Sex dependent difference in oxidative stress in the heart of rats with type 2 diabetes

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Type 2 diabetes mellitus is known to double mortality from cardiovascular diseases (CVD), in which oxidative stress plays an important role. It is suggested that the impact of diabetes on CVD risk may vary depending on gender. The aim of the study was to assess oxidative stress parameters in the heart of 12 weeks old male and female Wistar rats with type 2 diabetes mellitus (T2DM) induced by high-calorie diet followed by intraperitoneal streptozotocin injections. The level of advanced oxidation protein products, superoxide dismutase, glutathione reductase and glutathione peroxidase activity in the isolated heart mitochondria and NADPH-oxidase and xanthine oxidase activity in the post-mitochondrial supernatant fraction were determined. It was shown that T2DM induced more pronounced oxidative stress confirmed by the increased level of advanced oxidation protein products in the heart mitochondria of males than females. The data obtained indicate that the main reason of oxidative stress in the heart of diabetic males is the activation of non-mitochondrial sources of reactive oxygen species. While in the heart of diabetic female rats it is the decrease in antioxidant enzymes activity in mitochondria. These results justify the necessity of gender-specific therapy for the prevention and management of diabetic CVD.

Keywords: heart mitochondria, oxidative stress, gender dependance, diabetic rats.

Diabetes mellitus is a chronic non-communicable disease of high prevalence and the seventh most common cause of death worldwide. According to the World Health Organization, there are currently approximately 150 million people with diabetes. With the increasing prevalence of physical inactivity and obesity, the burden of diabetes is predicted to increase to 700 million by 2045 [1].

Type 2 diabetes mellitus (T2DM) is associated with a twofold increase in the risk of all-cause mortality and death from cardiovascular disease (CVD). CVD is the leading cause of morbidity and mortality for individuals with diabetes, which accounts for > 50% of all deaths [2].

However, increasing evidence indicates that sex may modify the effects of diabetes at risk of CVD. Many studies have shown that premenopausal women have a lower prevalence of T2DM when compared to men or postmenopausal women [3]. This evidence suggests a potential protective role of estrogens in the development of diabetes. It was revealed a 3-5 fold increased risk of coronary heart disease (CHD) in non-diabetic men versus premenopausal females [4]. However, when women are diagnosed with diabetes, this protective factor is disappeared as shown by a recent meta-analysis that concluded that women with diabetes have a 40% increased risk of developing CHD compared to men [5].

It is well established that oxidative stress is linked to the tissue damage and the development of diabetic cardiovascular complications. Reactive oxygen species (ROS), including superoxide anion, hydroxyl radical, and hydrogen peroxide, are critical signaling molecules with important roles in both cardiac physiology and disease. Both cytosolic sources, including NADPH oxidases (NOX), xanthine oxidase (XO), cyclooxygenases, cyto-
chrome P450 enzymes, and mitochondrial sources, such as the respiratory chain, monoamine oxidases and NOX4, contribute to the intracellular ROS pool. Under physiological conditions, cardiac ROS signaling regulates the heart development and cardiomyocyte maturation, cardiac calcium handling, excitation-contraction coupling, and vascular tone. However, pathological conditions of unregulated ROS production leading to elevated ROS levels can result in oxidative stress through oxidative damage to DNA, proteins, and lipids, as well as activation of the mitochondrial permeability transition pore, mitochondrial dysfunction, and cell death [6]. Indeed, dysregulated ROS production and oxidative stress have been implicated in a lot of cardiac diseases, including diabetic cardiomyopathy [7].

It was shown that there are sex-associated differences in oxidative stress and that estrogens protect females against its burden [8]. Thus, oxidative stress could be one of the targets underlying the protective effect of estrogens in diabetes. While progress has been made towards elucidating the main mechanisms of sex differences in the risk of diabetic cardiovascular complications, many uncertainties remain. Future research devoted to understanding these mechanisms could increase awareness of the sex-specific risk factors and eventually lead to more personalized diabetes care.

Therefore, the aim of the study was to assess the impact of sex on oxidative stress in the heart of rats with T2DM.

**Materials and Methods**

**Chemicals.** All chemicals used were of analytical reagent grade quality and purchased from Sigma Chemical Co. (St. Louis, MO, USA). All spectrophotometry was performed using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan).

**Experimental design.** The present study was approved by the bioethics committee of the “V. Danilevsky Institute of Endocrine Pathology Problems National Academy of Medical Sciences of Ukraine” (Kharkiv, Ukraine) and performed in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986).

