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PROTECTIVE EFFECTS OF MELATONIN ON MATURE OVARIAN FOLLICLES' STRUCTURES IN ADULT MICE TREATED WITH BUSULFAN

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ABSTRACT

Busulfan is one of specific drugs to treat cancer that has many side effects. Among them the ovarian tissue damage and reproductive disorders can be pointed out. Melatonin is the epiphysis hormone that has been known as an antioxidant. Considering its protective role, the study aims to examine the effects of melatonin on mature ovarian follicles' structures in mice treated with busulfan, with the help of histological evaluation of ovarian tissue. The present study was conducted on 60adult female mice (5-6 months). Animals were randomly divided into4groupsafterestrus synchronization. The group included:

1. Control group that received 0.1mlnormal saline for 5 days, intraperitoneally.
2. The second group received a single dose of20 mg / kg busulfan, intraperitoneally.
3. The third group 10mg / kg melatonin for 5 days, intraperitoneally.
4. The fourth one received busulfan and melatonin for 5 days, daily10mg / kg melatonin. On the second day20mg / kg busulfan was injected along with melatonin,intraperitoneally.5 days after the start of treatment, all the animals' ovarian were removed and some parameters in the mature follicles including size mature follicle, the oocyte area and its nucleus, the thickness of the zonapellucida and theca follicular, as well as the number of mature atrophied and

health follicles were analyzed using ANOVA test and findings were presented as mean and standard deviation and P<0.05 was considered meaningful. The results of this study demonstrated that busulfan caused a very significant increase in the number of mature atrophied follicles, as well as a very significant decrease in the number of mature non-atrophied follicles compared with the control group (P<0.01), while coincident treatment with busulfan and melatonin reduces the number of mature atrophied follicles; so, in chemotherapy with busulfan, melatonincan minimize damage to the ovarian tissue by reducing the number of mature atrophied follicles.

Keywords: Busulfan, Mature ovarian follicle, Epiphysis gland, Melatonin

INTRODUCTION

Chemotherapy and radio therapy exert many changes in the reproductive system. Many factors are involved in the changes, among which are the drugs with alkylated property that has the greatest side effects on the organ [10]. Today, especially in developing countries, successful treatments of malignancies and hope to live have been highly increased and after recovery most of these people, especially young people, regain fertility and tend to have a child. Among alkylating chemotherapeutic agents it can be named busulfan which is administered to treat the chronic leukemia, ovarian cancer and bone marrow transplantation in cancer patients [14]. Busulfan can cause long-term improvement in patients with cancer, but many problems are created in the oligo-ovulation process and cause the destruction of the ovaries, infertility and liver toxicity and the most adverse effects on ovary [12]. Due to its alkylating effect on DNA, Busulfan effects adversely on cells that have more division power; so, its most negative effects can be applied on the oocytes. Therefore, the fertility can be maintained by inhibiting cell differentiation during chemotherapy, the patients were maintaining fertility. Although studies show that the all oocytes don't lost followed by using busulfan [2]. The use of the drug in pregnant women may lead to gonadal dysfunction and decreased testicular germ cells and somatic cells in newborns [11]. In studies conducted on human, combination therapy of busulfan with cyclophosphamide reduces the activity of the gonads and endocrine disorders [8]. Studies show that treatment with busulfan and cyclophosphamide increases the gonadal amount as well as tropine levels of LH, FSH, which increases the follicle proliferation and sensitivity of the ovary to chemotherapy drugs.

Research has shown that chemotherapy drugs such as busulfan, increase the production of oxidants and oxidativestress [7] on one hand and decrease natural antioxidants levels such as glutathioneon the other hand, and finally lead to cell death [5]. It seems that one of the ways in which busulfan damage germ cells is apoptosis induction in which the cells shrink and DNA is fragmented in a systematic process and the plasma membrane becomes bubbly [4]. Melatonin is one the epiphysis secretions that is effective in regulating some physiological phenomena. Melatonin has a neuronal-hormonal function as well as a regulatory function in reproduction, safety and temperature. In addition, melatonin has an effect on cell proliferation and differentiation. Also, its anticancer and anti-aging effects have been reported [14].

Melatonin receptors were expressed on various tissues including brain, retina, cardiovascular system, gastrointestinal tract, granulosis cells of ovarian follicles, uterine myometrium cells and the male reproductive system [3].

