Original Research Article

Investigation of TAp63 gene expression and follicle count using melatonin in cisplatin-induced ovarian toxicity

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ABSTRACT

Background: Premature ovarian failure is among the most important side effects of chemotherapy during reproductive period. Preserving ovarian function is gradually gaining importance during oncologic treatment. The present study aims to investigate the potential of melatonin to protect from cisplatin-induced ovarian toxicity in rats.
Methods: Twenty-nine female rats were divided to three groups: Saline control group (group 1), cisplatin group (group 2), and cisplatin and melatonin group (group 3). While the rats in groups 2 and 3 were administered 5 mg/kg single dose of cisplatin via intra-peritoneal (IP) route, the rats in group 3 were started on melatonin (20 mg/kg IP) before cisplatin administration and continued during 3 consecutive days. Ovaries were removed one week after cisplatin administration in all groups. Blood samples were obtained before the rats were decapitated. Histological evaluation, follicle count, and classification were performed. TAp63 mRNA expression was evaluated using mRNA extraction and real-time polymerase chain reaction (PCR) method. Serum estradiol (E2) and anti-Mullerian hormone (AMH) values were measured with enzyme immune-assay technology.
Results: While primordial follicles were seen to decrease in group 2 as compared to group 1 (p=0.023), primordial follicle count was observed to be preserved significantly in melatonin group as compared to group 2 (p=0.047). Moreover, cisplatin-induced histo-pathological morphology was preserved in favor of normal histology in melatonin group. A significant difference was not observed between groups with regard to mean serum AMH and E2 values (p=0.102 and p=0.411, respectively). While TAp63 gene expression significantly increased in group 2 as compared to control group (p=0.001), we did not detect a statistically significant difference in cisplatin and melatonin group, although gene expression decreased (p=0.34).
Conclusions: We conclude that concurrent administration of melatonin and cisplatin may protect from ovarian damage.

Keywords: Cisplatin, Melatonin, TAp63, Primordial follicle, Ovarian reserve

INTRODUCTION

The most important and common side effect of chemotherapy (CT) is premature ovarian failure and the infertility because CT-induced ovarian damage, which is progressive and irreversible. Germ cell pool is limited and defined in intra-uterine life.[1] Prevention of CT-induced ovarian damage, preserving ovarian follicle pool

and improvement of quality of life continue to draw attention as the recent developments in cancer CT enables to achieve a long, disease-free interval.

Fertility preserving strategy is cryopreservation of an amount of ovarian tissue or premature oocytes. However, it may be used limitedly due to the worries about time, costs, and re-introducing of cancer cells.[2] In addition, re-implantation of cryopreserved tissue did not bring menstrual cycles back or resulted in amenorrhea again within one to two years in more than half of the cases.[3,4]

Cisplatin (cis-diamminedichloroplatinum) is a platinum-based alkylating agent that is widely used for treating solid tumors and gynecologic cancers [5], and similarly with the other CT agents, it induces apoptosis leading to DNA breaks as the result of DNA cross-links.

TAp63 is the oocyte-specific isoform of p63, which is a member of the p53 family, that is a tumor suppressor gene found in high concentrations in mouse and human primary oocytes; it plays an important role in oocyte death following genotoxic agent use.[6] TAp63 that is found in oocyte nucleus is responsible for DNA damage and apoptosis pathway in primordial follicle, and the regulator of apoptosis of primordial follicle cells in case of exposure to cisplatin.[7,8] Highly expressed TAp63 makes ovarian reserve excessively susceptible to DNA damage in mouse oocytes.[6,9] Cisplatin induces dormant follicle development as well as large follicle apoptosis.[10]

Cisplatin also increases free oxygen radicals and leads to a decrease in ovarian antioxidant capacity.[11,12] The ovarian reserve-preserving effect of anti-oxidants has been investigated in many studies. In vivo animal studies have shown the organ-preserving effect of oxidative stress blockage.[11,13]

