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The Effects of Antioxidant Melatonin on Rat Ovary Neonatal Exposure to Bisphenol A

Antioksidan Melatoninin Neonatal Dönemde Bisfenol A Uygulanan Rat Ovaryumları Üzerine Etkisi

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ABSTRACT

Aim: The aim of this study was to investigate the effects of melatonin on the rat ovary against to neonatal exposure to Bisphenol A (BPA).

Material and Methods: In this study, 24 Wistar Albino female newborn rats were divided into four groups (n=6). Rats were injected with (sesame oil + ethanol) and (100 mg/kg) BPA by subcutaneously (sc) between postnatal days (PND0-PND10), in control and BPA groups, respectively. Melatonin group rats were injected daily with (10 mg/kg) melatonin (PND20-PND30) and BPA+melatonin group rats were injected daily with (100 mg/kg) BPA (PND0-PND10) and (10 mg/kg) melatonin (PND20-PND30). Histological and morphometric investigation were performed. Furthermore cell cycle marker Cdc-2 expression was evaluated by using IHC.

Results: Histological findings showed that neonatal exposure to BPA results in degenerative alternations in ovary. It was found that there were no statistically significant changes in the body weight gains and in the average weights of ovarian tissue of rats between all groups. However, there was an increase in the average weights of ovarian tissue and body weight gain of rats from BPA administrated group. We also found that immunoreactivity of cdc-2 was lower in BPA group rats compared to others.

Conclusion: It was concluded that neonatal exposure to BPA disrupts folliculogenesis that causes degenerative changes in ovarian follicles and melatonin may have positive effects on rat ovary damaged by BPA.

Keywords: Bisphenol A (BPA); neonatal; melatonin; ovary; ovarian follicles.

ÖZ

Amaç: Bu çalışmanın amacı neonatal dönem Bisfenol A (BPA) uygulanmasına karşı melatoninin rat ovaryumları üzerindeki etkisinin araştırılmasıdır.

Gereç ve Yöntemler: Bu çalışmada Wistar Albino cinsi, 24 adet, yenidoğan dişi rat 4 gruba ayrılmıştır (n=6). Kontrol ve BPA grubu ratlara, sırasıyla, susam yağı + etanol ve BPA (100 mg/kg) postnatal 10 gün boyunca (PNG0-PNG10) subkutan olarak enjekte edilmiştir. Melatonin grubu ratlara günlük (10 mg/kg) melatonin (PNG20-PNG30) ve BPA+melatonin grubu ratlara ise günlük (100 mg/kg) BPA (PNG0-PNG10) ve (10 mg/kg) melatonin (PNG20-PNG30) 10 gün boyunca, subkutan olarak enjekte edilmiştir. Histolojik ve morfolojik analizler yapılarak ovaryum dokuları incelenmiştir. Bunlara ek olarak oosit hücre siklusu marker CDC-2 immunohistokimyal yöntem kullanılarak tespit edilmiştir.

Bulgular: Histolojik bulgular neonatal dönemde BPA maruziyetinin ovaryumda dejeneratif değişikliklere neden olduğunu göstermiştir. Morfolojik analizler sonucunda, vücut ağırlık artışları ve ovaryum dokusu ağırlıkları gruplar arasında istatistiksel olarak anlamlı bir fark saptanmazken BPA grubunda diğer gruplara kıyasla kilo alımının ve ovaryum dokusu ağırlığının daha fazla olduğu saptanmıştır. Bunlara ek olarak, immunohistokimyasal bulgularımızda, Cdc-2 immunreaktivitesinin BPA grubu ratlarda diğer gruplarla karşılaştırıldığında daha düşük olduğu saptanmıştır.

Sonuç: Neonatal dönemde BPA maruziyetinin folikülogeneze zarar verdiği, bu durumun da ovarium foliküllerinde dejeneratif değişikliklere neden olduğu ve melatonin de BPA tarafından hasar verilen rat ovaryumları üzerinde pozitif etkisi olabileceği kanısına varılmıştır.

Anahtar kelimeler: Bisfenol A (BPA); neonatal; melatonin; ovaryum; ovaryum folikülleri.