Due tothe small size and highlipophilic properties, melatonin passes the cell membrane easily, spreads across the cell, and protects DNA from damaging factors [15]. Moreover the role of melatonin receptors, it has been revealed that melatoninorits metabolites can neutralize the free radicals. Furthermore, It has been shown that besides antioxidant and protective activity melatonin increases expression of genes involved in the synthesis of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase [6].

Studies show that melatonin directly controls the ovarian activity and decreases follicle- stimulating hormone (FSH) and luteinizing hormone (LH), in other words causes some changes in the pituitary-hypothalamic–gonadal axis [13].There is no available report about the combined use of melatonin and busulfan and their combined effect on ovarian changes. The aim of this study was to evaluate the protective effects of melatonin on histological changes in the ovaries of adult mice undergoing chemotherapy with busulfan.

MATERIAL AND METHODS

Animals and treatment

In this study, 60 Swiss Albino adult female mice were bought from animals keeping center of Tabriz Medical Sciences University. They were kept in the animal house for two weeks with free access to pet food and water, standard environmental conditions and 12:12circadian cycle. The mice were 5 to6-monthsold weighing about 40±10g. Animals was randomly divided into4groups of 15 animals in each group. The Groups were called controls, treatment1, 2, 3 respectively. In order to have the highest rate of estrus synchronization in all 4 groups we use two doses of 0.5 µg cloprostenol (Hipra, Spain) with three days interval, intraperitoneally and one dose of3 µg progesterone (Aburaihan, Iran) subcutaneously, with the first injection of cloprostenol [17]. After estrus synchronization, control group received0.1 ml of normal saline for 5 days, intraperitoneally; that, the first dose of normal saline was administered with the second injection of cloprostenol. Treatment group1 received 20mg / kg busulfan (Pierre Fabre, French), 24hours after the second injection of cloprostenol, in a single dose, intraperitoneally. Treatment group2 received 10 mg / kg intraperitoneal melatonindaily (Sigma, USA) for 5days that its first dosage was coincident with the second injection of cloprostenol. Treatment group3received daily 10mg / kgmelatoninfor 5 days, thaton the second day they received 20 mg/kg busulfan, intraperitoneally. According to the circadian cycle of melatonin secretion, the groups that received melatonin were placed in a modified circadian rhythm in which an artificial darkness started one hour before the darkness onset and exogenous melatonin was injected [16].According to the study conducted by Winiarska, normal saline was used in order to solubility of melatonin [22].

Table 1: Injection stages

Injection days	Control group	Treatment group1	Treatment group2	Treatment group3
1	normal saline 0.1 ml	-	Melatonin 10mg/kg	Melatonin 10mg/kg
2	normal saline 0.1 ml	Busulfan20mg/kg	Melatonin 10mg/kg	Melatonin 10mg/kg + Busulfan20mg/kg
3	normal saline 0.1 ml	-	Melatonin 10mg/kg	Melatonin 10mg/kg
4	normal saline 0.1 ml	-	Melatonin 10mg/kg	Melatonin 10mg/kg
5	normal saline 0.1 ml	-	Melatonin 10mg/kg	Melatonin 10mg/kg

Animals were anesthetized with chloroform 5 days after treatment. The ovaries were removed via abdominal incision and were transferred immediately to the fix at or containing bottles often percent formalin. The samples immersed in fixative solution at room temperature for72 hours. After three days of fixation and ensure the stability of ovarian tissue, xylel and liquid paraffin were used with ascending grades of ethanol passage to study thematurefollicles.5 μ- thickness serial sections were prepared using a rotary microtome device (Leitz, Germany) and 10 slides were prepared from these sections, and then were stained using H&E method. In histological and histo-morphometry studies of the mice ovarian, the biggest mature follicle was selected from the slide that was obtained from the ovarian middle part, then Motic device software (Motic, China), and optical microscope (Olampus, Japan) were used for quantitative evaluation of parameters. The mature follicle size and the cafollicular as well as zonapellucida thickness, oocyte area and its nucleus were calculated by 100× and 400× magnification, respectively. The number of mature atrophied and mature non-atrophied follicles were counted in all 10 slides of ovarian samples.

Statistical analysis

In this study, the morphological results of optical microscopic of the samples were compared and all quantitative parameters were analyzed with ANOVA test, and findings are presented as mean and standard error values in which $p< 0.05$ was considered significant. Also, Tukey test was used to assess significant differences among groups and Kruskal-Wallis test was conducted just about the number of mature atrophied follicles.

RESULTS

Quantitative parameters of mature follicles such as the mature follicle size, the area of oocyte and its nucleus, the thickness of zonapellucida and theca follicular, the number of mature atrophied and non-atrophied follicles.

