization was observed in some cells and eosinophilic increase in the cytoplasm of some cells Figure 1.

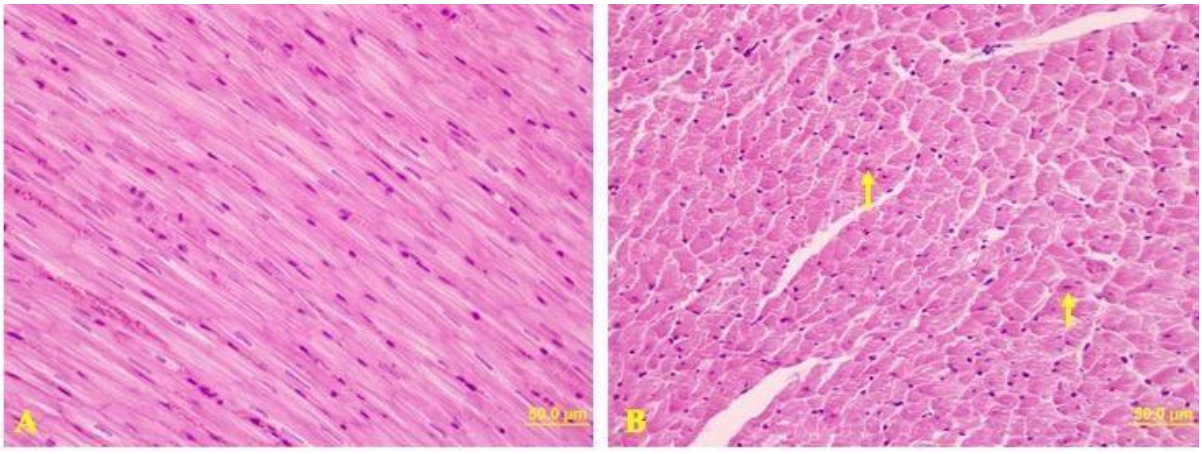

Figure 1. H-E staining of heart tissue. A) Control group, B) Diabetes group. The yellow arrows indicate increased eosinophilia. Scala bar 50 □m. Abbreviation: H-E; Haematoxylin-eosin.

Immunohistochemistry results

Expression of nNOS and iNOS in heart tissue sections was observed in both the control and diabetes groups. Both nNOS (p <0.097) and iNOS (p <0.079) expressions decreased in the diabetes group compared to the control group. The decrease in both enzyme expression was not statistically

significant. Expression of enzymes was mostly observed in the cytoplasm of heart muscle cells. Pictures and graphics of nNOS and iNOS immunoreactivity are given in Figure 2.