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INTRODUCTION

Bisphenol A (BPA) is an endocrine-disrupting chemical, widely used in plastic industry including in baby feeders, food packages, water bottles, dental treatment materials, detergent, the inner surface of metal box, construction supplies, pesticides (1). BPA has been detected in maternal and fetal plasma, and in human placenta (2). Exposure to BPA in fetal or neonatal period in mice affects gamete production, fertility, development of reproductive tract and the different stages of oogenesis (3). An experimental dose of BPA, 100 mg/kg body weight, was selected as a toxic dose, recent studies has been observed that 50 mg/kg body weight BPA has no adverse-effect on the reproductive system (4). Higher than 50 mg/kg body weight dose of BPA affect the reproductive system and result in histological alterations (5).

Melatonin is a neurohormone which mostly synthesized and secreted by the pineal gland (6). Melatonin has an important role in the regulation of many functions including sexual maturation, reproduction, thermoregulation, circadian rhythms and immunity. Melatonin acts as a potential free radical scavenger (7) and has the ability to reduce reactive species formation (8,9). Melatonin stimulates the gene expression of other antioxidant enzymes, thus prevents oxidative stress damage (10).

Melatonin plays an important role in oocyte maturation, embryonic development and luteinizing. Further studies on infertile women revealed that increased intrafollicular melatonin concentration reduced intrafollicular oxidative damage and also increased fertilization and incidence of pregnancy (11).

Mammalian oocytes in ovarian follicles are arrested at the first meiotic prophase, their meiosis progress until second meiotic metaphase after hormonal stimulation (12,13).

The meiotic maturation of oocytes is regulated by maturation promoting factor (MPF), a complex of catalytic subunit Cdc2 (Cdk1) and regulatory subunit cyclin B (14). It has been shown that the Cdc 2 level low or absent in small oocytes of mice and goats and the level increases according to oocyte growth (15-18). According to given information above, the aim of our study was to investigate the possible effects of prepubertal administration of melatonin against to rat ovary exposure to BPA during neonatal period.

MATERIAL AND METHODS

Animals and Experimental Design

In this study, we used 24 female Wistar albino newborn rats. Animals were obtained from the Animal Breeding and Experimental Research Laboratory of Gazi University. This study was approved by the Ethical Committee of Gazi University (Dated 18.03.2013 and numbered 67-6163). The animals were housed under (12 h light/dark cycle, 20-25°C) designed laboratory conditions for (65-110 days) until they became mature. They feed water and food. Rats were divided equally into four groups (n=6). In control, rats were injected with 100mg/kg sesame oil and 10mg/kg 1% ethanol for 10 days between postnatal (PND0-PND10) and (PND20-PND30) respectively.

Rats in BPA group, were injected daily with 100 mg/kg BPA (Sigma, LOT MKBH2096V, USA) dissolved in sesame oil subcutaneously (sc), for 10 days (PND0-PND10). Melatonin group rats were injected daily with 10 mg/kg melatonin (Sigma, LOT SLBC 7539V, USA) dissolved in 1% ethanol sc, consideration the circadian rhythm, at 4pm, for 10 days between (PND20-PND30).

BPA and melatonin group rats were injected with 100 mg/kg BPA dissolved in sesame oil, for 10 days (PND0-PND10) and 10 mg/kg melatonin dissolved in 1% ethanol consideration the circadian rhythm, at 4pm, for 10 days (PND20-PND30). When the rats became sexual maturity, about 70th day, rats were sacrificed by using anesthesia with ketamine hydrochloride (40 mg/kg) (Ketalar, Eczacibasi, Istanbul, Turkey) and xylazine hydrochloride (5 mg/kg) (Rompun, Bayer, Istanbul, Turkey) and the ovaries were removed.

Histological and Morphometric Investigation

Ovarian tissues were fixed in 10% formalin and embedded in paraffin after routine histological procedures were performed. 4 µm sections were obtained from each paraffin block and stained with hematoxylin-eosin (H&E). The slides were evaluated under light microscope (LeicaDM4000, Germany).

Follicular and stromal degeneration and edema were scored from 0 to 3 according to the injury severity, where 0 represented no pathologic findings, and 1, 2 and 3 represented pathologic findings of less than 33%, 33% to 66%, and more than 66% of the ovarian tissue section, respectively. The histomorphologic changes were examined in six microscopic fields for each slide at magnification, ×400.

