ORIGINAL ARTICLE:

Effects of per oral cypermethrin exposure on Bcl-2 expression in granulose cells and antral follicle count of *Rattus norvegicus* ovaries

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ABSTRACT

Objectives: To determine the effect of oral cypermethrin exposure on the decrease of Bcl-2 expression in granulosa cells and antral follicle count in *Rattus norvegicus* ovary.
Materials and Methods: This was a true experimental study using post-test control group design. This study used 24 *Rattus norvegicus* which were divided into 4 groups, the control group, treatment group (P1) exposed to cypermethrin 10 mg/kg BW, treatment group (P2) exposed to cypermethrin 15 mg/kg BW and treatment group (P3) exposed to cypermethrin 20 mg/kg BW. The treatment groups were exposed to cypermethrin for 28 days. A surgery was conducted in proestrus phase and ovarian organs were taken. Bcl-2 expression examination was performed by using immunohistochemical method and the antral follicle count was assessed with the Hematoxillin Eosin (HE)
Results: This study showed the decrease of Bcl-2 expression in cypermethrin-exposed group compared to control group. There was a decrease in antral follicles count in cypermethrin-exposed group compared to control group.
Conclusion: Cypermethrin can decrease Bcl-2 expression and antral follicles count in *Rattus norvegicus* ovaries.

Keywords: Bcl-2; antral follicles; cypermethrin

ABSTRAK

Tujuan: Untuk mengetahui pengaruh paparan cypermethrin per oral terhadap penurunan ekspresi Bcl-2 pada sel granulosa dan jumlah folikel antral.pada ovarium *Rattus norvegicus.*
Bahan dan Metode: Desain penelitian yang digunakan true experimental (eksperimental sesungguhnya) dengan dipilih pendekatan post test only control group design. Menggunakan 24 ekor *Rattus norvegicus* yang dibagi dalam 4 kelompok yaitu kelompok kontrol, kelompok perlakuan (P1) dipapar cypermethrin 10 mg/kg BB, kelompok perlakuan (P2) dipapar cypermethrin 15 mg/kg BB dan kelompok perlakuan (P3) dipapar dengan cypermethrin 20 mg/kg BB. Kelompok perlakuan dipapar dengan cypermethrin selama 28 hari. Kemudian dilakukan pembedahan pada saat fase proestrus dan diambil organ ovarium. Dilakukan pemeriksaan ekspresi Bcl-2 dengan metode imunohistokimia dan jumlah folikel antral dengan pewarnaan Hematoksillin Eosin (HE)
Hasil: Terjadi penurunan ekspresi Bcl-2 pada kelompok yang dipapar cypermethrin dibandingkan kelompok kontrol. Terdapat penurunan jumlah folikel antral pada kelompok yang dipapar cypermethrin dibandingkan dengan kelompok kontrol.
Simpulan: Cypermethrin dapat menurunkan ekspresi Bcl-2 dan jumlah folikel antral pada ovarium *Rattus norvegicus.*

Kata kunci: Bcl-2; folikel antral; cypermethrin

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pISSN:0854-0381 ● eISSN: 2598-1013 ● doi: http://dx.doi.org/10.20473/mog.V26I32018.123-127
● Maj Obs Gin. 2018;26:123-127 ● Received 3 Sep 2018 ● Accepted 14 Nov 2018

INTRODUCTION

WHO data shows globally that 8 - 10% infertility rate in Europe equals to 14%. In Indonesia, infertility incidence rate is approximately 11% or occurs in 3 to 4.5 million couples. Infertility is defined as a failure to become pregnant after 12 months or more despite regular sexual intercourse two to three times a week without using contraceptives. One study showed that the cause of infertility is related to problems in female partenr, such as the problems in the tube (27.4%), menstrual problems (20%), uterus (9.1%), ovary (3.6%) and sexual disorders (2.7%).[1]

Pesticides are chemicals with unsafe usage which can affect a person's health. There are 44.4% farmers who use pesticice dose higher than recommended, while those who use the recommended dosage are 36.4%. Some farmers even increase the dose up to two times, while recommended dose recommendation is 12.1%. This is because a fear that the use of recommended doses will not be effective anymore in controlling the pests. Pesticides have a trait that it cannot be easily eradicated and leaving residue, so that its usage will affect a person's health.

