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Negative Effect of Cigarette Smoke: Black Garlic Opportunities for Prevention of Ovulation Disorders

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Abstract: Women's issues are primarily related to infertility. This study aimed to demonstrate that ovarian healing in wistar rats exposed to cigarette smoke was impacted by the administration of extracted black garlic. This research uses True Experimental methods in vivo in the laboratory to identify the cause and effect of the variables being tested. This method involves replication, randomization, and control. The design chosen was a post-test-only control group design, where the experimental group received treatment and the control group did not, without random selection. The results of the study showed that there was no effect due to exposure to cigarette smoke with an increase in cortisol and a decrease in the number of secondary follicles in the ovaries of female wistar rats as well as the administration of Black Garlic (Allium sativum) extract. Black garlic extract unaffected cortisol levels and the number of secondary follicles or repair of the ovaries due to exposure to cigarette smoke. Further research is needed in dosing black garlic extract or combining it with other ingredients to provide effective results.
Keywords: Black garlic extract; cortisol levels; follicle count; infertility.

INTRODUCTION

According to data from the World Health Organization (WHO), one worldwide problem in global health is infertility. The total number of ten percent women experience health issues related to infertility (World Health Statistics, 2019). If a couple has not been pregnant in more than two years, they are considered to be infertile in Primary Infertility. However, infertility may also develop if a couple is unable to conceive for more than a year. In this instance, a married couple's attempt to conceive was unsuccessful due to their failure to take contraception. This is called Secondary Infertility (Rahmadiani D, 2021). According to a survey, wives account for 64% of infertility and husbands for 36%. From WHO's perspective, women are responsible for 36% of Fallopian tube, 33% of ovulation, 6% of endometriosis, and 40% of other unexplainable or idiopathic conditions. Conversely, Oligozoospermia (16% multifactoral), endocrinologist (20%), and immunologic 2% are from the husband's side (World Health Organization, 2018), subfertility is the term used to describe the effects of lifestyle on exposure to environmental pollution, which often results in abnormal pregnancies. Smoke from cigarettes is one of the major contributing causes of ovulatory disorders.

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Ovulatory disorders stand in the second position as infertility disorders owned by women (Irmawati, 2021a.b). An imbalance in hormones caused by the suppression of FSH and LH hormone release is stated to be the cause of this ovulation issue. This hormone exhibits barriers as a result of hypothalamic and pituitary dysfunction. If problems with either hormone's release arise, the follicles' ability to develop will be hampered, which will impact the ovulation process.

A variety of intricate chemicals included in cigarette smoke create huge impacts on various reproductive systems (De Angelis et al., 2020). Tobacco elements in smoke that contain nicotine have the potential to alter the body's hormone balances. This is contingent upon the quantity and duration of exposure to cigarette smoke. Reproductive function will be interfered with by any exposure to cigarette smoke. The substances included in cigarettes cause ovarian cell illness which affects estrogen levels. If this occurs, then genetic abnormalities will be more likely to affect female eggs, or oocytes (Nur Halimah & Winarni, 2018).

The smoke from cigarettes contains a lot of reactive oxygen species (ROS). Free radicals are defined as less stable molecules that are reactive and able to harm tissue because they can release electrons(Legowo, 2015). Oxidative stress will be impacted in this situation by an imbalance between pro-oxidants and antioxidants. Mitochondrial malfunction results in oxidative stress, which leads to defects in chromosomal segregation, as well as failures in maturation and conception (Ramdiana; Legiran, 2023). Cortisol from the adrenal glands is one of the glucocorticoid hormones produced by the body in response to stress. Gonadotropine-Releasing Hormone (GnRH) hormone released under stress inhibits the Hypothalamic-Pituitary-Gonadal pathway, which in turn impairs reproductive function. The influence of cortisol is a side effect of GnRH secretion inhibition at the pituitary level. It can take advantage of the ovarian level's lowered production of Follicle Stimulating Hormone (FSH) and cortisol, which can obstruct the creation of steroid hormones and cause apoptosis (Setiyono et al., 2015), it has been demonstrated that follicles contain cortisol and Corisol-Binding Protein (CBP). This stimulates a variety of pathways including oocyte quality and direct steroid genesis effects. The number of follicles that mature from the primordial to the antral stage depends on how they respond to the hormone FSH. Follicles develop and grow in response to FSH stimulation until they reach the mature state. Consequently, it may be said that cortisol may disrupt the GnRH pulse, lowering FSH hormone levels and inhibiting the growth of new follicles.

