Effects of melatonin on lipid peroxidation and antioxidant enzymes in streptozotocin-induced diabetic rat testis

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Abstract

Aim: To examine the effects of melatonin treatment on lipid peroxidation (LPO) and the activities of antioxidant enzymes in the testicular tissue of streptozotocin (STZ)-induced diabetic rats. Methods: Twenty-six male rats were randomly divided into three groups as follows: group I, control, non-diabetic rats (n = 9); group II, STZ-induced, untreated diabetic rats (n = 8); group III, STZ-induced, melatonin-treated (dose of 10 mg/kg·day) diabetic rats (n = 9). Following 8-week melatonin treatment, all rats were anaesthetized and then were killed to remove testes from the scrotum. Results: As compared to group I, in rat testicular tissues of group II, increased levels of malondialdehyde (MDA) (P < 0.01) and superoxide dismutase (SOD) (P < 0.01) as well as decreased levels of catalase (CAT) (P < 0.01) and glutathione peroxidase (GSH-Px) (P > 0.05) were found. In contrast, as compared to group II, in rat testicular tissues of group III, levels of MDA decreased (but this decrease was not significant, P > 0.05) and SOD (P < 0.01) as well as CAT (P < 0.05) increased. GSH-Px was not influenced by any of the treatment. Melatonin did not significantly affect the elevated glucose concentration of diabetic group. At the end of the study, there was no significant difference between the melatonin-treated group and the untreated group by means of body and testicular weight. Conclusion: Diabetes mellitus increases oxidative stress and melatonin inhibits lipid peroxidation and might regulate the activities of antioxidant enzymes of diabetic rat testes. (Asian J Androl 2006 Sep; 8: 595–600)

Keywords: melatonin; antioxidant enzymes; lipid peroxidation; oxidative stress; diabetes mellitus; testis

1 Introduction

Melatonin (N-acetyl-5-methoxy-tryptamine) is synthesized mainly by the pineal gland and is suggested to have antioxidant and prophylactic properties [1]. The direct effects of melatonin on the male reproductive system and testosterone synthesis from Leydig cells have also been examined in studies on animals [2]. Because melatonin binding sites have been detected in the reproductive system of different species, it seems reasonable to assume that melatonin exerts its actions through direct interaction with the steroidogenic cells of the reproductive organs [2].

Diabetic testicular dysfunction might be transient or permanent depending on the degree and duration of the disease. Erectile dysfunction (ED) is a well-recognized complication of diabetes mellitus (DM). Infertility among
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Diabetic men is a less well-examined problem and the evaluation of the gonadal state in these cases is not clearly established. The low incidence of diabetes in infertile patients might be the reason for the limited amount of current research [3]. However, an altered neuroendocrine and testicular axis was noted in experimental studies [3]. Seethalakshmi et al. [4] found that testicular weight, sperm count and motility are significantly decreased in diabetic rats. Moreover, Cameron et al. [5] defined increasing tubule wall thickness, germ cell depletion and Sertoli cell vacuolization in diabetic human testicular biopsies and in diabetic rats.

Enhanced oxidative stress and changes in antioxidant capacity are considered to play an important role in the pathogenesis of chronic DM [6, 7]. Although the mechanisms underlying the alterations associated with DM are presently not well understood, hyperglycemia lead patients to increased oxidative stress because the production of several reducing sugars (through glycolysis and the polyol pathway) is enhanced [8]. These reducing sugars can easily react with lipids and proteins (nonenzymatic glycation reaction), increasing the production of reactive oxygen species (ROS) [8].

Ultimately, the aim of the present study is to study the effect of DM on rat testicular tissue and to determine the effects of melatonin on diabetic rat testes. To our knowledge, this is the first study that investigated the role of melatonin in STZ-induced diabetic rat testes.

2 Materials and methods

2.1 Animal model

Twenty-six male Spraque–Dawley rats (11 weeks old) obtained from the Laboratory Animal Production Unit of Selcuk University were used in the present study. They were kept in an environment of controlled temperature (24–26°C), humidity (55–60%) and photoperiod (12:12 h light: dark cycle) for 1 week before the start of the experiment. A commercial balanced diet (Hasyem, Isparta, Turkey) and tap water were provided ad libitum. All animals were treated in compliance with the present institutional guidelines.

