Cypermethrin triggers apoptosis, depletes granulosa cells, and induces endometrium thinning in female rats

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ABSTRACT

Objective: To analyze the effects of subchronic cypermethrin on the ovary and endometrium as well as the involvement of apoptosis in the toxicity of cypermethrin.

Methods: A total of 32 female Wistar rats were randomly divided into four groups, with 8 rats in each group. The control group received no treatment, and the other three groups received oral cypermethrin at 10, 15 or 20 mg/kg body weight for 28 days (sub-chronic). The granulosa cells were calculated histopathologically. The apoptotic index was determined by *in situ* technique. Histopathological examination was performed on the uterus and ovary.

Results: There was no significant difference in the number of primary follicular granulosa cells between the treatment groups and the control group ($P>0.05$). However, the number of secondary and tertiary follicle granulosa cells in the treatment groups was significantly decreased compared to that of the control group (P all<0.05). The apoptotic index of primary follicular granulosa cells increased significantly in the groups treated with cypermethrin compared with the control group ($P<0.05$). The secondary, tertiary, and endometrial granulosa cell apoptosis index was significantly higher in all treatment groups compared to the control group ($P<0.05$). The higher the dose of cypermethrin was, the higher the apoptotic index of secondary, tertiary and endometrial granulosa cells was. There was a significant decrease in endometrial thickness in the three treatment groups compared to the control group ($P<0.05$). Thinning of the endometrial layer was seen in the cypermethrin exposure groups.

Conclusions: Exposure to cypermethrin can suppress the number of secondary and tertiary follicular granulosa cells, and trigger thinning of the endometrium through induction of apoptosis.

1. Introduction

At this present time, pyrethroid pesticides are still used in various parts of the world to control grasses, pests, and vectors. Residue from these pesticides has persisted in the environment and has been found in soil as well as in comestibles such as fruit and vegetables. Humans, as well as other organisms, have therefore exposed to these pesticides.

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Exposure in mammals will result in toxic accumulations in various organs; consequently, these accumulations may disrupt lipid profiles, serum proteins, and organ function itself[1–4].

Cypermethrin is one of the pyrethroid pesticides used to increase agricultural production and veterinary medicine. It has been proven that cypermethrin is found in consumption agricultural products, including vegetables and fruits, in developed and developing countries[5–8]. Accumulation in food products causes oral exposure. Against the reproductive hormone system, cypermethrin suppresses the production of the hormones, estradiol, luteinizing hormone, and follicle-stimulating hormone (FSH). Against the reproductive organs, cypermethrin interferes with the structure and function of the ovary, causes follicular atresia, and lowers estradiol levels[9–11].

Apoptosis is a programmed cell death that eliminates infected cells or damaged cells so that they do not become cancer cells[12]. Apoptosis is controlled by several modulators, such as genes, proteins (caspases) and organelles[13]. Caspase proteins work together to destroy cell structure and function resulting in apoptosis. Caspases 2, 8, 9 and 10 are initiator caspases, and 3, 6 and 7 are executioner caspases, which are activated by an initiator caspase[14,15]. Several studies have evaluated the effects of cypermethrin on the apoptosis of tissue and cells. Exposures to both low and high doses of cypermethrin trigger caspases 3 and 8 upregulation in carp brain tissue[16]. Cypermethrin also triggers apoptosis in mouse bone marrow cells and human neuroblastoma cells[17,18]. In addition to apoptosis, cypermethrin also induces cell necrosis[19].

The formation of fertile eggs and endometrial receptivity are two very important factors in natural conception[20]. Mammalian ovarian follicles contain oocytes that undergo a series of events, such as ovulation, fertilization, and embryo formation. These ovarian follicles are surrounded by granulosa and theca cells, which in turn, produce signals and hormones that spur the development of the competent oocytes[21]. In the ovaries, the primary functional cells are the granulosa cells. The proliferation and differentiation of these cells will affect the growth, development, and physiological processes of the follicles[22]. Granulosa cells, the primary components of ovarian follicles, are mainly responsible for the synthesis of steroid hormones and other regulatory factors in oocyte growth[23,24]. Granulosa cells are more sensitive to apoptosis than theca cells and cumulus cells. The higher rate of granulosa cell death will have significant impacts on follicle development and will suppress oocyte growth[25]. Previous studies have demonstrated that the administration of cypermethrin at the doses of 1.38; 2.76; and 5.52 mg/kg for 6 and 12 weeks will result in the decrease in follicle types[26]. The administration of cypermethrin at a dosage of 50 mg/kg (1/5 median lethal dose) for 4 weeks showed degenerative ovarian changes in the form of follicular atresia and a decrease in concentrations of protein, lipid, phospholipid, and cholesterol[27].

