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Green Tea Extract Increases the Number of Granulosa Cells and E2 Serum Levels of Female Rats Exposed by Cypermethrin

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ABSTRACT

Green tea extract (GTE), Camellia sinensis, known as a very effective antioxidant to repair the damage of organs exposed by stress oxidative. Cypermethrin (CYP) is a harmful substance that can accumulate in the body and cause oxidative stress. This study aimed to demonstrate the effect of GTE in increasing the number of granulosa cells and E2 serum levels. Female rats (Rattus norvegicus) were randomly selected and divided into 5 groups, control group (KN), a group given only CYP 20 mg/kg body weight/day (KP), groups given CYP 20 mg/kg body weight/day and then treated with GTE 7, 14, 28 mg/kg body weight/day (P1, P2, and P3 respectively). Treatment was given for 28 days. The number of granulosa cells was counted by HSel software, E2 serum levels were determined via ELISA ASSAY. Treatment results with GTE 7, 14, and 28 mg/kg body weight/day significantly ($p < 0.05$) increased the number of granulosa cells and E2 serum levels relative to KN. Administration of GTE 28 mg/kg body weight/day caused the greatest increase in the number of granulosa cells and E2 serum levels. Our result suggests that GTE has potential prevention to stress oxidative by CYP exposure in granulosa cells and E2 serum.

Keywords: Cypermethrin, E2, Granulosa Cells, Green tea

INTRODUCTION

Background

Cypermethrin (CYP) is a class of pesticides. According to WHO, CYP belongs to class II pyrethroid(1). CYP is widely used for pesticides in vegetables and fruits. CYP becomes very dangerous and affects health(2) if that consumed by humans still contain residual CYP(3).

CYP is metabolized in the liver through hydrolytic ester cleavage and oxidative mechanisms by the CYP-450 enzyme forming 3-Phenoxybenzoid acid(4), then goes into the bloodstream and into the cells target, where it will further be metabolized to form cyanohydrins(5), and further decomposes into various toxic compounds such as radicals hydroxyl, cyanides and aldehydes(6) resulting in reactive oxygen species (ROS). ROS causes stress oxidative and reacts directly with cellular biomolecules, destroying fat tissue, proteins, and DNA causing the cells died(7).

In mammals, CYP can accumulate in fat tissue, liver, kidneys, adrenal glands, ovaries, lungs, blood, and heart. CYP is a highly hydrophobic compound and this suggests that its action in the biological membrane may be related to phospholipid proteins(7). In the reproductive organs, especially ovaries can lead to atresia of ovarian follicle, increase ovarian fat tissue and alkaline phosphatase(8), decrease number of estrus cycles per month, low corpus luteum(9), decrease levels of E2(10), apoptosis in ovarian granulosa cells(11), and autophagy of granulosa cells(12).

In the reproductive system, CYP causes adverse effects. Some of the effects of CYP on reproduction have been reported in recent years, such as decreased sperm count in mice, decreased implantation sites, number of live fetuses and fetal weight in rabbits(9). Research in laboratory showed that CYP exposure in that pregnant animals can affect for offspring. Several organ abnormalities and muscle bone have also been observed in the offspring of

the pregnant rabbits because exposed by CYP (13). Other research showed that oral CYP at a dose of 50 mg/kg shows atresia follicular, decrease estradiol and progesterone serum levels[8].

Decreased levels of estradiol as a result of the influence of ROS in the brain of the hypothalamus that can interfere with the performance of Gonadotropin Releasing Hormone (GnRH) so that the production of Follicle Stimulating Hormone (FSH) decreases. Decreased of FSH will result in decreased secretion of 17β estradiol in granulosa cells of the ovarian follicle. As a result of the low 17β estradiol, the granulosa cells can not proliferate as normal. So the number of secondary ovarian follicle granulosa cells is low[14]. Research conducted by Al-Hamdani (2017) showed the effect of CYP on ovaries. Female mice was given CYP with various doses 1.38 mg/kg body weight (low dose); 2.76 mg/kg body weight (medium dose), and 5.52 mg/kg body weight (high dose). The results of this study showed that the weight of mice fell 5.77% at low doses, 8.16% in moderate doses, and 12.34% at high doses. The number of healthy follicles decreased significantly after 6-weekly CYP was given in moderate and high doses. 17β estradiol levels decreased by 27.4% and 32.9%[9].