2.2 Experimental design

Twenty-six male rats were randomly divided into three groups (each animal placed into a separate stainless-steel cage) as follows: group I, control non-diabetic rats (n = 9); group II, STZ-induced, untreated diabetic rats (n = 8); group III, STZ-induced, melatonin-treated diabetic rats (n = 9) which were injected daily with melatonin. Melatonin (Merck–Schuchardt, Hohenbrunn, Germany) was given at a dose of 10 mg/kg/day i.p. [9] for 3 days following STZ treatment and continued until rats were killed. In control rats, isotonic saline solution (equal to the volume of melatonin) was given intraperitoneally. STZ dissolved in sodium citrate buffer (pH 4.5) was administered i.p. at a single dose of 35 mg/kg. Blood glucose levels were measured with a Gluco-meter (Roche Diagnostic, Manheim, Germany) in all rats after 3 days of STZ treatment. Prior to initiating the experiments, it was determined that animals with blood glucose levels < 300 mg/dL would be excluded; however, none was excluded. After 8-week melatonin treatment, the rats were anaesthetized with an intramuscular injection of 50 mg/kg ketamine hydrochloride (Ketalar, Eczacibasi, Istanbul, Turkey) and then the testes were removed from the scrotum. The specimens were harvested and stored at −20°C until biochemical assays were performed.

2.3 Biochemical procedure

The frozen tissue samples of testes were weighed and homogenized (Ultra Turrax T25, Staufen, Germany), in 50 mmol/L phosphate buffer (pH 7.4) kept in an ice bath. The homogenate and supernatant were frozen at −20°C in aliquots until used for biochemical assays. The protein content of the supernatant was determined using the Lowry method [10].

2.4 Determination of malondialdehyde (MDA)

Malondialdehyde (MDA) level, an indicator of free radical generation, which increases at the end of lipid peroxidation, was estimated using the double heating method of Draper and Hadley [11]. The principle of the method is the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid (TBA) with MDA. For this purpose, 2.5 mL of 100 g/L TBA solution was added to 0.5 mL supernatant in each centrifuge tube and the tubes were placed in a boiling water bath for 15 min. After cooling in tap water, the tubes were centrifuged at 1000 × g for 10 min and 2 mL of the supernatant was added to 1 mL of 6.7 g/L TBA solution in a test tube and the tube was placed in a boiling water bath for 15 min. The solution was then cooled in tap water and its absorbance was measured using a spectrophotometer (Shimadzu UV-1601, Kyoto, Japan) at 532 nm. The concentration of MDA was calculated by

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the absorbance coefficient of the MDA–TBA complex \( \varepsilon = 1.56 \times 10^5 \text{ cm}^{-1}\text{mol}^{-1} \) and is expressed as nanomoles per gram of protein.

### 2.5 Determination of superoxide dismutase (SOD) activity

Total (Cu-Zn and Mn) superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to the method of Sun et al. [12]. The principle of the method is based, briefly, on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine/xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the supernatant after 1.0 mL ethanol/chloroform mixture (5/3, v/v) were added to the same volume of sample and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. Activity was expressed as units per milligram protein.

### 2.6 Determination of catalase (CAT) activity

CAT (EC 1.11.1.6) activity was measured according to the method of Aebi [13]. The principle of the assay is based on the determination of the rate constant \( k \) (dimension: \( s^{-1} \)) of hydrogen peroxide decomposition. By measuring the absorbance changes per minute, the rate constant of the enzyme was determined. Activities were expressed as \( k \) (rate constant) per gram protein.

### 2.7 Determination of glutathione peroxidase (GSH-Px) activity

GSH-Px (EC 1.6.4.2) activity was measured using the method of Paglia and Valentine [14]. The enzymatic reaction in the tube that contained reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced glutathione (GSH), sodium azid and glutathione reductase was initiated by the addition of hydrogen peroxide (H\(_2\)O\(_2\)) and the change in absorbance at 340 nm was monitored by a spectrophotometer. Activity was given in units per gram protein. All samples were assayed in duplicate.

### 2.8 Statistical analysis

Data were presented as mean ± SD. SPSS 9.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. The one-way analysis of variance and post hoc multiple comparison tests were performed on the data of biochemical variables to examine the differences among groups. \( P < 0.05 \) was considered statistically significant.