The endometrium is a uterine layer that functions in implantation through complex processes involving autocrine, paracrine, and endocrine signals and intracellular signals[28]. Appropriate endometrial thickness is needed for the success of pregnancy. Various studies suggest that in thin cases of endometrium, the pregnancy rate is often low[29,30]. Iatrogenic thin endometrium can be caused by surgical or drug administration[31]. Until now, the effect of cypermethrin on the thinning of the endometrium has never been revealed.

To the best of our knowledge, there have been no studies evaluating the effects of medium dose of exposure to cypermethrin on the ovary and endometrium as important organs in determining fertility. Therefore, in this study, we aim to investigate the effects of medium dose cypermethrin exposure on the ovary and endometrium and the involvement of apoptosis in the effects of cypermethrin on these two organs.

2. Materials and methods

2.1. Animals

Thirty two female Wistar rats (*Rattus norvegicus*) of reproductive age (12-15 weeks), with body weight of 150-200 g, which were healthy, not pregnant and not disabled were used and divided into four groups, with 8 rats in each group. These included a control group (without any treatment), a second group which received a dose of 10 mg/kg cypermethrin orally, a third group which received a dose of 15 mg/kg body weight (b.w.) cypermethrin orally, and a fourth group which received a dose of 20 mg/kg b.w. cypermethrin orally. The median lethal dose (LD_{50}) of cypermethrin in rats was 640 mg/kg body weight[32]. This study determined a dose of 1/64th of the LD_{50}, 1/42nd of the LD_{50}, and 1/32nd of the LD_{50} as medium dose of cypermethrin. The duration of administration of cypermethrin was 28 days. This duration was in accordance with previous studies[33,34].

Prior to treatment, the rats were acclimatized for 1 week under laboratory conditions. Each rat was kept in a 45.0 cm × 35.5 cm × 14.5 cm plastic cage with a strong wire lid. The base of the cage contained husks ranging in thickness from 0.5 to 1 cm which was replaced every 3 days. The room was illuminated in 12-hour intervals, followed by 12 h of darkness, and the ambient temperature in the room ranged between 27 ℃-28 ℃. The rats were provided standard feed in the form of pellets (Comfeed®, Japfa Comfeed Indonesia Tbk, Jakarta, Indonesia), distributed once in the morning and once in the afternoon at 40 g/day/rat, and drinking water was provided *ad libitum*.

2.2. Cypermethrin preparation

Cypermethrin used here was found in the Rhizotin 100 EC brand, and was quantified as an active ingredient at the proportion of 100 mg/mL. It was administered orally to the rats in the treatment groups using probes, once daily for 28 days, which corresponded to the duration of the previous study[33,34].

2.3. Body weight measurement

Bodyweight measurement was done with an electronic scale (Ohaus, MA, USA). Weight measurement was performed before and after treatment.

2.4. Calculating number of granulosa cells

Before dissection, the rats were injected with 1% intramuscular ketamine. The dissection was made by opening the abdominal cavity and peritoneum. The right and left ovaries were removed by cutting the ligaments binding them. Hysterectomy was also performed. The ovaries and uteri were cleansed with 0.9% NaCl and weighed in the analytical balance. The organs were then put in a plastic bag containing 10% formalin buffer fixative solution and left for 12-24 h. After dissection was completed, the remains of the rats were properly buried. The ovaries were stained using hematoxylin-eosin. The granulosa cells were cuboid-shaped cells located around the follicles and they had the following characteristics: Primary follicles had 1-2 granulosa cell layers, minimal pellucid zones, and no antrum. The secondary follicles had 2-6 granulosa cell layers, a small antrum, and a small pellucid zone. The tertiary follicles had more than 6 granulosa cell layers, a large antrum, and a considerable number of follicular fluids and matured oocytes (clean oocytes, moderate cytoplasmic granules, and clear pellucid zone colors). The observation was conducted under a light microscope (Olympus type MFD 5X51) at 400 times magnification.