The number of mature atrophied and non-atrophied follicles

Quantitative studies of mature follicles in the treatment group1show that the one dose injection of busulfan destroys ovarian follicles and mature atrophied follicles increase. So, a very significant difference is observed in the number of mature atrophied follicles compared with the control group ($P<0.01$).While the injection of 5 doses of melatonin in the treatment group 2 causes the decrease of mature atrophied follicles, In treatment group 3 that received busulfan together with melatonina very significant increase in the number of mature atrophied follicles was observed compared with the control group ($P<0.01$). The number of mature non-atrophied follicles was decreased in all three treatment groups and there was a significant difference compared with the control group ($P<0.01$).

Nucleus, oocyte and mature follicle size

Nucleus, oocyte and mature follicle size of three treatment groups has no significant difference compared with the control group, except nucleus size difference that is meaningful intreatment group1 compared to the treatment group2($P<0.05$).

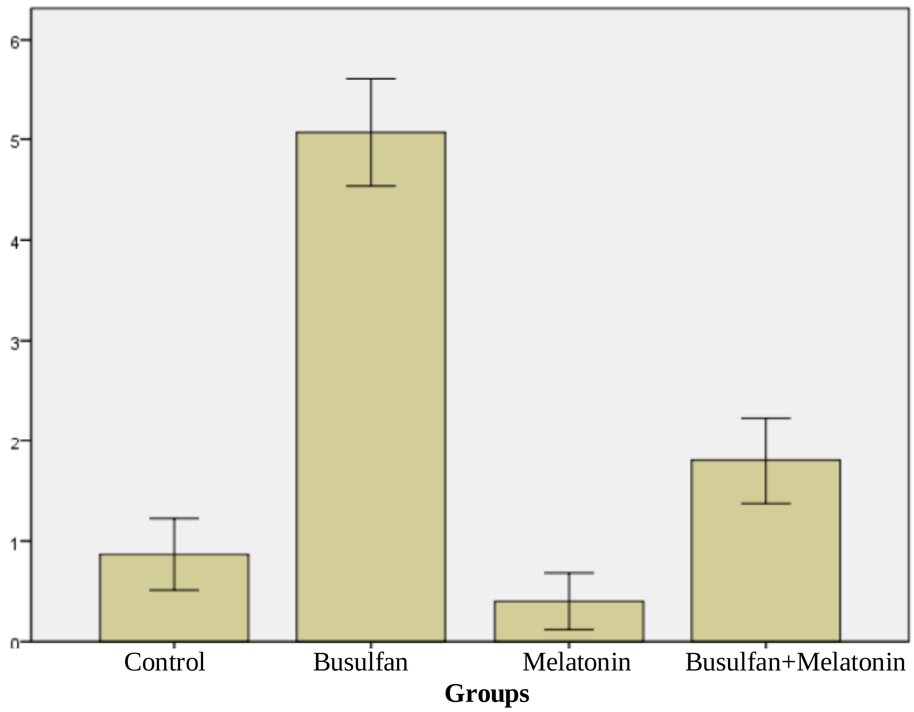
Zonapellucida thickness and Theca follicle

The thickness of zonapellucida and theca follicular has no significant difference in all three treatment groups compared with the control group.

Table 2: Comparison of the factors related to mature ovarian follicles' structures of mice treated with busulfan and melatonin

Factor Type / Treatment	Maturefollicle area (μm^2)	Oocyte area (μm^2)	Nucleus area (μm^2)	Zonapellucida thickness (µm)	Theca folliclethickness (µm)	Atrophied mature follicles number	Non-Atrophied mature follicles number
	Mean± SE N	Mean± SE N	Mean± SE N	Mean± SE N	Mean± SE N	Mean± SE N	Mean± SE N
Control	1.38×10^5± 10745.86 17	5.39×10^4 ±1378.4 11	5548.6±71/64^{a} 10	11.72±0.43 12	26.02±1.12 20	0.87±0.165a 15	7.80±0.200a 15
Busulfan	1.19×10^5 ±6428.45 20	5.54×10^4 ±1868.1 12	5277.1±290.27^{ab} 10	10.48±0.25 12	24.30±0.80 20	5.07±0.248b 15	4.93±0.153b 15
melatonin	1.34×10^5 ±10830.84 19	5.76×10^4 ±2460.1 10	6154.3±142.10^{ac} 10	11.70±0.35 10	24.45±1.08 20	0.40±0.131ac 15	3.67±0.211c 15
Busulfan+melatonin	1.29×10^5 ±11482.65 24	5.65×10^4 ±1366.8 10	5657.5±186.60^{a} 10	11.25±0.35 12	24.95±1.08 20	1.80±0.200d 15	5.93±0.284d 15
P value	P=0.625	P=0.546	P=0.012	P=0.055	P=0.680	P<0.01 P=0.320(a-ac)	P<0.01

