Effects of Blue Light on Puberty and Ovary in Female Rats

Kılıç Uğurlu A et al. Effects of Blue Light on Rats Puberty

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ABSTRACT

Purpose: This study was planned to examine the effect of blue light exposure and exposure time on puberty.

Methods: Eighteen 21-day-old female Sprague Dawley rats were divided into three groups consisting of six rats in each group: Control Group (CG), Blue Light-6 hours (BL-6), and Blue Light-12 hours (BL-12). CG rats were maintained with 12/12-hour light-dark cycles. The rats of BL-6 and BL-12 were exposed to blue light (450-470 nm) and irradiance level 0.03 uW/cm² for 6-hours and 12-hours, respectively. Rats were exposed to blue light until the first signs of puberty. Serum FSH, LH, Estradiol, and leptin concentrations were measured by ELISA method. Ovaries and uteri were dissected for histomorphological examination.

Results: The medians of the pubertal entry days of the CG, BL-6, and BL-12 were 38th, 32nd, and 30th days, respectively (p=0.001). The FSH, LH, Estradiol, DHEA-S, and leptin concentrations of all groups were similar. However, LH and estradiol concentrations of BL-6 were higher compared to CG. There was a negative correlation between blue light exposure, exposure time, and melatonin concentrations (r=-0.537, p<0.048). Ovarian tissue was compatible with the pubertal period in all groups. As the blue light exposure time increased, capillary dilatation and edema in the ovarian tissue increased. Prolonged exposure caused polycystic ovary-like (PCO) morphological changes and apoptosis in granulosa cells. Our study is the first to show the effects of blue light on puberty.

Conclusion: Our study showed that exposure to blue light and the duration of exposure lead to early puberty in female rats. As the duration of blue light exposure increased, PCO-like, inflammation, and apoptosis were detected in the ovaries.

Keywords: Apoptosis, blue light (470 nm), early puberty, melatonin, rat

INTRODUCTION

Sunlight contains red, orange, yellow, green, and blue light with different wavelengths. Light entering the eye’s retina is transmitted to the suprachiasmatic region of the hypothalamus and regulates circadian rhythm by controlling the body’s biological clock. This mediates the timing of various aspects such as core body activity, body temperature, and the sleep-wake cycle. Evening light exposure causes a decrease in the release of the hormone melatonin, leading to disruption of the circadian rhythm and reduction in the antioxidative effects of melatonin (1–5). Blue light exposure during daylight increases alertness and promotes memory and cognitive functions (4,5). However, it is known that exposure to blue light at night has the most significant melatonin-suppressing effect (6).

In addition to the sun, electronic mobile devices emit high-energy, short-wavelength blue light (7). In the last century, blue light sources such as fluorescent and LED lighting and television became common in our daily lives. However, over the past ten years, the use of touch-screen devices such as tablets and smartphones has increased in all age groups (8). Blue light exposure is more intense with these devices because of the shorter eye-screen distance. In recent years, the age of children using these devices has rapidly decreased (9). Since the COVID-19 pandemic, screen exposure in children and adolescents has increased substantially due to remote education and more screen time at home during lockdowns (10–12). And also, an increase in the incidence of precocious puberty was observed during the pandemic period compared to the pre-pandemic period (13–15).

One of the factors that initiate puberty is the decrease in melatonin. In children living near the equator with lower melatonin concentrations because of the long daylight hours, puberty occurs earlier than in those at higher latitudes (16). We know that the light-exposed at night has a suppressive effect on melatonin. It was shown that blue light suppressed melatonin production more significantly than any other color (7).
However, the impact of this exposure on the pubertal process are unclear. Our study aimed to examine the effects of blue light exposure on rat’s puberty.

MATERIALS AND METHODS

2.1. Animals

Eighteen immature 21-day-old female Sprague Dawley rats weighing 35-50 g were procured from the Experimental Animal Center of Gazi University (Ankara, Turkey). The study groups were isolated from male rats after postpartum 21 days. The rats were housed in polysulfone cages (42.5 × 26.6 × 18.5 cm in size; 3 rats per cage) at 21-24°C and 40-45% humidity at the Laboratory Animals Breeding and Experimental Research Center of the Faculty of Pharmacy, Gazi University (Ankara, Turkey). The animals were fed a standard pellet diet and water ad libitum during the experimental period. All the animals were maintained by the Guide for the Care and Use of Laboratory Animals(17), and the experimental procedures were approved by the Experimental Animal Ethics Committee of Gazi University (G.U.ET-21.052).

