Measurement of asymmetric dimethylarginine, nitric oxide levels and total antioxidant capacity in experimental diabetic rats

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Summary

The aim of this study is to investigate the status of oxidative stress and nitric oxide related parameters in diabetic rats. In experimental animals (12 rats) diabetes was induced by intraperitoneal injection of a single 50 mg/kg dose of streptozotocin. At the end of the experiment, in the blood serum of the control and experimental animals ADMA, NO was measured by using ELISA test. Serum levels of GSH, MDA and ceruloplasmin as well as TAC were detected in all animals. Compared to the control animals serum MDA levels in diabetic rats were remarkably and significantly ($P < 0.05$) higher ($26.38 \pm 1.94 \mu\text{mol/L}$ and $42.23 \pm 1.24 \mu\text{mol/L}$, respectively). On the other hand, a statistically significant ($P < 0.05$) decrease from $4.39 \pm 0.15 \text{ mg/dl}$ to $4.00 \pm 0.09 \text{ mg/dl}$ was detected in serum GSH levels of the diabetic rats. Compared to the serum ceruloplasmin levels of healthy rats ($35.05 \pm 2.79 \text{ mg/dl}$), diabetic rats showed a significant decrease ($P < 0.001$) in their serum ceruloplasmin concentration ($23.10 \pm 1.65 \text{ mg/dl}$). Likewise, serum ADMA concentration of experimental animals ($7.26 \pm 0.86 \text{ ng/ml}$) was higher than that of controls ($5.59 \pm 0.75 \text{ ng/ml}$) but not significantly ($P > 0.05$). As to serum TAC, compared to the control rats a statistically significant ($P < 0.05$) decline was observed in diabetic rats. Serum TAC of diabetic rats reduced from $1.01 \pm 0.06 \text{ mmol trolox equivalent/L}$ to $0.87 \pm 0.03 \text{ mmol trolox equivalent/L}$. Serum NO concentration of experimental animals ($30.42 \pm 2.48 \mu\text{M/ml}$) was significantly ($P < 0.05$) less than that of controls ($62.28 \pm 10.74 \mu\text{M/ml}$).

Keywords: Diabetic rats, total antioxidant capacity, asymmetric dimethylarginine

Diabetes is one of the systemic chronic metabolic diseases accompanied by hyperglycemia, dyslipidemia, glycosuria and much clinical and biochemical evidence (18). In diabetes, both acute and chronic complications can be seen in all organs. This is suggested to be caused by the tissue damage formed with autooxidation of glucose, protein glycation and auto-oxidative glycation with free radicals (23).

In studies showing the relationship between reactive oxygen species and diabetes and complications of diabetes, it has been emphasized that the tissue damage induced by non-enzymatic glycation, metabolic stress caused by changes in energy metabolism, hypoxia and ischemia-reperfusion increases free radical production and alters the antioxidant defense system (9).

Asymmetric dimethylarginine (ADMA) is a proteolytic by-product of arginine methylation found in the plasma, urine and tissues. ADMA is guanidino analogue of L-arginine and synthesized endogenously

and metabolised by dimethylarginine dimethylaminohydrolases (DDAHs). In the protection of vascular tone and its structure, vasoactive mediators released from endothelial play an important role and nitric oxide (NO) is one of the most important of these mediators. Nitric oxide plays an important role in the regulation of vascular tone and platelet adhesion and aggregation. On the other hand, arginine and ADMA-DDAK pathway play an important role in the regulation of nitric oxide synthesis. ADMA is determined to be inhibiting the NOS enzyme (29).

Lipids are biomolecules that are most vulnerable to the effects of free radicals. Unsaturated bonds of cholesterol and fatty acids of cell membranes create peroxidation products by easily entering reaction with free radicals (6).

