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Research Article

The Melatonin Attenuates Alloxan Induced Post-Diabetic Testicular Damage and Oxidative Effects in Rats

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Abstract

The aim of this study was to examine the protective effect of melatonin (MLT) on the alloxan-induced post-diabetic testicular damage in rats. Forty-eight Sprague Dawley male rats were divided into four groups (n=12). Group C received 0.9% saline for 45 days, as control. Group DM (Diabetes Mellitus) was injected by 120 mg/kg single dose alloxan intraperitoneally (IP). Group MLT was treated by 10 mg/kg/d MLT IP for 45 d. Group DM+MLT was treated by 10 mg/kg/d MLT IP for 45 days following alloxan injection. Six animals on days 15 and the remaining 6 on days 45 were sacrificed in each group after the initiation of the study. Testis tissues and blood samples were collected to investigate blood glucose, oxidant and antioxidant statues, sperm parameters, and histopathological and 8-hydroxy-2'-deoxyguanosine (8-OHdG)-immunopositive cell examinations. MLT treatment of diabetic rats prevented the increases in malondialdehyde (MDA) levels and 8-OHdG concentrations, and the decreases in the GSH and SOD levels observed in DM groups. Alloxan administration depressed the sperm concentration, the number of progressively motile spermatozoa, premature acrosome reaction (AR) and calcium ionophore A23187-induced AR rates, without any change in dead and/or abnormal sperm rates in DM group. Histopathological and immunohistochemical observations also revealed that MLT-treated diabetic rats had an enhanced histological architecture and DNA damage in testis. These findings suggest that MLT treatment prevents the testicular damage by declining the oxidative stress, but it did not recover the depressed sperm parameters in diabetic rats.

Keywords: *Diabetes, Melatonin, Rat, Testis*

Melatonin Ratlarda Alloxan İle İndüklenen Diyabet Sonrası Oluşan Testiküler Hasarı ve Oksidatif Etkileri Hafifletir

Özet

Bu çalışmanın amacı, ratlarda alloxan ile oluşturulan diyabet sonrası gözlenen testiküler hasar üzerine melatonin'in (MLT) koruyucu etkisini incelemekti. Araştırmada kullanılan 48 adet Sprague Dawley ırkı erkek rat 4 gruba ayrıldı (n=12). Kontrol grubu olan Grup C'ye 45 gün süreyle %0.9 serum fizyolojik verildi. Grup DM'ye (Diabetes Mellitus) tek doz alloxan (120 mg/kg) intraperitoneal (IP) olarak enjekte edildi. Grup MLT'ye 45 gün süreyle 10 mg/kg/gün dozda MLT IP olarak enjekte edildi. Grup DM+MLT'ye alloxan enjeksiyonunu takiben 45 gün süreyle 10 mg/kg/gün dozda MLT IP olarak uygulandı. Her gruptan 6 hayvana araştırma başladıktan 15 gün sonra, diğer 6 hayvana ise 45 gün sonra ötenazi uygulandı. Kan glikoz seviyesi, oksidan ve antioksidan durumları, sperm parametreleri ve histopatolojik ile 8-hydroxy-2'-deoxyguanosine (8-OHdG)-immunopozitif hücre incelemeleri için testis dokusu ve kan örnekleri alındı. Diyabetik ratlara yapılan MLT uygulamasının, grup DM'de görülen malondialdehyde (MDA) seviyesi ile 8-OHdG yoğunluğundaki artışı ve glutathion (GSH) ile superoxide dismutase (SOD) seviyelerindeki azalmayı önlediği belirlendi. Alloxan uygulaması grup DM'de sperm yoğunluğu, motil sperm sayısı, prematüre akrozom reaksiyonu (AR) ve calcium ionophore A23187 ile indüklenen AR oranlarını deprese ederken, ölü ve/veya anormal sperm oranını değiştirmedi. Ayrıca, histopatolojik ve immunohistokimyasal muayeneler, diyabetik ratlara yapılan MLT uygulamasının histolojik yapı ve DNA hasarında iyileştirici bir etki gösterdiğini ortaya çıkardı. Sonuç olarak, MLT uygulamasının diyabetik ratlarda oksidatif stresi azaltarak testiküler hasar oluşumunu önlemesine rağmen, sperm parametrelerinde oluşan bozulmaları engelleyemediği gözlendi.

Anahtar sözcükler: *Diyabet, Melatonin, Rat, Testis*

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INTRODUCTION

Diabetes Mellitus (DM), with a chronic nature; i) is characterized by hyperglycaemia, ii) alters metabolic processes of carbohydrate, protein and lipid, and iii) increases the risk of complications arise from cardiovascular diseases [1]. It is associated with a high risk of atherosclerosis, thus having a destructive effect on the organs of renal, nervous, and ocular systems. Moreover, reproductive impairment is a common complication of DM in both genders [2,3]. Streptozotocin (STZ) is used in the treatment of neoplastic β-cell and in the formation of DM type-1 animal model. In virtue of the oxidative stress induced by hyperglycemia and histologic changes in testis, one feature providing the surpassing the STZ's reproductive activity has also occurred [4].