2.2. Light exposure protocol

A blue LED strip (FSHI. 1048.B020.6012, HI-LED, FLEX honor) providing blue light at a wavelength of 450-470 nm was placed approximately 20 cm above the center of each cage in the experimental groups (Figure 1). In the experimental setup, the blue light source was used at an irradiance level that lowered rat melatonin concentrations but would not cause retinal damage (18–20). This was determined to be an irradiance level of 0.03 uW/cm² at the rats’ eye level. The irradiance in the centre area of the cage where the rats were housed was measured with a spectroradiometer and adjusted to the same level.

2.3. Experimental design

The rats were randomly divided into three groups of six rats: the Control Group (CG), Blue Light-6 hours (BL-6), and Blue Light-12 hours (BL-12). CG rats were maintained under standard conditions with 12/12-hour light-dark cycles (light time 6:00 a.m.-6:00 p.m.; dark time 6:00 a.m.-6:00 p.m.; blue light time 6:00 p.m.-12:00 p.m.; dark time 12 p.m.-6:00 a.m.); for 12 hours (light time 6:00 p.m.-6:00 a.m.; blue light time 6:00 p.m.-6:00 a.m.) respectively. The rats were weighed at the beginning and end of the experimental procedure, and the percentage weight gain was calculated with the formula of Weight gain (%) = (Last day – First day)/First day

2.4. Vaginal examination and cytology

The vaginal examination was one of the external signs of puberty in rodents(21). The rats were examined daily, starting at 22 days of age, to detect vaginal opening. After vaginal opening, vaginal smear samples were collected to determine the estrous stage. To do this, a moistened cotton swab was inserted into the vagina. Cells from the vaginal lumen and walls were gently taken and transferred to a glass slide. After the samples were allowed to air-dry, they were stained by Giemsa stain and examined under a light microscope.

The stages of the estrous cycle were classified as proestrus (oval nucleated epithelial cells), metestrus (fragmented, cornified epithelial cells and smaller, darker stained leukocytes), and diestrus (nucleated epithelial, predominante leukocytes)(22). Rats were exposed to blue light until the first estrus stage after vaginal opening.

2.5. Termination of the experimental procedure

At the first estrus stage, all the rats were sacrificed by taking blood from the heart at 8.00 am, according to the peak melatonin rhythm of rats (23) under general anesthesia (10 mg/kg xylazine hydrochloride and 50 mg/kg ketamine hydrochloride). After the anesthesia procedure, blood samples were obtained by the intracardiac puncture. The blood samples were centrifuged at 3000 rpm (906xg) for 15 minutes and the serum was separated. The serum samples were stored at -80°C until analysis. The height of the ovarian tissues was measured by a micrometer, and the uterine and ovarian tissues were dissected and weighed.

1.6. Determination of Biochemical Parameters

The collected blood was centrifuged at 3000 rpm for 10 minutes at +4°C and stored at -80°C. The serum concentration of the follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, testosterone, Dehydroepiandrosterone sulfate (DHEA-S), leptin, and melatonin were evaluated by enzyme-linked immunosorbent assay (ELISA; Rats@ Bioassay Technology Laboratory, China).

1.7. Histopathological method

The right and left ovaries and the uterus were weighed, fixed, and then sectioned, then the ovaries and the uterus were fixed in Bouin’s fixative and embedded in paraffin blocks using standard procedures. Sections of 4-micron thickness were taken from the prepared paraffin blocks and stained with hematoxylin and eosin. The samples were examined for histomorphological changes by light microscopy in the Leica DM4000 (Germany) computer-assisted image system, and images were obtained using the Leica-Qwin program.