Measurement of Body Weight Gain and Ovarian Tissues

The body weights of six rats from each group were measured during the experiment and recorded once daily. Ovarian tissue weights were measured in each group after the rats were sacrificed. Ovarian weight was calculated by averaging ovarian weights in each animal.

Immunohistochemical Procedure

The avidin-biotin peroxidase method was used for the immunohistochemical (IHC). Following the deparaffinization process, the cross sections were subsequently incubated in citrate buffer (pH: 6.0) (Lab Vision, Thermo Scientific, Fremont) and 3% hydrogen peroxide (Lab Vision, Thermo Scientific, Fremont). Ultra V block (Lab Vision, Thermo Scientific, Fremont) was applied for blocking. Tissue sections were incubated with Cdc2 primer antibody (Santa cruz Cat: sc-954, Lot: 12210) in 1:100 dilution +4 C overnight. Then, the tissue sections were incubated with secondary antibody (Lab Vision, Thermo Scientific, Fremont) for 10 min. The reaction was revealed by streptavidin peroxidase complex (Lab Vision, Thermo Scientific, Fremont) with DAB. Mayer's hematoxylin was used for background staining. The slides were evaluated under light microscope with computer imaging system (Leica DM 4000, Germany).

Positive immunoreactivity for cdc-2 were scored from 0 to 3 according to the injury severity, where 0 represented no pathologic findings, and 1, 2 and 3 represented pathologic findings of less than 33%, 33% to 66%, and more than 66% of the ovary section, respectively. The histomorphologic changes were examined in six microscopic fields for each slide at magnification, ×400.

Statistical Analysis

All statistical analyses were performed using SPSS statistical software (SPSS for windows, version 17.0). The differences between the groups were statistically evaluated using the Kruskal-Wallis test (post hoc: Mann-Whitney U test with Bonferroni corrected). p values less than 0.05 were accepted as statistically significant.

RESULTS

Evaluation of Body Weight of the Female Rats

The body weight of rats from each group were measured, at the first and the last days of the experiment and the mean values of body weight gains were given in Table 1. There was no statistically significant changes in body weight gains of rats between all groups (p=0.257).

Evaluation of Ovarian Tissue Weight of the Female Rats

The weights of ovarian tissues were measured at the end of the experiment and the average weights of ovarian tissue of female rats from each group were given in Table 1. There was no statistically significant increase in the average weights of ovarian tissue between the groups (p=0.888). However, there was an increase in the average weights of ovarian tissue of rats from BPA administrated group (0.18±0.08g). In contrast, the average weight of ovarian tissue in melatonin (0.14±0.03g) and BPA+melatonin (0.15±0.05g) were similar to those of controls (0.15±0.04g).

Histopathological Investigation of Ovary

The comparison of follicular degeneration, stromal degeneration and edema scores of all experimental groups were summarized in Table 2. Follicular degeneration ($p<0.001$), stromal degeneration ($p<0.001$) and edema ($p=0.001$) were significantly increased in the ovary of BPA group rats when compared with the control group. There was a significant decrease in follicular ($p=0.003$) and stromal degeneration ($p=0.002$) in the BPA+Melatonin group compared to BPA group.

Histopathological examination showed that normal histological features in the ovary of the control (Figure 1.a,b) and melatonin groups (Figure 1.e,f). In control, the ovary was surrounded by germinal epithelium and underline tunica albuginea. Primordial follicles, consist of an oocyte is surrounded by a single layer of squamous follicular cells, primary follicles that consists of an oocyte surrounded by a layer or layers of cuboidal follicular cells were observed. When the follicular cells proliferate into a stratified epithelium known as granulosa cell layer. Secondary follicles with the appearance of a follicular antrum within the granulosa layer were observed. In the melatonin group, secondary and primary follicles were seen in the ovarian cortex (Figure 1.e,f). Atretic follicles and ovarian follicles that could not be determined at what stage of development were seen in the ovarian cortex of BPA group rats. The spaces between follicular cells, cell infiltrations and degenerative changes were seen in this group (Figure 1.c,d). In BPA+melatonin group rats, primary follicles were observed in the ovarian cortex. In contrast to BPA group rats, there were not seen any spaces between follicular cells in ovarian cortex. (Figure. 1.g,h).

