BJPS

Brazilian Journal of
Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902022e20945

Article

Effects of melatonin on both testicular regeneration and recovery of spermatogenesis in busulfan-treated rats

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Testicular damage is one of the most hazardous effects of chemotherapy as it is frequently associated with oligozoospermia and azoospermia. This study aimed at evaluating the protective effect of melatonin in a rat model of busulfan-induced testicular injury. Rats were divided into four groups: control, melatonin, busulfan, busulfan plus melatonin. After 15 days, the semen was collected from the epididymis and testes were assessed. Sperm removed from cauda epididymis and analyzed for sperm count and viability. Testis tissues were also removed, fixed in formalin and were embedded in paraffin. Sections of testis tissue were stained with hematoxylin-eosin for histological examination and prepared for TUNEL (Terminal deoxynucleotide transferase dUTP Nick End Labeling) assay to detect apoptosis and PCNA (proliferating cell nuclear antigenassay) to detect proliferation cells. Serum and testes supernatants were separated to detect testosteron level and oxidative stress parameters. In histological examination, degenerative changes in seminiferous tubules were observed in the experimental groups. In biochemical examination, the total oxidant status (TOS) levels in Busulfan group were significantly higher than in the control group while the total antioxidant status (TAS) levels of all the groups were similar. In conclusion, the beneficial properties of melatonin treatment by its potent anti-oxidants may reduce adverse effects of chemotherapy in the reproductive system in a rodent system.

Key words: Busulfan. Melatonin. Spermatogenesis. Testis. Rat.

INTRODUCTION

Busulfan is a widely used cancer therapy agent in the treatment of chronic diseases such as leukemia and lymphoma as well as prior to bone marrow transplantation, but a concerning consequence of the use of this drug is temporary or permanent sterility in male patients (Dehghani *et al*., 2013; Hosseini *et al*., 2014). Further adverse effects of busulfan include damage to the bladder, the liver, the skin, and the organs of the nervous system as well as reproductive conditions such as oligospermia, azoospermia, low testis weight, changes in the serum levels of male sexual hormones, and increases in apoptotic sperm (Nasimi *et al*., 2016; Vahdati *et al*., 2015). Busulfan inhibits cell division by sticking to one of the DNA strands (Iwamoto *et al*., 2004), leading to sterility and the loss of spermatogonia (Intarapat, Sailasuta, Satayalai, 2016). The organs that contain cells with high proliferation capacity, such as the testes, are more susceptible to the adverse effects of busulfan (Dun, Aitken, Nixon, 2012). However, the busulfan-induced depletion of spermatogenic germ cells is reversible, and animals generally regain fertility, albeit to varying degrees, because of the active reproliferation of surviving germ cells (Choi *et al.,* 2004).

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Melatonin (*N*-acetyl-5-methoxytryptamine), the most important hormone of the pineal gland, regulates testicular function through the hypothalamic–adenohypophyseal axis (Reiter, 1993). In addition to the direct effects, the indirect effects of melatonin on somatic cells of the testis include modulation of cellular growth, proliferation, and the secretory activity of several testicular cell types (Rossi *et al*., 2014). For instance, melatonin acts as a local modulator of the endocrine activity of Leydig cells (Gonzalez-Arto *et al.,* 2016) and influences cellular growth, proliferation, energy metabolism, and the oxidation state of Sertoli cells; therefore, melatonin may regulate spermatogenesis (Yang *et al.,* 2014).

Malignant diseases might cause gonadal dysfunction through hormonal changes. However, the negative effects of cytotoxic drugs on spermatogenesis are more powerful. Therefore, the aim of the present study was to evaluate busulfan-induced histopathological and biochemical changes, melatonin-induced changes to spermatogenesis, and the effects of melatonin on these changes in rat testes.

