Effects of melatonin treatment on the spermatogenesis and serum inflammatory cytokine levels in diabetic rats

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Abstract

Aim: Diabetes mellitus (DM) is known to have side effects on sexual and reproductive functions in males. The aim of current study was to investigate the effect of melatonin treatment on spermatogenesis and serum inflammatory cytokine levels in diabetic rats.
Material and Method: Eighteen adult Wistar albino male rats were divided randomly into three groups each containing 6 rats; control group (group 1), diabetic group (group 2), diabetic treated with melatonin group (group 3). Experimental diabetes was obtained by administrating 50 mg/kg streptozotocin. Melatonin treatment was administered intraperitoneally for 7 days at a dose of 20 mg, daily. Serum levels of interleukin-1 beta (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) were assayed. Spermatogenesis was evaluated according to Johnsen score at immunopathological examination.
Results: The Johnsen scores in control, diabetic and treatment groups were 9.78, 8.91, and 9.61, respectively. Spermatogenesis was negatively affected in diabetic rats and significantly improved with melatonin therapy (p<0.05). The serum levels of TNF-α in diabetic rats were observed to be increased compared to the control group (p<0.05). The levels of serum IL-1β and TNF- α were significantly decreased with melatonin treatment. IL-6 levels were not different among the three groups (p=0.248).
Conclusions: In the present study, intraperitoneal melatonin treatment was determined to have a positive effect of on spermatogenesis and caused a decrease in serum inflammatory cytokine levels in diabetic rats.

Keywords: Cytokines; Diabetes Mellitus; Melatonin; Rats; Spermatogenesis.

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease caused by insulin resistance or defective insulin secretion. Streptozotocin (STZ)-induced DM is characterized by hyperglycemia and chronic inflammatory conditions resulting from insulin deficiency (1,2). Clinical and experimental studies conducted with rats have shown that DM reduces semen volume, number of spermatozoa and their motility (3-6). Oxidative stress is considered to be an important factor in many diseases including DM (7). It has been reported that in STZ-induced diabetic rats, spermatogenesis is adversely affected due to oxidative stress (5,8,9). Although the precise mechanism of DM has not yet been clarified, the major cause for damage to spermatogenesis has been reported to be the increased production of free radicals (10).

Spermatogenesis is a complex process controlled by endocrine and testicular paracrine/autocrine factors (11,12). It has been reported that testicular cytokines and growth factors such as Interleukin-1 (IL-1), Interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), leukemia inhibitory factor (LIF), and stem cell factor (SCF) affect germ cell proliferation, and the functions and secretion of Leyding and Sertoli cell (12-15). These cytokines and growth factors are produced by a variety of testicular cells in immunocytes and interstitial and seminiferous tubular compartments (e.g., Sertoli, Leyding, pertitubular cells,

spermatogonia, differentiated spermatogonia, and even spermatozoa) (16). TNF alpha in the testis is produced by pachytene spermatocytes, round spermatids, and testicular macrophages plays a significant role in acute inflammation and anti-tumor immunity (13,17). IL-1 and IL-6 produced by Sertoli cells may potentially act as a physiological paracrine factor on lymphocytes and other testicular cells (18). IL-1, IL-2, interferon-gamma (IFN-γ) and TNF-α have been shown to regulate (induce / inhibit) Leyding cell steroidogenesis and secretion of transferrin by Sertoli cells (12-14,20). Some pro-inflammatory cytokines such as TNF-α and IL-1β have also been reported to have important roles in testicular homeostasis (13,21,22).

Melatonin is a hormone released from the pineal gland and has antioxidative and anti-inflammatory effects (23). Studies have shown that melatonin sweeps away free radicals by in vitro detoxifying highly reactive hydroxyls (OH) (24,25). Several studies on rats have reported the positive effects on the reproductive system (26,27). However, there are discrepancies concerning the results of some studies on the antioxidant activity of male germ cells (28). Recent studies reported that melatonin increases the development of spermatogenetic cells of seminiferous tubules (7,28,29).