Oxidative stress can be prevented by giving exogenous antioxidants such as green tea extract (GTE). The benefits of GTE to ward off various diseases due to the high content of polyphenols is a high catechin compound. Seven different forms of catechin are (Epicatechin-gallate (ECG), Epicatechin (EC), Gallocatechin (GC), Catechin (C), and Catechin-gallate (CG)[15]. Research conducted by El-Beshbishty et al. (2011) showed that polyphenols contained in GTE were also able to increase the activity of Superoxide Dismutase (SOD) as an endogenous antioxidant capable of protecting organ damage through antioxidant, anti-inflammatory and anti-apoptotic mechanisms[16]. The benefits of GTE may also reduce the risk of epithelial ovarian cancer[17]. EGCG contained in GTE catechins can inhibit proliferation by inhibiting the activation of Extracellular Signal-Regulated Kinase (ERK), angiogenesis and inducing apoptosis in endometriosis[18]. The result of these study showed that GTE has the promising effect on increasing the number of granulosa cells and E2 serum levels exposed to CYP.

Purpose

This study proposed to demonstrate that GTE can increase the number of granulosa cells and E2 serum levels on female rat exposed to CYP.

METHODS

This study was conducted using a true experimental with post-test only with control group design. This study used animal laboratory was taken care at the Pharmacology Laboratory, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia. This study used famale rat and the sample determination was done by random. All experiments were conducted according to the principles of guide for the care and use of laboratory animals in Indonesia and were approved by The Ethical Committee Universitas Brawijaya, Malang, Indonesia (No.15/EC/KEPK-S2/02/2018). These experiments were done from February to March 2018.

Animals and Study Groups

The rats were kept under standard conditions, temperature 25-27^{0}C, relative humidity (55±5%) and 12h/12h light/dark cycle. Rats were given normal drinking water ad libitum during the experimental periods. The female rats used were 10-12 weeks old (reproductive age) and 150-300 grams of body weight. The female rats were housed 2-3 in a cage. Twenty-fifth of the female rats were randomly selected and divided into 5 groups, control group (KN), a group given only CYP 20 mg/kg body weight/day (KP), groups given CYP 20 mg/kg body weight/day and then treated with GTE 7, 14, 28 mg/kg body weight/day (P1, P2, and P3 respectively). The CYP and GTE were given orally once a day with different doses per week in accordance with the body weight per week. All treatments were given 28 days.

Cypermethrin and Green Tea Extraction

CYP was an insecticide under the trademark RIZOTIN 100 EC with the active ingredient of cypermethrin 100 g/L administered at a dose of 20 mg/kg for 28 days.

GTE was prepared in Pharmacology Laboratory of Universitas Brawijaya by mixing 100 gram simplicia leaf green tea with 900 mL of 96% ethanol, to separate ethanol solution with active substance green tea so that extract ethanol green tea in the form of paste done evaporation process at temperature 90^{0}C during ± 1.5-2 as much and produced green tea paste as much as ± 20.5 grams. Furthermore, it can be used for dose exposure in rats. To retain the compounds contained in green tea extract then stored at a temperature of 4^{0}C.

The Number of Granulosa Cells

After rat termination, ovarian organs were taken and colored with hematoxylin eosin (HE) in the Pathology Anatomy Laboratory of the Faculty of Medicine, Universitas Brawijaya. The result of HE staining is in the form of slides, then scanned with dotSlide microscope with magnification 400 times per field of view. The selection and calculation of granulosa cells numbers in secondary ovarian follicles was consulted to Department of Pathology Anatomy Universitas Brawijaya/Syaiful Anwar Hospital. Furthermore, granulosa cells were calculated using HSel software.

E2 Serum Levels

E2 serum levels were taken from heart blood of rats in proestrus phase were then calculated by ELISA ASSAY at 450 nm length and E2 serum levels were pg/L in accordance with the guidance provided on the kit (Cusabio Rat Estradiol (E2) Elisa kit. Catalog number: CSB-E05110r). This assay is taken place at Central Biomedical Laboratory, Faculty of Medicine, Universitas Brawijaya Malang, Indonesia.