Cypermethrin is a form of natural synthesis derived from pyrethrin, a type of insecticide and is widely used worldwide. The use of cypermethrin has reproductive health effects. Its effect on the ovary may lead the follicle reserve to decrease. Cypermethrin has the effect of damaging the ovaries through hormonal changes.[2] There was a significant decrease in LH, FSH and estrogen levels. Such decrease indicate that cypermethrin may obstruct ovarian function.[3] Another study have shown that cypermethrin exposure can affect ovarian damage to tissue structure as well as in the ovarian function.

In ovarian apoptosis, the cellular mechanism removes follicle growth when it is ovulated. Apoptosis in antral follicle granulosa cells is controlled by hormones that act as signals. The results showed that decreased levels of gonadotropin caused ovarian apoptosis.[4] The apoptotic pathway in normal ovary is regulated by p53 and Bcl-2.[5]

MATERIALS AND METHODS

This study was a true experimental study with the post test only control group design. We used experimental animals of Wistar strain *Rattus norvegicus* from the Islamic State University of Maulana Malik Ibrahim Malang which were transferred to Physiology Laboratory, Brawijaya University, Malang for their maintenance. The inclusion criteria were female and healthy rats, aged 10-12 weeks, weight 100-150 grams, not pregnant and no anatomical abnormalities. The sample number was 24 rats. The rats were divided into 4 groups and each group consisted of 6 rats. The control group only was only given with distilled water, while the treatment group 1 (P1) was given with apermethrin in a dose of 10 mg/kg BW, treatment group 2 (P2) in a dose of 15 mg/kg BW and treatment group 3 (P3) in a cypermethrin dose of 20 mg/kg BW.

The experimental animals were exposed to cypermethrin for 28 days orally via sonde. The weight of the rats were was measured every week, starting from the first week to the fourth week of exposure. On day 29, vaginal swab was performed to observe the estrus cycle. The rats were dissected during proestrus phase, anesthetized by injection of 1% ketamine on rat thighs in a dose of 0.2 ml. The ovarian organs were taken then placed into a container containing 1% formalin buffer fixation solution. The ovaries were used for immunohistochemical examination and hematoxillin Eosin staining.

The ovarian tissue was transversally cut in a thickness of 2-3 millimeters and then fed into the buffer's nettral solution. Then the tissue was cut in a thickness of 3-5 microns for histopathology examination using Hematoxillin Eosin (HE) and immunohistochemical staining. Imunohistochemical examination was conducted to determine Bcl-2 expression by using Bcl-2 brand R & D antibody. Bcl-2 observation was conducted by using HPF (High Power Field) with 1000x magnification observed under light microscope in 20 fields of view. Immunohistochemical count was rated as negative if there was no brown granulosa cell and the result was positive if a brown granulosa cell was found in the cell nucleus analyzed by enumeration using Fiji application.

Enumeration of the antral follicle count was performed using a light microscope with 400 times magnification to the entire ovarian field. The features of the antral follicle have more than one layer of cells namely granulosa cells, theca cells and the formation of cavities containing follicular fluid.

Before the statistical test was conducted, the normality tests of Shapiro Wilk and One Way Anova test s had been applied to find whether the data were normally distributed. Then, it was continued with multiple comparison test with Least Significant Difference (LSD) test to find the most influential cypermethrin doses to the expression of Bcl-2 and the number of antral follikel of female Wistar strain *Rattus norvegicus*.[6]

RESULTS AND DISCUSSION

Bcl-2 expression was observed with immunohistochemical examination and was detected in the cytoplasm. Expression of Bcl-2 was observed by microscope using High Power Field (HPF) with 1000x magnification (Figure 1)

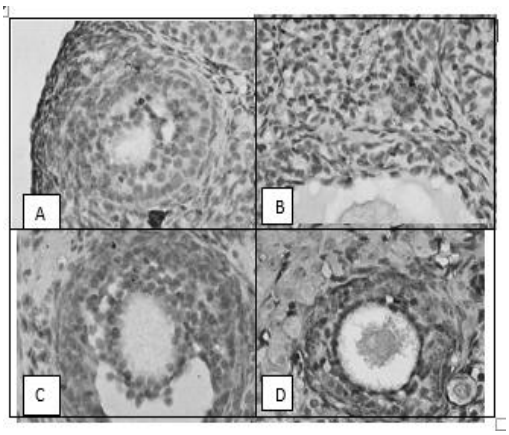

Figure 1. Bcl-2 expression. Notes: A. Bcl-2 expression in the control group, B. Bcl-2 expression in the treatment group of 10 mg dose, C. Bcl-2 expression in the treatment group of 15 mg dose, D. Bcl-2 expression in the treatment group of 20 mg dose