2.5. Analysis of granulosa cells' apoptotic index

The granulosa cell apoptotic index was the percentage of granulosa cells in primary, secondary and tertiary follicles exhibiting apoptosis in 1 000 granulosa cells. The analysis was carried out using an *In Situ* Cell Death Detection Kit (Roche Diagnostics, Switzerland, POD CAT No. 11684817910). The analytic procedure was performed by precisely following the instructions included in the kit. The apoptotic granulosa cells were characterized by their shrinking nucleus, DNA fragmentation, and dark brown nuclei. The observation was conducted under a light microscope (Olympus type MFD 5X51) at 1 000 times magnification.

2.6. Histopathological examination

Histopathological examination was performed on uterus and ovary according to a previous study[35]. Hematoxylin-eosin staining was used. After scanning the histological section, the thickness of the endometrium layer was evaluated. This evaluation was done by two independent observers using OLYMPUS XC10 microscope (magnification 400×) and dot slide software.

2.7. Ethics

This study was approved by the Ethics Committee of Medical Research, under the auspices of the Faculty of Medicine, University of Brawijaya, Malang, East Java, Indonesia (approval No.222/EC/Kep/S2/06/2017).

2.8. Statistical analysis

An analysis of variance test was employed to analyze the data using mean±standard deviation (mean±SD) to differentiate between treatment groups utilizing the program SPSS 23.0 Statistical Package for Windows. A probability value (P) less than 0.05 was considered statistically significant.

3. Results

3.1. Body weight

The value of body weight at the beginning and end of the study can be seen in Figure 1. In all groups, a tendency to increase body weight at the end of treatment was found, although no significant improvement had been found ($P>0.05$).

3.2. Number of primary follicular granulosa cells

Figure 2 indicated the number of primary follicular granulosa cells from different treatment groups. There were no significant differences in the number of primary follicular granulosa cells in all groups ($P>0.05$).

3.3. Number of secondary and tertiary follicular granulosa cells

As shown in Figure 3, the number of secondary and tertiary follicle granulosa cells from the groups treated with cypermethrin at the 20 mg/kg b.w. was the lowerest followed by those from groups treated with the 15 mg/kg b.w. and 10 mg/kg b.w. cypermethrin (P both <0.05). Among the latter two groups, the number of secondary and tertiary follicle granulosa cells of the group treated with the 15 mg/kg b.w. cypermethrin was also significant lower than that of the group treated with 10 mg/kg b.w. ($P<0.05$).