Data Analysis

The data of the effect of GTE on the number of granulosa cells of female rats exposed by CYP and the data of the effect of GTE on E2 serum levels of female rats exposed by CYP were analyzed statistically by One-Way ANOVA ($p \leq 0.05$) and post hoc Least Significance Different (LSD). The data of the correlation between the number of granulosa cells and E2 serum levels of female rats exposed by CYP was analyzed statistically by Pearson Correlation. All of the analysis data used SPPS 25.

RESULTS

The Effect of GTE on the Number of Granulosa Cells of Female Rats Exposed by CYP

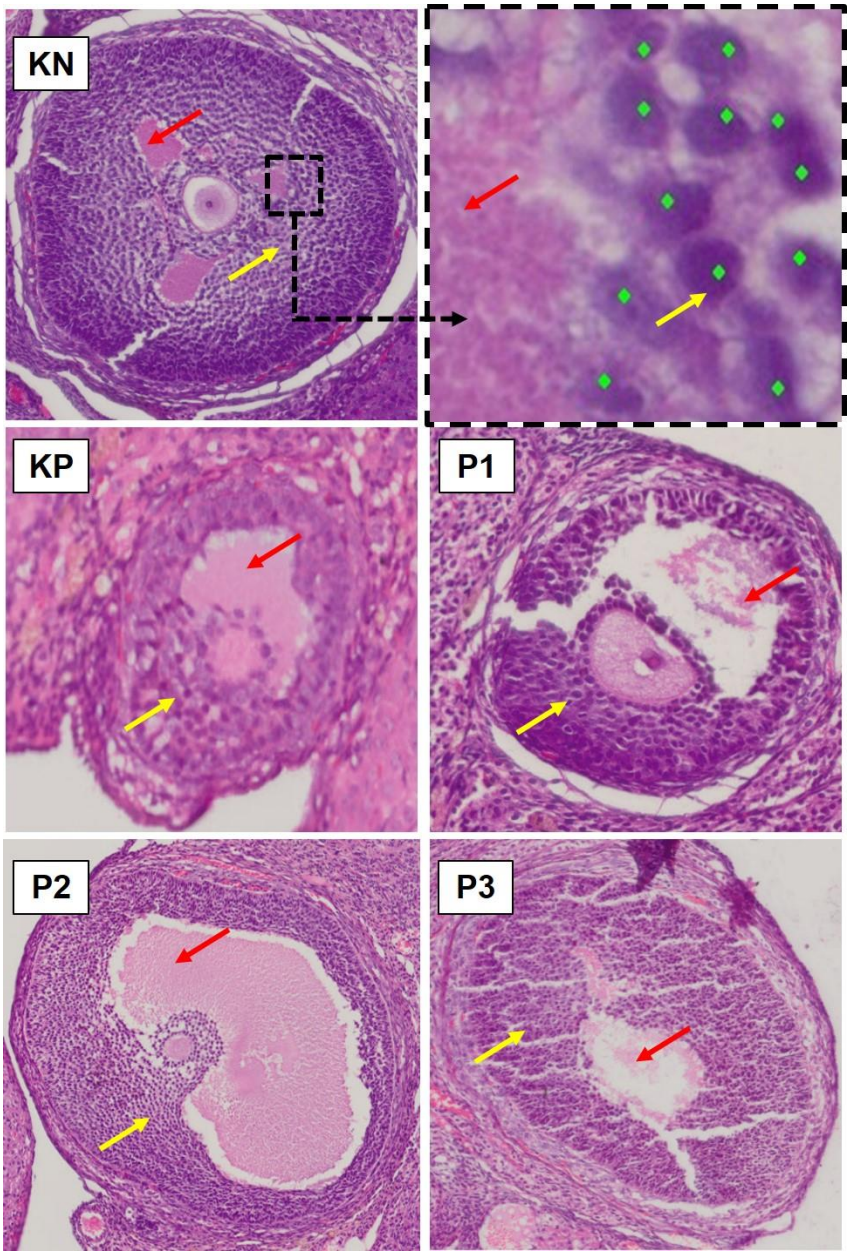
Count of granulosa cells was by selecting secondary follicles, dividing secondary follicles into four viewing fields, specify the area of granulosa cells thickness in the follicle, the thinnest area of the granulosa cell, and the other of two areas. Then the cells were calculated with HSel software and calculated the average. The result of this study as in figure 1 described that KN groups had the largest number of granulosa cells compared with KP, P1, P2, and P3 groups while the KP groups were seen to have the least number of granulosa cells compared to the other.