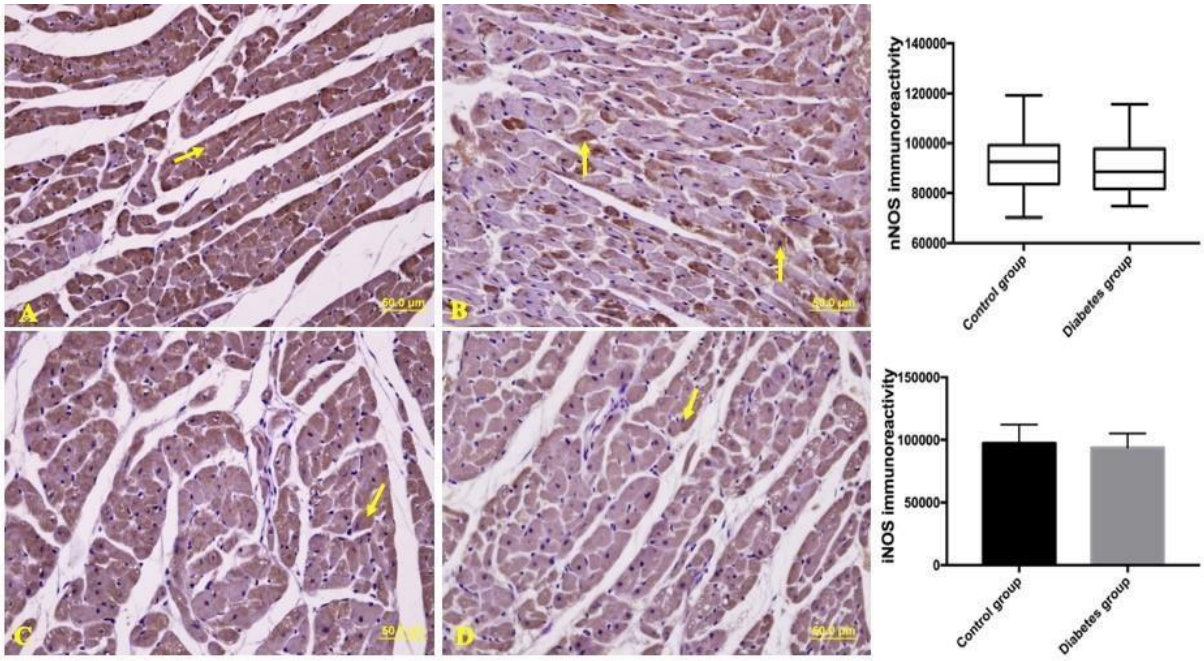

Figure 2. nNOS and iNOS immunohistochemistry staining. A) Control group, B) Diabetes group nNOS expression and immunoreactivity graph. C) Control group, D) Diabetes group iNOS expression and immunoreactivity graph. Yellow arrows indicate nNOS and iNOS expressions. Scala bar 50 □m. Abbreviations: nNOS; neuronal nitric oxide synthase, iNOS: inducible nitric oxide synthase.

Discussion

In the study, we found that DM causes myocardial damage in the heart tissue and changes in the expression levels of nNOS and iNOS. It has been reported that cardiomyocyte disintegration and cells with pyknotic nuclei are observed after the damage caused by diabetes in the heart tissue (12, 13). We found similar findings in this study, including in our previous study that we created experimental diabetes with streptozotocin (9).

DM causes a decrease in cardiac output, arterial blood pressure and heart rate, especially due to hyperglycaemia. Especially in endothelial cells, by suppressing the response to vasoactive agents, it triggers endothelial dysfunction and increases the risk of cardiac disease almost five times (14, 15). DM causes myocardial damage due to oxidative stress with increased reactive oxygen and nitrogen types. Three major free-radical sources in diabetic myocardium are mitochondria, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and NOS (15). NOS enzyme family is necessary for the production of nitric oxide (NO) from L-arginine (16). NO is a free-radical since it has an unpaired electron in its final orbit and can be covalently bonded with other molecules (17). NOS enzymes are nNOS, iNOS and eNOS and are available in a variety of cell and tissue types. NO especially passes from the endothelium and reaches to smooth muscle cells in the

vascular wall, increasing the formation of cGMP and causing relaxation and vasodilation of smooth muscles (18). DM triggers endothelial dysfunction in the emergence of vascular diseases by reducing this bioavailability of NO (19). Decreased NO bioavailability causes an increase in reactive oxygen species in the cell, triggering an increase in oxidative stress. Briefly, DM causes a breakdown in the electron transport chain that occurs in mitochondria due to hyperglycaemia and increased fatty acid in the cell. Thus, decreased ATP production leads to decreased NOS enzyme activity and this decrease in NOS enzyme activity leads to dysfunction in the cell by causing decreased NO production and increased superoxide radicals (20). In our study, we used the heart tissues of rats exposed to diabetes for 12 weeks. According to our immunohistochemistry staining results, there was a decrease in nNOS and iNOS immunoreactivity in diabetic heart tissue compared to the Control group. We think that nNOS and iNOS synthesis decreases in the cell due to increased hyperglycaemia. In our previous experimental diabetes study, all three NOS isoforms (nNOS, iNOS, and eNOS) were reduced in the diabetes group (9). A decrease in both serum NO and constitutive NOS (cNOS) (nNOS and eNOS) levels has been reported after diabetes induced by applying a high-fat diet (21). Contrary to our study, there are studies showing that plasma NO and iNOS gene expression increases (22). Diabetic rats have been reported to have endothelial dysfunction associated with decreased aortic NO and cNOS activity, in contrast to increase NO and iNOS reactivity in cardiac tissue (23). Accordingly, the decrease in both nNOS and iNOS expression in the heart tissue indicates that endothelial dysfunction is triggered in the tissue. In this case, increased NOS enzyme levels in the heart tissue does not indicate that endothelial dysfunction started in the tissue. On the contrary, due to the reduction of these enzyme levels, NO bioavailability is eliminated and oxidative stress increases in the heart tissue. According to our results, chronic diabetes causes a decrease in both nNOS and iNOS enzyme levels in the heart tissue, inhibiting the production of beneficial NO, causing diabetic vascular complications in the heart tissue.

Conflict of interest

The authors declare that no conflict of interest exists.

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