MATERIAL AND METHODS

Animals

All animals were obtained from the Experimental Animal Research Laboratory at Bezmialem Vakif University, Istanbul, Turkey. Animals had free access to food and water at controlled room temperature (22-25°C) under a 12:12-h day/night cycle for the duration of the study. The experiments were performed in accordance with the guidelines for animal research from National Institute of Health and were approved by the Committee on Animal Research at Bezmialem Vakıf University, Istanbul, Turkey (Ethics number:2017/129)

Experimental Design

Twenty-eight 6 months old male Wistar albino rats were used. The rats were randomly divided into the following four groups: Group I (n=7), control group (C); Group II (n=7), Melatonin group (Mel); Group III (n=7), Busulfan group (Bus); and Group IV (n=7), Busulfan+Melatonin group (Bus+Mel). The rats received busulfan (Sigma Cat: B2635 USA) intraperitoneally (ip.) at a single dose of 20 mg/kg/day (Ohira *et al.*, 2014), and received melatonin (Santa Cruz Cat: sc-207848) ip. at a dose of 10 mg/kg/day (Cui *et al.*, 2017) for 15 days. At the end of the experiment period, general anesthesia was administered to all the rats using 80 mg/kg ketamine (Ketalar, Pfizer, Turkey) and 5 mg/kg xylase HCl (Rompun, Bayer, Turkey). The semen was collected from the epididymis and testes were dissected for biochemical and histological analysis. Blood samples were collected from the left ventricle with an injection under anesthesia. Serums were obtained after whole blood centrifugation (3000 g, 20min, at 4°C). Tissue and serum samples were stored at −45°C in deepfreeze until analysis were performed. After 15 days, the semen was collected from the epididymis and the testes were assessed.

Histological Evaluation

The testis tissues were fixed in 10% formalin and following routine tissue preparation processes were embedded in paraffin. Sections of testis tissue were stained with hematoxylin-eosin (H-E). 100 tubules were classified as intact, atrophic, and degenerated. Tubules including a few or no germ cells were classified as atrophic tubules. Tubules with anormal cells or interrupted spermatogenic cells at various stages of spermatogenesis were classified as degenerated tubules. The sections were examined by a blind observer using a Nikon Eclipse i5 light microscope with a Nikon DS-Fi1c camera and the Nikon NIS Elements version 4.0 image analysis systems (Nikon Instruments Inc., Tokyo, Japan).

Immunocytochemistry For Cell Death

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed by using in situ cell death detection kit (Merck Millipore, S7100). The procedure was as follows: every section was incubated with proteinase K for 5 mins, washed with distilled water and incubated with 3% hydrogen peroxide in PBS for 5 min. The sections were then washed with PBS, put in the equilibrium buffer for 30 min, and incubated in

TdT enzyme at 37 °C for 1 h. The sections were agitated in washing buffer for 15 s, washed in PBS, put into anti-digoxigenin conjugate for 30 min and then washed with PBS. After incubation with peroxidase for 6 min, they were washed with distilled water, stained with Mayer's hematoxylin and covered with mounting medium. In each section, seminiferous tubules stained with 3 or more TUNEL positive cells were counted at 200× magnification.

Immunocytochemistry For Cell Proliferation

Cell proliferation analysis was performed on paraffin-embedded sections using the PCNA Staining Kit (ThermoFisher Scientific, Cat: 93–1143) following the manufacturer's protocol. Paraffin-embedded testis sections were dewaxed by treating the slides in 2 changes of xylene for 5 minutes each and were rehydrated. Endogenous peroxidase activity was quenched by using 3% hydrogen peroxide in PBS for 20 min. The slides were incubated with ready-to-use blocking solution for 10 min, and then incubated with biotinylated monoclonal anti-PCNA antibody (ready-to-use) for 1 h. After washing, sections were incubated with streptavidin-peroxidase (ready-to-use) for 10 min at room temperature, followed by incubation with 3, 3′-diaminobenzidine (DAB) for 5 min. Slides were finally counterstained with Mayer's hematoxylin and covered with mounting medium. The number of PCNA positive cells were calculated in 10 different seminiferios tubules of each section at 200× magnification.