Antioxidants are essential for balancing reactive oxygen species (ROS) in the body and preventing degenerative illnesses including cancer, cardiovascular disease, and premature aging. Antioxidants can interrupt a chain reaction without impairing the operation of normal cells, proteins, or lipids. They also inhibit free radicals and prevent harm to these structures (Halliwell & Gutteridge, 2007). Antioxidants and secondary metabolic chemicals are not produced by the body. It comes from diet, including fruits, vegetables, and spices. Plant-based antioxidants offer several health advantages to the body and are made up of bioactive substances such as flavonoids, phenolic compounds, tannins, phenolic diterpenes, and vitamins (Ibroham et al., 2022).

It has been shown that insulin can increase basal and GnRH levels, which in turn enhance LH and FSH levels in pituitary cells. Polycystic ovarian contraceptive tablets can decrease LH production and lower free androgen levels by raising Sex-Hormone-Binding Globulin (SHBG). However, there are some drawbacks to using this medication, such as increased insulin resistance, negative effects on lipids as inflammatory indicators, increased body weight that exacerbates hormonal disorders, and increased risk factors for various cardiac diseases(Zettira, Z; Nisa, 2015).

Other pharmacological treatments for infertility related to ovulatory dysfunction include clomiphene citrate, metformin, aromatase inhibitors, and glucocorticoids. Anti-androgens, glucocorticoids, gonadotropin-releasing hormone agonists, and oral conceptions with ethinyl estradiol are among the medications used to treat androgen-related symptoms. However, the drawbacks of hormonal therapy include the induction of ovarian hyperstimulation syndrome (OHSS), lower fertility, and very high expenditures (Maggyvin et al., 2019).

Herbal supplements are a popular preventative therapy option that is more practical and devoid of negative effects for maintaining health. In the opinion of the World Health Organization, between 70% and 80% of people worldwide accept traditional medicine for basic care, and over 50% of modern clinical medication contains natural substances, which has a substantial influence on medical advancement (Moshfegh et al., 2016).

Traditional medicine has recommended garlic as a treatment for inflammatory and metabolic conditions, such as diabetes mellitus, hypertension, cardiovascular disease, and cancer (Falahatian et al., 2022). According to the previous study, the fraction of dichloromethane extract had the greatest total flavonoids of 55.68 mg QE/ g which was fermented by black garlic, or Allium sativum extracts. Black garlic's dichloromethane extract's IC50 value is 361.07 µg/mL, making it a moderate antioxidant-level substance.

A product called "black garlic" is created by heating garlic to a high temperature. Processed garlic loses its fresh scent and turns black. As a result, it tastes sweeter and somewhat more sour. One of the bioactive substances found in black garlic is S-allyl cysteine, a derivative of the chemical that makes up garlic itself. This compound is created by an enzymatic process and functions as an antioxidant. Treatment with S-allyl cysteine (SAC) can raise the enzymes that mediate cell death, demonstrating protection against free radicals that cause cell damage(Handayani, A R; Romsiah; Rikmasari, 2020).

To prevent conditions that impair granulosa cell function, which can lead to decreased oocyte quality and ovulation disorders in the form of abnormal ovarian follicle growth and infertility, the study intends to assess the possible impact of administering black garlic extract (Allium sativum). Ovulation abnormalities caused by genetic, environmental, and lifestyle factors which can manifest as infertility (Kicińska et al., 2023).

Previous research on female Wistar rats showed that black garlic extract contains flavonoids which can increase antioxidant activity which is said to reduce oxidative stress and thus inhibit the decline in the number of follicles (Amida et al., 2021). Other research states that stress levels in women cause an increase in cortisol which affects the working system of the hypothalamus, and pituitary ovaries, resulting in a reduction in the number of follicles and the formation of dominant follicles will decrease (Setiyono et al., 2015). There is still limited research that reveals the effect of black garlic extract on ovarian repair, so this research aims to prove the effect of black garlic extract on the number of follicles and cortisol levels in female Wistar rats exposed to cigarette smoke. Should be replaced with this sentence: Previous research on female Wistar rats showed that black garlic extract contains flavonoids which can increase antioxidant activity which is said to reduce oxidative stress and thus inhibit the decline in the number of follicles (Amida et al., 2021). Other research states that stress levels in women cause an increase in cortisol which affects the working system of the hypothalamus, and pituitary ovaries, resulting in a reduction in the number of follicles and the formation of dominant follicles will decrease (Setiyono et al., 2015).