Melatonin (N-Acetyl-5-methoxytryptamine) is a tryptophan derivative that is primarily produced and secreted in peripheral reproductive organs, placenta, and ovaries.[14] Melatonin is lipophilic, antioxidant, and free radical scavenger.[15]

Presence of many melatonin receptors has been shown in primordial follicles in immune-fluorescence analyses.[16]

Melatonin, which is found in high amounts in follicular fluid, was shown to improve oxidative stress and oocyte quality, inhibit tumor growth through suppressing angiogenesis, improve immune system through increasing cytokine production, and prevent DNA damage and apoptosis through p53 gene.[17-19]

The present study aims to investigate the protective effect of melatonin in cisplatin-induced ovarian damage in rat model. We believe that target therapies will be promising when the molecular mechanism of cytotoxic drug-related ovarian damage is understood better.

METHODS

Ethics committee approval was obtained from Çanakkale 18 Mart university ethics committee of animal tests (council number: 2019/01 and decision number: 2019/01-13). A total of 29 healthy, adult female Wistar rats (aged 3-4 months and weighing 200-250 g) were used. Animals were kept in 12 hours of light and dark cycle, in a room at 24±2°C with sufficient air conditioning. Standard food and water were given ad libitum. A total of 29 rats with menstrual cycles were divided to three groups: control group (saline group, group 1, n=7), cisplatin group (group 2, n=11), and cisplatin and melatonin group 3, n=11). While rats in groups 2 and 3 were given single dose of 5 mg/kg cisplatin (Koçak Farma drug company, İstanbul, Turkey) via intra-peritoneal (IP) route, the rats in group 3 were given 20 mg/kg melatonin (Sigma-Aldrich chemical) IP route 30 minutes (min) before cisplatin administration and continued for 3 consecutive days. The rats in group 1 were given only equal dose of saline IP. Melatonin dose was estimated based on the protective effect in previous studies.[20] 1 week after chemotherapy, each rat was given ketamine (50 mg/kg IM) and xylazine (10 mg/kg IM) anesthesia and underwent bilateral oophorectomy through a 3-cm midline incision; intra-cardiac blood samples were obtained and the rats were sacrificed with cervical dislocation thereafter.

Parenteral administration rules were followed during IP administration to avoid direct toxic effect of CT agent.

Histological examination

The rat ovaries tissues were fixed for 24 h in 10% formalin solution. The tissues were dehydrated in graded series of ethanol and embedded in paraffin. After fixation in paraffin blocks, 4-µm serial sections were cut from ovary blocks with a microtome, and the sections were stained with routine hematoxylin and eosin (H and E). All the sections were analyzed and photographed using Zeiss Axio scope. A1 light microscope (Carl Zeiss 3708, Göttingen, Germany). At least five randomly selected ovarian sections were examined for histopathologic examinations from each group.

Follicles were classified based on the Pedersen and Peter's morphological criteria, as described previously, in primordial, primary, secondary, antral, and atretic follicles.[21] Additionally, these follicles were classified individually as histologically normal, when an intact oocyte was present and surrounded by granulosa cells that were well-organized in one or more layers and had no pyknotic nuclei. To avoid double counting of the same follicles, a follicle was counted on the first section in which the centrally located nucleus of oocyte appeared.

Hormonal assays

E2 and AMH were quantitatively estimated in rat serum sample using enzyme immunoassay technology (EIA)

kits (catalog number: 33540 and 813127 respectively; Beckman Coulter Acces II device and Beckman Coulter Inc., USA).

RNA extraction and real-time PCR methodology

In the present study, rat ovary samples were contained in a -80° C refrigerator. RNA extraction was performed on these samples with Thermo Scientific® GeneJET RNA Purification kit according to their manual. Extracted RNA's were used to synthesize cDNA with Thermo Scientific® Revert aid first strand cDNA synthesis kit, immediately afterward.