Assessment of Sperm concentration

Immediately following euthanisation, with a laparotomy the left epididymis was removed and weighed. The cauda of each epididymis was cut and minced separately in 2 ml of normal saline and filtered through nylon mesh for collection of spermatozoa (Haron *et al.,* 2010). An aliquot of the epididymal suspension was then used for sperm count and morphology using a Makler counting chamber (Sefi Medical Instruments LTD, Haifa, Israel).

Sperm concentration was measured using Makler counting chamber (depth 10 μm, Sefi Medical Instruments, Haifa, Israel) based on a WHO (1999) method. The right epididymis was finely minced by anatomical scissors in 1mL of isotonic saline in a petri dish and sperm samples were diluted with physiological solution (10 μl). Sperm specimen was mixed thoroughly, and a small drop was then pipetted onto the centre of the disc area of a Makler chamber. The number of spermatozoa in a strip of 10 squares was counted under a light microscope at 200× magnification. The calculated number represented the concentration in million per ml.

Assessment of Sperm Viability

Sperm viability was determined by a routine gold standard method suggested by WHO (1999). Approximately 20 μL of sperm suspension was mixed first with two drops of 1% eosin (Merck, Darmstadt, Germany) for 30s and then with three drops of 10% nigrosin (Merck, Darmstadt, Germany). After drying the smear in room temperature, the slides were examined under a light microscope at 40× magnification. Alive spermatozoa were unstained (white) but dead cells were stained red. After counting at least 100 sperm cells, the percentage of dead and alive spermatozoa was calculated.

Biochemical Evaluation

Tissue samples were homogenized with PBS (Phosphate Buffer Saline, pH:7.4) and total protein concentration was measured by Bradford method (Bradford, 1976).

Oxidative Stress Determination

Serum total oxidant status (TOS) was determined according to the novel automated spectrofotometric measurement method developed by Erel (2004) using a commertial kit (REL0024, Rel Assay, Turkey).

Assay was performed at Perkin Elmer, 1420 Victor 3 instrument. Oxidants in the sample oxidize the ferrous ion chelator complex to ferric ion. The oxidation reaction is prolonged by enhancer molecules, which are abundantly present in the reaction medium. The ferric ion makes a color complex with chromogen in an acidic medium. The color intensity which can be measured spectrophotometrically

is related to the total amount of oxidant molecules of the sample. Results were expressed as µm $H_2 0_2$ Equiv/L.

Serum total antioxidant status (TAS) was determined according to the novel automated spectrofotometric measurement method developed by Erel (2005) using a commertial kit (Cat Num: REL0017, Rel Assay, Turkey). Assay was performed at Perkin Elmer, 1420 Victor 3 instrument. Antioxidants in the sample reduce dark blue green colored ABTS radical to colorless reduced ABTS form. The change of absorbance at 660 nm is related with total antioxidant level of the sample. Results were expressed as mmol Trolox Equiv/L of samples.

Hormon Analysis

Testosterone ELISA kit (Cat num: 201-11-5126, Sunrenbio Company, Shangai) was used for the quantitative measurement of testosterone in defrosted tissue samples. Samples and standards were added to appropriate wells which is pre-coated with Anti-Human monoclonal antibody before incubation. Biotin was added to all wells, and combined with Streptavidin-HRP to form immune complex. Following the incubation again, wells were washed for removing the uncombined enzyme. Then chromogen Solution A, B were added for color change. Under the effect of acid, the blue color finally turned to yellow. Optical density was read on a standard automated plate reader at 450 nm (Thermo Scientific Microplate Reader). The detection range of kit is between 0.05 ng/ml-15 ng/ml.