Based on the average calculation of the number granulosa cells in each group in figure 2 KN group had the highest average of the number granulosa cells (329.53 ± 26.25) while KP group had the lowest average of the number granulosa cells (149.14 ± 20.63). When compared to three treatments given by CYP and GTE in various doses for 28 days, P3 (CYP 20 mg/Kg body weight/day + 28 GTE mg/Kg body weight/day) had the highest average of the number of granulosa cells while P1 (CYP 20 mg/Kg body weight/day + GTE 7 mg/Kg body weight/day) had the lowest average of the number of granulosa cells.

According to statistical analysis by One-Way ANOVA, it showed significant value ($p = 0.000$). All of the data had normal distribution ($p>0.05$) and homogenous ($p>0.05$). Meanwhile, to know the dose significantly different, it could be continued with LSD.

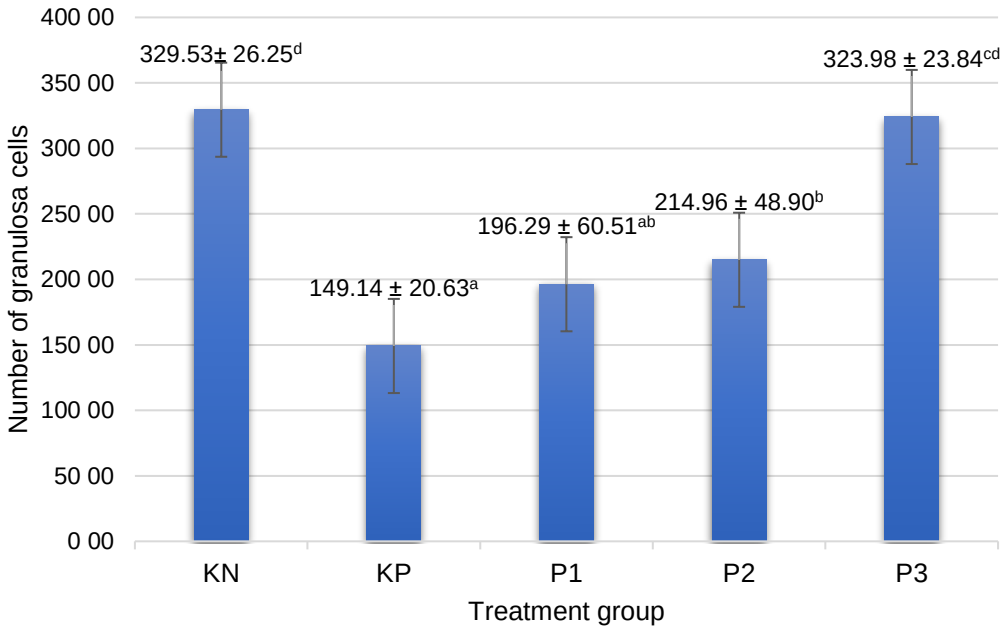
KP group (149.14 ± 20.63^{a}) differed significantly with P2 group (214.96 ± 48.90^{b}), P3 group (323.98 ± 23.84^{cb}), KN group (329.53 ± 26.25^{d}), but differed not significantly with P1 group (196.29 ± 60.51^{ab}). KN group (329.53 ± 26.25^{d}) differed significantly with KP group (149.14 ± 20.63^{a}), P1 group (196.29 ± 60.51^{ab}), and P2 group (214.96 ± 48.90^{b}) but differed not significantly with P3 group (323.98 ± 23.84^{cd}). The average of the number of granulosa cells in P3 group were almost same as KN group. It interpreted that P3 group with GTE 28 mg/Kg body weight/day could increase the number of granulosa cells in the ovary follicle of female rats near normal such as KN group.