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MATERIALS AND METHODS

This research is done by using true experimental which is conducted in vivo. Post-test Only Control Group Design was selected as the study design. The experimental group and the control group were compared in this design, which were not chosen randomly. While the control group did not get therapy, the experimental group did. The independent variables in this study were cigarette smoke and black garlic extract, while the dependent variables were cortisol and secondary follicles. They were collected from Brawijaya University's Pharmacology Laboratory. Single-type black garlic was obtained from producers in Magelang Regency and processed in a rice cooker at a temperature of 340-380 C and incubated for 15 days. The reason the researchers chose this single type of black garlic was because this product was sold and consumed by the public and CA patients, so the researchers applied it to experimental animals. The black garlic extraction process and DPPH antioxidant test were carried out at the Batu Medika Herbal Laboratory UPT and stated that the IC50 of Black Garlic was 173 ppm. The research was conducted for 3 months starting from December 2023 to March 2024 divided into 3 stages, namely 1 week for the acclimatization process, 4 weeks (30 days) for treatment of the samples, and the remaining time for surgery and data analysis. The research was carried out after ethical permission from the ethical commission had been issued No.14/ EC/ KEPK-S2/ 01/ 2024. Before treatment, a rat vaginal swab was performed, and each mouse's estrus cycle was identified by looking at the vaginal smear findings under a light microscope. For ten days, a vaginal swab was taken daily to track the rats' estrous cycles for two consecutive cycles(Akpantah A.O., Oremosu A.A., Noronha C.C., Ekanem T.B. & A.O., 2005).

The study will employ mice with a typical estrous cycle lasting four to five days. By multiplying the number of replications by the number of groups, one may get the sample size. The aforementioned computation yielded five replications for each group, to which 20% was added for the error sample. This resulted in 5 + 0.95 = 5.95, which was rounded to six white rats. 30 heads made comprised the whole sample that was used for the 5 groups. Five groups of thirty sample rats were given the following information:

KO: Normal Control Group That is a group of rats that did not get treatment, consisting of 6 heads that were only given standard feed.

KN: Negative Control Group, namely a group of rats exposed to cigarette smoke up to 2 cigarettes/day for 4 weeks (30 days) but not given black garlic extract (*Allium Sativum*) consisting of 6 mice.

P1: Treatment Group 1 is a group of rats exposed to cigarette smoke up to 2 sticks/day and given a black garlic extract (*Allium sativum*) dose of 50 mg/kg body weight/day for 4 weeks (30 days) consisting of 6 mice.

P2: Treatment Group 2 is a group of rats exposed to cigarette smoke up to 2 sticks/day and given black garlic extract (*Allium Sativum*) dose 100 mg/kg body weight/day for 4 weeks (30 days) consisting of 6 mice.

P3: Treatment Group 3 is a group of rats exposed to cigarette smoke up to 2 cigarettes/day and given black garlic extract (*Allium Sativum*) dose 200 mg/kg body weight/day for 4 weeks (30 days) consisting of 6 mice.

The giving of black garlic extract (Allium Sativum) was carried out in the second to fifth weeks using a probe (sonde) according to the dose determined by each group. The extract was diluted with sterile distilled water with a suspension of 40 mg/ml.
Exposure to cigarette smoke was carried out in the second to fifth weeks every day with 2 cigarettes per day in the morning and afternoon using a smoking pump.
Surgical tests were carried out in the 4th week after previous treatment by conducting vaginal swabs on mice to determine the estrus cycle. Taking heart blood (aortic blood vessels) to examine cortisol concentrations using the ELISA method and ovaries to examine the number of secondary follicles using the HE (Hematoxylin Eosin) staining method.

