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Effect of Extra Virgin Olive Oil on the Apoptosis Index and the Number of Antral Ovarian Follicles in Rats Exposed to Cigarette Smoke

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ABSTRACT

Infertility is a global problem. Risk factors for infertility are cigarette smoke. Cigarette smoke contains harmful substances that can have adverse effects on follicle development. Oxidative stress due to cigarette smoke can activate proinflammatory cytokines in ovarian blood. Alternative natural treatments are needed that have antioxidant and anti-inflammatory properties. Extra virgin olive oil is pure oil from olives rich in antioxidants. Its polyphenol content has been proven to be high in anti-inflammatory and antioxidant properties. This study aims to determine the effect of extra virgin olive oil on the apoptosis index and the number of antral ovarian follicles in mice exposed to cigarette smoke. The design of this study was a true experiment with a post-test-only control group design. The experimental animals used white mice aged 2-3 months, weighing 150-250 grams. Cigarette smoke was given a dose of 4 cigarettes/day for 28 days. The EVOO doses used were 1.5 ml/KgBB, 3 ml/KgBB, and 4.5 ml/KgBB. Data were analyzed using one-way ANOVA. The results showed a significant difference ($p<0.05$) in the apoptosis index and the number of antral follicles. This study concluded that extra virgin olive oil showed differences in the apoptosis index and the number of antral follicles in mice compared to the positive control group that was only exposed to cigarettes.

Keywords: antral follicles, apoptosis index, cigarette smoke, EVOO

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BACKGROUND

Infertility is a condition experienced by married couples who have been married for one year with regular sexual intercourse without using contraception but do not achieve pregnancy (Kusmirah, 2014). Infertility is a global problem that is often encountered, the number of couples experiencing infertility is around 42 million and has increased to 48 million couples of fertile age (Mascarenhas et al, 2012). The prevalence of women experiencing primary infertility in Indonesia is around 6% with an age range of 25-49 years (POGI, 2013). Risk factors for infertility include age over 35 years, alcohol consumption, obesity, mental disorders/mental stress, smoking, sexually transmitted infections, and genetics (Nordqvist, 2019). Smoking is a bad habit that is often encountered, this activity is often done by various groups regardless of class, age, or religion because it is easy to do. According to WHO, data on cigarette users in 2014 was around 5.8 trillion. Indonesia is one of the fourth largest cigarette users in the world, after China, Russia, and the USA (Eriksen et al, 2015).

Cigarettes contain chemical compounds that can be harmful to health. Cigarette smoke contains around 6,000 components that are harmful to health such as nicotine, carbon monoxide, and several metal compounds that can be antigens, cytotoxic, mutagens, or carcinogens in the body (Arnson et al, 2010). The dangerous content of cigarette smoke such as nicotine, BaP, and DMBA can have a bad effect on the development and number of follicles (Budani et al, 2020). Other bad effects of exposure to cigarette smoke can affect the formation of free radicals due to increased production of ROS (Reactive Oxygen Species) and disrupt the antioxidant mechanism in the body. This mechanism causes an imbalance between antioxidants and prooxidants so that it can produce oxidative stress (Dechanet et al, 2020). Oxidative stress from cigarette smoke can activate proinflammatory cytokines in the blood and organs such as the ovaries. Cigarette smoke can cause increased apoptosis in cells and increased oxidative stress in the ovaries, thereby affecting ovulation (Sobinoff et al, 2013). Antral follicles are marker follicles for infertility because they can play a role in the folliculogenesis process and be used as the best estimate of the immune response. The number of antral follicles below normal can provide a poor prognosis for the ovulation process (Lai et al, 2013).

The adverse effects of increased inflammation cause high rates of gynecological disease and fertility disorders. Handling these adverse effects can also have negative reactions on other body organs and require high costs (Lundberg et al, 2016). Therefore, alternative natural medicines are needed that have anti-inflammatories and antioxidants.

One of the fruits with high antioxidants is olives. Ripe purple-black fruit is squeezed to extract the oil known as olive oil. Olive oil is known as a healthy oil because it has a high content of unsaturated fats, oleic acid, and polyphenols (Kala et al, 2017). Olive oil is divided into 2 categories, namely through a refining process and raw. Virgin and extra virgin olive oil types are included in the raw category, while those labeled light or pure olive oil have undergone a refining process (Dewi, 2012).