Statistics

SPSS 26 program was used for statistical analysis. The "Kruskal-Wallis" test was used when comparing the medians of three independent groups in the data that did not follow the normal distribution, and the "Mann-Whitney U" test were used when comparing the medians of two independent groups. While investigating the associations between non-normally distributed and/or ordinal variables, the correlation coefficients and their significance were found using the Spearman test. All data are given as mean ± SD Bonferroni correction was used in post hoc tests. Statistically, p < 0.05 was considered significant. A power analysis was performed using GPower version 3.1.9.7 to determine the minimum sample size required to test the study hypothesis. Results indicated that a sample size of n=18 is required to achieve 80% power for detecting a large effect at a significance of α =0.05.

RESULTS

The mean initial weight of the female rats in CG, BL-6, and BL-12 were 42.5±4.7, 42±3.4, and 42±2.7 gr, respectively (p=0.91). The median age at puberty onset was 38±3, 32±3, and 30 days. Puberty onset was significantly earlier in BL-12 compared to CG (p=0.001) (Table 1). The age of onset of puberty decreased as the duration of blue light exposure increased (r=0.910, p<0.001). The mean±SD weight of onset of puberty in BL-6 were 85.1±9.7 g, 91.6±5.5 g, 80.5±5 g in CG, BL-6, and BL-12. The weight of puberty onset in BL-6 were more than in BL-12 (p=0.04). Median percentage weight gain in CG, BL-6, and BL-12 was 110%, 117%, and 93%, respectively. Percentage weight gain was higher in BL-6 compared to CG, while rats in BL-12 had the least weight gain (p=0.05) (Table 1). Serum concentrations of FSH, estradiol, testosterone and DHEA-S in the blue light-exposed groups were similar to those of controls (p>0.05) (Table 2). LH concentrations were higher in BL-6 than in CG (p=0.027). The high concentrations of LH and estradiol in BL-6 can be attributed to the ovulatory peak during estrus. The estrus stage in the BL-12 group was observed in the earlier hours of the day and the time between the estrus stage and sacrifice was longer. Therefore, we could not detect LH and estradiol surges. On the contrary, we detected LH and estradiol surges in the BL-6 group due to the shorter estrus stage and sacrification time.

Serum concentrations of leptin showed no significant difference among the groups (p>0.05) (Table 1). There was no correlation between percentage weight gain, leptin, and the day of puberty onset (p=0.05).
Melatonin levels were 144 (126-197) ng/L in CG, 143.7 (132-152) ng/L in BL-6, and 121(116-151) ng/L in BL-12 (p<0.05). As, melatonin levels decreased as the duration of exposure to blue light increased (r=−0.537, p=0.048).

The ovarian size, ovarian weight, or uterine weight of groups were similar (p>0.05) (Table 3). On histologic examination, ovarian tissue was compatible with pubertal period in all groups. Primary, antral, tertiary follicles and corpus luteum were observed in CG (Figure 2) and BL-6 groups. Edema and congestion in the medulla increased as the exposure time to blue light increased when the ovarian tissue of BL-6 was examined, a lower antral and Graafian follicle density and higher preantral follicle density were noted in BL-6 than in the CG. Perivascular edema and capillary dilation were prominent in the medulla of the BL-6 group (Figure 3a). At high magnification, extracellular edema in the granulosa cells in the preantral follicles and the presence of apoptotic cells with pyknotic nuclei in the granulosa layer of antral follicles were noted (Figure 3b). The most remarkable finding in the examination of BL-6 ovarian tissue was the presence of the corpus luteum covering most of the organ. Unlike the other groups, numerous Graafian follicles were noted in BL-12 group (Figure 4a). At high magnification, it was pointed out that the Graafian follicles had a thinner granulosa layer than in the CG. This appearance was consistent with polycystic ovary (PCO). Prolonged exposure caused PCO-like morphological changes in the ovary. In addition, the extracellular edema in the granulosa cells and the presence of apoptotic cells with pyknotic nuclei in the granulosa layer of the antral follicles were more pronounced in BL-12 than in BL-6 (Figure 4b).

The control group's FSH, LH, and estradiol levels demonstrated that the hypothalamus-adenohypophysis-axis activity was more pronounced in BL-6 than in the CG (Figure 5). In BL-12, the uterine and gland epithelium in the proliferative phase uterus was that the uterine epithelium size increased and became high prismatic epithelium, according to CG. Histological examination of uterine tissue from control rats revealed normal (Figure 6). In BL-12, the uterine and gland epithelium in the proliferative phase uterus had a similar histological appearance as BL-6. The most prominent change in this group was vessel dilation in the endometrial lamina propria and the capillaries reaching the surface (Figure 7). This finding indicated that the endometrium was entering the secretory phase. In conclusion, histological results indicate that the secretory phase had started, with changes consistent with ovulation.