Cortisol testing via ELISA, specifically the Competitive ELISA method, was utilized in this research due to its accuracy and safety in hormone measurement (Gholib et al., 2021). Initially, all reagents, standard solutions, and samples were prepared, and the number of strips needed for testing was determined and placed in the frame. For the blank control, substrate solutions A and B, along with a stop solution, were added. Subsequently, 50 µL of diluted standard was added to the standard well, 50 µL of sample to the sample well, and 50 µL of biotinylated antigen to each well, followed by thorough mixing. The plate was then covered with a sealer and incubated at 37°C for 60 minutes. After incubation, the sealer and fluids were removed, and the wells were washed five times with 300 µL wash buffer, followed by turning the plate over and shaking it to remove the liquid. Each well was aspirated and washed five times using an automatic washer. The plate was then placed on absorbent material, and 50 µL of avidin-HRP was added to the standard and sample wells. The plate was covered with a sealer and incubated again at 37°C for 60 minutes, followed by a similar washing procedure. Next, 50 µL of substrate solution A and 50 µL of substrate solution B were added to each well, and the plate was incubated with a new sealer in the dark at 37°C for 10 minutes. After incubation, 50 µL of stop solution was added to each well, changing the color from blue to yellow. The Optical Density (OD) value of each well was measured using a microplate reader set to 450 nm, 10 minutes after the stop solution was added.

The procedure for preparing ovarian samples involves several stages. Initially, in the fixation stage, the test material is soaked overnight in a 10% Neutral Buffered Formalin solution, then cut horizontally into 2-3 millimeter sections. These sections are placed in labeled cassettes, further soaked in formalin, and then processed into paraffin blocks. The tissue is cut to a thickness of 3-5 microns using a microtome. In the dehydration stage, the cut tissue is baked at 70-80°C for 30 minutes, placed in xylol for 20 minutes, then successively in ethanol solutions of 70%, 80%, 90%, and 100% for three minutes each, followed by a rinse in running water. During the hydration stage, the tissue is alternately immersed in ethanol solutions from 100% down to 70% for varying durations, totaling 14 minutes. In the coloring stage, the tissue is soaked in Harris Hematoxylin for five minutes and then in 1% Eosin for 3-5 minutes for staining. Rehydration involves immersing the tissue in ethanol solutions from 70% to 100% for durations ranging from two to five minutes. In the clearing stage, the tissue is placed in xylol solution three times for 35 minutes each. Finally, in the mounting stage, entelan liquid is applied to the tissue on the slide, which is then covered with a cover glass. The number of secondary follicles, identified by oocytes surrounded by two to three layers of stratum granulosum, is counted using a Leica Biosystems Aperio CS2 light microscope at 400x magnification across the entire ovarian cross-section (Aryani, HP. et al., 2019). The analysis techniques carried out are by normality test, one-way ANOVA test and post hoc test to use SPSS.

RESULTS AND DISCUSSION

This research used 30 experimental animal samples in the form of female Wistar rats that met the criteria. During the journey, one rat was found that died during treatment on the 19th day of exposure, namely sample no. 7 groups P1 with exposure to cigarette smoke 2 cigarettes/day and black garlic extract at a dose of 50 mg. The percentage calculation results for each group were then analyzed using the ANOVA method which is used in research using more than 2 groups. The test is carried out as a comparison of the means of various sample groups. Before carrying out the ANOVA test, the Shapiro-Wilk test was first carried out to prove that the data obtained had a normal distribution and the homogeneity of variance for each group was tested using Levene's test.

Table 1. Results of Average Cortisol and Number of Secondary Follicles

Observation Group	Cortisol			Secondary Follicles		
	Mean (%)	Standard Deviation	Notation	Mean (%)	Standard Deviation	Notation
KO	7.42450	0.920834	a	5.00	2.966	a
KN	6.85533	1.184239	a	5.00	2.449	a
P1	6.33340	1.752497	a	4.20	1.095	a
P2	5.96717	0.519012	a	6.17	2.858	a
P3	7.25233	1.701386	a	7.00	3.033	a
Sign Normality	= 0.994			= 0.224		
Sign Homogenity	= 0.072			= 0.272		

The ELISA technique, which is a sensitive, precise, and user-friendly way to assess cortisol levels in plasma, was used to investigate cortisol levels in this investigation. This approach is popular for measuring cortisol hormone because it uses an enzyme-based ELISA kit (Cat. No. EA0010Ge) that is based on using enzymes as conjugates.