Apart same letters are indicators of the meaningful difference at the level of α=0.005



Error bars: 95% Cl

Figure 1: Mean Atrophied mature follicles number

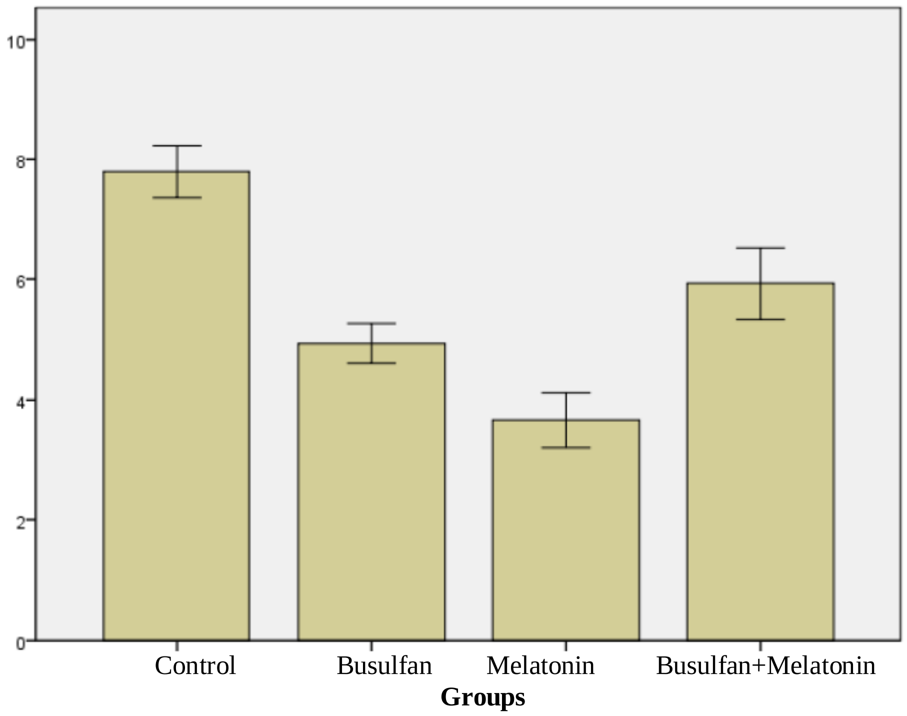