The aim of this study was to investigate the effect of melatonin treatment on spermatogenesis and determined the changes in the serum inflammatory cytokine levels in diabetic rats.

MATERIAL and METHODS

Animals
A total of 18 Wistar albino male rats (aged 6-8 months and weighing 220-240 g) were used in the study. The animals were kept under standard conditions with a 12-hour light/dark cycle (7 a.m. to 7 p.m.) at constant room temperature (22 ± 2 °C) and humidity (40-60%) and were provided ad libitum access to food and water. All the experiments were approved by the local animal care committee on of Ataturk University, Erzurum, Turkey and performed in accordance with the national guidelines for the use and care of laboratory animals (Ethics Committee Number: 2013/105).

Experimental design and procedures
The rats were randomly divided into three groups each consisting of 6 rats: the healthy control group (Group 1), diabetic group (Group 2) and diabetes+melatonin treated group (Group 3).

STZ is one of the most important diabetogenic chemical components in experimental research on diabetes (30). In this study, diabetes was induced in rats through the intraperitoneal (i.p.) administration of freshly prepared STZ (Sigma, St. Louis, USA) within 0.1 M sodium citrate solution (pH: 4.50) at a dose of 50 mg/kg/day. Five days after the administration of STZ, blood glucose levels of the rats were measured using a blood sugar measurement device (Glucomax Ultra TD 4227, Germany). The rats with a blood glucose level higher than 300 mg/dL were considered to have diabetes. On the 28th day, the diabetic rats were randomly divided into two groups (Group 2 and Group 3) with the latter being treated with melatonin (20 mg/kg/day, i.p.) for seven days. Melatonin (Sigma, St. Louis, Mo., USA) was dissolved in absolute ethanol and diluted with physiological saline. At the end of day 36, all the rats were sacrificed under anesthesia with thiopental sodium (5mg/kg), their testis tissues were placed in paraffin blocks and treated with routine histological methods.

Histopathological Evaluation
The testes were fixed in 10% formaldehyde solution. The tissues were processed to be paraffin-embedded and 5 µm sections were stained with hematoxylin and eosin. The sections were analyzed in terms of spermatogenesis. The seminiferous tubules were graded according to the Modified Johnsen scoring system. For each testis, 100 tubular sections were evaluated and their average Johnsen score was calculated. The normal average Johnsen score was accepted as >9.39 (31,32).

Histological criteria in Modified Johnsen scoring
Full sperматogenesis (Score 10); slightly impaired spermatogenesis, many late spermatids, disorganized epithelium (Score 9); less than five spermatozoa per tubule, few late spermatids (Score 8); no spermatozoa, no late spermatids, many early spermatids (Score 7); no spermatozoa, no late spermatids, few early spermatids (Score 6); no spermatozoa or spermatids, many spermatocytes (Score 5); no spermatozoa or spermatids, few spermatocytes (Score 4); spermatogonia only (Score 3); no germinal cells, Sertoli cells only (Score 2); no seminiferous epithelium (Score 1).

Cytokine Levels Analysis
Blood samples were obtained from the vena cava inferior of all the rats. Blood-containing tubes (Vacutainer SST, Becton Dickinson, USA) were centrifuged for 10 min at 3,000 ×g. The serum samples were stored at −80 ºC until used for the ELISA assays.

Serum IL-1β (BMS630, eBioscience, San Diego, CA, USA), IL-6 (BMS625, eBioscience, San Diego, CA, USA) and TNF-α (BMS622, eBioscience, San Diego, CA, USA) levels were quantified by using ELISA kits. All assay procedures were carried out according to the manufacturer's instructions. The detection range of the ELISA kit was 31.25−2000 pg/mL for IL-1β and IL-6 and 39.1-2500 pg/mL for TNF-α. The absorbance of standards and samples were obtained at 450 nm wavelength using Epoch spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Statistical analysis
Descriptive statistics were obtained as a mean ± standard deviation. After checking the assumptions of normality and homogeneity of variance (Shapiro-Wilk and Levene's test, the variables were evaluated. Data analysis was performed using one-way analysis of variance (ANOVA). For those variables that were found not to meet the requirements for the parametric tests, the Box-Cox transformation was applied. In cases that still did not meet the criteria for the

parametric tests, a non-parametric test, Kruskal Wallis, was utilized. The variables with significant differences were evaluated using the adjusted Bonferroni test. The data was analyzed by SPSS software (version 17, Chicago, IL, USA). The statistical significance was accepted as α = 0.05.