**DISCUSSION**

To the best of our knowledge, this study is the first to demonstrate the effects of blue light exposure on puberty. Blue light is the dominant light in increasing the screen brightness of mobile devices. The wavelength of blue light is between 450 and 490 nanometers (nm), but the main problem here is that we are exposed to light at high brightness and illumination level with the reflection of eye-screen distances (7,24). In the last decade, several studies suggested that nighttime exposure to blue light emitted by devices such as computers and tablets can impair the normal function of the biological clock, alter sleep-wake cycles, and induce metabolic changes (25-28). This effect of prepubertal exposure to blue light on puberty was one of the unknown topics in the literature. Our study showed that blue light was associated with early puberty in female rats. The results indicated that blue light exposure and exposure time accelerate puberty onset. The control group's FSH, LH, and estradiol levels demonstrated that the hypothalamus-adenohypophysis-axis activity was more pronounced in BL-6 than in the CG (Figure 5). In BL-12, the uterine and gland epithelium in the proliferative phase uterus was that the uterine epithelium size increased and became high prismatic epithelium, according to CG. Histological examination of uterine tissue from control rats revealed normal (Figure 6). In BL-12, the uterine and gland epithelium in the proliferative phase uterus had a similar histological appearance as BL-6. The most prominent change in this group was vessel dilation in the endometrial lamina propria and the capillaries reaching the surface (Figure 7). This finding indicated that the endometrium was entering the secretory phase. In conclusion, histological results indicate that the secretory phase had started, with changes consistent with ovulation.

Another factor contributing to puberty with exposure to blue light could be decreased melatonin secretion. We observed that melatonin levels decreased with increased the blue light exposure time and puberty withdrew earlier. The melatonin release pattern during the human lifespan involves an increase in melatonin concentration from the neonatal period to the pubertal period, followed by a decrease at the onset of puberty (30). Neuroendocrine control of the sexual maturation process is influenced by the pattern of melatonin secretion resulting from the light-dark cycle. High melatonin concentrations were thought to have an inhibitory effect on the gonadotropin-releasing hormone (GNRH) (31). In vitro studies of cultured prepubertal rat pituitary cells demonstrated that melatonin plays a role in the timing of developmental stages by inhibiting the release of GNRH and, therefore, LH (32). A study comparing girls with precocious puberty and age-matched controls found that lower melatonin concentrations were associated with early puberty (31). Lee et al. (33) found that blue light exposure in the evening suppressed melatonin more in children and adults, even if the exposure was shorter. A study examining the effect of light on the circadian system of children in early puberty and normal puberty showed that children in early puberty were more sensitive to evening light and their melatonin concentrations were more suppressed (31). During the COVID-19 pandemic, online education via electronic mobile devices and the increased time at home resulted in longer screen exposure in a younger age group. Studies have shown an increase in precocious puberty and accelerated puberty during pandemic-related lockdown compared to the pre-pandemic period (13), (14). Among these studies, Stagi et al. (13) compared data from the pandemic period and the two years before the pandemic and reported that the incidence of newly diagnosed precocious puberty cases increased, and the rate of puberty was accelerated. They found a significant increase in body mass index (BMI) and pre-sleep screen device usage time among patients diagnosed and followed up during the pandemic.

Signs (7,34) from Italy reported an increase in precocious puberty cases during the pandemic compared to the corresponding months of the previous year. Although there was no difference in BMI between the groups, the authors observed an increase in the duration of screen use and a decrease in physical activity. Our study demonstrated the effects of blue light exposure on puberty and the relationship with increased exposure time.

Early-light stress exposure is one of the common risk factors for psychopathology and deviations in pubertal timing. Several studies have demonstrated that stress promotes puberty in girls and female rats(35,36). Exposure to blue light may have induced stress in the rats. Stress may also contribute to early puberty.