Under normal conditions, organisms have an antioxidant defense system struggling with free radicals caused by endogenous or exogenous reasons and

oxidative stress developed due to these free radicals. Therefore, oxidative stress can also be viewed as an imbalance between the prooxidants and antioxidants in the body. The concentration of different reductant-oxidant markers is considered an important parameter for assessing the prooxidant status in the body tissues. Several indicators of *in vivo* redox status are available, including the ratios of GSH to GSSG, NADH to NAD⁺, as well as the balance between reduced and oxidized thioredoxin. The measurement of total antioxidant status may provide more valuable information compared to individual measurements of antioxidants. Total antioxidant capacity (TAC) reflects the total effect of all antioxidants in plasma and body fluids (11).

The aim of this study is to investigate the status of oxidative stress and nitric oxide related parameters in diabetic rats.

Material and methods

Experimental design and animals. This study was conducted on a total of 24 Sprague- Dawley rats with an age range of 8-10 weeks and 245.50 ± 14.5 g in weight. These rats were kept under standard light (12 hours of daylight/12 hours of darkness) and temperature (22°C) with sufficient water and food at the Experimental Animals Laboratory of Adnan Menderes University, Faculty of Veterinary Medicine, and then adapted to the environment by taking them one week before the start of the study. The animals used in the study were divided into 2 groups: as control (n = 12) and diabetic (n = 12) animals. The study was evaluated by the Local Ethics Committee of Animal Experiments in Adnan Menderes University and approved by the ethics committee on 01/01/2011 with decision number 2011/026.

Sample collection and biochemical assays. In order to create experimental diabetes, 0.01 M sodium citrate buffer (pH: 4.5) was injected to the experimental group with streptozotocin 50 mg/kg intraperitoneally. Streptozotocin injection may result in fatal hypoglycemia related to massive insulin release. To prevent hypoglycemia, rats were kept on a 5% glucose solution diet for 24 h after the injection. Similar to the experimental group of animals, a 0.01 M sodium citrate buffer was given to the control group intraperitoneally. After 28 days following the injection, the animals were fasted for 12 hours and serum glucose levels were measured. Rats with blood glucose levels > 11 mmol/L (higher than 200 mg/dl) were considered diabetic and were used for the study. After diagnosing all animals in the experimental group with diabetes, blood samples were collected from their heart under ether anesthesia and then they were euthanized by cervical dislocation. Blood was then centri-

fuged at 4,000 g for 10 min to remove red blood cells and recover serum.

Serum asymmetric dimethylarginine (ADMA) was manually tested in the ELISA device by Elisa commercial kit (Cusabio Biotech, Cat No: CSBE08896r). Serum nitric oxide (NO) levels were measured by 'Nitric Oxide Colorimetric Assay' (Roche Cat. No: 1756281) method. The value of nitrogen monoxide was determined by the measurements of serum nitrite. Plasma total antioxidant capacity (TAC) levels were studied by the method developed by Erel (10) with Rel Assay (Rel Assay Diagnostics, Türkiye, lot no: RL002) kits.

The lipid peroxidation of serum was measured by the Tris-Boric Acid (TBA) (the method described by Yoshioka et al. (33)). Malondialdehyde (MDA), formed from the breakdown of polyunsaturated fatty acids, was considered as an index for the peroxidation reaction. The absorbance of the action product of MDA with TBA was measured at 532 nm. Quantization was based upon a molar extinction coefficient of 1.56×10^5 M⁻¹ cm⁻¹.

Serum glutathione (GSH) concentration was assayed by the method of Beutler et al. (5). GSH concentration detected in the serum was measured spectrophotometrically at 412 nm.

The serum ceruloplasmin levels were determined by measuring p-phenylenediamine oxidase activity as described previously by Sunderman and Nomoto (27). Briefly, 5 ml phenylenediamine substrate (pH5.6) was added to the curve and test tubes. One microlitre sodium azide solution was then added into the curve tube only. This was followed by the addition of 0.1ml of sera to both the curve and test tubes. Samples were mixed and kept at 37.8°C for 15 min. Finally, 1 ml of the sodium azide solution was added to the test tube only, and all samples were then incubated at room temperature for 15 min. The optical density was measured at 546 nm using a spectrophotometer (Shimadzu, UV-160).