IHC Results

In our study, we evaluated Cdc-2, cell cycle marker expression through IHC analysis (Table 3). Cdc-2 immune-expression ($p<0.001$) were significantly decreased in BPA group when compared with the control group. On the other hand, Cdc-2 immune-expression were significantly higher in BPA+Melatonin than BPA group ($p<0.001$).

When we compare the groups between each others, we observed that high expression of Cdc-2 was in mainly situated in primary follicles, granulosa cell layer of secondary follicles, teca layer, lutein cells of corpus lutheum, endothelial cells of blood vessels in the medulla of the rats in the control group (Figure 2.a,b). In melatonin group the immunoreactivity of Cdc-2 in cortex and medulla (Figure 2.e,f) was higher than BPA and BPA+melatonin groups (Figure 2.c,d and g,h).

Table 1. The mean values of weight gain and ovarian weights of female rats for all groups

		Control (n=6)	BPA (n=6)	Melatonin (n=6)	BPA+Melatonin (n=6)
Body weight (g)	Mean±SD	121.86±8.66	146.62±24.40	129.90±15.54	146.56±21.70
	Median	118.5	145.3	126.0	142.4
	Min-Max	111.60-133.60	111.20-176.50	111.20-146.20	118.00-175.10
Ovary weight (g)	Mean±SD	0.15±0.04	0.18±0.08	0.14±0.03	0.15±0.05
	Median	0.15	0.19	0.13	0.13
	Min-Max	0.10-0.21	0.17-0.76	0.31-0.48	0.18-0.86

SD: Standard deviation, Min-Max: Minimum-Maximum

Table 2. Histopathologic evaluation scores of the ovarian tissues in all experimental groups

		Control (n=6)	BPA (n=6)	Melatonin (n=6)	BPA+Melatonin (n=6)
Follicle degeneration	Mean±SEM	0.47±0.09	1.75±0.15[a]	0.47±0.09	1.13±1.12[b]
	Median (IQR)	0.00 (1.00)	2.00 (1.00)	2.00 (1.00)	1.00 (1.00)
	Min-Max	0.00-2.00	0.00-3.00	0.00-3.00	0.00-3.00
	p	<0.001	0.001	<0.001	<0.001
Stromal degeneration	Mean±SEM	0.36±0.08	1.69±0.14[a]	0.75±0.11	1.02±0.14[b]
	Median (IQR)	0.00 (1.00)	2.00 (1.00)	1.00 (1.00)	1.00 (2.00)
	Min-Max	0.00-1.00	0.00-3.00	0.00-2.00	0.00-3.00
	p	<0.001	0.001	<0.001	<0.001
Edema	Mean±SEM	0.13±0.05	1.13±0.15[a]	0.44±0.09	1.19±0.13
	Median (IQR)	0.00 (0.00)	1.00 (1.75)	0.00 (1.00)	1.00 (1.00)
	Min-Max	0.00-1.00	0.00-3.00	0.00-2.00	0.00-3.00
	p	<0.001	<0.001	<0.001	<0.001

[a]: Significant increase vs control group, [b]: Significant decrease vs BPA group, SEM: standard error of the mean, vs: versus, IQR: Interquartile range

Table 3. The mean density of Cdc-2 immunostaining in all experimental groups

		Control (n=6)	BPA (n=6)	Melatonin (n=6)	BPA+Melatonin (n=6)
Positive immunoreactivity of cdc-2	Mean±SEM	2.16±0.12	0.63±0.09[*]	1.63±0.11	1.50±0.09[**]
	Median (IQR)	2.00 (1.00)	1.00 (1.00)	2.00 (1.00)	2.00 (1.00)
	Min-Max	1.00-3.00	0.00-2.00	1.00-3.00	0.00-2.00

[*]: Significant decrease vs control group, [**]: Significant increase vs BPA group, SEM: standard error of the mean, vs: versus, IQR: Interquartile range