RESULTS

Evaluation of the results on hematoxylin-eosin staining
The analysis of the results was undertaken by an expert (SG) that also performed Johnsen scoring. In the control group (Group 1), complete spermatogenic series were observed in the seminiferous tubules in the testis tissues (Figure 1). In diabetic rats, the structure of the seminiferous tubules was disorganized and there was a significant reduction in the spermatogenetic cell series (Figure 2).

The number of spermatogenic cells was found to be higher in the diabetic group that was treated with melatonin compared to the non-treated diabetic rats (Figure 3). The results of the Periodic acid−Schiff (PAS) staining indicated that the baseline thickness of the membrane of the seminiferous tubules was normal in all three groups. The Johnsen score was found to be 9.78, 8.91 and 9.61 in the control, diabetic and melatonin groups, respectively. Table 1 presents the results of all the groups according to the Johnsen criteria. The mean Johnsen score was significantly lower in diabetic rats compared to the control group (p<0.05). However, it was significantly higher in diabetic rats that were treated with melatonin (p<0.05) compared to the non-treated diabetic group. No statistical difference was observed between the control group and the melatonin group in terms of the Johnsen scores (p<0.05). In all groups, the polymorphonuclear leukocytes in the testis tissue were at a normal level.
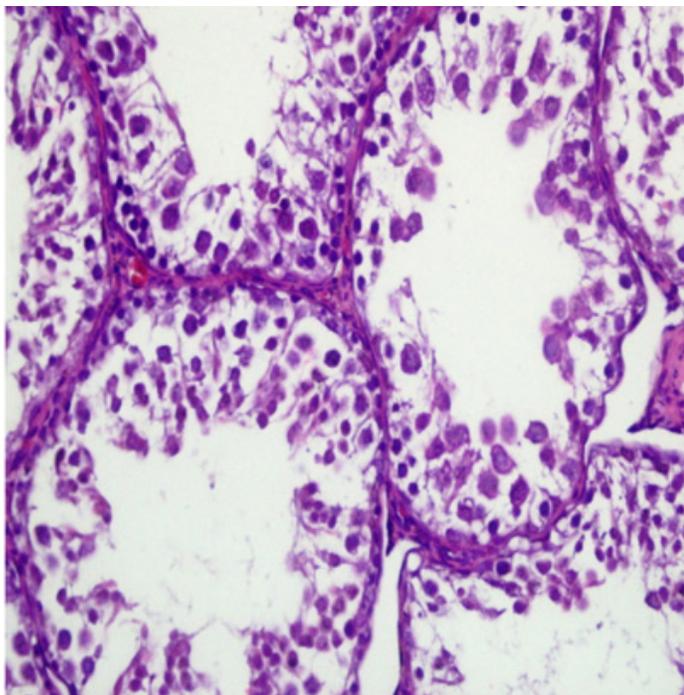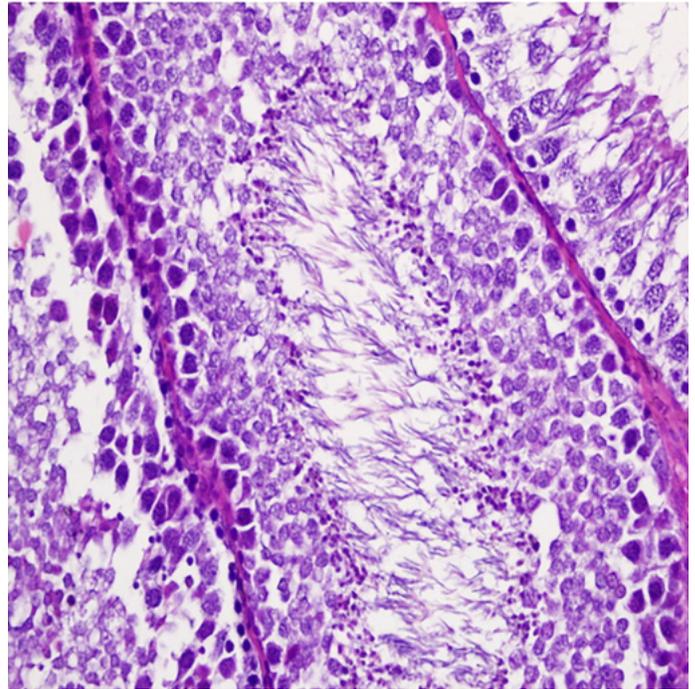