Reactive oxygen species (ROS) (e.g. hydrogen peroxide (H_2O_2), singlet oxygen (O_2^-) and superoxide anions and lipid peroxides) have been associated with the pathogenesis of many serious systemic diseases (e.g. cancer, rheumatoid arthritis, ischemic diseases, neurological disorders and diabetes). Also, the pancreatic β-cells are very sensitive to injury caused by the free radicals [5-8]. The most striking example of β-cell damage is the severe injury induced by alloxan or STZ. It has several destructive effects, including ROS generation, DNA methylation and protein modification. In all the living animal cells, there is a balance between the oxidants and antioxidant defence. When an imbalance occurs within the cells, the ROS initiate the cell death and histopathological changes. The 8-hydroxy-2′-deoxyguanosine (8-OHdG) enzyme reaction is one of the predominant forms of free radicals, and used as a biomarker of oxidative stress [9].

Dietary supplementation with antioxidants such as medicinal plants, vitamins, and flavonoids has been used to prevent the occurrence of DM [5-7]. The MLT is a free-radical scavenger and wide-spectrum antioxidant, and it stimulates the gene expression of antioxidant and pro-oxidant enzymes. MLT treatment after alloxan-induced β-cell destruction has been reported recently [10-14]. These studies suggest that MLT treatment in DM regulates the antioxidant enzymes and decreases the degenerative changes. However, no marked changes were reported in glucose level and β-cell apoptosis, while high MLT levels lead to decreased insulin release from the β-cells [15-19]. Recenly, diabetes in male rats has been induced via the STZ, but the effects of alloxan-induced diabetes on male fertility have not yet been investigated.

Herein, we aimed to determine the effect of MLT against the oxidative damage caused by alloxan injury of the testis, by measuring spermatological, testicular and biochemical parameters, and by conducting immuno-histochemical (IHC) examinations for apoptotic cells in diabetic Sprague-Dawley rats.

MATERIAL and METHODS

Animals and Groups

Forty-eight adult, 8 weeks old, male Sprague Dawley rats weighing (body weight, b.w.) about 230 g (ranging 200-250 g) were used. All procedures were carried out in accordance with the protocol approved by the Atatürk University Faculty of Veterinary Sciences, Local Board of Ethics for Animal Care and Use (Decision No: 2015/58). Pairs of rats were placed in cages under a 12 h daylight/darkness cycle with an ambient temperature of 23±2°C and humidity of 55±10% throughout the study. Standard rat chow and drinking water were provided *ad libitum*. Animals were divided into four groups (n=12, each) as follows: Control group (group C), receiving intraperitoneal (IP) saline only; MLT group, MLT injection (10 mg/kg/d, IP); DM group, diabetes induction + receiving IP saline (negative control group); DM+MLT group, diabetes induction + MLT injection (10 mg/kg/d, IP) for the 15th and 45th d. On these time points, all rats were anaesthetised with an IP injection of 60 mg/kg sodium pentobarbitone, then they were killed by taking blood samples from the intracardiac site. Thereafter, their testes and cauda epididymes were removed immediately.

Induction of Diabetes

In overnight-fasted rats, diabetes was induced by IP injection of alloxan (Sigma, St. Louis, Mo., USA) prepared freshly at a dose of 120 mg/kg b.w. in 0.9% NaCl. The inductions were verified after 24 h and non-diabetic animals (with a fasting blood glucose level lower than 220 mg/dl) were injected with the second dose of alloxan (as above). The animals were allowed 7 d for the stabilisation of blood glucose level. Blood samples were collected from the tail vein. Prior to the experiment (at zero h), the glucose levels were measured by a glucometer and test strips (On Call Pluss) on the 15th and 45th d. At the 8th d, animals having a fasting blood glucose level higher than 220 mg/dl were considered as 'diabetic'.

Drug Administration and Analyses

The MLT was supplied from Sigma (St. Louis, MO, USA). The pineal indole was dissolved freshly in ethanol and diluted with a sterile saline to give a final concentration of 0.01% ethanol and administered IP with a daily dose of 10 mg/kg. MLT treatment was commenced on the 14th d following the alloxan injection and this was considered as 1st d of treatment. This treatment is continued in the MLT and DM+MLT groups. The levels of glucose on the 15th and 45th d were measured using the commercial kits by an autoanalyser (as given above).

The right testis were removed, washed with physiological saline solution, and stored at –80°C until the analyses. The method, as described by Ohkawa et al.[20], was

used for determining the MDA levels in testis tissues. Measurements of tissue glutathione (GSH) level and superoxide dismutase (SOD) activities were performed according to the methods described previously by Tietze [21] and Sun et al.[22], respectively. The results were expressed as nmol/g protein for the MDA, nmol/g protein for the GSH and U/g protein for the SOD levels.

Sperm Collection and Evaluation

Collections of cauda epididymal sperm and the assessment of sperm parameters (progressive motility, live-dead sperm and abnormal sperm rates) were performed [under a phase contrast microscope (Carl Zeiss Axio Scope. A1) on a warm stage at 35.5±0.5°C] by modification of the method described by Akman and Aksoy [23]. The sperm concentration was determined with a Neubauer counting chamber, using modified method of Türk et al.[24], while the modified method of Larson and Miller [25] was used for acrosome reaction (AR) and staining procedures.