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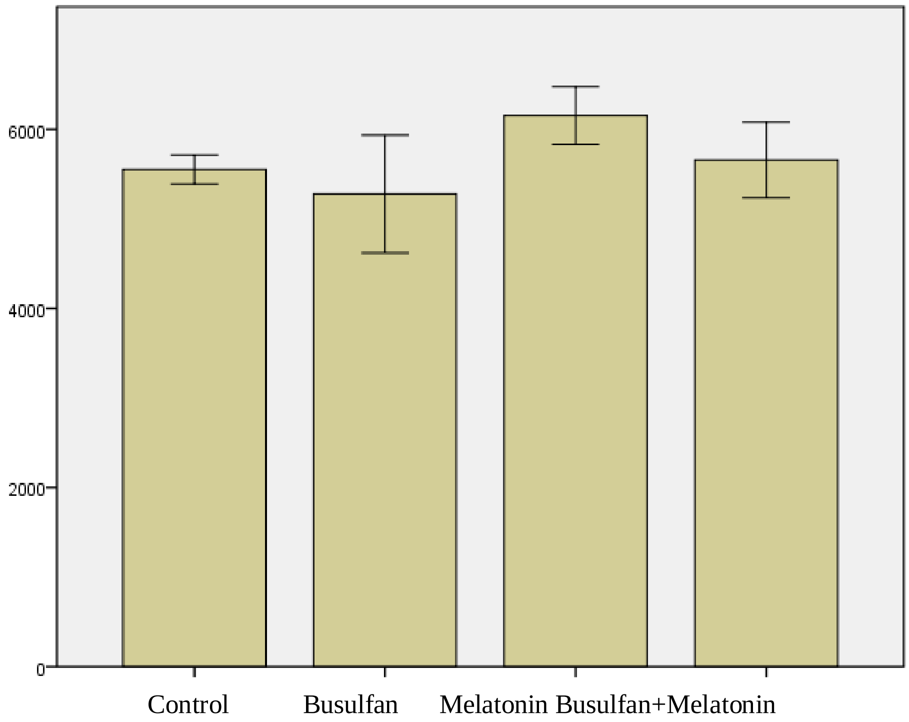
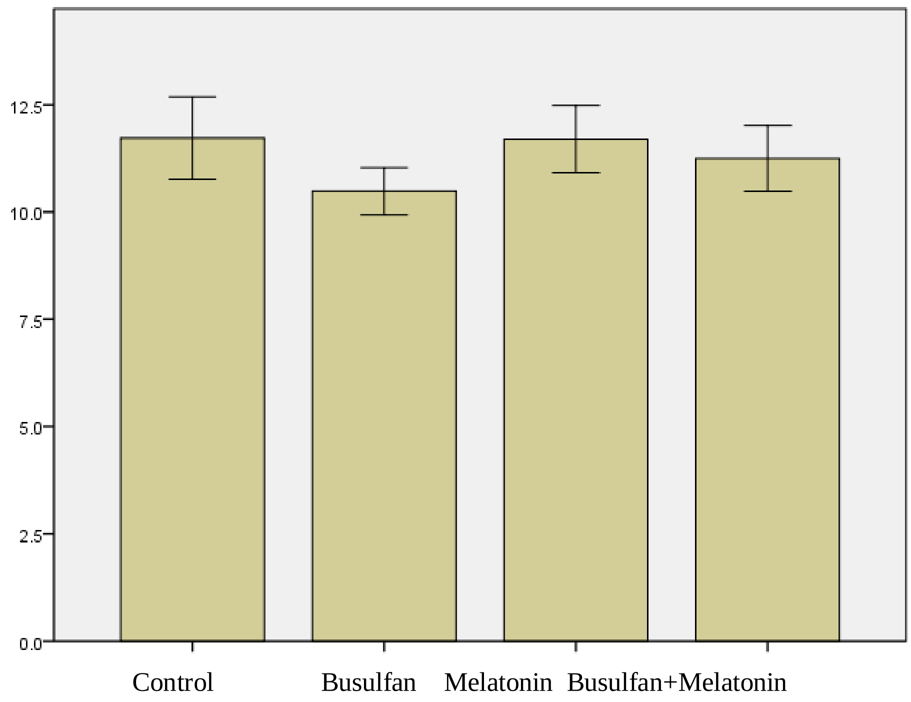
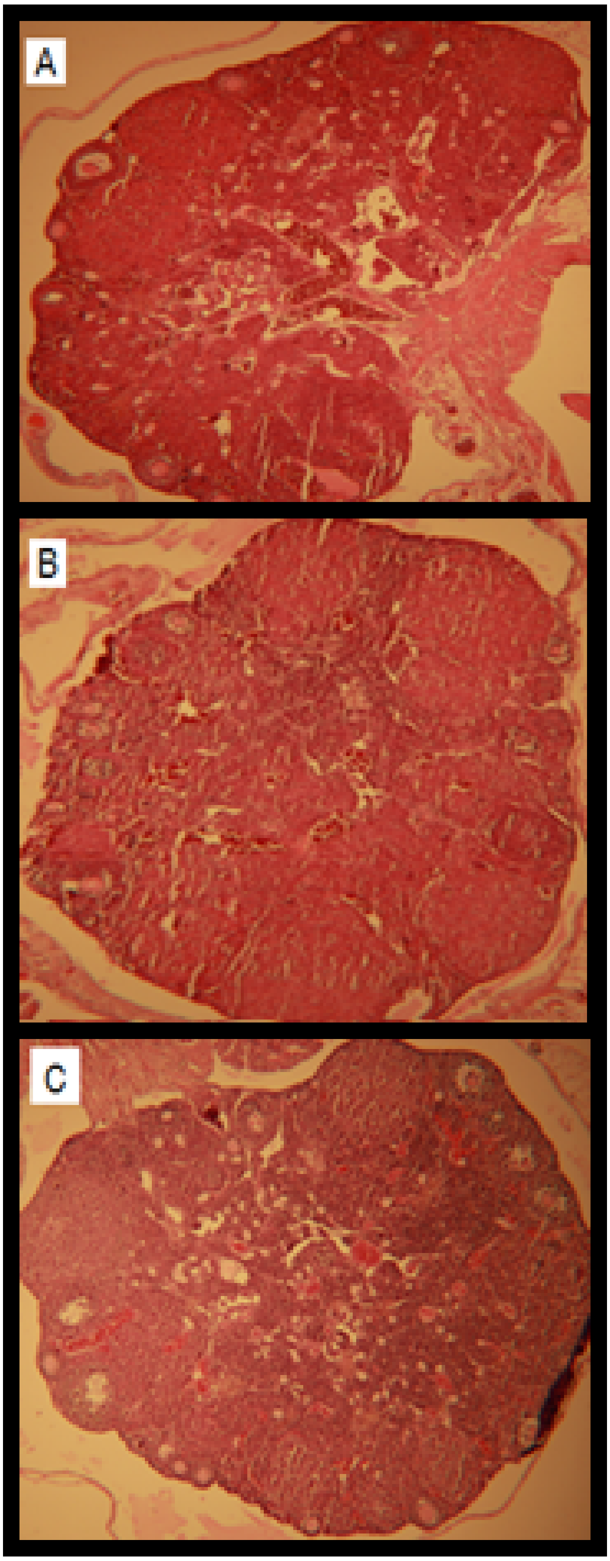
Figure 2: Mean Non-Atrophied mature follicles number