Statistical analysis of the relationship between doses of GTE and the number of granulosa cells in female rats ovarian used nonparametric statistic (Spearman rank) because the normality test for doses of GTE was not normally distributed. Spearman rank test results got correlation coefficient value (0.843) and $p = 0.000$. The correlation coefficient (0.843) indicated a positive and very strong correlation between the doses of GTE and the number of granulosa cells. This meant that the higher the dose of GTE given to female rats exposed by CYP made the higher the number granulosa cells. Otherwise, the less dose of GTE made the number of granulosa cells also increased slightly. From the above description, giving GTE with a dose of 28 mg/Kg body weight/day was a dose that more able to increase the number of granulosa cells of the secondary follicle of female rats exposed by CYP.



The yellow arrows were the granulosa cells while the red arrows were the antrum. These viewed by a microscope with 400x magnification. These were appeared to be a difference in the number of granulosa cells. The lowest number was KP and more increasing on P1, P2, and P3 appeared almost the same KN. The green dots were cells description calculated by HSel software.

Figure 1. Cross-section granulosa cells in secondary folicle of female rats ovary



The average and the standard deviation of the number of granulosa cells in secondary follicles of rats ovary, the result of One-Way ANOVA, and the result of LSD seen by a histogram. KN had the highest average followed by the P3, P2, P1, and the last was KP.

Figure 2. Average the Number of Granulosa Cells of Secondary Follicle of Female Rats Ovary

The effect of GTE on E2 Serum Levels of Female Rats Exposed by CYP

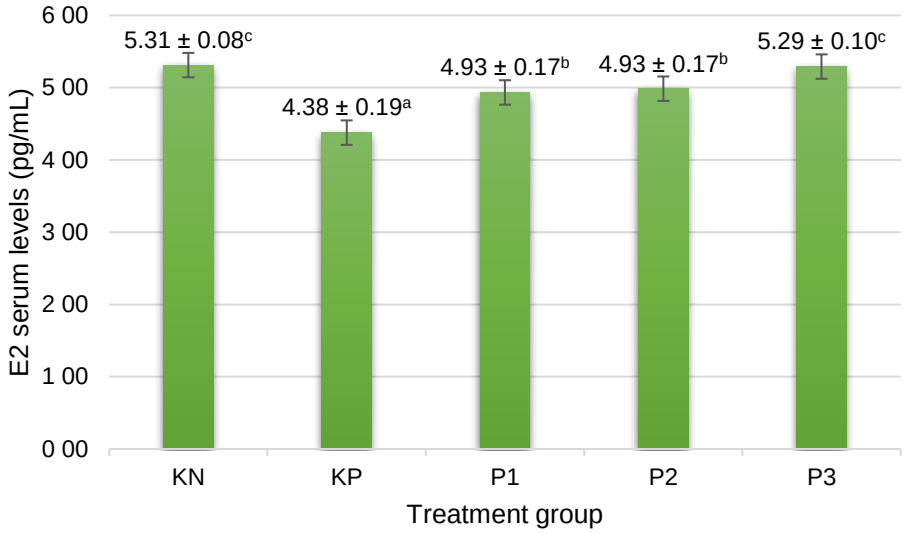
Based on the result of calculation of the average of serum E2 level in each group hence got figure 3. KN groups had highest average E2 serum levels (5.31 ± 0.08 pg/mL) meanwhile KP groups had lowest average E2 serum levels (4.38 ± 0.19 pg / mL). When compared to the three treatments that were given CYP 20 mg/Kg body weight/day and GTE of various doses for 28 days then P3 groups (CYP 20 mg/Kg body weight/day + GTE 28 mg/Kg body weight/day) had highest average E2 serum levels, while the P1 groups (CYP 20 mg/Kg body weight/day + GTE 7 mg/Kg body weight/day) had lowest average E2 serum levels.