Histochemistry and Immunohistochemistry of Testis Tissues

The samples of left testis were fixed in Bouin's fixative fluid and processed routinely for embedding in paraffin. The series of 5-6 µm thick sections prepared from the blocks were stained by Mallor's triple staining for histopathological examination, while streptavidin-biotin-peroxidase staining method (Ventana BENCHMARK GX, automatic staining instrument) was used for immunohistochemical staining of the oxidative cells. For the former examinations, Johnsen's criteria were used to determine the spermatogenesis in testis [26]. For the latter examinations of sections, 8-hydroxy-2'-deoxyguanosine (*8-OHdG, S*anta Cruz- 66036) primary antibody was used for oxidative enzyme activity [9]. Finally, all staining sections were evaluated by high-power light microscopic examination. Image analysis system used in this study consisted of a personal computer with hardware and software camera (Kameram SLR, 1.6.1.0, Mikro Sistem Ltd. Sti., Turkey) and an optical microscope (Nikon i50).

Statistical Analysis

Results were presented as means±standard error of means (S.E.M.). One-way ANOVA and Tukey's post hoc test were used to determine differences between the groups. Paired-t test was used for the assessments between the data on the 15th and 45th d. The mean values were considered significant when P<0.05. The SPSS/PC programme (Version 10.0; SPSS, Chicago, IL) was used for the analyses.

RESULTS

Blood Glucose and Weights

On d zero, 15 and 45, the glucose levels of rats in DM group displaying hyperglycemia symptoms were significantly (P<0.05) higher (320-550 mg/dl) compared with those of the control rats (80-140 mg/dl) *(Table 1)*. Furthermore, MLT treatment significantly reduced the levels (160-300 mg/dl) in the DM+MLT group, compared to the DM group.

Table 1 shows the weights of body, testis and the cauda epididymis. The final body weights of diabetic rats were significantly lower than those of the controls on d 15 and 45. The weights were significantly improved by the MLT treatment compared with those of the DM rats.

Biochemical Results

Table 2 represents the levels of lipid peroxidation (MDA), and antioxidant enzymes (SOD and GSH) in testis of control and experimental groups. On d 15 and 45, the MDA levels were significantly (P<0.05) higher in the testis of animals that were administered alloxan alone (DM group) compared with that of the control group. This increase was attenuated by MLT treatment. Significant reductions in the GSH and SOD levels were seen in the testes of DM group

Table 1. *Blood glucose levels and the weights of body, testes and cauda epididymis (means ± S.E.M.)*
Tablo 1. *Kan glikoz seviyesi ile vücut, testis ve cauda epididimis ağırlıkları (ort. ± S.E.M.)*

Groups	Days	Glucose (mg/dl)	Total Weights		
			Body (g)	Testes (mg)	Cauda epididymis (mg)
C	15	97±10^{c}	263±25^{a}	2705.00±196.2	380.67±27.76
	45	101±10^{z}	300±28^{x}	2761.50±245.36	451.25±24.85
MLT	15	98±15^{a}	244±19^{a}	2582.25±85.07^{t}	315.75±35.35
	45	97±12^{x}	267±27^{x}	2306.80±73.40	292.80±17.33
DM	15	512±32^{a}	210±24^{b}	2044.25±378.06	318.75±90.35
	45	525±25^{x}	176±13^{z}	2176.00±520.96	319.67±87.82
DM+MLT	15	436±17^{b}	232±17^{b}	2624.00±143.42	425.50±60.71
	45	337±21^{y}	240±15^{y}	2333.40±229.13	320.00±67.02

Different superscripts a, b, c within the same column indicate significant differences for group 15 d, and x, y, z in the same column indicate significant differences for group 45 d, respectively (n=6). P<0.05; "t" on C, MLT, DM and DM+MLT groups, shows statistical change between the 15th and 45th d (n=6). P<0.05; ***C*** *= Group C;* ***MLT*** *= Group MLT;* ***DM*** *= Group DM;* ***DM+MLT*** *= Group DM+MLT*

compared with that of the controls. Treatment of diabetic rats with the MLT alleviated these DM-induced decreases. MLT treatment resulted in a significant increase in the GSH levels as accompanied with a significant decrease in the MDA and SOD activities in the MLT group as compared with that of the controls.

Spermatological Results

Table 3 shows the results of the evaluation of spermatologic parameters. On the 45th d, the sperm concentration of the DM and DM+MLT groups was found significantly (P<0.01) lower than that of group C.

The number of progressively motile sperm cells in the group DM was significantly lower than that of the group C on d 15 (P<0.05). The number of progressively motile spermatozoa in the groups DM and DM+MLT was lower than that of the group C on d 45 (P<0.01).

The premature AR rate on the 15th and 45th d significantly increased in treatment (MLT, DM and DM+MLT) groups as compared to that of the controls. Also, the rate within the MLT group significantly decreased on the 45th d as compared to that of the 15th d.

On the 15th d, the A23187-induced AR rate of C group, as similar to that of DM group, was significantly (P<0.05) higher than those in the MLT and DM+MLT groups. Further, on the 45th d, the rate in C group became significantly higher than that of all treatment groups.