The experiments were performed on 16 male and 16 female Wistar rats (12 week-old, 200–230 g body weight), which were housed in Plexiglas cages (3 animals per cage) at a temperature of (22 ± 1)°C, in a constant 12-hour light/dark cycle.

The animal model of T2DM induced by a high-calorie diet combined with multiple low-dose streptozotocin (STZ) injections was used. This model provides the development of two main features of T2DM: the high-calorie diet initiates insulin resistance and the low-dose STZ induces a mild impairment of insulin secretion. Thus, this model mimics the natural history of the disease events (from insulin resistance to β cell dysfunction) and the metabolic characteristics of human T2DM [9].

Control intact groups (8 male and 8 female rats) were fed a standard diet ad libitum for 14 weeks. Experimental groups (8 male and 8 female rats) were fed the high-calorie diet, containing 16% fat, 28% carbohydrates, 6% proteins for 14 weeks. All groups had free access to water. In four weeks, rats of experimental groups were injected intraperitoneally with small doses of STZ (25 mg/kg b.w.) twice per week [10]. All animals were sacrificed according to the protocol of the ethics committee.

**Measurement of glucose homeostasis.** The intraperitoneal glucose tolerance test (IPGTT) was performed on overnight fasted rats. Blood glucose concentrations were initially measured at the basal condition (0 min), then the animals were administered an intraperitoneal injection of glucose (3 g/kg b.w.). Subsequently, tail blood glucose levels were measured at 15, 30, 60, 90 and 120 min after the glucose load [11].

The short insulin-sensitive test was performed on overnight fasted rats by intraperitoneal injection of insulin 1.0 U/kg (Actrapid, Novo Nordisk, Agsvaerd, Denmark). Blood glucose levels were determined immediately before and 30 min after the injection. The insulin sensitivity index (ISI) was calculated as ((Glc)\(_{0\text{ min}}\)−(Glc)\(_{30\text{ min}}\))/(Glc)\(_{0\text{ min}}\)×100 % [12].

Tail blood glucose levels were measured using a glucose analyser (Exan-G, Analita, Vilnius, Republic of Lithuania).

**Mitochondria isolation.** Mitochondria were isolated by conventional procedures. Freshly excised rat hearts were homogenized in a medium containing 0.18 M KCl, 10 mM EDTA, 0.5% bovine serum albumin (BSA), 10 mM HEPES, pH 7.4. Homogenates were cleared from debris and nuclei by two times centrifugation at 500 g (10 min at 4°C). Mitochondria were pelleted from the supernatants at 10000 g (15 min at 4°C) and resuspended in isolation buffer.

The post mitochondrial supernatant fractions were used for the determination of XO and NOX activity. To remove EDTA and albumin, mitochondrial
pellets were washed two times at 10000 g (15 min, 4°C) and resuspended in wash buffer containing 0.18 M KCl, 10 mM HEPES (pH 7.4) [13].

*Measurement of enzymes activity.* Measurement of enzymes activity was performed using a spectrophotometer (Shimadzu UV-1800, Japan).

Glutathione reductase (GR) activity was measured by tracking the appearance of NADPH at 340 nm. Mitochondria from the heart (0.1 mg protein) were incubated for 10 min at 37°C in 2 ml of assay buffer: 20 mM Tris-HCl pH 7.4, 0.25 mM EDTA, 10 μM FAD, 3 mM oxidized glutathione and 0.1 mM NADPH. The blank sample did not contain oxidized glutathione [14].

The enzymatic reaction was used for measurement glutathione peroxidase (GPX) activity. Reaction mixture (3 ml, pH 7.0) contained: 0.1 mg of mitochondrial protein, 0.56 mM NADPH, 1.0 U glutathione reductase, 7.5 mM sodium azide, 5 mM GSH, 5 mM EDTA and 0.05 M phosphate buffer. Tert-butyl hydroperoxide (23 mM) as a substrate was added to initiate the enzymatic reaction. The rate of GS(G) formation was determined by decreasing the reaction mixture absorbance at 340 nm [15].

The superoxide dismutase (SOD) activity in mitochondrial suspension was detected by the inhibition of nitroblue tetrazolium (NBT) reduction from the change in absorbance at 295 nm (ε = 9500 M⁻¹·cm⁻¹) [18].