For this study, gene act (Actin) was chosen as the control gene. ActB forward primer, ActB reverse primer, TP63 prob, TP63 forward primer, and TP63 reverse primer (Sigma-Aldrich®) was used. For target gene, new primers and probes were designed accordingly. The following mix was used for real-time PCR, 1 µl of the selected probe; 1 µl of selected forward primer; 1 µl of selected reverse primer; 2 µl ddH2O; and 2x LightCycler® 480 probes master was added. 15 µl of this mix was put in LightCycler® Capillers and 5 µl of cDNA was added with a pipette. For real-time PCR, the following protocol was used, 10 min at 95° C for denaturation; 45 cycles of 10 s at 95° C; 30 s at 60° C; 1 s at 72° C for amplification; and 30 min of 40ºC of cooling. The cycle was performed in LightCycler® 2.0 instrument (Mannheim, Germany); absolute quantification module of LightCycler2 software 4.1.1.21 was used for analysis.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) and median (minimum-maximum) due to nonnormality. The Shapiro-Wilk test was used to assess the normality. The Levene test was used to evaluate homogeneity of variances. Analysis of variance (ANOVA) and Kruskal-Wallis H test was run to determine if there were differences between groups. Pairwise comparisons were performed using Dunn's procedure with a Bonferroni correction for multiple comparisons. Adjusted p values are presented. All statistical analyses were performed using SPSS 19.0. All p values of less than 0.05 were considered to indicate statistical significance.

RESULTS

While normal histological structure, including centrally located follicle groups and intact basal membrane and surrounding granulose cells, is seen in rats in control group (Figure 3A), swelled and disorganized granulose cells, vascular congestion, interstitial, vacuolated oocytes, and picnotic nucleus were observed in group 2 (cisplatin group) (Figure 3B). In group 3 (cisplatin and melatonin), cisplatin-induced follicular damage was seen to decrease and morphology was seen to be similar with that of control group (Figure 3C).
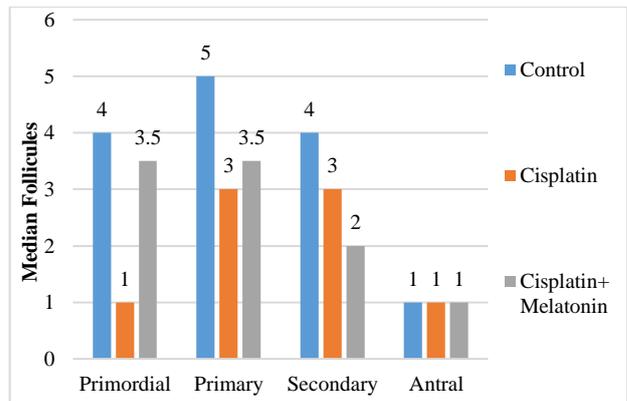

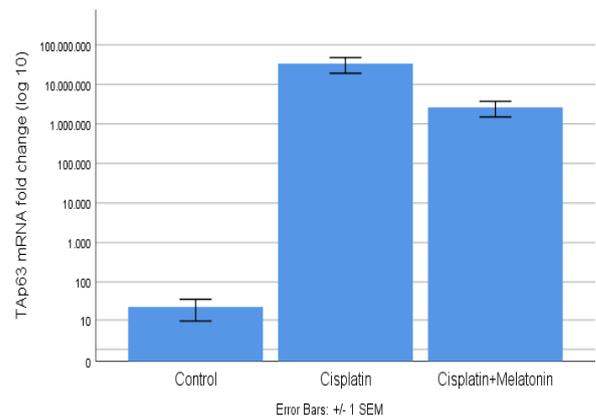**Figure 1: Median follicle values of the groups.**