On d 15 and 45, there were significant decreases in the sperm concentration, number of motile spermatozoa, and rate of A23187-induced AR in rats administered alloxan alone (DM group) as compared with that of the controls (P<0.05). This decrease was exacerbated by MLT treatment in DM+MLT rats *(Table 3)*.

Histochemical and Immunohistochemical Findings

The Johnsen's criteria of spermatogenesis and 8-OHdG immunopositivity in the testes of control and experimental groups are presented in *Table 4*.

Table 2. *The levels of MDA and activities of SOD and reduced GSH in testes tissues (means ± S.E.M.)*
Tablo 2. *Testiküler MDA, SOD ve GSH seviyeleri (ort. ± S.E.M.)*

Groups	Days	MDA (nmol/g protein)	SOD (U/g protein)	GSH (nmol/g protein)
C	15	13.13±1.50^{a}	975.69±55.75^{a}	27.30±3.50^{a}
	45	8.37±0.75^{x}	929.04±60.35^{x}	10.03±1.30^{x}
MLT	15	6.36±1.20^{b}	679.45±90.25^{b}	36.98±2.25^{b}
	45	2.94±0.70^{y}	825.60±75.45^{y}	34.51±3.25^{y}
DM	15	29.07±1.25^{c}	780.52±42.25^{b}	11.28±2.80^{c}
	45	35.10±0.50^{z}	650.20±38.75^{z}	12.50±2.25^{x}
DM + MLT	15	7.70±1.50^{b}	881.79±40.75^{a}	25.39±2.50^{a}
	45	3.92±0.50^{y}	773.36±35.50^{y}	27.62±3.20^{z}

Different superscripts a, b, c within the same column indicate significant differences for group 15 d, and x, y, z in the same column indicate significant differences for group 45 d, respectively (n=6). P<0.05; ***C*** *= Group C;* ***MLT*** *= Group MLT;* ***DM*** *= Group DM;* ***DM+MLT*** *= Group DM+MLT*

Table 3. *The cauda epididymal sperm parameters (means ± S.E.M.)*
Tablo 3. *Cauda epididimal sperm parametreleri (ort.±S.E.M.)*

Groups	Days	Cauda Epididymis		Rates (%)				
		Sperm Concentration (x10^{6})	Number of Progressively Motile Sperm (x10^{6})	Dead Sperm	Mid-piece Abnormality	Premature AR	Total AR	The A23187-Response Rate of Sperm
C	15	64.58±8.38	37.86±5.16^{a}	42.30±2.70	0.00±0.00	11.87±2.87^{a}	42.90±1.8	31.03±2.91^{a}
	45	74.41±7.21^{x}	41.49±3.25^{x}	44.77±2.53	2.05±1.20	14.10±0.83^{x}	49.35±4.50	35.25±5.07^{x}
MLT	15	41.99±10.53	22.11±5.76^{ab}	46.08±1.46	5.30±0.40	36.10±1.82^{b}	48.95±0.90	12.90±0.97^{b}
	45	39.06±4.36^{xy}	25.39±1.95^{xy}	36.26±1.71^{t}	1.64±1.00^{t}	30.76±1.01^{y}	46.36±1.76	15.62±1.24^{y}
DM	15	39.06±17.47	10.27±6.12^{b}	55.53±5.80	1.37±1.37	37.80±9.79^{b}	55.73±9.27	17.95±2.12^{ab}
	45	25.78±2.34^{y}	5.83±2.42^{y}	63.47±3.42	0.00±0.00	36.50±2.10^{y}	48.90±3.90	12.37±1.82^{y}
DM+MLT	15	29.49±8.07	14.96±3.49^{b}	47.46±3.99	1.03±1.03	31.15±3.49^{b}	47.33±4.20	16.23±3.35^{b}
	45	21.88±9.08^{y}	10.84±4.08^{y}	49.67±6.23	5.68±1.62	28.94±3.15^{y}	44.62±2.70	11.21±2.40^{y}

Different superscripts a, b, c and x, y, z within the same column indicate significant differences between groups for 15 d and 45 d, respectively (n=6). P<0.05; "t" on C, MLT, DM and DM+MLT groups, shows statistical change between the 15th and 45th d (n=6). P<0.05; ***C*** *= Group C;* ***MLT*** *= Group MLT;* ***DM*** *= Group DM;* ***DM+MLT*** *= Group DM+MLT*

Table 4. *Johnsen's criteria of spermatogenesis and 8-OHdG immunopositivity in testis (means ± S.E.M.)*
Tablo 4. *Testis dokusunda Johnsen'in spermatogenezis kriterleri ve 8-OHdG immunopozitifliği (ort. ± S.E.M.)*

Groups	Days	Johnsen's Criteria	8-OHdG Immunopositivity					
			Spermatogonia	Leptotene/ Zygotene Spermatocytes	Pachytene Spermatocytes	Spermatid	Sertoli Cell	Leydig
C	15	9.8±0.1[a]	++	+	-	-	-	-
	45	9.7±0.2[y]	++	+	-	-	-	-
MLT	15	9.7±0.1[a]	++	+	-	-	-	-
	45	9.8±0.1[x]	+	+	-	-	-	-
DM	15	7.8±0.3[c]	+++	+++	++	-	+	+
	45	7.2±0.1[z]	++++	+++	++	+	+	+
DM+MLT	15	8.4±0.2[b]	+++	++	+	-	+	+
	45	8.6±0.3[y]	++	+	-	-	+	+