Figure 2. Presence of disorganized structure of the seminiferous tubules and reduced spermatogenic cell series in diabetic rats

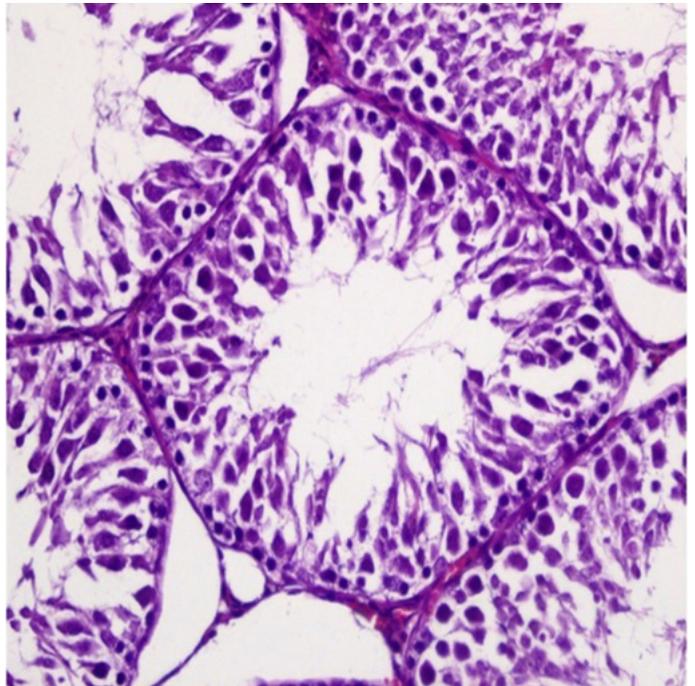**Figure 3.** Increased number of spermatogenic cells in diabetic rats following melatonin treatment

Figure 1. Presence of complete cell series of the seminiferous tubules in the testis tissues of the control group

Table 1. Johnsen score levels				
	Control Group	Diabetic Group	Diabetes + Melatonin Treated Group	p
Johnsen Score	9.78±0.57	8.91±1.79[ab]	9.61±0.78[b]	<0.05
p<0.05; a: Different from the treated group; b: Different from the control group				

Serum inflammatory cytokines

When the serum levels of the inflammatory cytokine were examined, TNF-α was found to be higher in the diabetic group than the control group with the difference being non-significant. The levels of TNF-α were lower in the melatonin group compared to the control and diabetic groups (p<0.05). When compared to the control group, the levels of IL-1β and IL-6 were found to be lower in the diabetic and melatonin groups. Furthermore, the level of IL-1β was significantly lower in the melatonin group compared to the control and diabetic groups (p=0.036). However, no significant difference was found between the groups in terms of the IL-6 levels (p=0.248) (Table 2).

Table 2. Serum cytokine levels				
	Control Group	Diabetic Group	Diabetes + Melatonin Treated Group	p
IL-1β (pg/mL)	1652.8±66.5	1455.5±118.0	743.3±628.8	0.036
IL-6 (pg/mL)	127.9±22.8	118.1±14.4	104.7±7.0	0.248
TNF-α (pg/mL)	199.4±60.8	220.6±65.6	78.0±64.3	0.008
Interleukin-1 beta (IL-1β); Interleukin-6 (IL-6); Tumor necrosis factor-alpha (TNF-α)				

DISCUSSION

This study had two objectives; the first was to demonstrate that DM has a negative effect on spermatogenesis in male rats and melatonin treatment prevents the loss of spermatic cell series thus corrects functional deficiencies. The second objective was to explore the relationship between the melatonin treatment and serum cytokine levels associated with spermatogenesis. According to the results, the levels of IL-1β and TNF-α were significantly lower in the melatonin group compared to the control group.