Statistical analysis

All values were presented as mean + SEM. Statistical analyses were performed using one-way ANOVA and post hoc Tukey's honestly significant difference test. Histopathological results were compared with Kruskal–Wallis variance analysis. Differences were considered to be significant at $p < 0.05$. A computer program SPSS 11.0 (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis.

RESULTS

Histolopathological Alterations

Seminiferous tubules containing all stages of spermatogenesis were observed in control and Mel groups (Figure 1A, 1B). However, degenerative changes in seminiferous tubules were obvious in the experimental groups. Some of the tubules were atrophic. A significant germ cell loss was detected in these atrophic seminiferous tubules (Figure 1C). Arrested spermatogenic cells at various stages of division were observed in some of the degenerated seminiferous tubules (Figure 1D). In Bus group, 71.57±2.6% of tubules were intact, 16.57±2.19% of tubules were atrophic, and 11.85±2.20% of tubules were degenerative. In Bus+Mel group, 75.71±2.2% of tubules were intact, 14.28±3.16% of tubules were atrophic (Figure 1E) and 10.00±2.43% of tubules were degenerative (Figure 1F). In Bus group, the number of affected seminiferous tubules were found to be significantly increased when compared with control group ($P<0.05$). On the other hand, the number of affected seminiferous tubules in Bus+Mel group was lower than in Bus group with no statistically significant difference. Mean tubule numbers of the groups (intact, degenerated and atrophic) are given in Table I.

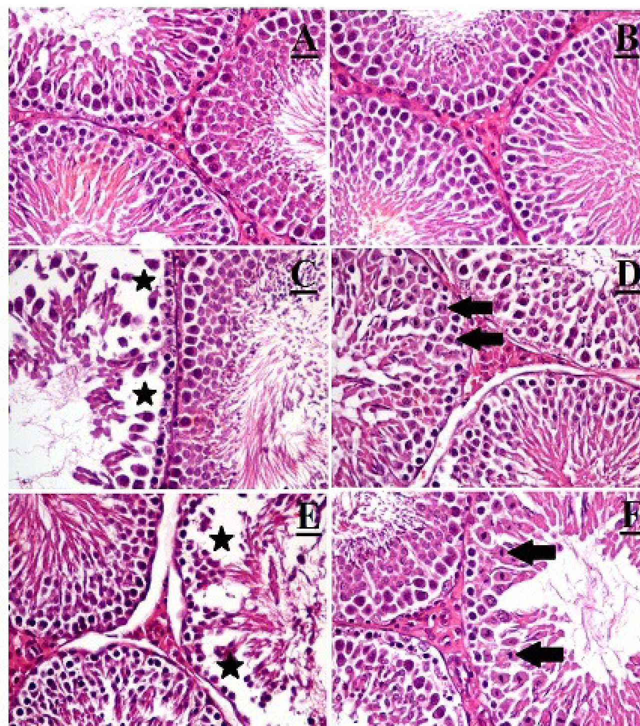

FIGURE 1 - Control (A) and Mel (B) groups. The seminiferous epithelium is structurally intact and shows normal association of germ cells. **Bus group** revealing atrophic tubule (asterisks) **(C)**, and arrested spermatocytes in different stage of division (arrows) **(D). Bus+Mel group** revealing atrophic tubule (asterisks) **(E),** and arrested spermatocytes in different stage of division (arrows) **(F)** H-E; X 400.

TABLE I - The mean numbers of seminiferous tubules of the groups

Parameters	Control	Mel	Bus	Bus+Mel
Intact tubule (%)	92.14±1.01	91.42±0.9	71.57±2.6[a]	75.71±2.2[a,b]
Atrofic tubule (%)	4.28±0.71	4.28±0.71	16.57±2.19[c]	14.28±3.16[c,b]
Degenerated tubule (%)	3.57±0.9	2.14±1.01	11.85±2.20[d]	10.00±2.43 [d,b]

[a]Significant decrease (P =0.0001), vs. control group
[b]Not significant change (P >0.05), vs. Bus group
[c]Significant increase (P =0.0001), vs. control group
[d]Significant increase (P =0.002), vs. control group