3.4. Apoptosis of primary follicular granulosa cells

The apoptotic index of primary follicular granulosa cells in the different treatment groups was shown in Figure 4. The apoptotic index of the primary follicle granulosa cells increased significantly in all groups treated with cypermethrin compared with the control group ($P<0.05$). The apoptotic index of the primary follicular granulosa cells treated with 15 mg/kg b.w. cypermethrin did not differ significantly compared with groups treated with 20 mg/kg b.w. cypermethrin ($P>0.05$), but the apoptotic index of both 15 mg/kg b.w. and 20 mg/kg b.w. cypermethrin treatment groups were significant higher compared with the 10 mg/kg b.w cypermethrin treatment group ($P<0.05$).
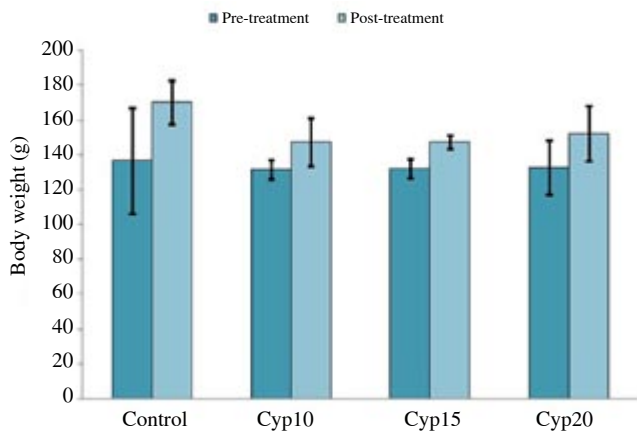
Figure 1. Body weight in the control group and groups exposed to cypermethrin at different doses. Note: there are no significant differences among the cypermethrin treatment groups (*P*>0.05). The control group: without any treatment; the Cyp10 group: receiving a dose of 10 mg/kg cypermethrin orally; the Cyp15 group: receiving a dose of 15 mg/kg cypermethrin orally; the Cyp20 group: receiving a dose of 20 mg/kg cypermethrin orally. Cyp: cypermethrin.
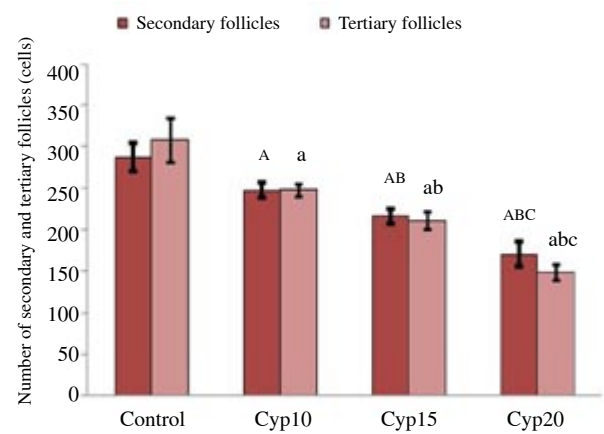

Figure 3. Number of secondary and tertiary follicles in the control group and groups exposed to cypermethrin at different doses. Note: a/A: *P*<0.05 secondary follicles /tertiary follicles compared with the control group; b/B: *P*<0.05 secondary follicles /tertiary follicles compared with the group exposed to cypermethrin at the 10 mg/kg dosage level; c/C: *P*<0.05 secondary follicles /tertiary folliclescompared with the group exposed to cypermethrin at the 15 mg/kg dosage level.
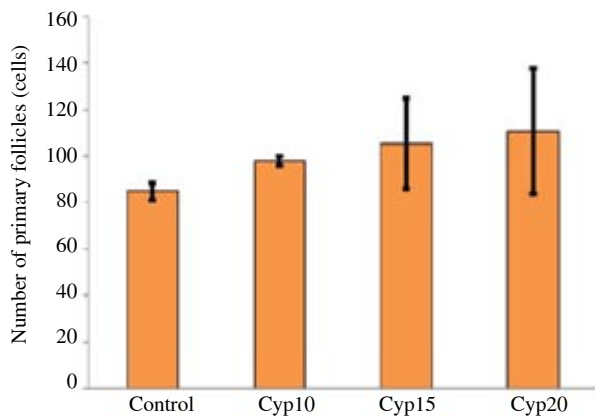

Figure 2. Number of primary follicles in the control group and groups exposed to cypermethrin at different doses. Note: there are no significant differences among the treatment groups (*P*>0.05).
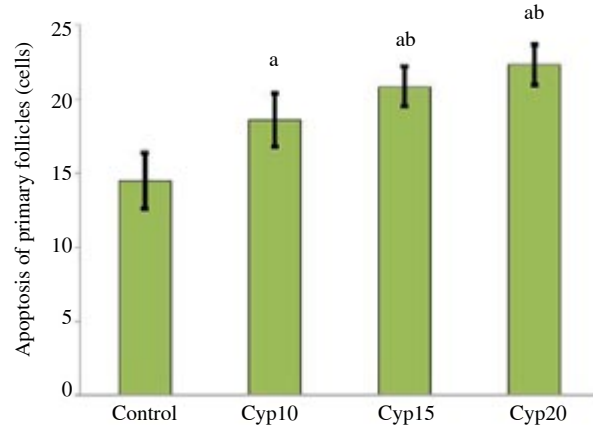

Figure 4. Apoptotic index of primary follicles in different treatment groups. Note: a: *P*<0.05 compared with the control group; b: *P*<0.05 compared with the group exposed to cypermethrin at the 10 mg/kg dosage level.