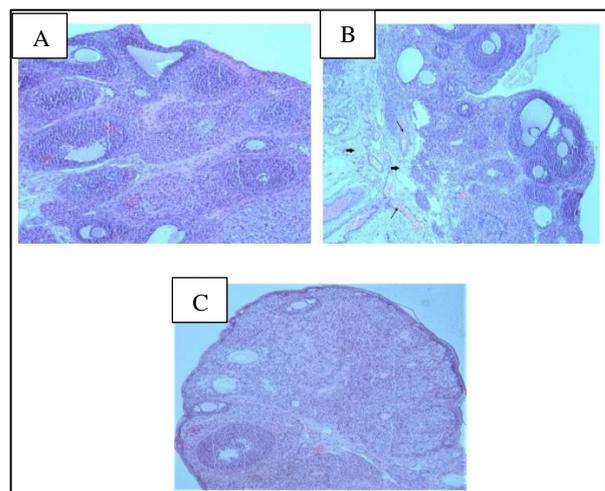**Figure 2: Mean Tap63 mRNA expression of groups.**

Figure 3: Light microscopic images of ovarian sections of our study groups. (A) Normal ovarian tissue belonging to control group. (B) Appearance of histopathological damage with interstitial edema, vascular congestion and leukocyte infiltration in cisplatin group. (C) In the melatonin and cisplatin group, the damage caused by chemotherapy is almost disappeared, its similarity to the normal control group is remarkable (H and E staining, 200X). Thin arrow, vascular congestion; thick arrow, interstitial edema and leukocyte infiltration.

There was a significant difference between groups with regard to median primordial follicle count (p=0.009). In comparative analysis, primordial follicle count was seen to decrease significantly in cisplatin group compared to control group (p=0.023). In cisplatin and melatonin group, while primordial follicles were preserved as compared to cisplatin group (p=0.047), no significant difference was observed between groups as regards median primary, secondary, and antral follicle counts (Table 1).

Table 1: Median follicle values of the groups.

Groups		Primordial	Primary	Secondary	Antral
Group 1	n=7	4 (2-12)	5 (0-22)	4 (0-8)	1 (0-2)
Group 2	n=11	1 (0-10)	3 (1-10)	3 (0-7)	1 (0-4)
Group 3	n=10	3.5 (2-13)	3.5 (1-8)	2 (1-9)	1 (0-2)
P value		<0.009*	0.711	0.969	0.730

Data presented in median (min-max); *Significant pairwise comparisons: Group 1 versus group 2 (p=0.023); Group 2 versus Group 3 (p=0.047)

No significant difference was found between groups with regard to serum AMH and estradiol (E2) values (p=0.102 and p=0.411, respectively) (Table 2).

Table 2: E2 and AMH levels of the groups.

Groups		E2	AMH
Group 1	n=7	38.4183±7.1816	12.8783±4.4534
		(27.13-45.87)	(4.96-17.09)
Group 2	n=11	43.4686±6.8166	9.6011±4.2173
		(35.09-53.38)	(3.25-15.36)
Group 3	n=10	44.4470±10.730	8.5120±2.8944
		(33.59-64.61)	(4.50-12.99)
P value		0.411	0.102

Data presented in mean ± SD (min-max)

While TAp63 mRNA expression was very low in control group, it was observed to increase significantly in cisplatin group (p=0.001). Although gene expression decreased in cisplatin and melatonin group, this reduction was not statistically significant (p=0.34) (Table 3).

Table 3: Mean Tap63 mRNA expression of the groups.

Group 1	Group 2	Group 3
n=7	n=6	n=6
22.61±13.20	33263772.37± 14205736.17	2609242.05± 1112895.74
P values		
Group 1 vs Group 3	0.032	
Group 1 vs Group 2	0.001	
Group 3 vs Group 2	0.343	

Data presented in mean±std. error of mean

DISCUSSION

The present study has revealed that melatonin administered together with cisplatin preserved primordial follicle count in ovaries and decreased TAp63 apoptosis gene expression.

AMH is produced in granulose cells of growing preantral and small antral follicles and reflects primordial follicle count.[22] AMH regulates early follicle development through negative stimulator effect on follicle maturation and inhibiting follicle-stimulating hormone sensitivity of growing follicles.[23]

AMH's reflecting follicle population and being more stable than fluctuating gonadotrophins enables to use it as a reliable marker for evaluating ovarian reserve, and it is frequently used for assessing ovarian damage.[24] Furthermore, AMH shows a more rapid decline than estradiol during CT.[25]

Enzyme-linked immunosorbent assay (ELISA) and Western blot analyses showed that cisplatin treatment was shown to significantly decrease AMH-positive follicle count and ovarian AMH protein level as compared to control group.[24,26] We could not find a significant difference between AMH and E2 levels of the groups. AMH was being secreted from primarily growing follicles and not finding a significant difference in primordial follicles in our study may support this result. Another reason may be our giving cisplatin as the single agent and in a single dose.