Results of TUNEL method

TUNEL staining detected apoptotic cells in the testis tissue (Figure 2). The mean TUNEL (+) cell number was 68.71±7.93 in control group, 57.28±6.74 in Mel group 192.42±21.3 in Bus group and 96.57±9.47 in Bus+Mel group. There were only a few TUNEL-positive cells per tubule in the control and Mel groups (Figures. 2A, 2B). Germ cells degeneration was obvious in Bus group. Therefore plenty of TUNEL-positive germ cells were detected in this group (Figure 2C). On the other hand, the number of TUNEL (+) cell in Bus+Mel group was statistically significantly lower than that of Bus ($P<0.05$) (Figure 2D). The mean number TUNEL + cells of the groups are shown in Table II.

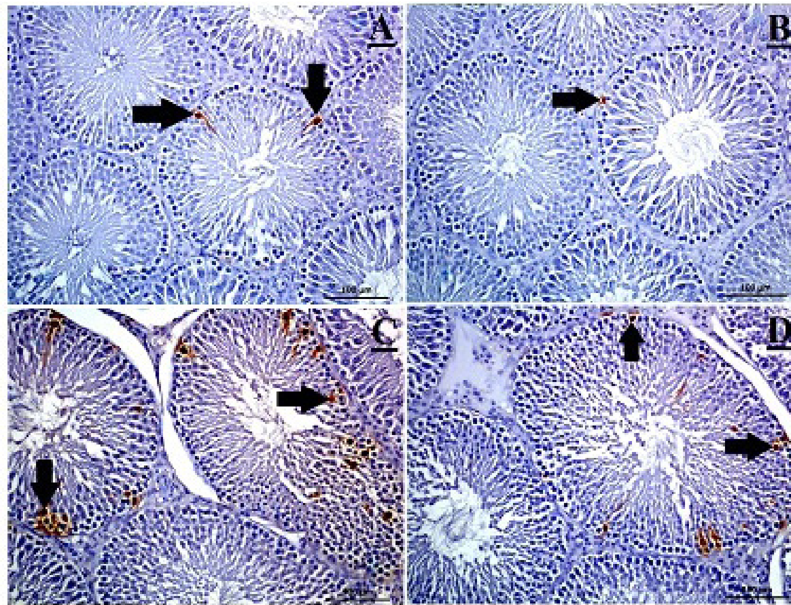

FIGURE 2 - The distribution of TUNEL + cells in **control (A), Mel (B), Bus (C)**, and **Bus+Mel group (D)** (arrows) is shown. TUNEL immunohistochemistry; X 200.

TABLE II - Mean numbers of TUNEL and PCNA + cells of the groups

Parameters	Control	Mel	Bus	Bus+Mel
TUNEL (+) tubul cell	68.71±7.93	57.28±6.74	192.42±21.3[a]	96.57±9.47[b]
PCNA (+) tubul cell	1891.2 ±258.9	1805.5 ±197.4	990.8±79.42[c]	1632.4±131.5[d]

[a]Significant increase (P =0.0002), vs. control group
[b]Significant decrease (P =0.0002), vs. Bus group
[c]Significant decrease (P =0.0002), vs. control group
[d]Significant increase (P =0.0002), vs. Bus group

Results of PCNA method

The control and Mel groups revealed showed strong nuclear reaction for PCNA (orange to brown color) in many of the spermatogenic cells (Figures 3A, 3B). In Bus group, since there was a obvious degeneration of the germinal epithelium in most of the seminiferous tubules, the positive reaction was not obvious (Figure 3C). Mel -treated testis showed strong nuclear reaction in most spermatogonia, and moderate reaction in the nuclei of other spermatogenic cells (Figure 3D). The mean number PCNA (+) cells were 1891.2 ±258.9 in control group, 1805.5 ±197.4 in Mel group, 990.8±79.42 in Bus group, and 1632.4±131.5 in Bus+Mel group. In Bus group, PCNA (+) cells were found to be significantly decreased when compared with control and Mel groups ($P<0.05$), However, the mean number of PCNA (+) cell in Bus+Mel group was significantly increased versus that of Bus group ($P<0.05$). The mean number PCNA + cells of the groups are shown in Table II.