Furthermore, the normality and homogeneity tests showed that the data were the normal distribution (p> 0.05) and homogeneous (p> 0.05). One-Way ANOVA test results showed significant (p = 0.000). Post hoc test (LSD) showed the result of KP Group (4.38 ± 0.19^{a}) significantly different with P1 group (4.93 ± 0.17^{b}), P2 group (4.99 ± 0.14^{b}), P3 group (5.29 ± 0.10^{c}), and KN group (5.31 ± 0.08^{c}). KN group (5.31 ± 0.08^{c}) differed significantly with KP group (4.38 ± 0.19^{a}), P1 group (4.93 ± 0.17^{b}), and P2 group (4.99 ± 0.14^{b}) but different not significant with P3 group (5.29 ± 0.10^{c}).

The average of E2 serum levels in P1 and P2 groups were lower than KN group but higher than KP group. Giving GTE at dose 7 mg/Kg body weight/day and 14 mg/Kg body weight/day proved able to increase E2 serum levels of female rats exposed by CYP 20 mg/Kg body weight/day. In contrast to P3 group, it was almost similar to KN group. It could be interpreted P3 group with GTE the dose of 28 mg/Kg body weight/day could increase E2 serum levels of female rats near normal such as KN group.

The Spearman Rank test was also used to analyze the correlation between the doses of GTE and E2 serum levels. The result was a correlation coefficient value (0.853) with a probability (0.000). The Value of correlation coefficient showed a positive and very strong correlation between the dose GTE and E2 serum levels. That was the dose of GTE was given higher that higher E2 serum levels in female rats exposed to CYP. Otherwise, giving GTE at the lower doses that lower E2 serum levels in female rats exposed to CYP.

It could be concluded, giving GTE with the dose of 28 mg/Kg body weight/day was a better dose to increase E2 serum levels of female rats exposed by CYP 20mg/Kg body weight/day compared to other groups.



The average histogram and standard deviation of E2 serum level of female rats and the LSD test. The KN group has the highest average followed by the P3 group then P2, P1 and the last is the KP group.

Figure 3. Average of E2 serum levels

Analysis of Correlation between of Granulosa Cells Ovarium with E2 Serum Levels of female rats. Because of the data of the number of granulosa cells and E2 serum level had the normal distribution ($p > 0.05$) so that the statistic analysis used Pearson Correlation. The result was correlation coefficient = 0.744 and $p = 0.000$. The correlation coefficient indicated there was a positive and strong correlation between the number of granulosa cells and E2 serum levels. This meant that the higher of the number of granulosa cells that the higher the serum E2 level. Otherwise, the number of granulosa cells was lower then the E2 serum level was lower.

DISCUSSION

GTE Increased the Number of Granulosa Cells of Female Rats Ovary Exposed by Cypermethrin

Based on the result of one-way ANOVA test showed significant value ($p = 0.000$), could be measured in various doses given to the number of ovarian secondary follicle granulosa cells in female rats. The correlation test between doses of GTE and the number of granulosa cells resulted in correlation coefficient value = 0.843 and $p = 0.000$. it meant the higher dose given the higher the number of granulosa cells or vice versa. The dose of GTE given at 28 mg/Kg body weight/day in this study can be expressed as the most capable dose to restore the number of granulosa cells as in the negative control group. Provision of GTE for 28 days orally along with CYP of 20 mg/Kg body weight/day had been able to prevent the decrease in the number of granulosa cells.

When compared to the KN group and the KP group, there is a very significant decrease in the number of granulosa cells. This study is in accordance with research[8] which CYP had an influence on the ovarian follicle atresia, granulosa cells apoptosis[11] and granulosa cells autophagy[12]. In other studies[9] mentioned that cypermethrin had given orally at doses of 2.76 mg/kg body weight and 5.52 mg/Kg body weight/day could cause damage to the reproductive organs i.e the decrease in the number of follicles significantly after given for 12 weeks. esearch conducted by Molavi, Morteza (2014) suggests that cypermethrin gave at doses of 75 mg / KgBB for 7, 14 and 24 days showed decreased levels of FSH, granulosa cell RNA damage and rodent ovarian mouse cell. Decreased levels of FSH can disrupt its function as a stimulator for granulosa cells to secrete E2. The failure of FSH to perform its function leads to undeveloped ovarian follicles caused by the small number of granulosa cells[8].