A closer striking finding in our study was histological results of ovary. There was an increase in edema and capillary dilation in the ovarian medulla, granulosa cell apoptosis, and histomorphological changes consistent with PCO in the ovarian tissue of the group with prolonged blue light exposure. Polycystic ovary syndrome (PCOS) is a common endocrine disorder in adolescence. There are genetic abnormalities, lifestyle, prenatal hormonal imbalances, and environmental factors in etiology of the PCOS (37). Simon et al. (38) demonstrated that the effects of circadian rhythm disruption are among the environmental factors that may contribute to PCOS in humans (38). In a PCOS study in which 6-week-old rats were exposed to continuous light for four weeks, PCOS-like results were discovered in the ovarian tissue of the rats. The authors emphasized that...
constant light exposure is an essential environmental factor in PCOS development. Hormonally, they detected no differences in serum concentrations of FSH, LH, estradiol, or testosterone (39). In another rat study, 6-week-old rats were exposed to 600 lux light for 16 weeks. The study was conducted to model PCOS in rats and revealed PCOS-like histological findings in the ovaries and increased testosterone concentrations (40). In both studies, it was observed that prolonged light exposure to mature rats caused the appearance of PCOS. In our study, BL-12 showed a thin granulosa layer in the Graafian follicles, which is one of the PCO-like findings (41,42). The absence of other signs of PCO and the lack of difference in androgen concentrations may be related to the duration of blue light exposure, irradiance level, and early sacrifice of the rats. Short-term exposure to blue light during the prepubertal period induced PCO-like symptoms in the ovaries. In our study, the increased edema and capillary dilation in the ovarian medulla and apoptosis of the granulosa cells observed in the rats exposed to blue light. There are no previous publications associating blue light exposure with apoptosis in the granulosa cells of ovarian tissue. However, increased edema and capillary dilation in the ovarian medulla may have been triggered in the experimental groups due to reduced melatonin and the subsequent increase in the pro-inflammatory process (43).

As one of the limitations of our study, groups exposed to daylight could have been included. However, in our study, the groups were determined according to the 3R rule. Previous studies have shown that blue light is the light that suppresses melatonin the most compared to other wavelengths of light. In a study in which light, dark cycle and light cycle during the day were applied to female rats, the average day of puberty onset was determined to be between 34 and 35 days, consistent with the rats' physiology (44). The time in which the rats were sacrificed was determined to catch the melatonin concentration at the optimal level. Rats were sacrificed at the time interval when melatonin levels were highest (23). However, at this point, we could not capture the gonadotropin peaks in the cycle due to the difference between the puberty onset time and the sacrificed time. We could not show a direct effect of melatonin on Kisspeptin and GnRH. And one of the limitations, hormonal measurements were performed with ELISA. Liquid chromatography–mass spectrometry/ mass spectrometry or high-performance liquid chromatography methods could make differences better for interpretation.

CONCLUSION
The blue light exposure and duration of exposure caused earlier onset of puberty. With increased blue light exposure duration, signs of PCO, inflammation, and apoptosis were detected in the ovaries. In the future, human studies are needed to demonstrate that blue light accelerates puberty onset and determine its short- and long-term effects on ovaries.

Conflict of Interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding
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REFERENCES

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Table 1. Puberty onset, weight gain(%) and leptin concentrations of the groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>BL-6 (30-34)</th>
<th>BL-12 (30-32)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puberty Onset (day)</td>
<td>39 (38-40)</td>
<td>33 (30-34)</td>
<td>30 (30-32)</td>
<td>*0.001</td>
</tr>
<tr>
<td>Weight Gain (%)</td>
<td>110 (67-133)</td>
<td>117 (98-152)</td>
<td>93 (56-110)</td>
<td>0.09</td>
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<tr>
<td>Leptin (ng/mL)</td>
<td>3.1 (2.6-4.1)</td>
<td>3.2 (2.5-4.4)</td>
<td>3.4 (2.7-11.3)</td>
<td>0.51</td>
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</tbody>
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Values represent median (minimum-maximum)
*Control vs. BL-12 p=0.001

Table 2. The hormone and oxidative-antioxidant marker concentrations of the groups