Extra virgin olive oil is first pressed with the cold press method so that the olives do not go through a heating process, without chemicals. Using quality olives, Extra Virgin Olive Oil has a natural and complete vitamin, antioxidant, and mineral content to be consumed directly. The polyphenol content has been proven to be a high anti-inflammatory and antioxidant. The four main phenolic compounds found in olive oil, namely hydroxytyrosol, oleuropein, hydroxytyrosol enolate, and dihydroxyphenylene elenolic acid dialdehyde, significantly provide a protective effect on red blood cells against oxidative damage (Hashmi et al, 2015).

In traditional medicine, olive oil can also therapeutically reduce blood sugar, cholesterol, and uric acid, to treat diabetes, hypertension, inflammation, diarrhea, respiratory tract and urinary tract infections, stomach and intestinal diseases, asthma, hemorrhoids, rheumatism, laxatives, mouth cleanser, and as a vasodilator (15). The normal dose consuming EVOO is 25-40 ml or 8-70 grams per day (15). Consumption of EVOO dose of 30 ml can be used as a laxative, while consumption above 30 ml gives side effects of diarrhea and skin allergies (Sanchez et al, 2011).

METHODS

The research design used in this study is a true experimental study with a post-test-only control group design conducted in the laboratory in vivo. In this study, the treatment or intervention carried out by the researcher was by providing exposure to cigarette smoke and giving extra virgin

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olive oil in various doses to white rats (Rattus norvegicus). The conditions observed after the intervention in this study were the apoptosis index and the number of ovarian antral follicles.

The experimental animal used was the Wistar strain of white rats (Rattus norvegicus). The sampling technique in this study used systematic random sampling based on inclusion criteria. The condition rats used as research samples were female, aged 2 - 3 months, weighing 150 - 250 grams, healthy, and moving actively. This research used experimental animals (rats) and was conducted in the Laboratory at the Faculty of Medicine, Brawijaya University in July-August 2024.

The intervention before starting the treatment, the mice were acclimatized for a week for adaptation. The researchers determined five groups (five mice per group), namely the negative control group (KN) which was not exposed to cigarette smoke or extra virgin olive oil, the positive control (KP) which was exposed to cigarette smoke without being given extra virgin olive oil, treatment group 1 (P1) mice with exposure to cigarette smoke and extra virgin olive oil at a dose of 1.5 ml/KgBW/day, treatment group 2 (P2) mice with exposure to cigarette smoke and extra virgin olive oil at a dose of 3 ml/KgBW/day, and treatment group 3 (P3) mice exposed to cigarette smoke and extra virgin olive oil at a dose of 4.5 ml/KgBW/day. Exposure to cigarette smoke was given as many as 4 cigarettes a day and extra virgin olive oil once a day, for 28 days. Synchronization of the estrus phase for 3 days. Vaginal smear examination aims to determine the estrus phase in mice as a marker that the mice will be terminated on the last day of the study in the proestrus phase. Measurement of parameters for the apoptosis index using the tunnel assay and for the number of antral follicles using histology preparations with Hematoxylin Eosin staining**.**

The data on the cell apoptosis index and the number of antral follicles will be presented in the form of graphs and tables. The data normality test uses Shapiro-Wilk and the hypothesis test uses One Way Anova. If the results show a significant difference, it will be rated with a multiple comparison test, namely the Least Significant Difference (LSD) test. The purpose of using the LSD test is to find out at what dose the most significant effect is obtained. Data analysis was carried out using IBM SPSS Statistics software version 23.

The entire process in this study complied with ethical principles in accordance with the permission of the ethical committee of Research Institut Ilmu Kesehatan STRADA Indonesia, with a letter No. 001460/EC/KEPK/I/07/2024.