*Measurement of advanced oxidation protein products.* Determination of advanced oxidation protein products (AOPP) levels was performed by the modified method of Witko-Sarsat et al. [19]. KI (1.16 M) in volume 0.1 ml was added to 2.0 ml of mitochondrial suspension (0.1 mg protein/ml) in a phosphate buffer solution containing 0.1% Triton-X100. After 2 min, 0.2 ml of acetic acid was also added. The absorbance of the reaction mixture was immediately read at 340 nm against the blank. AOPP concentrations were expressed as nmol-equivalent chloramine T/mg protein.

*Measurement of protein content in the samples.* Mitochondrial protein was determined by the Lowry protein assay method with BSA as the standard [20].

*Statistical analysis.* Data normality were rated using the Shapiro-Wilk test, and all normally distributed data are expressed as the means ± standard error of the mean (SEM). Group comparisons of quantitative variables were performed by one-factor analysis of variance (ANOVA). The Newman-Keuls test was used for multiple comparisons of the groups. Values were considered significant at P < 0.05.

**Results and Discussion**

As shown in Table 1, the basal glycaemia in diabetic rats was significantly higher in comparison with control animals, and the level of basal hyperglycaemia was independent of sex.

T2DM caused the development of glucose intolerance and insulin resistance, as indicated by an increase in the area under the glycaemic curves during the IPGTT and a decrease ISI in both male and female rats compared to control groups. Moreover, a more pronounced decrease in glucose tolerance and insulin sensitivity was noted in diabetic males compared with diabetic females (Table 1, Table 2).

It was established that the oxidative stress, which was evaluated by the AOPP production in the heart mitochondria of the control animals, was higher in males than in females (P = 0.03). T2DM increased AOPP levels in the mitochondria of both sexes (P = 0.0001), but it was significantly lower in females (P = 0.02) (Fig. 1). AOPPs are recognized as a marker of oxidative damage of proteins and the intensity of oxidant-antioxidant imbalance and inflammation. The AOPP
Table 1. Indexes of glucose homeostasis in control and diabetic rats (n = 8)

| Group       | Basal glucose level, mmol/l | IPGTT, AUC (mmol/l)x×min |
|-------------|----------------------------|-------------------------|
|             | Male (M) | Female (F) | M vs F | Male (M) | Female (F) | M vs F |
| Control (C) | 5.00 ± 0.16 | 4.27 ± 0.20 | *p = 0.40 | 934.7 ± 51.2 | 982.9 ± 81.2 | *P = 0.73 |
| Diabetes (D)| 8.21 ± 1.14 | 8.10 ± 0.61 | *P = 0.99 | 1802.7 ± 76.1 | 1306.3 ± 163.8 | *P = 0.021* |
| C vs D      | *P = 0.003* | *P = 0.0005* |                     | *P = 0.0003* | *P = 0.03* |                     |

Data are shown as mean±SEM. M vs F – Male versus Female; C vs D – Control versus Diabetes; *significant differences

Table 2. The insulin sensitivity indexes in control and diabetic rats (n = 8)

| Group       | ISI, % |
|-------------|--------|
|             | Male (M) | Female (F) | M vs F |
| Control (C) | 31.33±2.64 | 32.92±1.15 | *P = 0.89 |
| Diabetes (D)| 14.27±0.82 | 22.81±0.91 | *P = 0.01* |
| C vs D      | *P = 0.006* | *P = 0.02* |                     |

Data are shown as mean±SEM. M vs F – Male versus Female; C vs D – Control versus Diabetes; *significant differences

It has been shown that there was no significant difference in the activity of NOX and XO in the post-mitochondrial fraction of the heart homogenate between control male and female rats (Fig. 5, 6).

T2DM caused an increase of NOX and XO activity in male cardiomyocytes in comparison with the control group (P = 0.0003 and P = 0.0001, respectively), while did not affect these parameters in females (Fig. 5, 6).

Increasing evidence suggests that sex and gender affect the pathophysiology, prevalence and response to therapy of many metabolic diseases, including diabetes. Sex differences have also been observed in the progression of cardiovascular diabetic complications, reinforcing the need for sex-specific approaches in diabetes management.