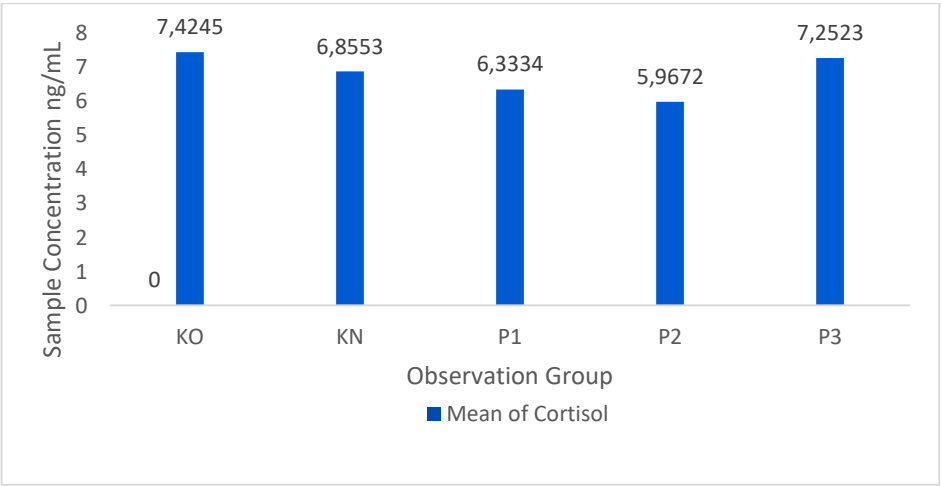

Figure 1. Average cortisol levels

The image above shows the average cortisol levels of mice without intervention (normal control), only exposure to cigarette smoke (control-), as well as three treatment groups exposed to cigarette smoke and Black Garlic extract at different doses, namely 50, 100, and 200mg. From these data, it can be seen that there is a

decrease from normal control to control (-). Then P1 dose of 50 mg and the P2 dose of 100 mg also decreased. Meanwhile, P3 with a dose of 200 mg experienced an increase.

At this stage, using the Shapiro-Wilk Test, the cortisol levels were normally distributed, where the p-value was > 0.05. The following are the results presented.

Table 2. Normality Test Results for Cortisol Levels

Variabel	P-value	Explanation
Cortisol	0.994	Homogen

From the table above, it is proven that the p-value is 0.994 > 0.05, which indicates that the data is homogeneous and can be continued with the ANOVA test. After carrying out the ANOVA parametric test, p=0.282 (p>0.05) was obtained. It is known that cortisol levels did not differ significantly between groups. Post-hoc tests are carried out to find groups that have the same or different means. Following are the results using the Tukey HSD Post-Hoc Test.

Table 3. Post-Hoc Test Results: Tukey HSD Cortisol

Observation Group	Average Difference ± SD	P-Value
Normal Control vs Control (-)	0.56917	0.937
Control (-) vs P1	0.52193	0.960
P1 (50 gr) vs P2	0.36623	0.989
P2 (100 gr) vs P3	1.28517	0.431
P3 (200 gr) vs Control (-)	0.39700	0.983

*P-value<0,05 is meaningful

Based on the table above, it is shown that there is a comparison of the average rat cortisol between groups. From the calculation results, it can be seen that normal control, control (-), P1, P2, and P3 have a mean that is not significant with a p-value > 0.05.

The growth of primary follicles into secondary follicles produces larger granulosa cells, which are distinguished by the presence of a vitelline membrane, a thin layer on the oocyte, and a zona pellucida (Aryani, HP. et al, 2019). Figure 3 is secondary follicles from each group taken from the results of painting using HE staining.

The research carried out homogeneity in the proestrus phase of the estrous cycle, namely that there were pre-antral to antral follicles (including secondary follicles). The HE staining slides were scanned using an Olympus SZ Microscope, 1 field of view with 30x magnification over the entire cross-section of the ovary, then the number of secondary follicles was counted which was characterized by the presence of a zona pellucida and a thin covering on the oocytes in each group. Furthermore, an illustration of the average number of antral follicles in mouse ovaries can be seen in the Figure 3.