Different superscripts a, b, c within the same column indicate significant differences for group 15 d, and x, y, z in the same column indicate significant differences for group 45 d, respectively (n=6). P<0.05; ***C*** *= Group C;* ***MLT*** *= Group MLT;* ***DM*** *= Group DM;* ***DM+MLT*** *= Group DM+MLT*

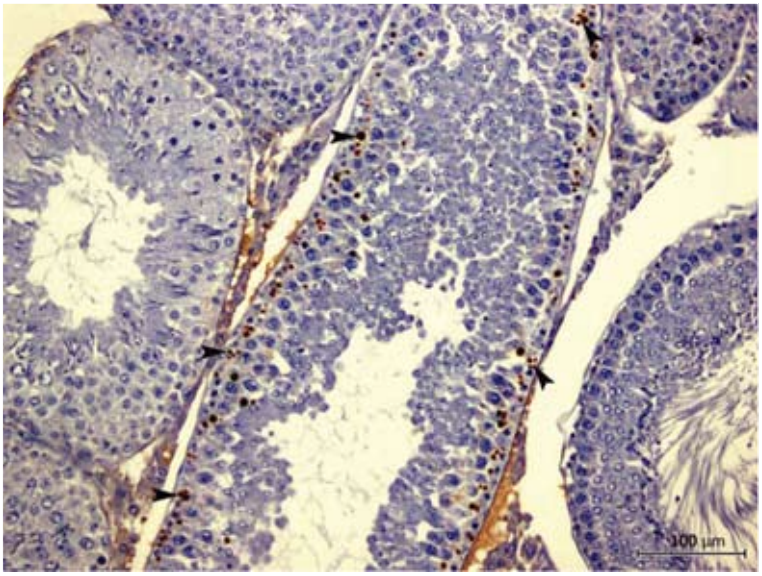


Fig 1. Oxidative stress marker 8-OHdG immunopositivity in the MLT group *(arrow heads)*. Immunopositivitiy in nuclei of leptoten or zygotene spermatocytes in the some seminiferous tubules. Streptavidin-biotin peroxidase staining

Şekil 1. MLT grubundaki oksidatif stres belirleyicisi olan 8-OHdG immunopozitiflik *(ok başları)*. Seminifer tubullerdeki leptoten veya zigoten spermatositlerin çekirdeğindeki immunopozitifliği göstermektedir. Streptavidin-biotin peroksidaz boyama

In all the groups, the seminiferous tubules (composed of germ cell layers) were ellipsoids to rounds in shape. Histochemical findings of testes showed that both the control and MLT groups had normal histological appearances that included the series of spermatogenic, Sertoli and Leydig cells *(Fig. 1)*. According to the Johnsen's criteria, the most consistent findings from the germinal cells of diabetic rats were desquamation and disorganisation, and there were considerable reductions in the spermatogenic cell series and especially in the spermatids at the metamorphose level *(Fig. 2)*. On the d 15 and 45, treatments of diabetic rats with the MLT led to a marked amelioration in the changes of seminiferous tubules as compared to those in the DM groups. The oxidative cells were demonstrated by the 8-OHdG immunohistochemical staining, located in both the nuclei and cytoplasm of germ cells. In the control and MLT groups, the 8-OHdG positivity was recorded only in the nuclei of leptotene and zygotene spermatocytes *(Fig. 1)*. However, in the DM and DM+MLT groups, the 8-OHdG concentrations were very strong in both the nuclei of leptotene and zygotene spermatocytes and the cytoplasm of pachytene spermatocytes, Sertoli and Leydig cells *(Fig. 2* and *Fig. 3)*. The 8-OHdG immunopositive cells increased in the DM groups as compared with those in the controls and MLT groups. Moreover, in the DM+MLT group, the number of 8-OHdG immunopositive cells decreased distinctly on d 45 as compared to d 15.

DISCUSSION

DM is a chronic disease characterized by hyperglycemia and markedly affects the functions of many organs and body systems. Further, it may lead to a decrease in libido and fertility, thus people's quality of life and life expectancy could become inevitablely reduced [3].