RESULTS

Table 1 The Effect of EVOO on Antral Follicles

Treatment Group	Antral Follicles	
	Mean ± SD	*p-value*
Negative control	10±2.35	
Positive control	3.2±0.83	
P1 (evoo 1.5 ml/KgBW/day)	5.2±0.82	0.000<α
P2 (evoo 3 ml/KgBW/day)	6.0±0.70	
P3 (evoo 4.5 ml/KgBW/day)	8.8±1.64	

Table 1 shows a histogram of the average number of antral follicles in rats exposed to cigarette smoke (positive control) which is lower than the average of rats not given anything (negative control). Furthermore, the average of three groups of Rattus norvegicus rats exposed to cigarette smoke and given extra virgin olive oil at a dose of 1.5 ml/KgBW/day, a dose of 3 ml/KgBW/day, and a dose of 4.5 ml/KgBW /day increased compared to rats exposed to cigarette smoke (positive control). There is a statistically significant difference between the average number of antral follicles in the ovaries of Rattus norvegicus rats between the negative control group (healthy rats without exposure to cigarette smoke) (10±2.35) and the positive control group (rats exposed to cigarette smoke) (3.2±0.83). Based on the average value, the number of antral follicles in the negative control group is greater than the average number of antral follicles in the positive control group. It appears that the mean value of the number of antral follicles in the positive control group is smaller when compared to the mean number of antral follicles in the negative control group. Likewise, the negative control group was significantly different from group P1 (5.2±0.82) and group P2 (6.0±0.70), but not significantly different from group P3 (8.8±1.64).

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The mean value in the negative control group was close to the mean value of the P3 group. There was a significant difference in the mean number of antral follicles in the ovaries between the positive control group (mice exposed to cigarette smoke) (3.2 ± 0.83) and the P1 group or the treatment group of cigarette smoke exposure + extra virgin olive oil dose of 1.5 ml / KgBW / day (5.2 ± 0.82). It appears that the mean value of the number of antral follicles in the positive control group is smaller when compared to the mean number of antral follicles in the P1 group.

There was a significant difference in the mean number of antral follicles between the positive control group (3.2 ± 0.83) and the P2 group or the treatment group of cigarette smoke exposure + extra virgin olive oil dose of 3 ml / KgBB / day (6.0 ± 0.70). This means that mice exposed to cigarette smoke + extra virgin olive oil 3 ml / KgBB / day will increase the number of antral follicles when compared to mice exposed to cigarette smoke alone.

From the results of the study, there was a significant difference in the average number of antral follicles between the positive control group (rats exposed to cigarette smoke) (3.2±0.83) and the P3 group or the treatment group exposed to cigarette smoke + extra virgin olive oil at a dose of 4.5 ml/KgBW/day (8.8±1.64). It appears that the average value of the number of antral follicles in the positive control group is smaller when compared to the average number of antral follicles in the P3 group.

Based on the results of the LSD test, the number of antral follicles in mice between the negative control group and the positive control group showed a statistically significant difference, the average value of the positive control group experienced a decrease in the number of antral follicles compared to the negative control group. This means that mice exposed to cigarette smoke will show a lower number of antral follicles compared to healthy mice. In other words, exposure to cigarette smoke in mice can reduce the number of ovarian antral follicles.

These results are supported by research conducted by Sobinoff et al (2013), confirming that exposure to cigarette smoke can reduce the number of antral follicles in mice. The number of antral follicles decreases due to apoptosis in the follicle granulate cells.

Table 2 The Effect of EVOO on Apoptosis Index

Treatment Group	Apoptosis Index	
	Mean ± SD	*p-value*
Negative control	15 ± 4,69	
Positive control	40,67 ± 6,53	
P1 (evoo 1.5 ml/KgBW/day)	29,67 ± 3,67	0,000 < α
P2 (evoo 3 ml/KgBW/day)	25 ± 2,45	
P3 (evoo 4.5 ml/KgBW/day)	16,67 ± 3,01	

Table 2 based on the results of the LSD test shows that there is a significant difference in the average apoptosis index between the positive control group (KP) and groups P1 (EVOO 1.5 ml/KgBB/day), P2 (EVOO 3 ml/KgBB /day) and P3 (EVOO 4.5 ml/KgBW/day). However, there was no significant difference between group P1 (EVOO 1.5 ml/KgBB/day) and group P2 (EVOO 3 ml/KgBB/day). In addition, there was no significant difference between the negative control group and group P3 (EVOO 4.5 ml/KgBB/day). The table shows a decrease in the percentage of apoptosis index along with the addition of the dose of extra virgin olive oil given. The lowest average apoptosis index value was in treatment group 3 (EVOO 4.5 ml/KgBW/day) which was close to the average value of the negative control group.