Based on one way Anova test result on Bcl-2 expression in *Rattus norvegicus*, there was significant difference (p=0.000) in mean of the fourth group. Figure 2 shows mean histogram ± standard deviation of Bcl-2 expression in the *Rattus norvegicus* in the groups treated with cypermethrin in doses of 10 mg /kg BW, 15 mg/kg BW, and 20 mg/kg BW. The average expression of Bcl-2 was highest in control group (without any treatment) (25.53 ± 1.30a%) and in the treatment group there was a decrease in the mean of Bcl-2 expression along with the increased dose of cypermethrin. Based on One Way Anova test result on the data of antral follicle count in *Rattus norvegicus*, there was significant difference (p=0.046) in the mean of four observed sample groups.

Antral follicle is the ovarian follicle that has an antrum/ cavity containing fluid follicles. The oocyte is surrounded by zona pellucida, consisting of several layers of cuboid-shaped granulosa cells and a cyber cell. Figure 3 shows antral follicle of the control group and the treatment group receiving various doses and stained with HE.

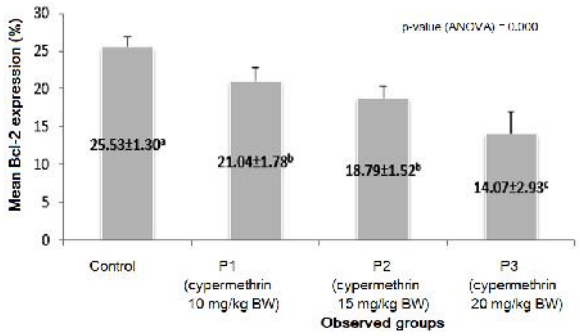

Figure 2. Mean of Bcl-2 expression

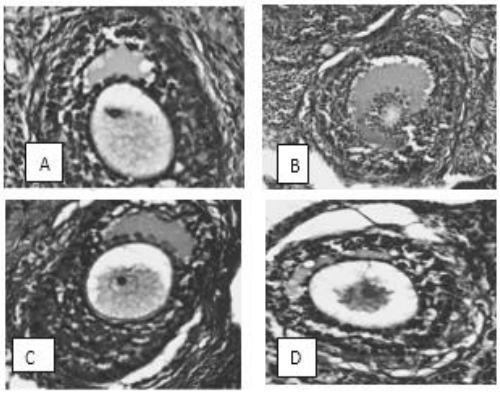

Figure 3. Ovarian antral follicles in control and treatment groups. Notes: A. Number of antral follicles in control group B. Treat-ment group of 10 mg dose, C. Treatment group of 15 mg dose, D. Treatment group of 20 mg dose.

Figure 4 shows mean histogram ± standard deviation of antral follicles count in *Rattus norvegicus* rats of four observed sample groups with treatment of cypermethrin with 10 mg dose/ kg BW, 15 mg/kg BW, and 20 mg/kg BW. The highest mean of antral follicles was in the control group, and there was a decrease in the mean of antral follicles count as the dose was added to the three treatment groups.

Based on data analysis from Pearson correlation test between Bcl-2 expression and antral follicles count of *Rattus norvegicus* treated with cypermethrin of 10 mg dose/kg BW, 15 mg/kg BW, and 20 mg/kg BW, there was significant correlation (p=0.000) between Bcl-2 expression and antral follicles count. High level of correlation was found as indicated by the correlation coefficient of 0.776.

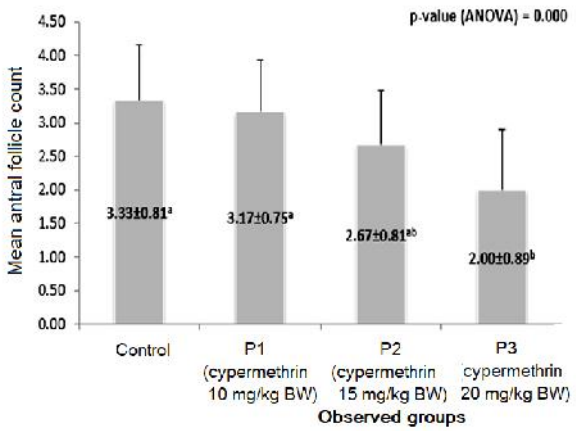

Figure 4. Mean histogram of antral follicles count. Notes: Mean antral follicles count in control group was higher than in the treatment groups.