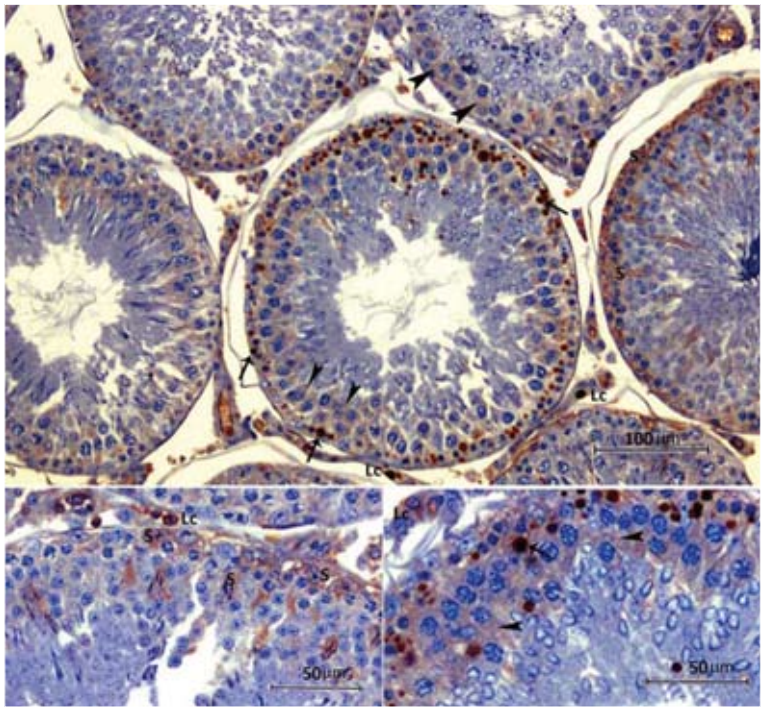
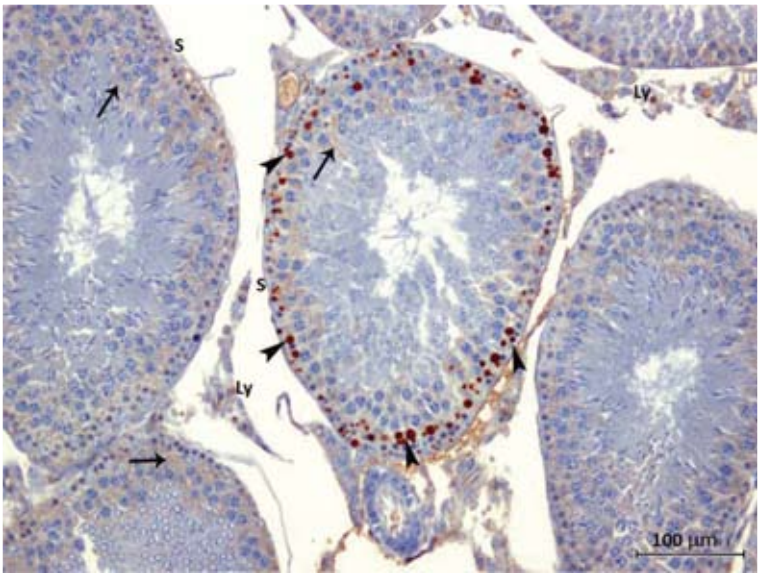


Fig 2. Oxidative stress marker 8-OHdG immunopositivity in the DM group *(arrows)*. Immunopositivity in nuclei of leptoten or zygotene spermatocytes *(arrows heads)*, immunopositivity in cytoplasm of pachytene stage spermatocytes, (S) Sertoli and (Lc) Leydig cells the some seminiferous tubules, desquamation and disorganisation in some seminiferous tubule. Streptavidin-biotin peroxidase staining

Şekil 2. DM grubundaki oksidatif stres belirleyicisi olan 8-OHdG immunopozitiflik *(oklar)*. Seminifer tubullerdeki leptoten veya zigoten spermatositlerin çekirdeğindeki immunopozitifliği *(ok başları)*, bazı seminifer tubullerdeki Leydig (Lc), Sertoli (S) hücreleri ile pakiten aşamasındaki spermatositlerin sitoplazmasındaki immunopozitifliği ve bazı seminifer tubullerdeki disorganizasyon ile deskuamasyonu göstermektedir. Streptavidin-biotin peroksidaz boyama

Fig 3. Oxidative stress marker 8-OHdG immunopositivity in the DM+MLT group *(arrows)*. Immunopositivity in nuclei of leptoten or zygotene spermatocytes *(arrows heads)*, immunopositivity in cytoplasm of pachytene stage spermatocytes, (S) sertoli and (Ly) Leydig cells the some seminiferous tubules. Streptavidin-biotin peroxidase staining

Şekil 3. DM + MLT grubundaki oksidatif stres belirleyicisi olan 8-OHdG immunopozitiflik *(oklar)*. Seminifer tubullerdeki leptoten veya zigoten spermatositlerin çekirdeğindeki immunopozitifliği *(ok başları)*, bazı seminifer tubullerdeki Leydig (Ly), Sertoli (S) hücreleri ile pachtene aşamasındaki spermatositlerin sitoplazmasındaki immunopozitifliği göstermektedir. Streptavidin-biotin peroksidaz boyama



The well-known effect of alloxan, used herein as diabetic agent, on the β-cells in pancreas is to cause the development of insufficient synthesis of insulin and, thus, increasing the blood glucose levels. Several researchers have reported that the MLT treatment in alloxan-induced diabetic rats alleviates some symptoms of diabetes, e.g. elevated blood glucose levels [27-29]. Herein, the mean levels of diabetic rats were markedly higher than those of the normal levels and this elevation in glucose was nearly constant during the course of diabetes, whereas in diabetic rats treated with the MLT, glucose level markedly reduced and become close to the normal levels ultimately.

Previous studies have shown that the ROS productions are responsible from the alloxan-induced destructive effects, particularly in the tissues of pancreas as well as kidney, retina, myocardium and testis [2,30]. These radicals could cause tissue damage as they react with the macromolecules (e.g. membrane lipids and proteins) within the testis. Herein, markedly higher MDA levels of testis were observed in the DM group than those in the controls. Treatment with the MLT markedly prevented the alloxan-induced lipid peroxidation in the testis tissue, indicating the antioxidant capacity of the MLT. This reduction in lipid peroxidation is likely to be the indication of a lesser damage of free oxygen radicals.