DISCUSSION

From the research results, the apoptosis index and the number of antral follicles in mice between the negative control group, the positive control group, and the treatment group showed a statistically significant difference, the average value of the positive control group experienced a decrease in the number of antral follicles compared to the negative control group. The treatment group experienced an increase in the number of antral follicles and a decrease in the apoptosis index compared to the positive control group. This means that mice exposed to cigarette smoke showed a lower number of antral follicles compared to healthy mice. In other words, exposure to cigarette

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smoke in mice can reduce the number of ovarian antral follicles. These results are supported by research conducted by Sobinoff et al., (2013) in their research, confirming that exposure to cigarette smoke in a group of mice was able to reduce the number of antral follicles compared to negative controls. The number of antral follicles decreases due to apoptosis in the granulosa cells of the follicles. The occurrence of apoptosis in the antral follicles is justified by evidence of apoptosis marker examination. The results showed that there were two markers, namely caspase 2 and caspase 3, which were found in the granulosa cells of the antral follicles.

Exposure to cigarette smoke is a collection of toxic chemicals produced from the burning of cigarettes by active smokers. One of the contents of cigarette smoke is nicotine which can inhibit the work of reproductive hormones such as estrogen and progesterone, in addition, cigarette smoke activates an increase in free radicals in target organs so that it damages the function of target organs such as the ovaries (Ghanbari et al, 2012).

Nicotine can release catecholamines into the bloodstream to the cellular level, it plays a role in increasing synthesis, the release of neurotransmitters and hormones, inducing oxidative stress, activating transcription factors and catecholamine tyrosine hydroxylase enzymes (Lazar, 2012). Oxidative stress produced by cigarette smoke can reach the ovaries and activate inflammatory cytokines that play a role in the process of cell regulation and cell apoptosis.

The ovary has almond-shaped characteristics and specific functions compared to other organs because the ovary can develop follicles until ovulation occurs every month. The folliculogenesis process is influenced by hormones, one of which is FSH which acts as a stimulus for antral follicles until they reach the Graaf follicles with the help of increased estrogen hormone secretion. Disruption of antral follicle development can occur due to one of the factors such as increased oxidative stress due to exposure to cigarette smoke. The oxidative stress process, DNA damage, lipid peroxidation, and protein peroxidase occur. This triggers the release of mitochondrial proteins into the cytoplasm. The release of mitochondrial proteins into the cytoplasm initiates apoptosis (Mcllwain, 2013).

One way to reduce oxidative stress is by administering antioxidants extracted from plants. One of the bioactive ingredients that can be used as antioxidants in reducing oxidative stress is polyphenols. One source of polyphenols is olives. Olives are processed into extra virgin olive oil by cold pressing. In the study, it was proven that EVOO can increase the number of antral follicles in mice exposed to cigarette smoke. Cytotoxicity and cell death caused by oxidative stress can be minimized by antioxidants and repair mechanisms in cells. The antioxidants contained in EVOO include phenolic compounds, tocopherols, squalene, pigments, and beta-carotene. EVOO is known to have a high polyphenol content, polyphenols are known as anti-inflammatories, antioxidants, and anticoagulants. The way these antioxidants work protects cells from oxidative damage caused by free radicals.

In this study, it was proven that administering extra virgin olive oil according to the specified dose was able to significantly reduce the apoptosis index and increase the number of antral follicles in mice exposed to cigarette smoke.

CONCLUSION

In this study, cigarette smoke was proven to cause an increase in the apoptosis index and a decrease in the number of antral follicles in the ovaries of white mice. The administration of extra virgin olive oil significantly reduced the apoptosis index and increased the number of antral follicles in mice exposed to cigarette smoke. There was a significant correlation between the dose of extra virgin olive oil with a decrease in the apoptosis index and an increase in the number of antral follicles.

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