<table>
<thead>
<tr>
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<th>Control</th>
<th>BL-6</th>
<th>BL-12</th>
<th>p value</th>
</tr>
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<tr>
<td>FSH (IU/mL)</td>
<td>16.6 (9.6-20.2)</td>
<td>19.4 (6.6-24.2)</td>
<td>9.2 (6.3-17.8)</td>
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<tr>
<td>LH (IU/mL)</td>
<td>67.5 (38.4-99.8)</td>
<td>106.9 (79-153.2)</td>
<td>82 (42-142)</td>
<td>0.02</td>
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<tr>
<td>Estradiol (pmol/L)</td>
<td>92.5 (57.5-166.2)</td>
<td>110.9 (89.6-150.5)</td>
<td>99.6 (47-127.5)</td>
<td>0.60</td>
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<td>DHEA-S (µmol/L)</td>
<td>1.13 (1.01-1.13)</td>
<td>1.26 (0.90-1.57)</td>
<td>0.99 (0.64-1.54)</td>
<td>0.15</td>
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<td>Testosterone (nmol/L)</td>
<td>335 (229-439)</td>
<td>243 (199-339)</td>
<td>261 (134-290)</td>
<td>0.30</td>
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<td>Melatonin (ng/mL)</td>
<td>144 (126-197)</td>
<td>121 (110-151)</td>
<td>121 (116-151)</td>
<td>0.11</td>
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Values represent median (minimum-maximum)
*Control vs. BL-6 p=0.027

Table 3. Ovary lengths / Ovary and uterus weights of the groups

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<tr>
<th></th>
<th>Control</th>
<th>BL-6</th>
<th>BL-12</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right ovary (mm)</td>
<td>4.5 (3.7-5.5)</td>
<td>3.8 (2.3-4.9)</td>
<td>4.9 (4.5-5.5)</td>
<td>0.06</td>
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<tr>
<td>Left ovary (mm)</td>
<td>4.5 (4.4-6.2)</td>
<td>4.3 (2.3-5.6)</td>
<td>4.5 (4.4-6.4)</td>
<td>0.43</td>
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<td>Ovary weight (mg)</td>
<td>120 (40-130)</td>
<td>120 (20-140)</td>
<td>110 (60-150)</td>
<td>0.31</td>
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<tr>
<td>Uterus weight (mg)</td>
<td>410 (210-820)</td>
<td>510 (300-650)</td>
<td>640 (250-900)</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Values represent median (minimum-maximum)
All developing preantral, antral, and Graafian follicle structures and stroma appeared normal.

正常结构和血管分布。
Figure 3a. Histological Findings of Ovary – BL-6 Group (H&E X40)
At low magnification, the lower AF and GF density and higher PF density were noted in the cortex. Small CL structures were observed. There was prominent capillary dilation (☉) and especially perivascular edema (◆).

Figure 3b. Histological Findings of Ovary – BL-6 Group (H&E X200)
Extracellular edema (☉) in the granulosa cells of the PF and apoptotic cells (◆) in the granulosa cells of the AF were prominent.
Figure 4a. Histological Findings of Ovary – BL-12 Group (H&E X40)
Pronounced thinning of the granulosa layer of the follicles (Φ) was noted. There was increased edema (⊕) in the medulla and pronounced capillary dilation (⊗).

PF: Preantral follicle, AF: Antral follicle, GF: Graafian follicle, CL: Corpus luteum

Figure 4b. Histological Findings of Ovary – BL-12 Group (H&E X200)
Pronounced thinning of the granulosa layer of the follicles (Φ) was noted. ⊗: capillary dilation, ⊕: perivascular edema
Figure 5. Histological Findings of Uterus -CG (H&E X40)
Uterine tissue from control rats revealed normal UE of proliferative phase endometrium, a small number of UG in the lamina propria, and spiral arterioles that had not yet reached the surface. The myometrial structure was typical.

Figure 6. Histological Findings of Uterus - BL-6 (H&E X40)
UE height was relatively increased. UG were more numerous and epithelial height increased. BV advanced towards the surface. The number of UG was also relatively increased in BL-6 group, and UG epithelial cells were larger than in CG. The endometrial vessels spread superficially. All these findings indicated that the uterus was entering the secretory phase.

Figure 7. Histological Findings of Uterus - BL-12 (H&E X40)
UE height was relatively increased, UG were more numerous and epithelial height increased. KD: BV extend to the surface and appear dilated.