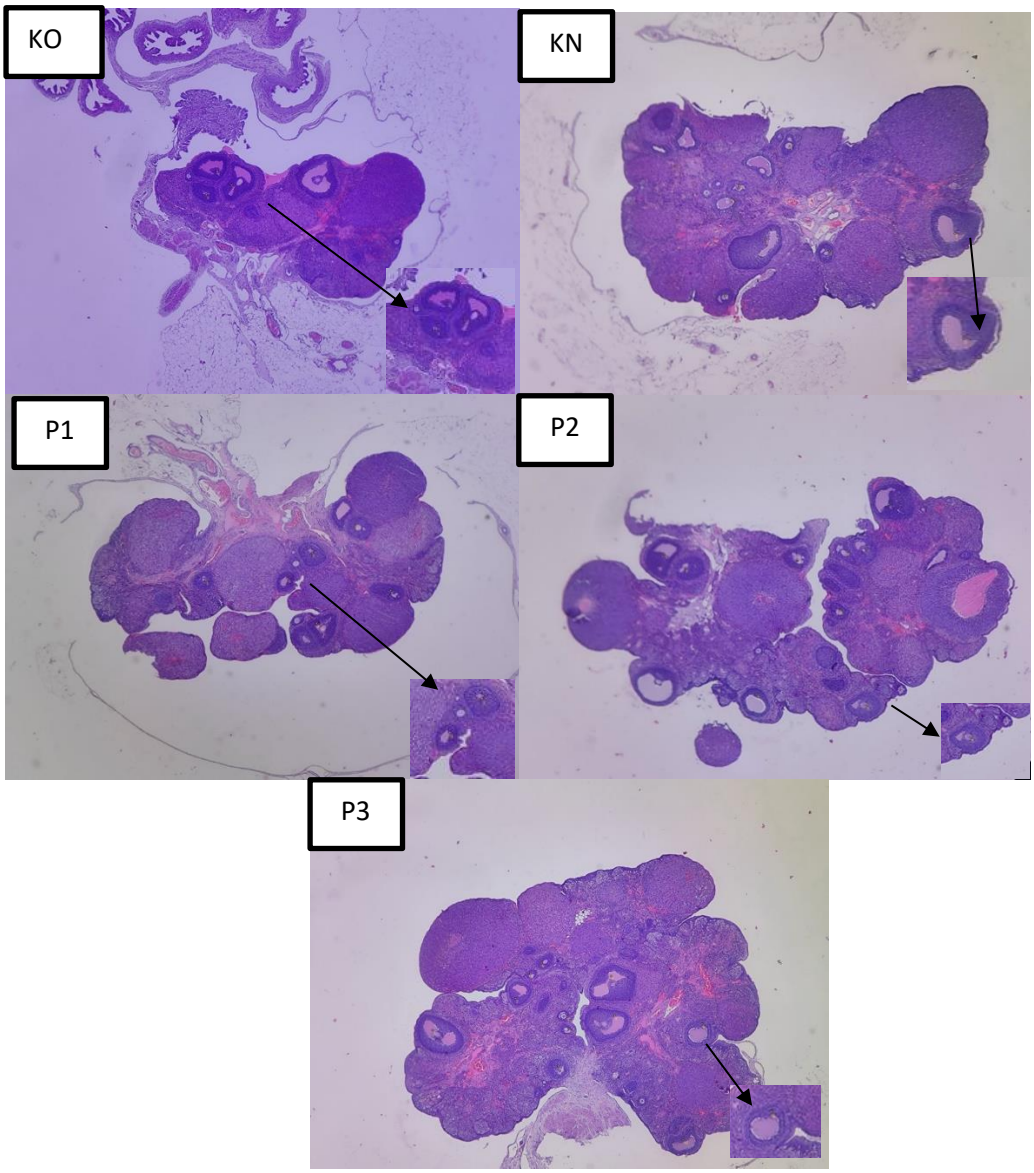

Figure 2. HE Staining Results of Secondary Follicles. KO:Normal Control; KN:Control Negative; P1:Treatment1; P2:Treatment2; P3:Treatment3. Yellow circles represent secondary follicles; Black arrows show enlarged images of secondary follicles on the ovaries.