### 3 Results

The mean body and testicular weights, and blood glucose levels of all three groups are given in Table 1. As compared to the control, rats body weight decreased in diabetic and melatonin-treated diabetic rats \( (P < 0.01) \). However, there was no significant difference between the melatonin-treated diabetic group and the untreated diabetic group by means of body and testicular weights \( (P > 0.05) \). The blood glucose concentration of STZ-treated group at the end of 8 weeks was considerably higher than that of the control group \( (P < 0.01) \). Melatonin did not significantly affect the elevated glucose concentration of diabetic group.

The level of MDA in the testes was increased in untreated diabetic group compared with that in the control group \( (P < 0.01) \). However, melatonin treatment reduced MDA level compared to the untreated-diabetic group, but this decrease was not significant (Table 2).

The SOD activity in the untreated diabetic group was significantly higher than that in other groups \( (P < 0.01) \). However, the SOD activity was significantly decreased in the melatonin-treated diabetic rats compared to that in the untreated diabetic group (Table 2). CAT activity was decreased in the untreated diabetic group compared to that in both the control group \( (P < 0.01) \) and melatonin-treated diabetic group \( (P < 0.05) \). Furthermore, melatonin treatment significantly increased CAT level compared to the untreated diabetic group (Table 2).

The activity of GSH-Px was decreased in untreated and treated diabetic rats compared with that in the con-
4 Discussion

DM is the most common endocrine disease that leads to metabolic abnormalities involving regulation of carbohydrate metabolism. These abnormalities produce pathologies including vasculopathies, neuropathies, ophthalmopathies and nephropathies, among many other medical derangements [15]. Oxidative stress plays a role in the development of diabetic complications [16]. In the diabetic state, lipid peroxidation (LPO) can be induced by protein glycation and glucose auto-oxidation that can further lead to the formation of free radicals [17]. The main free radicals that occur in this diseased state are superoxide (O\textsubscript{2}\textsuperscript{-}), hydroxyl (OH) and peroxyl (LOO\textsuperscript{-}) radicals. These free radicals all might play a role in DNA damage, glycation and protein modification reactions, and in lipid oxidative modification in diabetes [18]. The damage that these radicals inflict on cells might be quantitatively determined by measurement of levels of MDA, a product of LPO [19]. Certain enzymes play an important role in antioxidant defense, to maintain viable reproductive ability; a protective mechanism against oxidative stress is of importance [20]. These enzymes include SOD, GSH-Px, glutathione reductase (GSH-Rd) and CAT, which convert free radicals or reactive oxygen intermediates to non-radical products. SOD and GSH-Px are major enzymes that scavenge harmful ROS in male reproductive organs [20].

Melatonin is an important component of the antioxidant profile of many tissues and cells. Reiter et al. [21] documented that melatonin is an efficient scavenger of OH\textsuperscript{-}, peroxynitrite anion (ONOO\textsuperscript{-}), O\textsubscript{2}\textsuperscript{-}, nitric oxide radical (NO) and peroxy radicals. Moreover, it enhances the ability of cells to resist oxidative damage by inhibiting the pro-oxidant nitric oxide synthase [22].

The degree of LPO has been assessed according to MDA formation, which has been routinely used as an index of LPO. The increased MDA level in DM suggests that hyperglycaemia induces peroxidative reactions in lipids [23]. Furthermore, the results suggest that the antioxidative defense systems might have been increased as a response to the diabetic oxidative stress state. In our study, MDA levels in the testicular tissues from the melatonin-treated diabetic group were, however, reduced compared to the untreated diabetic group, but were not significant. This finding was contradictory to the findings in the erythrocytes of Vural et al. [9], which showed a significant return of MDA levels in the melatonin-treated group as compared to the untreated diabetic group to approximate levels of the control group. Oner-Iyidogan et al. [2] demonstrated a significant increase in MDA levels with acute administration of ethanol. However, these levels were significantly reduced with the successive administration of melatonin in the testicular tissue. In another study, the level of MDA was significantly lower in the melatonin treated group compared to the controls in exposed extracorporeal shock wave lithotripsy (ESWL) in the rabbit kidney [24]. Baydas et al. [25] compared vitamin E and melatonin effects on brain, liver and kidney MDA levels in streptozotocin-induced rats and found that MDA levels are more efficiently decreased with administration of melatonin compared to vitamin E, suggesting that melatonin seems to be a more potent antioxidant, especially in the brain and kidney. According to the present study, the level of MDA in testicular tissues is not significantly reduced. A possible explanation for this finding might be attributed to the longer duration of the current experiment (8 weeks). Furthermore, the specialized inherent structure of the testicular tissue used in the present study might have formed a blood-testicular barrier to melatonin uptake.