Statistical analysis. The findings of the study were analyzed by using an SPSS 11.5 (Statistical Package for the Social Sciences) program. The significance level of the differences were determined by using Independent Samples Test.

Results and discussion

The results of this study are shown in Table 1. As a result of this study, serum ADMA concentration of experimental animals (7.26 ± 0.86 ng/ml) was higher than that of controls (5.59 ± 0.75 ng/ml) but not significantly ($P > 0.05$). Serum NO concentration of experimental animals (30.42 ± 2.48 µM/ml) was significantly ($P < 0.05$) less than that of controls (62.28

Tab. 1. Control and experimental groups related to the serum ADMA, NO, MDA, GSH, ceruloplasmin and total antioxidant activity levels

Groups	ADMA (ng/ml)	NO (µM/ml)	TAC (mmoltrolox equivalent)	GSH (mg/dl)	MDA (µmol/L)	Ceruloplasmin (mg/dl)
Control (n = 12)	5.59 ± 0.75	62.28 ± 10.74	1.01 ± 0.06	4.39 ± 0.1	26.38 ± 1.9	35.05 ± 2.8
Diabetic rats (n = 12)	7.26 ± 0.86	$30.42 \pm 2.48^*$	$0.87 \pm 0.03^*$	$4.00 \pm 0.9^*$	$42.2 \pm 1.3^*$	$23.10 \pm 1.7^{**}$

Explanations: * $P < 0.05$, ** $P < 0.001$

$\pm 10.74 \mu\text{M/ml}$). As to serum TAC, compared to the control rats a statistically significant ($P < 0.05$) decline was observed in diabetic rats. Serum TAC of diabetic rats reduced from $1.01 \pm 0.06 \text{ mmol trolox equivalent/L}$ to $0.87 \pm 0.03 \text{ mmol trolox equivalent/L}$.

Compared to the control animals serum MDA levels in diabetic rats were remarkably and significantly ($P < 0.05$) higher ($26.38 \pm 1.94 \mu\text{mol/L}$ and $42.23 \pm 1.24 \mu\text{mol/L}$, respectively). On the other hand, a statistically significant ($P < 0.05$) decrease from $4.39 \pm 0.15 \text{ mg/dl}$ to $4.00 \pm 0.09 \text{ mg/dl}$ was detected in serum GSH levels of the diabetic rats. Compared to the serum ceruloplasmin levels of healthy rats ($35.05 \pm 2.79 \text{ mg/dl}$), diabetic rats showed a significant decrease ($P < 0.001$) in their serum ceruloplasmin concentration ($23.10 \pm 1.65 \text{ mg/dl}$).

Insulin-dependent diabetes is becoming more common today with the influence of living conditions and therefore it is a widely studied pathology with its treatment and mechanisms of action. The data obtained by the preliminary work on animal experiments in this field is used in applications in humans (28).

Streptozotocin (STZ) reduces biosynthesis and secretion of insulin by disrupting glucose oxidation (4). The severity and duration of hyperglycemia depends on the dosage of the drug and type of laboratory animals (28). Since we aimed to have animals with diabetes, the STZ method was selected and a single dose was applied. Our results show that the smallest dose of i.p. 50 mg/kg STZ is enough to have animals with diabetes which is in accordance with the literature (13).

Free radicals are continuously synthesized in the body during normal metabolic processes. As a result of protein glycation and auto-oxidation of glucose in diabetes, the formation of free radicals increases. Increased oxidative stress is thought to play an important role in the etiopathogenesis of chronic complications of diabetes (3). Oxidative stress is known to be associated with metabolic or vascular diseases (30). Increased concentrations of glucose in diabetes result in deterioration of the balance between antioxidants and prooxidants and thus lead to increased oxidative stress (12).