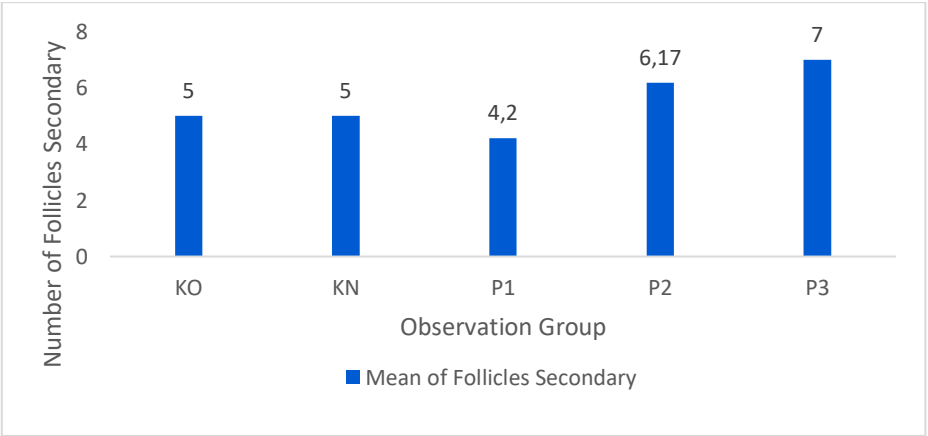

Figure 3. Average Number of Secondary Follicles

It was found that the average number of secondary follicles in the ovaries of mice without intervention (normal control), only exposure to cigarette smoke (control negative), and three treatment groups were exposed to cigarette smoke and Black Garlic extract at different doses, namely 50, 100 and 200 mg. From these data, it can be seen that the average number of secondary follicles between normal controls and controls (-). Then the number of secondary follicles in P1 with a dose of 50 mg decreased, while P2 with a dose of 100 mg and P3 with a dose of 200 mg increased.

At this stage, using the Shapiro-Wilk Test, the number of secondary follicles was normally distributed, where the p-value was > 0.05. The following are the results presented.

Table 4. Normality Test Results for Number of Secondary Follicles

Variabel	P-value	Explanation
Secondary Follicles	0.224	Homogen

From the table above, it is proven that the p-value is 0.224 > 0.05, which indicates that the data is homogeneous and can be continued with the ANOVA test. After carrying out the ANOVA parametric test, p = 0.427 (p>0.05). It is known that the number of secondary follicles did not differ significantly between each group. Post-Hoc tests are carried out to find groups that have the same or different means. Following are the results using the Tukey HSD Post-Hoc Test.

Table 5. Post-Hoc Test Results: Tukey HSD Secondary Follicles

Observation Group	Average Difference ± SD	P-Value
Normal Control vs Control (-)	0.000	1.000
Control (-) vs P1	0.800	0.986
P1 (50 gr) vs P2	1.967*	0.731
P2 (100 gr) vs P3	0.833*	0.981
P3 (200 gr) vs Control (-)	2.000	0.683

*P-value<0,05 is meaningful

Based on the table above, it can be seen that there is a comparison of the average number of secondary follicles in mice between groups. From the calculation results, it can be seen that normal control, control (-), P1, P2 and P3 have a mean that is not significant with a p-value > 0.05.

The Effect of Cigarette Smoke Exposure on Increased Cortisol Levels and Decreased Number of Secondary Follicles.

The research showed that there was no effect due to exposure to cigarette smoke with an increase in cortisol and a decrease in the number of secondary follicles in the ovaries of female Wistar rats. Research shows that there is no effect of exposure to cigarette smoke in increasing cortisol levels in Rattus norvegicus ovaries.

This is in line with research by Armalini & Friadi (2019) which proves that there is no significant effect of exposure to cigarette smoke on increasing cortisol in the ovaries of Rattus norvegicus. This could be because the work of cortisol is not solely influenced by exposure to cigarette smoke but also by several contributing factors such as physical and non-physical stress, for example, pain, fear, infection, trauma, hypoglycemia, and the influence of drugs such as corticosteroids. The occurrence of an increase in cortisol in normal controls compared to controls (-) is not significant, while the decrease in cortisol levels in KP 1 compared to the increase in cortisol levels

in KP 2 and KP 3 can be said to be a coincidence because the average shows a value of cortisol levels close to or almost the same as normal controls without intervention. So it could be said that this happened due to chance or it is possible that the cortisol levels in each mouse had different variations at the beginning before the treatment was carried out.

Normal serum cortisol levels range between 3.95 ng/mL - 27.23 ng/mL (Adisty et al, 2015). A community study said that women's stress levels cause cortisol levels to increase which will affect the working system of the Hypothalamus Pituitary Ovaries resulting in a small number of follicles. So if cortisol levels increase, the possibility of dominant follicle formation will decrease (Setiyono et al., 2015). In this case, the researchers continued previous research and tested directly on Rattus norvegicus mice and this was the first research.

It is not proven that there is an influence between exposure to cigarette smoke on reducing the number of secondary follicles in the ovaries of Rattus norvegicus in this study, seen from the results of table 5.1, this is in line with the results of other studies which state that cigarette smoke will not affect the fertility of Rattus norvegicus, which has more of an impact on ovarian weight (Margarisa et al., 2023). But this is contrary to other researchers who say the effects of cigarette smoke are bad for women's ovaries. Apart from that, the bad effects are determined by the amount and length of time a woman is exposed to cigarette smoke. It is also stated that cigarette smoke inhibits the function of the reproductive organs and causes premature menopause (Jumiati et al., 2012). Exposure to cigarette smoke has a negative impact on the body, especially the ovaries. Cigarettes that enter the organ system will damage every organ movement. Ovaries that are exposed to substances in exposure to cigarette smoke will also experience problems in their performance. In the Talakua & Unitly (2020) study, 10 cigarette smoke exposures were used over a period of 28 days, thus providing more effective results due to the large amount of exposure given.