Sufficient knowledge is not available in literature regarding which follicle stage is more susceptible to CT-induced damage; however, most studies are focused on primordial oocyte pool. Although Yücebilgin et al have reported that CT leads to a significant reduction in primordial follicle count, Borovskaya et al have stated that single-dose cisplatin reduces primordial follicle count in the ratio of 30%; Abir et al have shown the susceptibility to CT agents in more mature follicles such as preantral follicles in vivo and in vitro.[27-29] Similarly, Taşkın et al have emphasized the reduction in secondary and tertiary follicle count besides the reduction in primordial follicles.[30] In our study, we detected a significant reduction in only primordial follicle count

with single-dose cisplatin administration and observed the protective effect of melatonin against this reduction.

P63 plays a master regulator of primordial follicle in CT-induced DNA damage.[6,7] In a healthy oocyte, the protein is inactive so as not to be used. TAp63's being active through phosphorylation and elevated levels of it make the mature oocyte extremely sensitive to DNA damage and starts apoptosis.[6,9] Gonfloni et al have shown p63 accumulation in the oocyte and apoptosis through cisplatin administration.[8]

The agents that inhibit TAp63 phosphorylation are promising in preventing cisplatin-induced apoptosis.[8,31] From an alternative perspective, another question to be answered is whether the inhibition of apoptotic pathway reduces the success of anti-cancer therapy or not.

Majidinia et al have focused on the effect of melatonin in DNA damage and repair.[32] P53 was shown to suppress cancer cell proliferation and protect from DNA damage through being activated by melatonin use.[33,34]

Jang et al have presented the protective effect of melatonin against cisplatin-induced death of granulose cells in growing follicles and detected that excessive signaling increase of apoptosis decreases with immunofluorescence tests.[35] In our study, we also observed that cisplatin significantly increased TAp63 mRNA expression. Although melatonin use decreased the mean value of gene expression, a statistically significant difference could not be found; however, increasing sample size may change this.

Cisplatin increases free oxygen radicals and leads to a decrease in antioxidant capacity in the ovaries.[12] Many studies have shown the cisplatin-induced oxidative stress within the potential mechanisms of tissue toxicity.[11,36] Free oxygen radicals have a destructive effect against all cell components, primarily lipids. Melatonin clears toxic oxygen-based reactants and their derivatives including reactive hydroxyl radicals.[37] It also reduces free radical production through increasing the function of respiratory complex.[38] Therefore, melatonin is defined as an antioxidant cascade.[39] Tamura et al have shown that oocyte quality is impaired with oxidative stress and melatonin reduces oxidative damage by restricting free radical production.[17] Melatonin supplementation has improved oocyte quality and prevented cisplatin-induced testicular toxicity and the reduction in sperm motility in males.[40,41] Interest to antioxidant molecules has increased for prevention of CT-induced ovarian tissue damage. Cisplatin-induced ovarian toxicity was reported to reduce with the use of anti-oxidants such as mesna, mirtazapine, and sildenafil.[42,43,30] Curcumin and capsaicin were shown to reduce tissue oxidative stress markers and improve histological parameters in cyclophosphamide administered rats.[44]

The main limitations of our study are short duration of cisplatin and melatonin use and not matching with another potential treatment group.

CONCLUSION

In brief, the use of melatonin together with CT was shown to be able to prevent cisplatin-induced primordial follicle loss in rat ovaries. Better understanding of the molecular mechanisms of TAp63 that triggers CT agents-induced oocyte apoptosis would enable to develop novel pharmacologic treatments.

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