Groups
Error bars: 95% Cl
Figure 3: Mean Nucleus area



Groups
Error bars: 95% CL
Figure 4: Mean Zonapellucida thickness

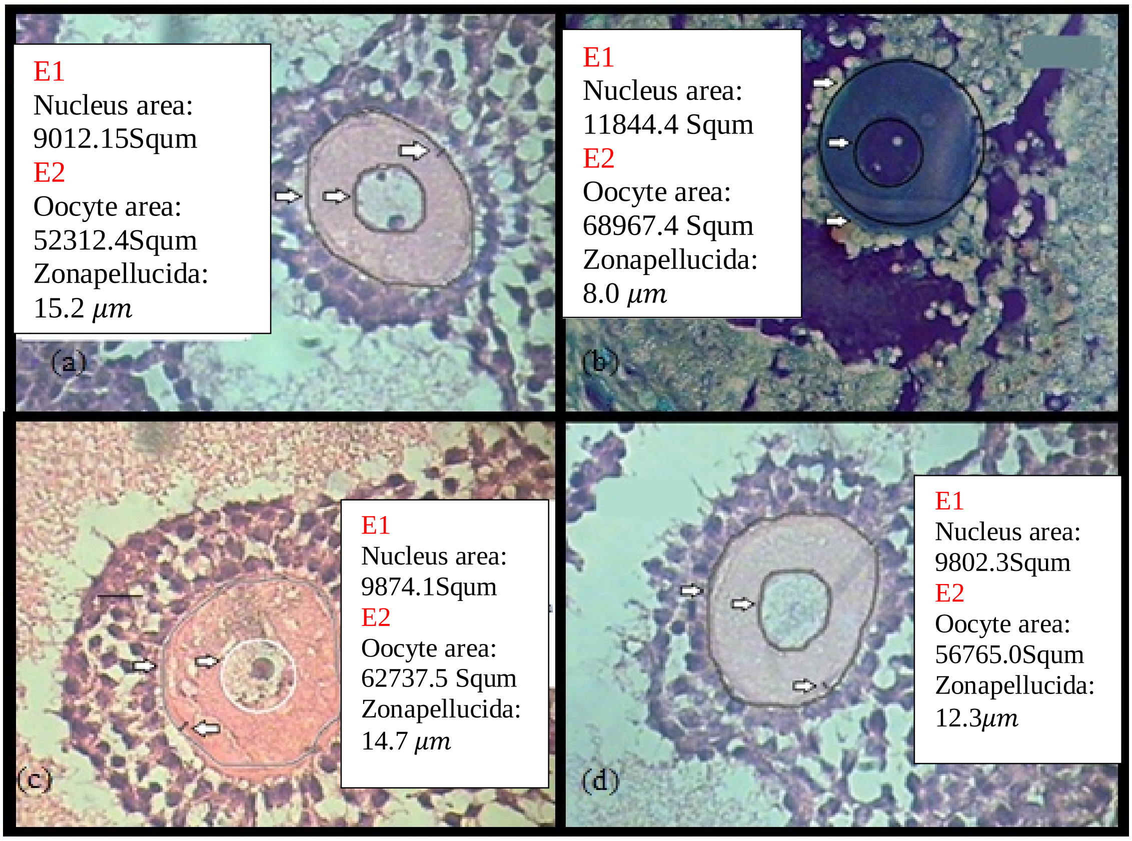


A. There was a significant increase in the number of mature atrophied folliclesin busulfan-treatment group

B. There was a significant reduction ofmature follicles in melatonin-treatment group

C. There was a significantincrease in the number of mature non-atrophiedfollicles and there was a significant reduction ofmature atrophied follicles inBu/Me-treatment groupcompared with the busulfan-treatment group