Bcl-2 is one member of Bcl-2 protein family. Bcl-2 is anti-apoptosis that acts to disrupt the action of Bax/Bak. Apoptotic regulation is highly dependent on the ratio between Bcl-2 and Bax to determine whether the cells will apoptosis or survive. If the anti-apoptotic protein is lower than the pro-apoptotic protein then apoptosis will occur. Apoptosis in the ovary in the presence of a fetus involves oocytes and in adulthood it involves granulosa cells.

Decreased expression of Bcl-2 as an anti apoptotic protein can be caused by the mechanism of cypermethrin action. Cypermethrin enters the body through the mouth and will be absorbed in the body, then it is metabolized in the blood and will affect some organs. One of them is the hypothalamus and pituitary that can cause a decrease of LH, FSH and estrogen. FSH and estrogen decrease triggers follicles to apoptosis and becomes atresia. A study by Sanga et al (2013) showed that cypermethrin doses of 50 mg resulted in severe atresia follicles caused by apoptosis. This can be caused by the decrease in Bcl-2 expression or increased Bax expression.[2] According to a study by Filali et al (2009), women with FSH and LH therapy showed increasing Bcl-2 expression and no apoptosis in ovaries.[7]

Differences in Bcl-2 expression are also caused by increasing ROS. Cypermethrin can induce oxidative stress, which will produce an imbalance of oxidants and antioxidants. Oxidative stress can lead to cell structure damage, and can induce DNA damage and apoptosis. DNA damage and apoptosis are caused by the dose-dependent exposure to cypermethrin. Such apoptosis occurrs through an intrinsic pathway or mitochondrial pathway caused by signals in cells involving the Bcl family, such as Bcl-2 and Bax. Apoptosis in the ovaries can occur in germ cells, granulosa cells, internal cataracts and in luteal cells. Bcl-2 has an important role in the occurrence of apoptosis in the ovaries.

In this study with these model rats, Bcl-2 expression in granulosa cell treatment groups decreased with the increasing doses of cypermethrin. Decreased expression of Bcl-2 in the exposed group showed that cypermethrin can induce apoptosis in intrinsic pathways or mitochondrial pathways, despite the low doses of 10 mg/kg BW. However, this study did not investigate whether it can also run through an extrinsic pathway by testing the TNF.

Exposure to oral cypermethrin will be absorbed by the body through the digestive tract, metabolized in the blood and distributed throughout the body including to reproductive system. Cypermethrin can accumulate in body fat, skin, liver, kidneys, adrenal glands, ovaries, lungs, blood and heart. Accumulation of cypermethrin can also affect the function of many organs and one of them is the reproductive organs. One of reproductive organs the ovary whose one of its functions is producing follicles. The accumulation of cypermethrin can damage the ovaries, manifested in the decrease in the number of follicles. This is in accordance with a study by Mukadam (2014). In groups that had been exposed to cypermethrin for 30 days, follicular wall fractured and ruptured, the oocytes showed cytoplasmic fragmentation and follicular shrinkage occurred.[8] The presence of damage to the reproductive organs, especially the ovaries, should have been confirmed by examining the MDA levels in the ovaries, while it was not performed in this study.

The function of the reproductive organs is influenced by the hormones of the hypothalamus-pituitary-ovarian azis. Cypermethrin affects the secretion of hormones secreted by the pituitary. There was a decrease in LH, FSH and estrogen hormones. It showed that cypermethrin can disrupt ovarian function. Decreased secretion of LH and FSH hormones and from the anterior pituitary and estrogen of the ovaries was caused by the direct action of cypermethrin on the anterior pituitary. Decreased FSH hormone will inhibit the growth and development of the follicles in the ovaries. It is also in accordance with a study by Solati et al (2010), who found that the dose of cypermethrin 10 mg/kg, 15 mg/kg and 20 mg/kg exposed in mice for 30 days significantly decreased sexual behavior and FSH and LH hormone levels, compared with control group.[9]

In this study, a decrease in follicles count cypermethrin-treated group of 20 mg/kg BW was caused by a feed-

back mechanism of hypothalamus - pituitary – ovarian axis. Decreased FSH hormone was caused by the exposure to cypermethrin in a dose of 20 mg/kg BW which caused the follicles not developing and caused many follicles to become atresia. It could also be caused by the follicle that did not proliferate, so that the antrum was not formed.

CONCLUSION

Cypermethrin can decrease Bcl-2 expression and antral follicles count in *Rattus norvegicus* ovaries.

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