It has been reported that diabetes causes the vacuolization of the Sertoli cells, resulting in an increase in apoptosis of spermatocytes and spermatogenic cells in the seminiferous tubules of male rats (33). According to the research, male reproductive disorders in diabetic animal models include a reduction in semen quality, testicular weight, and sperm amount and motility as well as the induction of abnormal sperm and oxidative stress in the testes [34]. Similar to the results of previous studies, in the present study, the Johnsen score of the diabetic rats was found to be lower 9.39 indicating the presence of testicular damage (31).

The uncontrolled and unnecessary apoptosis that occurs during spermatogenesis can result in testicular dysfunction, and this plays a critical role in the pathogenesis of testicular dysfunction in diabetic men. DM has previously been shown to increase oxidative stress in the testes and lead to the development of apoptosis (7,8). Increased oxidative stress causes cellular damage through various mechanisms including the lipid peroxidation of reactive oxygen species, and the oxidative damage to DNA and proteins (35). Guneli et al. reported

the Johnsen scores to be significantly lower in diabetic rats compared to the control and treatment groups. In the same study, the authors evaluated apoptosis and found it to be significantly higher in the diabetic group than the melatonin treatment group (7). Other studies have also demonstrated that melatonin is a powerful antioxidant agent, which directly cleans the toxic hydroxyl radicals and protects DNA against oxidative and nitrosative changes (25,36). Hemadi et al. (26) reported that melatonin is effective in reducing apoptosis during cell proliferation and Nasiraei-Moghadam et al. (29) underlined the protective effects of melatonin on spermatogenesis. Furthermore, melatonin has been found to reduce the effect of toxic agents such as STZ that cause testicular damage (7). It is also suggested that due to its antioxidant and anti-apoptotic features, melatonin should be used to protect the sperm, epididymis and seminal vesicles (37).

Cytokines have been shown to have a significant role in the regulation of the testicular function (13,22). TNF-α has several biological effects by regulating inflammatory responses in the germ or somatic cells in the testes as well as other organs. Depending on the receptor types and the presence of target proteins and specific adaptors in the cells, these effects may be stimulating, inhibiting, pro-apoptotic, pro-inflammatory or destructive (38). This is supported by the results of our study which demonstrated an increase in the TNF-α level in the diabetic group. Furthermore, TNF-α is an important cytokine for the regulation of testicular function. In the present study, through the antioxidant and anti-apoptotic effects, the melatonin treatment resulted in a significant decrease in the levels of TNF-α and IL-1β.

The statistical decrease in the TNF-α and IL-1β levels in the treatment group shows the important role of these levels in the regulation of testicular function. Other studies have reported that TNF-α and IL-1β may play a role in various physiological functions in the male reproductive system and their levels are increased in cases of inflammatory diseases that elevate oxidative stress (13,21,22). Furthermore, in the present study, the IL-6 level was found to be lower in the diabetic and melatonin groups compared to the control group. However, this difference was not statistically significant, which can be attributed to IL-6 not being as effective in spermatogenesis as TNF-α and IL-1β.

The limitations of this study include the use of only one scoring system, Johnsen scoring, to assess the testicular damage due to diabetes, the lack of evaluation of apoptosis, absence of a non-diabetic control group and the absence of a testis biopsy prior to forming the experimental diabetes model.

CONCLUSION

In conclusion, the antioxidant and anti-apoptotic effects of the melatonin treatment, the serum levels of inflammatory cytokines were reduced and the Johnsen scores were increased. Based on these results, it is considered that

melatonin can be used to reduce testicular damage as well as confirm and follow the positive effects of serum cytokine levels.

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