There is currently no consensus regarding antioxidant enzyme levels in various organs during the diabetic diseased state. Whereas some studies measuring activities of SOD and CAT in DM show the reductions in the levels of these enzymes [26], other studies report the

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA (nmol/g protein)</th>
<th>SOD (U/mg protein)</th>
<th>CAT (k/g protein)</th>
<th>GSH-Px (U/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 9)</td>
<td>4.22 ± 0.90</td>
<td>0.096 ± 0.008</td>
<td>0.155 ± 0.020</td>
<td>4.101 ± 0.970</td>
</tr>
<tr>
<td>Diabetic (n = 8)</td>
<td>5.51 ± 0.94</td>
<td>0.125 ± 0.017\textsuperscript{c}</td>
<td>0.090 ± 0.010\textsuperscript{c}</td>
<td>3.501 ± 0.840</td>
</tr>
<tr>
<td>Diabetic + melatonin (n = 9)</td>
<td>4.82 ± 0.92</td>
<td>0.097 ± 0.012</td>
<td>0.112 ± 0.020</td>
<td>3.783 ± 0.790</td>
</tr>
</tbody>
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increases in the activities of both enzymes with the STZ-induced diabetes [27–29]. SOD catalyzes the conversion of superoxide radical to H2O2. It protects the cell against the toxic effect of superoxide radicals. In the present study, the increase in the activity of SOD was significant in the testicular tissue of the untreated diabetic rats. The increased SOD activity might be another sign of the increased oxidative stress in the testicular tissue. Melatonin might be a scavenger for the free oxygen radicals. Therefore, it might prevent the elevation of the activities of SOD enzymes in diabetic rat testes.

CAT activity is increased significantly in the melatonin-treated diabetic group. Hydrogen peroxide is often metabolized by CAT and GSH-Px; when CAT activity is decreased, as in the present study, H2O2 is reduced to a very highly oxidizing OH radical in the presence of Fe2+ or other transition metals. The OH radical cannot be enzymatically removed from cells but a free radical scavenger can detoxify it.

Despite the increased SOD and the decreased CAT activities in diabetic rat testes, the activity of GSH-Px was not significantly changed. There are discrepancies in the activity of GSH-Px in diabetic rats. Both decreases [28] and increases in the activity of GSH-Px are reported in diabetes [23]. GSH-Px catalyzes the reduction of H2O2 by reduced glutathione. The resulting glutathione disulfide is reduced by NADPH. Therefore, the reduction of the GSH-Px (dependent on H2O2 degradation) observed in endothelial cells might be a result of high glucose concentration. This abnormality might be associated with the increased cellular damage following an exogenous exposure to H2O2 [30]. Furthermore, superoxide radicals could inhibit the activity of GSH-Px [31, 32]. In the current study, it has been demonstrated that melatonin treatment increases the activity of GSH-Px in diabetic testicular tissue, but this increase is not significant.

In summary, our study demonstrates that the diseased diabetic state increases MDA activity, which is mitigated by melatonin administration; however, this decrease is not significant. In addition, the diabetic testicular tissue SOD activity is increased and level of CAT is decreased, whereas activity of GSH-Px is not altered. As a result, we believe that STZ-induced DM induces testicular damage and melatonin treatment might affect antioxidant enzyme quantity and/or activity.

In the light of our results and those of others, it can be concluded that DM increases oxidative stress and melatonin inhibits LOP and regulates antioxidant enzymes of diabetic rat testes. Further molecular and histopathologic investigations are needed to prove the protective role of melatonin in DM-induced oxidative testicular damage.

References

17. Mullarkey CJ, Edelstein D, Brownlee M. Free radical generation by early glycation products: a mechanism for accelerated