Many aspects of glucose metabolism and energy balance are regulated differently in males and females and influence their predisposition to T2DM. It is known that males are more likely to develop obesity, insulin resistance and hyperglycemia than females. Women are also characterized by higher ca-

![Fig. 1. Levels of AOPP in the isolated heart mitochondria of male and female rats. Data are shown as mean ± SEM. *M vs F, P < 0.05; #C vs D, P < 0.05](image1)

![Fig. 2. Superoxide dismutase activity was detected by the inhibition of NBT reduction. One arbitrary unit (a.u.) of SOD means the enzyme amount causing 50% inhibition of NBT reduction rate. Data are shown as mean ± SEM. *M vs F, P < 0.05; #C vs D, P < 0.05](image2)
Glutathione reductase activity was measured by tracking the appearance of NADPH in the isolated heart mitochondria of male and female rats. Data are shown as mean ± SEM. *M vs F, P < 0.05; #C vs D, P < 0.05.

Glutathione peroxidase activity was measured in the isolated heart mitochondria of male and female rats with tert-butyl hydroperoxide. Data are shown as mean ± SEM. *M vs F, P < 0.05; #C vs D, P < 0.05.

NOX activity was measured as superoxide production in the post-mitochondrial fraction of heart homogenate of male and female rats. Data are shown as mean ± SEM. *M vs F, P < 0.05; #C vs D, P < 0.05.

Xanthine oxidase activity was assayed in air-equilibrated PBS solution after addition of xanthine by measurement of uric acid production in the post-mitochondrial fraction of heart homogenate of male and female rats. Data are shown as mean ± SEM. *M vs F, P < 0.05; #C vs D, P < 0.05.

We revealed a more pronounced decrease in glucose tolerance and insulin sensitivity in diabetic males compared to diabetic females. The reason for these differences, at least partially, could be explained by the effect of gonadal hormones.

Clinical and experimental observations confirm the protective actions of endogenous estrogens in diabetes, mainly through estrogen receptor α (ERα) activation in various tissues, including the brain, the liver, skeletal muscle, adipose tissue and pancreatic beta cells [23].

Oxidative stress is thought to be a major factor contributing to the development and progression of diabetic complications, insulin resistance and impaired insulin secretion resulting in the development of T2DM. Thus, oxidative stress in T2DM constitutes an important factor implicated not only in the development of diabetic complications but also in the development of T2DM itself [24]. Oxidative stress is defined as a state in which ROS overproduction in vivo exceeds the buffering capacity of antioxidant enzymes and antioxidants, thus resulting in a local imbalance between ROS production and destruction. It was shown that ROS in appropriate amounts acts as signal transduction molecules providing cell protection, while in large excess, they modify bio-
logical macromolecules, thus inducing cell dysfunction or death. Recent research has shown that ROS signaling pathways are complex, compartmentalized and in many cases essential for normal cardiovascular physiology [25]. However, ROS overproduction has been implicated systemically or acutely in a variety of cardiovascular diseases and conditions including atherosclerosis, diabetic vascular disease, myocardial infarction, hypertrophy, cardiomyopathy and heart failure [26]. Sources of ROS production in the tissue include the mitochondrial electron transport system, NOX, xanthine oxidase, uncoupled nitric oxide synthase, and arachidonic acid metabolism pathways, but they vary in their pathological role and importance depend on the disease and the organ. The mitochondrial electron transport chain, NOX, and xanthine oxidase are thought to be primary sources of ROS production in cardiomyocytes [27]. It is suggested that mitochondrial ROS are a major cause of oxidative stress associated with T2DM [28].

We established that oxidative stress in the heart mitochondria, which was evaluated by the AOPP production, was higher in diabetic males than diabetic females (\(P = 0.02\)). The protection against oxidative stress in females versus males may be also a result of estrogen action.

The antioxidant role of estrogen has been abundantly demonstrated [29]. Recent molecular studies have shown that 17\(\beta\)-estradiol (E2) plays a regulatory role in mitochondrial function, including ATP production, generation of mitochondrial membrane potential, mitochondrial biogenesis, and regulation of calcium concentrations [30]. E2 may also influence mitochondrial function by altering mitochondrial ROS formation and is thought to induce antioxidant responses [31].

Previous studies also reported that hepatic mitochondrial lipid oxidation levels were lower in diabetic females when compared with diabetic males [32] and that estradiol was responsible for this protection against oxidative stress [33].