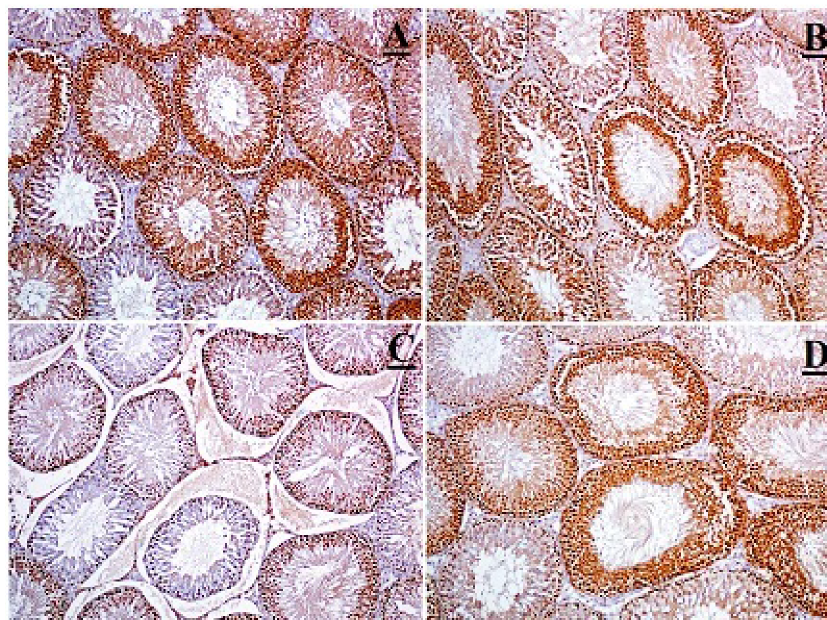

FIGURE 3 - The distribution of PCNA + cells in **control (A), Mel (B), Bus (C)**, and **Bus+Mel group (D)** (arrows) is shown. PCNA immunohistochemistry; X 100.

Assessment of Epididymal Sperm Parameters

The sperm concentration and viability of the left epididymis of the rats from Bus and Bus+Mel groups were significantly decreased versus those of control and Mel group ($P<0.05$). The mean numbers of unstained sperm (vital) were 69.14±5.43 in control group, 60.71±4.72 in Mel group, 39.57±2.32 in Bus group, and 46.57±4.12 in Bus+Mel group. The mean sperm viability in Bus+Mel group was higher than that of Bus group without statistical significance.

Sperm concentration degrees were 481.7±36.7 in control group, 485.2±43.7 in Mel group, 311.2±22.9 in Bus group and 357.5±21.8 in Bus+Mel group. Mean sperm concentration of Bus group was significantly decreased when compared with that of control group ($P<0.05$), The difference was not statistically significant between Bus and Bus+Mel group ($P > 0.05$). The mean sperm concentration and viability degrees are shown in Table III.

TABLE III - Sperm parameters of all groups

Parameters	Control	Mel	Bus	Bus+Mel
Sperm concentration (million/g)	481.7±36.7	485.2±43.7[a]	311.2±22.9 [a]	357.5±21.8[b]
Viability (Eosin Test; %)	69.14±5.43	60.71±4.72	39.57±2.32[c]	46.57±4.12[b]

[a]Significant decrease ($P = 0.0002$), vs. Control group
[b]Not significant change ($P >0.05$), vs. Bus group.
[c]Significant decrease ($P = 0.0003$), vs. Control group

Biochemical Results

Oxidative stress determination results

Low levels of TOS were observed in control and Mel group. The mean TOS level of Bus group was significantly higher than that of control group (p <0.05). The mean TOS level of Bus+Mel group was lower than that of Bus group with no significant difference. The mean TAS levels in control, Mel and Bus+Mel group were high, but no significant differences were detected among groups. The mean TAS level of Bus+Mel group was higher than that of Bus group. The mean TOS and TAS levels of all groups are shown in Table IV.