3.5. Apoptosis of secondary and tertiary follicular granulosa cells

Figure 5 demonstrated the apoptotic index of the secondary follicular granulosa cells of the control and treatment groups. The apoptotic index of the secondary and tertiary follicular granulosa cells increased significantly in all groups treated with cypermethrin compared with the control group (*P*<0.05). The apoptotic index of secondary or tertiary granulosa cells was the highest in group treated with 20 mg/kg b.w. cypermethrin followed by those from groups treated with the 15 mg/kg b.w. and 10 mg/kg b.w. cypermethrin (*P* both <0.05). The apoptotic index of secondary or tertiary granulosa cells in group treated with the 15 mg/kg b.w. cypermethrin was also significant higher than that of the group treated with 10 mg/kg b.w. (*P*<0.05).

3.6. Histology of endometrium

Endometrial histology in all groups were presented in Figure 6. In the control group, the thick and intact endometrial lining was obtained, and the endometrial cavity looked minimal. In the cypermethrin exposure groups, thinning of the endometrial layer was seen, and the endometrial cavity was wide. The higher the dose of cypermethrin, the wider the endometrial cavity.

3.7. Apoptosis of endometrium

The endometrial apoptosis index was seen in Figure 7. The endometrial apoptosis index in the cypermethrin treatment groups was significantly higher than that of the control group (*P*<0.05). The apoptosis index was the highest in group treated
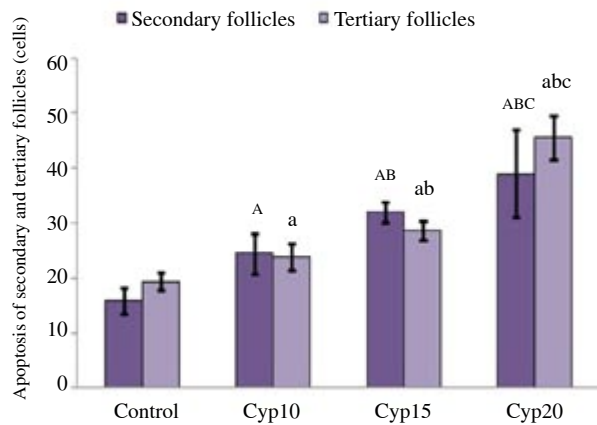
Figure 5. Apoptotic index of secondary and tertiary follicles in different treatment groups. Note: a/A: *P*<0.05 secondary follicles /tertiary follicles compared with the control group; b/B: *P*<0.05 secondary follicles /tertiary follicles compared with the group exposed to cypermethrin at the 10 mg/ kg dosage level; c/C: *P*<0.05 secondary follicles /tertiary folliclescompared with the group exposed to cypermethrin at the 15 mg/kg dosage level.
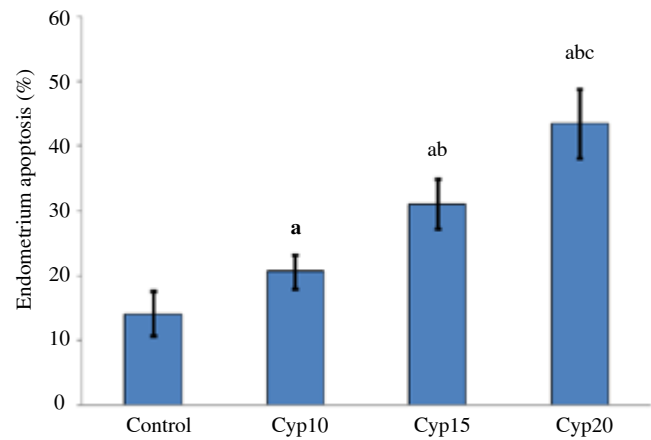