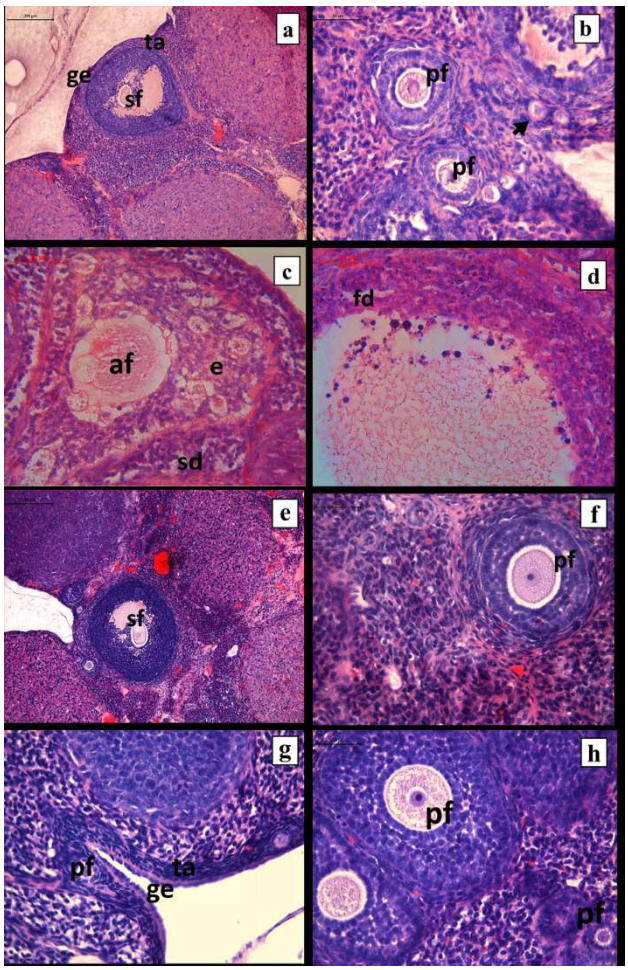

Figure 1. Histopathological examinations of ovary

In control, normal microscopic appearance of ovary that surrounded by germinal epithelium (ge) and underline tunica albuginea (ta). Primordial (arrow), primary (pf) and secondary (sf) ovarian follicles were seen in the ovarian cortex of rats from control (a-b). In BPA group, atretic follicles (af), stromal (sd) and follicular degenerations (fd) and edema (e) were seen (c-d). Normal histological futures of ovary with the present of secondary (sf) and primary follicle in the ovarian cortex of melatonin group rats (e-f). In BPA+melatonin group (g-h) primary follicles were observed in the ovarian cortex. H&E.