CYP is metabolized in the liver by hydrolytic ester cleavage and the oxidative pathway by the enzyme CYP-450 producing ROS, which is responsible for oxidative stress in mammals. ROS reacts directly with cellular biomolecules, destroying fat tissue, proteins, and DNA causing the cells to die[7]. CYP is involved in oxidative stress by mediating neurotoxicity. The main contributors to oxidative stress are the overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in cells or tissues exposed by CYP. Oral CYP produces oxidative stress in the nervous system. Cytochrome P450 2E1 (CYP2E1) is recognized as one of the major

contributors involved in the metabolism of CYP leading to ROS generation and oxidative stress through oxidation function[19].

The occurrence of ROS in the brain of the hypothalamus that can interfere with the performance of Gonadotropin-Releasing Hormone (GnRH) so that the production of Follicle-Stimulating Hormone (FSH) decreases. This decrease in FSH will result in decreased granulosa cells of the ovary follicle so that the secretion of E2 also decreases and then the ovarian follicle granulosa cells can not proliferate as normal. So the number of secondary ovarian follicle granulosa cells is low[14].

To ward off free radicals is necessary to have enough antioxidants and strong so it can neutralize free radical groups in the body. Green tea can be made antioxidant because it has a high catechin content when compared with other natural materials[15]. Antioxidant activity of green tea is related to its polyphenol content as its main component. According to Chacko (2010) green tea as an antioxidant can act as anticancer, anti-inflammatory, antiarthritis, antibacterial, antiangiogenic, antivirus, protect the nerve work and prevent the occurrence of heart disease[20].

Catechins and polyphenols in tea are effective as anti-reactive oxygen physiologically and nitrogen species in vitro, including superoxide radicals (O2-), peroxyl radicals, oxygen, peroxynitrite (ONOO-) and hypochlorite acid. The ability of tea polyphenols in chelation metal ions, such as iron and copper, can contribute to the antioxidant activity by preventing the active transition of the reduction of free radical catalyst formation. Catechins and tea polyphenols can inhibit the activity of redox-sensitive factors, nuclear factor κB (NF-κB) and protein-1 activator (AP-1), on cell culture. Although other antioxidants may also inhibit this redox-sensitive transcription factor. Research showed that tea catechins and polyphenols act as kinase inhibitors in complex signaling pathways. So green tea can break the oxidant group and provide antioxidants to the organs in the hypothalamus-pituitary-ovarian pathway[21].

GTE Increased the E2 Serum Levels of Female Rats Exposed by CYP

Results of the One-Way ANOVA test showed significant value (p = 0.000). So it was stated there was an effect of GTE in various doses to the E2 serum levels of female rats. Correlation test between doses of GTE and E2 serum levels had correlation coefficient value = 0.853 and p = 0.000. It could be interpreted the higher dose of GTE given then the higher E2 serum levels and vice versa. GTE at a dose of 28 mg/Kg body weight/day administered orally in this study was the most capable dose to restore E2 serum levels as in the KN group. This study also confirmed giving GTE for 28 days can prevent the decrease of E2 serum levels of female rats exposed by CYP.

This study is consistent with several studies. Research conducted at the University of Mysore, India proved that CYP administration with 5.52 mg/Kg body weight for 6 and 12 weeks was able to decrease the number of monthly estrus cycles, healthy follicles, corpus luteum, and E2 serum levels[9].

Beside CYP can cause oxidative stress, CYP can effectively suppress the opening of voltage-gated chloride channels (VGCC) and inhibit GABA. GABA neurotransmitters are one of the most dominant neurotransmitters, which regulate the chloride channels in the brain. To continue hypothalamic stimulation requires neurotransmitters to be able to secrete GnRH (19). In addition, CYP, which has metabolic pathways similar to mammals, produces metabolite chemicals similar to E2[22].

Free radicals can be counteracted by providing powerful and sufficient antioxidants to release free radical groups in the body. One of the strongest antioxidants is green tea, where green tea has a high flavor of flavonoids (Suzuki at al., 2016). According to article review by Roychoudhury (2017), strong antioxidants possessed by green tea can improve the health of reproductive organs[23].

CONCLUSION

In conclusion, green tea extract at a dose 28 mg/kg body weight/day is useful to increase the number of ovarian granulosa cells and E2 serum levels in female rats exposed by cypermethrin.

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