There are numerous natural or synthetic products that have a reasonable cytoprotective nature, presumably by influencing the antioxidant systems. The GSH is a critical

enzyme for sustainable cell viability. It modulates the cell responses to redox changes connected with the generation of reactive oxygen and nitrogen species, detoxifies the metabolites of drugs and regulates apoptosis [2]. In the presence of cytoplasmic and mitochondrial thiols such as the GSH, the alloxan produces free oxygen radicals in a redox reaction with its reduction product, dialuric acid. Oxidation of dialuric acid generates superoxide and hydrogen peroxide radicals and, in a final Fenton's reaction step, the hydroxyl radicals, as being the most reactive and toxic one. The latter radicals are responsible for final destruction of the β-cells, having a particularly low antioxidative capacity, and ensuing the state of insulin-dependent 'alloxan diabetes'. The marked decrease in the GSH levels promoted by alloxan represents a change in the cellular reduction or oxidation reactions, suggesting that the cells could have become more sensitive to the ROS. This situation would cause to a reduction in the protective effect of antioxidant defense system [31,32].

The most effective defense mechanism of the cell against various viruses, bacteria or other substances is the release of O_2^- and OH. The SOD is a key scavenging enzyme that eliminates the toxic ROS induced by the alloxan. It converts the superoxide radicals into H_2O_2 and molecular oxygen. In this respect, the decrease in SOD activity in DM group might have resulted from the inactivation either by glycolisation of the enzyme or by hydrogen peroxide [33,34]. In our study, the activities of both GSH and SOD enzymes markedly decreased in testis tissues of alloxan-treated rats. This result might be due to the increase in free radical generation or a decrease in the amounts of protecting enzymes against lipid peroxidation. However, treatment with the MLT ameliorated the alloxan-induced testis damages due to free radical production. These results suggested that the MLT might have a free radical scavenging activity and prevents pathological alteration caused by O_2^- and OH^-. Indeed, similar findings have been reported on testis tissue that alloxan injection led to lower GSH and SOD activities [35,36].

The DM is characterized by a sudden and distinct loss in body weight, a symptom as also observed herein. The decreases in body and testis weights of DM rats showed the damage or destruction of structural proteins related to diabetes [3]. When diabetic rats were treated with the MLT, a weight loss in the body was reversed. The capability of MLT to protect body weight losses seems to be a result of its ability to increase antioxidant capacity and reduce the hyperglycemia and ROS. In our study, the total testis weights (except that of the MLT group) did not show any difference between and within the groups studied, as reported by Abbasi et al.[37]. In the MLT group, total testis weight decreased markedly from 15th d to 45th d.

Alloxan-induced destructive effects lead to the productions of ROS. The 8-OHdG produced by ROS can be used as a biomarker of the DNA [38]. In normal mature rat testis, the 8-OHdG is observed in leptotene, zygotene, and pachytene spermatocytes, as immediately removed from the DNA by natural defense mechanisms within the testis [39]. In this study, histological sections from the testis of control and MLT groups were similar to histochemical structures, but the 8-OHdG level quantitatively measured in rat testis was relatively low in the MLT d 45 group as compared with those of the controls and MLT d 15 groups. However, the levels increased in the diabetic groups, and were drastically decreased by the MLT treatment in the DM+MLT group. The defense system (against the stress) by the MLT might have developed in pachytene stage spermatocytes in the DM+MLT group.

It was reported in some studies that the rats having undergone the STZ had decreased sperm motility, but without any marked change [4,37,40-44]. Similarly, herein, we did not find any difference neither between nor within the groups on the 15th and 45th d.

No marked change in total sperm number was observed by Abbasi et al.[37], while the daily sperm production in rats and the sperm concentration in mice and rats decreased in the STZ-induced diabetes [4,40-44]. Herein, the sperm concentration in the 45th d markedly decreased in the DM group as compared to those in the C group, but the MLT treatment of diabetic rats did not lead to recovering effect for a decrease in the sperm concentration.

In our study, the lower sperm concentration and the lower number of motile sperm in the DM group are consistent with the reproductive disorders [45,46]. Present findings with decreased sperm concentration in diabetic rats were similar to those of Bal et al.[4], while being slightly lower than those of Navarro-Casado et al.[43] and Scarano et al.[47]. The reduction in sperm concentration in diabetic rats might imply that the STZ had an adverse effect on the spermatogenesis [40,47]. Singh et al.[40] reported that the decrease in sperm density may be related to the decrease in the number of Leydig cells [4]. Some authors reported that the reduced epididymal and motile sperm concentrations in diabetic rats could arise from a combined effect of decreased Leydig cell function and the oxidative stress induced by diabetes [4,46,48,49]. It has been shown that the destructive effect of free oxygen radicals reduces the sperm motility and viability [49]. Shrilath and Muralidharan [44] noted that the sensitivity of sperm to the oxidative stress in the epididymis was increasingly evident around the 15th d after the STZ injection. Other researchers reported that spermatozoa are reasonably well protected by the Sertoli cells, but they are less protected against the oxidation in the epididymal milieu. Consequently, the oxidative damage to sperm may lead to DNA damage, alter the membrane functions and impair motility [50,51]. There are numerous studies that the MLT treatment increases testicular antioxidant capacity and decreases oxidative stress and thus diminishes diabetes-induced oxidative damage and leading to the restoration of sperm