Figure 7. Apoptotic index of endometrium in different treatment groups. Note: a: *P*<0.05 compared with the control group; b: *P*<0.05 compared with the group exposed to cypermethrin at the 10 mg/kg dosage level; c: *P*<0.05 compared with the group exposed to cypermethrin at the 15 mg/kg dosage level.
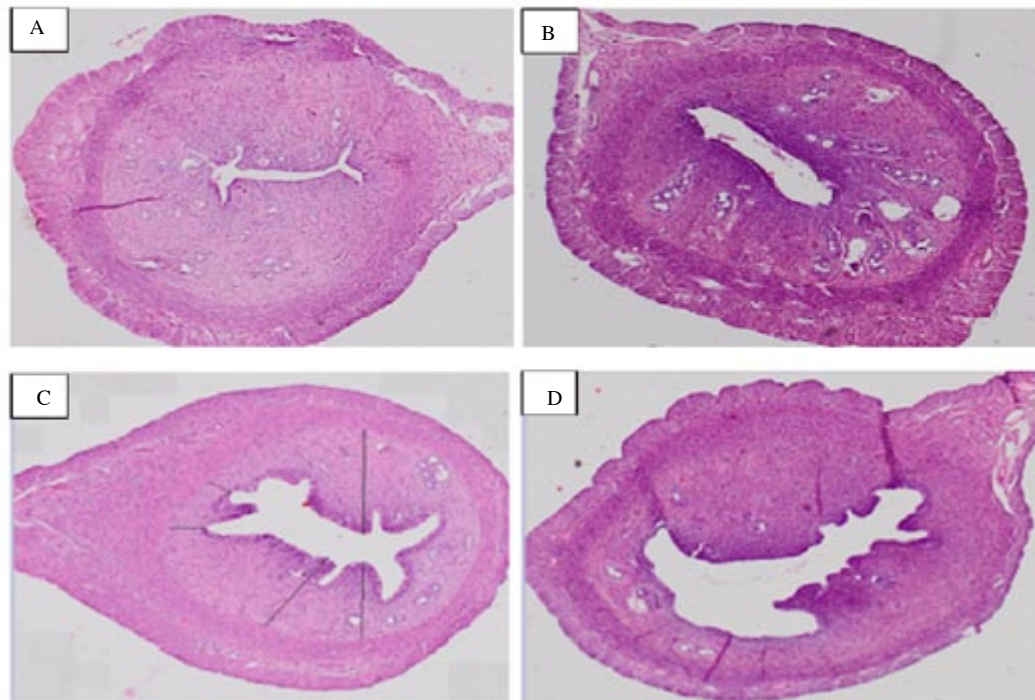

Figure 6. Endometrial histology in all groups (hematoxyline-eosin staining at magnification 100×). In the control group (A), the thick and intact endometrial lining was obtained. The endometrial cavity looks minimal. In the cypermethrin exposure groups (B, C, D), thinning of the endometrial layer was seen. The endometrial cavity was wider compared to the control group (B, C, D). The greater endometrial cavity was seen in the highest dose (D).

with 20 mg/kg b.w. cypermethrin followed by those from group treated with the 15 mg/kg b.w. and 10 mg/kg b.w. cypermethrin (*P* both <0.05). The apoptosis index in group treated with the 15 mg/ kg b.w. cypermethrin was also significant higher than that of the group treated with 10 mg/kg b.w. (*P*<0.05).