Figure 5: Effects of busulfan and melatonin treatment on histology of the Ovary



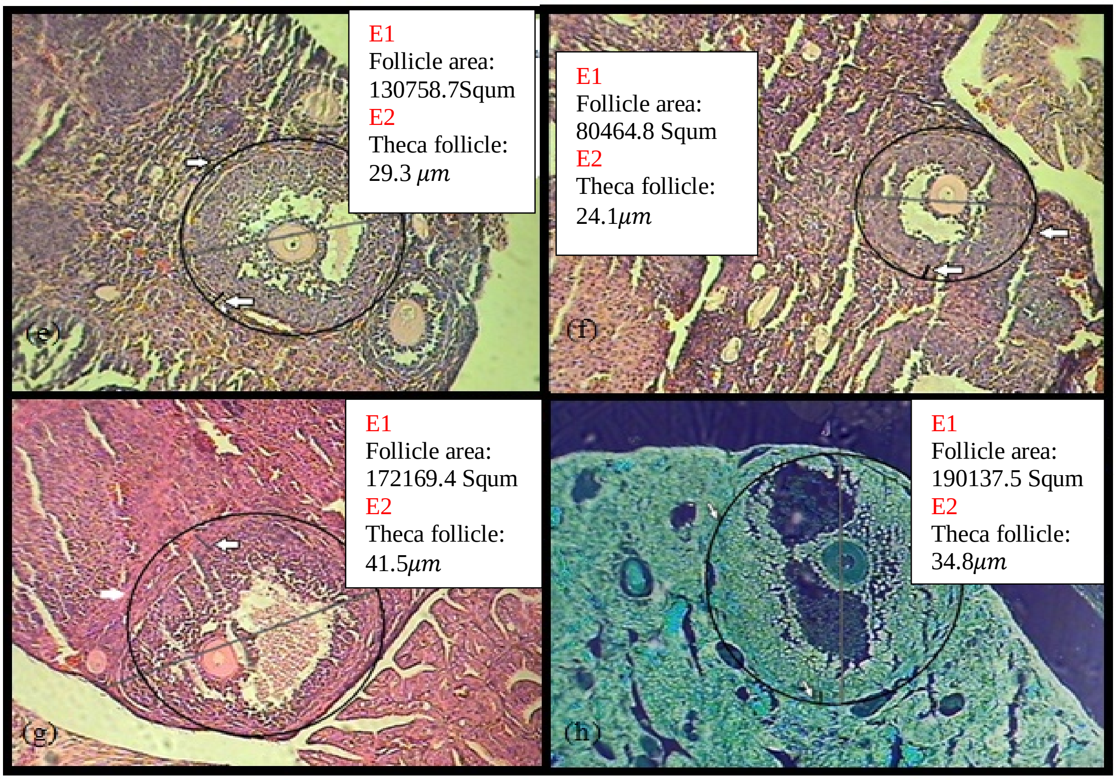


Figure 6: Comparison of the quantative parameters related to mature ovarian follicles' structures of mice treated with busulfan and melatonin; (a,b,c,d)The thickness of zona pellucida, oocyte area andnucleus in groups of control, busulfan, melatonin and Bu/Me respectively; (e,f,g,h) The thickness oftheca follicular andmature follicle area in groups of control, busulfan, melatonin and Bu/Me respectively.