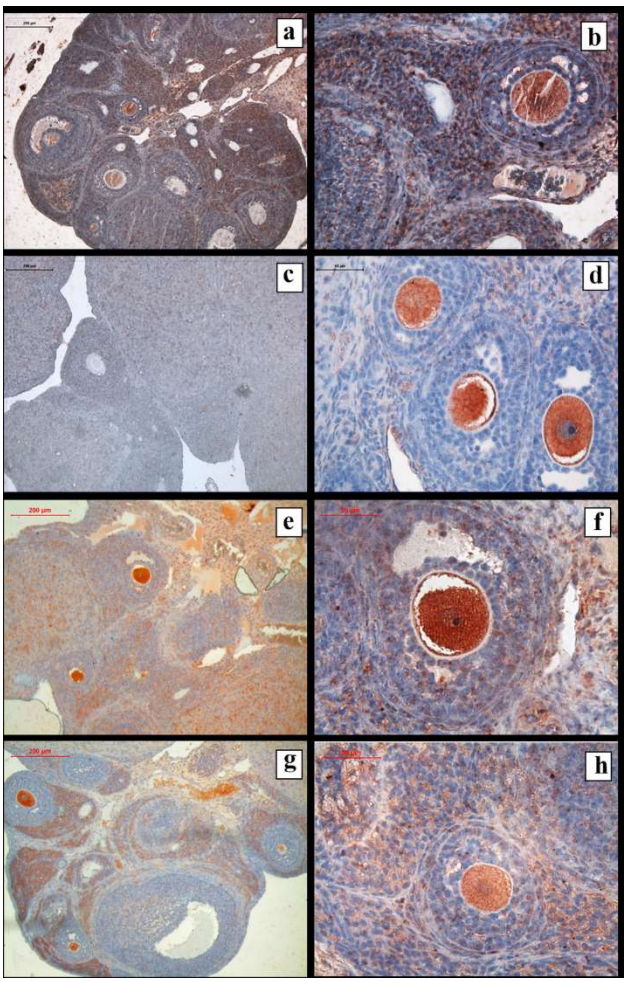

Figure 2. The immunoreactivity of Cdc-2 in rat ovary from each group

The immunoreactivity of Cdc-2 was lower in medulla and different stages of ovarian follicles in cortex of BPA group rats compare to control, melatonin and BPA+melatonin administrated groups. Control (a-b), BPA(c-d), Melatonin (e-f) and BPA+melatonin (g-h).

DISCUSSION

BPA has several effects on the ovary based on exposure time. Several studies conducted on rodents have revealed that exposure to BPA during early postnatal period caused decreasing in primordial follicle reserve (19). In a recent study conducted by Maffini et al. (20) on female rats expose to BPA results in ultrastructure changes, such as decreasing in the number of oocytes and increasing in uterine tissue weight and the expression of estrogenic receptors in endometrium. Fernandez et al. (21) investigated the effects of high dose BPA exposure on female rats and has reported that high dose BPA administration has negative effects on ovary, causes ovarian cyst formation that leads decrease in fertility.

In our study, we examined the effects of neonatal exposure to (100mg/kg) BPA and prepubertal (PND20-30) administration of (10mg/kg) melatonin on body weight gain and ovarian tissue weight. We found that there was no statistically significant changes in body weight gains between all groups. However, maximum body weight gain increasing was observed in BPA group, followed by the BPA+melatonin, melatonin, and control groups. We can suggest that neonatal BPA administration increased body weight gain and may be accelerated onset of puberty.

It has reported that neonatal exposure to BPA alters estrous cycle in female rats. Large secondary follicles with multi-oocytes, degeneration in ovarian follicles and a large number of antral-space-like formations in ovarian sections were observed in the microscopic findings of adulthood period. They also reported that there was a significant decrease in the number and size of corpus luteum in the ovary of BPA administred compare to the control group (22).

In our study, we used hematoxylin eosin staining to determine the histological changes induced by BPA. Normal histological features in ovary of rats from control and melatonin groups were observed. On the other hand, atretic follicles and ovarian follicles that could not be determined at what stage of development were seen in the ovarian cortex of BPA group rats. Furthermore the spaces between follicular cells, cell infiltrations and degenerative changes were seen in ovary of rats in this group. Neonatal exposure to 100mg/kg dose of BPA between PND0-10, disrupted follicular structure and caused structural changes and prepubertal (PND20-30) melatonin administration recovered its effects.

Chuffa et al. (23) examined the effect of long-term melatonin treatment on the ovaries. They have reported that melatonin improves ovarian function by increasing the number of secondary and Graaf follicles and corpus luteum and also protects oocytes and granulosa cells from nicotine damage (24).

In our study, we observed that the administration of melatonin had positive effects on BPA- damaged ovarian tissue, our H&E results showed us this positive effect.

Cdc2 p34 expression at m-RNA level was investigated in goat oocytes to determine the oocyte maturation. They found parallel increasing with the oocyte size and protein expression of p34 in the cytoplasm of oocytes (25).

In our study, we evaluated cell cycle marker Cdc-2 expression to determine the effect of BPA on oocyte growth, by using IHC. We observed that the immunoreactivity of Cdc-2 was lower in medulla and different stages of ovarian follicles in cortex of BPA group rats compare to control, melatonin and BPA+melatonin administrated groups. According to our IHC results, we can suggest that neonatal exposure to BPA results in low expression of cdc-2 in ovarian follicles. Furthermore, we found high expression of Cdc-2 in ovarian cortex of melatonin group rats compare to BPA and BPA+melatonin groups. These results suggest that melatonin administration may have positive effect on oocyte growth.

We can conclude that in female rats, neonatal exposure to BPA, causes histologically alternations in ovary, disrupts folliculogenesis results in degenerative changes of ovarian follicles and melatonin administration may have positive effects on ovarian damaged by BPA. Furthermore, the morphological changes in the ovaries induced by BPA were thought to affect the pubertal development.

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