TABLE IV - Biochemical results of all groups

Parameters	Control	Mel	Bus	Bus+Mel
TOS	4.54±0.64	6.26±1.33	9.14±1.72[a]	8.64±1.03[b]
TAS	1.56±0.30	1.75±0.30	1.36±0.32[c]	1.69±0.24[b]
Testosteron level	166.9±5.84	180.9±13.2	146.5±7.61[d]	163.6±7.01[b]

[a]Significant increase (P=0.024), vs. control group
[b]Not significant change (P >0.05), vs. Bus group.
[c]Not significant change (P >0.05), vs. control group
[d]Significant decrease (P=0.033), vs. control group

Hormon results

The mean plasma testosterone level of Bus group was significantly lower than those of control group (p <0.05) and Bus+Mel group. The mean plasma testosterone levels are given in Table IV.

DISCUSSION

Anticancer agents are useful for the treatment of cancer, but in the course of the treatment, various adverse effects occur, and those that target the male reproductive system are especially severe and constitute one of the main causes of male sterility (Ohira *et al.,* 2014; Panahi *et al*., 2015). In rodents, busulfan has been reported to be responsible for sterility stemming from the death of spermatogonial cells (Anjamrooz *et al*., 2007; Benavides-Garcia *et al*., 2015). The seminiferous tubules of the rats treated with different doses of busulfan (13–40 mg/kg) showed depletion of most spermatogonia and some spermatocytes (Bucci, Meistrich, 1987). Mohammad-Ghasemi *et al.* (2009) reported that busulfan administration of different single doses of 10, 20, and 40 mg/kg induce side effects on the male reproductive system. In the present study, we administered busulfan intraperitoneally at a single dose of 20 mg/kg and observed tubule degeneration associated with apoptotic cell death and tubule atrophy. In rodents, an increase in the number of spermatogonia undergoing apoptosis has been observed following busulfan treatment (Choi *et al*., 2004), and preventing sterility and infertility during the treatment with such cytotoxic agents is of great importance. Therefore, many recent studies have evaluated the protective or curative effects of foods or medicines against the cytotoxic effects induced by anticancer agents.

In this study, we examined the sperm parameters and histopathological status as well as the oxidative stress parameters of the testis to determine the toxic effect of busulfan and the protective effect of melatonin. The presence of degenerated and atrophic tubules with increased apoptosis and arrested spermatogenic cells at various stages of division in the busulfan-administered group (Bus group) proved the cytotoxic effect of that dose of busulfan on testis tissue; Dehghani *et al.* (2013) reported similar changes in seminiferous tubules in the testis after busulfan administration.

Apoptosis is a form of cell death characterized by morphological and biochemical changes such as

DNA fragmentation, formation of apoptotic bodies, and weak inflammatory response (Hikim, Swerdloff, 1999). Testicular apoptosis is found during normal spermatogenesis in mammals and is believed to be essential for the preservation of the correct ratio between Sertoli cells and gametes (Lee *et al.,* 1997). However, the relatively small increase in the percentage of germ cell apoptosis can result in defective spermatogenesis, leading to infertility (Moline *et al.,* 2000). Increases in the incidence of germ cell apoptosis often are observed as a result of various forms of physical or chemical injury to the testis (Richburg, 2000). In the present study, several apoptotic cells were observed in the control group.