Haluzik and Nedvidkova (14) detected increase in MDA concentration of kidney tissue and reduction in superoxide dismutase (SOD) and catalase activities in rats with diabetes induced by STZ. Yilmaz et al. (32) found an increase in the level of MDA in the liver tissue of 8-week-old rats with STZ diabetes, and suggested that the increased oxidative stress was responsible for diabetic cardiomyopathy, nephropathy and vascular complications. Akkaya and Çelik (2) investigated the relationship between free radicals and antioxidants in healthy and diabetic rats and reported an increased in the MDA levels and reduction in the homocysteine, leptin and vitamin C levels of diabetic rats compared to the rats in the control group. In this study, MDA levels of rats with diabetes were found to be increased

compared to the control group. These results are consistent with the results of earlier studies in the literature.

Opposite results were found in studies conducted on plasma ADMA levels in patients with type 2 diabetes mellitus. For example, Krzyzanowska et al. (16) reported increased ADMA levels in patients with type 2 diabetes, whereas Paiva et al. (22) found reduced ADMA levels in patients with type 2 diabetes and suggested that this may be caused by an increased glomerular filtration rate and poor glycemic control. In another study conducted by Lin et al. (17), the nitric oxide synthase pathway, which was deteriorated in Diabetes Mellitus, was examined and plasma ADMA levels were found to be higher in streptozotocin-induced diabetic rats.

Nitric oxide is a molecule that can play an important role as both pro-oxidant and anti-oxidant (30). In the literature, different results have been reported about NO levels in diabetes. Although Abou-Seif and Youssef (1) have found high plasma NO amounts in diabetic patients, Mohan and Das (19) reported that diabetes decreases plasma NO amounts in rats, and their NO levels can be increased by insulin and their increased MDA values can also be reduced.

Elabbady et al. (8) suggested a mechanism where NO may take place in the etiology of type I diabetes. Accordingly, macrophages rather CD4 T cells activated due to an autoimmune reason damage islet cells of the pancreas by causing large amounts of NO release. Khandelwal et al. (15) stated that synthesis of different isoforms of nitric oxide synthase enzyme may be increased or reduced depending on age, type of tissue and metabolic changes caused by diabetes in the tissue.

Measurement of the total antioxidant status may provide more valuable information compared to individual measurements of each antioxidant and, on account of this, total antioxidant capacity measurements that give the value of total antioxidants in the blood have become more common rather than individual antioxidant measurements. Therefore, in this study, we measured the total antioxidant capacity in rats with diabetes by using the most popular method developed by Erel (10).

Opara et al. (21) found serum total antioxidant levels lower in diabetes mellitus patients with and without proteinuria compared to the controls. Shin et al. (25) have found an inverse relationship between insulin resistance and plasma total antioxidant capacity, β -carotene and α -tocopherol levels. Dosoo et al. (7) have reported that the total antioxidant capacity in Non-IDMM patients reduced in an inverse proportion with fasting blood glucose.

Yanwen et al. (31) found that GSH levels were reduced in diabetic rats compared to the control group and likewise GSH levels were reduced in STZ-induced diabetic rats compared to the control group (24). Subramanian et al. (26) stated that ceruloplasmin levels were reduced in diabetic rats compared to the control group. In our study, GSH and ceruloplasmin levels were found to be reduced in diabetic rats compared

to the control group. These results are consistent with results of the earlier studies in the literature.

In this study, according to the findings obtained from the streptozotocin induced experimental diabetic model, increased glucose levels in diabetic rats cause oxidative stress and lipid peroxidation by increasing plasma MDA value and lead to a reduction in total antioxidant capacity. In addition, increased glucose levels cause an increase in the serum ADMA levels by affecting hyperglycemia ADMA metabolism and lead to a reduction in NO levels. DDAH is an endogenous inhibitor of NOS, so ADMA-DDAH pathway plays important role in control of NO *in vivo*. Although ADMA has been identified as a risk factor for endothelial dysfunction associated with DM, it is known that in diabetic patients with foot ulcers, decreased levels of NO and increased levels of ADMA in wound fluid are indicators of impaired wound healing. Since free radicals may play a role in the pathogenesis of diabetes and formation of complications, oral antidiabetic, insulin and various agents as well as antioxidant agents and vitamins can be used to prevent occurrence and spread of the disease.

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