3.8. Thickness of endometrium

Figure 8 showed the thickness of the endometrium in various

groups. Endometrial thickness in the three groups exposed to cypermethrin was significantly lower than that of the control group (*P*<0.05). Endometrial thickness in group treated with 20 mg/kg b.w. cypermethrin was significant lower that groups treated with 10 mg/ kg b.w. and 15 mg/kg b.w. cypermethrin. No significant differnece in endometrial thickness was found between the 10 mg/kg b.w. and 15 mg/kg b.w. groups (*P*>0.05).
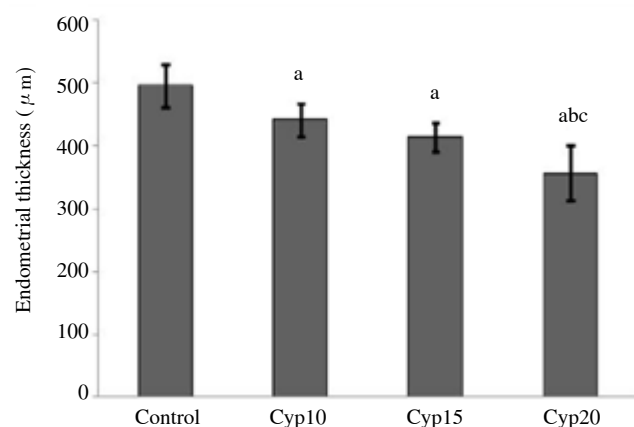
Figure 8. Endometrial thickness in the control and cypermethrin groups. Note: a: $P<0.05$ compared with the control group; b: $P<0.05$ compared with the group exposed to cypermethrin at the 10 mg/kg dosage level; c: $P<0.05$ compared with the group exposed to cypermethrin at the 15 mg/kg dosage level.

4. Discussion

In this study, cypermethrin orally for 28 days does not affect body weight. This shows that the administration of cypermethrin in female rats does not interfere with growth. This finding is not consistent with previous studies[35]. We suspected this was due to differences in dosage and type of experimental animals used.

In this study, there was no significant difference in the number of primary follicular granulosa cells in various treatment groups. This suggests that exposures to different doses of cypermethrin do not trigger a change in the number of primary follicular granulosa cells. On the other hand, there was a significant decrease in the number of secondary and tertiary follicles due to the administration of cypermethrin. The higher the dose of cypermethrin, the lower the number of secondary and tertiary follicles. This shows that cypermethrin exposure causes a normal disruption of secondary and tertiary follicle growth. In addition, secondary and tertiary follicles are more sensitive to cypermethrin than primary follicles. The mechanism of decline in secondary and tertiary follicles, at least through the mechanism of apoptosis, was evidenced in this study. When cypermethrin is metabolized, it is converted to cyanohydrin and then decomposed to cyanides and aldehydes. Both of these decomposition products can trigger the formation of reactive oxygen compounds[36].

Increased reactive oxygen compounds which are not accompanied by the antioxidant detoxification capacity will trigger oxidative stress. Previous studies have shown a decrease in endogenous antioxidant capacity and an increase in malondialdehyde due to exposure to cypermethrin in various dosage ranges[37,38]. Oxidative stress in severe degrees will trigger apoptosis. This study extends previous findings that cypermethrin can increase caspase-3 antral and pre-antral follicles[39], and abnormal development of endometrial

pinopods as endometrial receptivity indicators[35].

Interestingly, the apoptotic index of the primary follicles increased due to cypermethrin administration. However, the number of primary follicular granulosa cells did not decrease. This indicates that the primary follicles can maintain homeostasis (the role of a dormant factor and the stimulus of mammalian target of rapamycin or protein kinase B) in spite of external factors such as cypermethrin exposure. For secondary and tertiary follicles, the cypermethrin exposure has shown an apparent effect in the form of a decrease in a number of these follicles due to apoptotic effect. We hypothesized that cypermethrin will trigger the decrease of the FSH hormone and other paracrine factors[40]. This is supported by previous findings that cypermethrin causes a decrease in serum FSH level[38]. In addition, it is proven *in vitro* that oocytes and embryos are not instantly impaired and are sufficiently resistant to direct contact with cypermethrin[41].

In this study, it was also found significant depletion of endometrium in the group that was significantly exposed to cypermethrin compared to the control group. These findings indicate that exposure to cypermethrin causes suppression of normal endometrial growth. This inhibition of endometrial growth is caused by an increase in apoptosis, as evidenced in this study. This finding extends the earlier findings that thin endometrium is caused by normal growth inhibition, one of which is due to apoptosis[27].

It can be concluded that exposure to cypermethrin can suppress the number of secondary and tertiary follicular granulosa cells, and trigger thinning of the endometrium through induction of apoptosis.

Conflict of interest statement

The authors declare there is no conflict of interest.

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