Busulfan induces apoptosis in multiple organs, therefore increasing the percentage of TUNEL + germ cell number (Choi *et al.,* 2004; Mohammad-Ghasemi *et al.,* 2009; Vaisheva *et al.,* 2007). Busulfan-induced male germ cell apoptosis has been reported extensively in mice and murine rodents (Vaisheva *et al.,* 2007). In the Bus group, many apoptotic cells accompanied with tubule degeneration were detected. The number of apoptotic cells in the busulfan- and melatonin-administered group (Bus+Mel group) was statistically significantly lower than that of the Bus group ($p < 0.05$). Melatonin also protected the tissue from tubular degeneration and atrophy. As a matter of fact, the mean PCNA + cell number in the Bus+Mel group was higher than that of the Bus group. Immunolabeling the cells with PCNA was used to identify proliferating spermatogonia (D'Andrea *et al.,* 2008). Because busulfan caused degeneration of the germinal epithelium, many of the seminiferous tubules showed negative reactions.

Melatonin has been shown to modulate cellular growth, proliferation, and the secretory activity of several testicular cell types (Rossi *et al.,* 2014). It has been shown that, by binding specific receptors, melatonin directly regulates testosterone secretion (Frungieri *et al.,* 2005; Maitra, Ray, 2000).; Wu *et al.,* 2001), and this connection was obvious in our study as we analyzed the testosterone levels of the groups. The mean testosterone level of the Bus group was significantly lower than that of the control group ($p < 0.05$). Recently, Sasso-Cerri *et al.* (2017) reported that busulfan exerts a harmful impact on Leydig cells, peritubular myoid cells, and spermatogenic cells; we therefore conclude that melatonin is protective against busulfan- induced degeneration of the spermatogenic cells. Decreases in sperm count and viability following busulfan treatment in rats have been reported (Anjamrooz *et al.,* 2007; Vahdati *et al.,* 2017). In this study, epididymal sperm parameters, including sperm count and viability, were better in the melatonin-administered group than in the Bus group.

Another factor that may cause disruption of spermatogenesis is oxidative stress. Lipid peroxidation is a major measure of the damage to proximate lipids by free radicals (Agarwal, Prabakaran, 2005) and as an end effect of oxidative stress, it causes sperm cell death (Sharma *et al.,* 2017). In addition, high concentrations of H_2O_2 and ROS in sperm resulted in nuclear DNA fragmentation and cell membrane lipid damage (Liu *et al.,* 2006). Thus, the influence of busulfan on sperm cell death can be explained by DNA fragmentation and lipid peroxidation. Our evidence of TUNEL assays corroborated recent research studies and demonstrated that chemotherapy increases apoptosis following DNA and lipid damage in sperm and reproductive tissue of male mice. In the present study, we measured both TOS and TAS to conceive oxidative stress status. The mean TOS level of the Bus group was significantly higher and the mean TAS level was lower than those of the control group. It is obvious that busulfan-induced oxidative stress elevated ROS but decreased tissue antioxidant enzyme activity; the tissue TOS level of the Bus+Mel group was lower than that of the Bus group, but conversely, the TAS level of the Bus+Mel group was higher than that of the Bus group. Previous investigations have indicated that melatonin improves the quality of semen parameters and reduces ROS and lipid peroxidation in animals, which explains these results (Ashrafi, Kohram, Ardabili, 2013; Karimfar *et al.,* 2015).

As a conclusion, our results, related with the number of TUNEL + and PCNA (+) cell numbers, suggest that melatonin is able to induce spermatogonial mitotic activity. Melatonin is also effective for normalizing testosterone levels and oxidative stress status. However, at that dose and duration, melatonin has only a mild effect on histopathological alterations induced by busulfan. We therefore suggest that the beneficial properties of

melatonin treatment, via its potent antioxidants, may reduce the adverse effects of chemotherapy in the reproductive system in rodents.

Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was financially supported by a grant from Scientific Research Fund of Bezmialem Vakıf University.

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Received for publication on 24th October 2020
Accepted for publication on 03rd February 2021