parameters in the DM group rats [10-14]. The protective effects of MLT (used herein as an antioxidant) on the testis tissue are consistent with the previous results in DM models [11,12,44]. According to our results, the alloxan-induced DM in rats increases the MDA levels and leads to apoptosis in Sertoli and Leydig cells as well as spermatocytes (in leptotene, zygotene and pachytene stages) during the spermatogenesis. The sperm concentration on 45th d decreased in the DM group as compared to those in the C group, and this detrimental effect in diabetic rats was not inhibited or reversed by the MLT. Hence, we considered that the reason of low fertility in diabetic men might be due to the low sperm concentration, as observed herein with the cauda epididymal sperm in DM rats. For example, when a cow is desired to conceive by artificial insemination method, there has to be a certain number of motile sperm within the inseminate (contained within a single frozen-thawed straw). If this insemination dose within the straw is diminished per one-million sperm, the pregnancy rate in cows decreases by 2-3% from the normal values [52].

Morphological abnormalities of sperm observed herein were also reported similarly by Navarro-Casado et al.[43] unlike by Scarano et al.[47]. This discrepancy might be due to different assessment intervals used (2–3 weeks therein *vs.* 15 to 45 d herein). Clearly, their assessment intervals were too short for development of abnormalities during the spermatogenesis.

Cholesterol is a sterol playing a critical role in capacitation and contained within all mammalian sperm cells. For the capatitation and AR, a small amount of ROS is required. When cholesterol on the sperm plasma membrane is exposed to oxidation, its level diminishes, and the spermatozoon inevitably undergoes capacitation [53,54]. In a previous study, it was stated that as the temperature and time period are increased in culture medium during incubation, the rate of premature AR raises in rabbits, and that when cholesterol was added into sperm suspension, it prevented the spermatozoa from undergoing the premature AR [55]. Our study suggest that the DM might reduce the cholesterol levels of the plasma membrane because of its increasing effect on the ROS quantity within the cauda epididymis, so it might enhance the number of spermatozoa having undergone the premature AR. Lombardo et al.[56] noted that the reduction in ROS concentration by using antioxidants exerts adverse effect on male fertility. Some studies indicated that when antioxidants are used in oral pills and/or added directly into ejaculated spermatozoa, the rate of spermatozoa having undergone the AR increases [57,58]. Unlike the study of Lombardo et al.[56], herein, the increase in the premature AR rate in MLT group was found to resemble the findings of Comhaire et al.[57]. In this respect, since the MLT increased the premature AR rate as compared to those in the C group, MLT treatment in DM rats did not diminish this rate markedly as compared to those in the DM group, due likely to the outlined feature of antioxidants concerned.

Our hypothesis about the infertility of men with DM would be that the premature and A23187-induced AR rates might be critical. Indeed, a normal ejaculate must have enough spermatozoal quantity and be capable of undergoing the AR in the fertilisation area (oviduct) for the oocyte penetration in cattle [59]. In this study, the rate of spermatozoa having undergone premature AR in rats with DM was more evident than in the C groups. This case would decrease the number of sperm cells avaible for the induction (e.g. by A23187) of the AR. Since a sufficient number of sperm (with AR capability) would not reach to the oviduct, the penetration rate of fertilisable oocyte(s) might then reduce drastically, ultimately leading to male infertility.

In the experimental studies on the DM, it has been reported that the administration of some antioxidants increases the antioxidant capacity, while decreasing the oxidative stress restoring changes in the spermatological parameters [4,37]. Other antioxidants may not reverse the deterioration in these parameters, but they may restore the daily sperm production in testis, and alleviate the mitochondrial function and DNA damage [42]. Deterioration of spermatologic parameters due to the administration of various substances (with ROS generating activities within the testis), the MLT, used as an antioxidant, could ameliorate these parameters [60-63]. In our study, the MLT did not protect the sperm concentration, the number of progressively motile spermatozoa, the premature and the A23187-induced AR rates of the cauda epididymal sperm in rats with alloxan-induced DM. Possible reasons for dysfunction of MLT on the sperm parameters would be linked with; i) the adjuvant, used for solving the MLT, that might have adverse effects on spermatologic parameters [64], ii) the use of DM and MLT together that might have increased the adverse effects on spermatogenesis, and iii) the dose of MLT used that might have affected the results.

In summary, the results of this study suggest that the melatonin could reduce the blood glucose, lipid peroxidation marker (MDA levels), 8-OHdG and oxidant parameters in diabetic male rats. This may contribute to its protective effects on post-diabetic complications, such as in testicular damage. However, the melatonin did not improve the depressed sperm parameters in diabetic rats when used for the duration of 45 d. Nevertheless, it would contribute to a balanced oxidant-antioxidant status and provide a potential therapeutic choice to reduce the histologic damage of testis in diabetic patients.

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