DISCUSSION AND CONCLUSION

In the present study the effects of busulfan and melatonin on mature follicles parameters has been studied which are important indicators for measuring the quality of female fertility. In this study, administration of a single dose of20 mg / kg busulfan increased the number of atrophied follicles significantly, which refers the effect of busulfan alkylating property that has damaging effects on the proliferating cells. Having DNA alkylating properties, Busulfan impacts negatively on cells with greater division power. Studies show that treatment with busulfan and cyclophosphamide increases gonadotropin levels of FSH and LH, which increases the follicle proliferation and increased sensitivity of the ovarian tissue against the chemotherapy drug. Jiang et al, reported similar results and stated that the administration of busulfan and cyclophosphamide in cancer diseases caused a substantial decrease of primary follicles on day 30after treatment and a significant loss of growing folliclesat60 days after treatment [12]. Pelloux et al, examined the effects of busulfan on fetal ovarian follicle proliferation in the pregnant rats and concluded that the number of Germ cells and follicles were decreasedin12-day embryos that have received busulfan [18]. In the recent study, the number of mature atrophied and non-atrophied follicles increased and decreased, respectively ($P<0.01$).Researchers have shown that chemotherapy drugs such as busulfan, on the one hand create oxidative stress by increasing the production of oxidants[7] and on the other hand, it decreases the body's natural antioxidants such as glutathione levels and eventually leading to cell death [5].Another way in which Germ cells are destroyed by busulfan is the apoptosis induction in which the cells shrink and DNA is fragmented in a systematic way and the plasma membrane becomes bubbly [4]. Tienturrier et al, evaluated the ovarian function of 21 girls with a average age of 11-21 years old who received high-doses of busulfan aftera bone marrow transplant, and concluded that high-dose busulfanisa major cause of ovarian failure [21].In this study it was found that the quantities parameters of a mature follicle decrease following the use of busulfan which is indicative ofits harmful effects that increase the vulnerability of mature follicles and that are consistent with studies conducted by Tienturrier. In this study, the effects of melatonin administered alone and in combination with busulfan on the mature follicles also were studied.

In recent years the role of melatonin as a free radicals neutralizer, highly effective and strong antioxidant has attracted much attention. Due to its small sizeand high lipophilic property, melatonin passes the cell membrane easily, spreads across the cell, and protects DNA against damaging agents [15].Tamura et al, in 2012 identified melatonin as a potent antioxidant in ovarian follicles. They stated that melatonin has a direct role in oocyte maturation and growth of the fetus and reduces oxidative stress during ovulation. It also acts as an important drug to improve ovarian function and oocyt equality [20]. Tamuraet al, conducted a study in 2009 and demonstrated that follicular fluid contained high concentrations of melaton in compared to blood plasma and melatonin receptors are present in granulosa cells [19]. Studies conducted by Fouché court show that the antioxidant activity and protective effect of melatonin result in increased expression ofgenes involved inthe synthesis antioxidant enzymes such as superoxide dismutase, glutathione reductase and glutathione peroxidase [6].Similarly, the studies conducted by Johnstone show that there is an inverse relationship between melatonin and GnRH receptors in mice and that, it controls directly the ovarian activity and decreases secretion of FSH and LH [13]. Confirming previous reports, Mohammad Ghasemi's study shows that melatonin has anti proliferative effects on germ cells and other cells [14]. Thus, owing anti-proliferative properties, melatonin can reduce the damaging effects of busulfan by the two methods. Firstly, decreases the release of FSH and LH through the direct impact on hypothalamic-pituitary-gonadal axis, and secondly through indirect method that has anti proliferative effect on the germ cells and other cells. In this study as well, the number of mature non-atrophied follicles was decreased meaningfully ($P<0.01$), also the number of mature atrophied was decreased. Anti-apoptotic effects of melatonin on various tissues have been shown in various studies. Besides antioxidant and anti-proliferative effects of Melatonin, it has the anti apoptotic property; so, it can prevent germ cells destruction by preventing their apoptosis [1]. The results obtained in this study, reveals that quantities parameters of mature follicles in melatonin-treatment group the control group no significant difference have no meaningful difference compared with the control group. Melatonin reduces the damaging effects busulfan by reducing the number of mature atrophied follicles and maintaining its structures such as zonapellucida and theca follicular. The present study showed that the administration of 10 mg / kg melatonin with busulfan for 5days, maintains the quantative parameters of mature follicles, decreases mature atrophied follicles and thus reduce the side effects of busulfanin ovarian tissue. So it seems that maintaining the mature follicles' quantitative parameters in combination therapy group would be the result of the antioxidant, anti-proliferative and anti-apoptotic properties of melatonin. Melatonin dosage in this study was similar to the study conducted by Guneli and Atessahin [1,9].

SUGGESTIONS

In summary, the results of this study showed that administration of20 mg / kg busulfan, decreases quantative parameters of mature follicles and increases the number of atrophied follicles,

The administration of melatonin at a rate of10 mg / kg for 5days of chemotherapy onset has a substantial decreasing effect on the adverse impacts of chemotherapy on mature follicles,

And has a well protective effect on the ovaries. This study suggests that melatonin may be useful and important drug in the clinical application of ovarian functional disorders following the administration of anti-cancer drugs.

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