In addition, our results indicated different impact of T2DM on the activity of antioxidant enzymes in the heart mitochondria of male and female rats. Thus, the activities of SOD, GR and GPX were significantly higher (\(P = 0.0007\), \(P = 0.006\) and \(P = 0.0002\), respectively) in mitochondria of diabetic males compared to the control group. In contrast to diabetic males, an inhibition of these enzymes (SOD \(P = 0.0002\); GR \(P = 0.0002\); GR \(P = 0.02\)) activity in the cardiomyocytes mitochondria was observed in females with T2DM in comparison with control rats. However, the activity of SOD and GR was not significantly different in the heart mitochondria of diabetic males and females, while the GPX activity was significantly higher (\(P = 0.0002\)) in male rats with T2DM compared to diabetic females.

The obtained results suggest the pathogenic mechanisms of the changes in the redox balance of the diabetic heart mitochondria have significant modifications depending on the sex of experimental animals. These findings are confirmed by a more pronounced disturbance of glucose homeostasis which was accompanied by a greater intensity of oxidative stress in the cardiomyocytes mitochondria of diabetic males compared to diabetic females. It is known that a slight increase in ROS production by mitochondria leads to activation of redox-sensitive signaling pathways, like Keap1/NF-E2-related factor 2, which increases the expression of antioxidant enzymes in the nuclear genome, including the mitochondrial isoforms of SOD, GR, and GPX [34]. Thus, the maintenance of the redox balance is supported by negative feedback.

We suggest that the higher activity SOD, GR and GPX and lower levels of AOPP in the heart mitochondria of control female rats compared to males are most likely caused by the positive effect of estrogens on redox homeostasis in these organelles. However, inhibition of antioxidant enzymes in the heart mitochondria of diabetic female rats may be associated with a decline of the estrogens protective function in hyperglycemia states or insulin resistance conditions.

While mitochondria are considered as main source of ROS in the heart, the other most important one is NADPH oxidases, which are in cardiomyocytes in two isoforms – NOX2 and NOX4. NOX2 is localized in the plasma membrane and is involved in intracellular signaling of the angiotensin II receptor and some other receptors, while NOX4 is contained in mitochondria and exhibits constitutional activity [35, 36].

In our study, we revealed that T2DM causes an increase in NOX activity only in male cardiomyocytes in comparison with the control group (\(P = 0.0003\)), but did not affect these parameters in females. Other research also demonstrated that fibrosis and myocardial hypertrophy in mice with T2DM are accompanied by a rise of NOX2 expression and male rats have higher levels of the NOX subunits [37]. In addition, NOX activity in ovariectomized
rats was increased and treatment with 17b-estradiol restored enzyme activity [38]. These data suggest that estrogen may contribute to decreased oxidative stress, possibly via the regulation of NOX activity [37].

XO has been reported as an additional source of ROS in cardiomyocytes which generates the superoxide-anion as a by-product of the conversion of xanthine to uric acid. It was also shown that the activity of XO increases in diabetic (type 1) rats [39], and its inhibition can prevent oxidative stress-mediated atrial remodeling in alloxan-induced diabetic rabbits [40]. We found that XO activity was higher \((P = 0.0002)\) in the heart of diabetic males compared to diabetic females. Similar results were obtained in another study that demonstrated higher XO activity in plasma of diabetic Goto-Kakizaki male rats when compared with females [41].

Our data confirm that oxidative stress could be one of the main mechanisms of sex differences in the risk of diabetic cardiovascular complications and the target for protective effect of estrogens in diabetes.

**Conclusions.** The results of the present study confirmed a higher ROS production and a lower activity of antioxidant enzymes in the heart mitochondria of control male rats compared to females. But the activity of other non-mitochondrial sources of ROS in the cardiomyocytes (NOX and XO) was not affected by sex in control rats.

We revealed that T2DM induces more pronounced oxidative stress in the heart mitochondria of males compared to females that was confirmed by increasing AOPP levels in mitochondria. This may be associated with higher sensitivity of myocardium cells of males to hyperglycemia and insulin resistance. We suggest that the main reason for oxidative stress development in diabetic female rats is a decrease in the activity of antioxidant enzymes in the heart mitochondria, while in diabetic males, activation of non-mitochondrial sources of ROS may be a major contributing factor.

The question still remains whether these sex differences with oxidative stress explain the gender differences in diabetic cardiovascular complications, and more researches are needed to clarify it. This data justify the necessity of gender-specific therapy development for the prevention and management of diabetic macroangiopathy.
ензимів в мітохондріях. Результати досліджень підтверджують необхідність застосування гендерноспецифічної терапії для профілактики та лікування діабетичних ССЗ.

Ключові слова: мітохондрії серця, оксидативний стрес, гендерні особливості, діабетичні шури.

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