The number of secondary follicles in the ovaries of Rattus norvegicus and elevated cortisol levels following exposure to cigarette smoke were not related, according to the findings has been shown in Table 5.1.2. The results of this study are in line with Armalini & Friadi's research which states that there is no significant effect between cigarette smoke exposure to increased cortisol levels in the ovaries of *Rattus norvegicus(Armalini & Friadi, 2019)*. This can occur because of a variety of variables, such as physical and non-physical stress (pain, fear, illness, trauma, hypoglycemia, and the impact of medications like corticosteroids). It can affect cortisol levels in addition to exposure to cigarette smoke. The average showed cortisol levels that were nearly identical to a normal control group that did not receive any treatment. The occurrence of an increase in cortisol levels in the normal control group relative to the control group (-) became meaningless, while the decrease in cortisol levels in P1 relative to the increase in cortisol levels in P2 and P3 can be considered a coincidence. Therefore, it is possible to argue that this occurred by chance or that each mouse's initial cortisol level varied before the therapy was administered.

Serum cortisol levels should range between 3.95 to 27.23 ng/mL. According to community research, women who experience high levels of stress also have high blood cortisol levels, which can impact the pituitary ovary's hypothalamus axis by reducing the number of dominant follicles. The number of dominant follicles decreases with increasing cortisol levels (Setiyono et al., 2015). This study is one of the first studies which was conducted directly on Rattus norvegicus rats.

The findings also demonstrated that there was no correlation between exposure to cigarette smoke and a decline in the number of secondary follicles in Rattus

norvegicus ovaries. This assertion is consistent with the findings of earlier research, which found that ovarian weight had a greater bearing on Rattus norvegicus fertility than cigarette smoke does (Margarisa et al., 2023). However, the findings of other research indicate that exposure to secondhand smoke damages women's ovaries with the severity of the damage varying according to the quantity and duration of a woman's exposure. Cigarette smoke interferes with reproduction and causes women's menopause to occur sooner (Jumiati et al., 2012). In general, there is evidence showed smoking has a harmful effect on the body, particularly the ovaries. When cigarettes enter the body, they harm the organs' ability to move. Ovaries that are exposed to chemicals from cigarette smoke will also have issues with their functionality. Talakua & Unitly (2020) stated that exposure to cigarette smoke was used as much as 10 cigarettes in 28 days. This is done to provide more effective results due to the amount of exposure given.

This study was conducted based on research (Dewita, 2021) which stated that exposure to cigarette smoke in female Rattus norvegicus of 2 cigarettes/day for 56 days decreased the hormone estradiol, causing less than optimal growth of follicular cells in the ovaries and research (Nasution et al., 2016) who stated that the results of their research showed that a significant increase in MDA (*Malondialdehyde*) levels was obtained from exposure to cigarette smoke of 2 cigarettes/day for 28 days. So researchers assume that conducting research using 2 cigarettes/day for 30 days.

It was also stated in a study of variations in the length of exposure to cigarette smoke that the length of exposure was the main factor in increasing MDA levels. In this case, it can be concluded that the longer the exposure to cigarette smoke, the higher the MDA levels (Armadiyanti et al., 2018). Increasing MDA levels indicate a cell membrane oxidation process which causes non-stop fat peroxidation. MDA can interact with a variety of biological molecules which can ultimately cause organ damage. So the researchers concluded that the absence of this relationship was probably due to exposure to cigarette smoke that was less abundant and less long (Armadiyanti et al., 2018)

The Effect of Black Garlic (*Allium sativum*) Extract on Decreasing Cortisol Levels and Increasing the Number of Secondary Follicles in the Ovaries of *Rattus Norvegicus* Exposed to Cigarette Smoke.

The results showed that Black Garlic (Allium sativum) extract did not affect the decrease in cortisol due to exposure to cigarette smoke as shown in Table 5.1. Judging from the mean value, the decrease in cortisol levels in controls (-) compared to normal controls is not significant, while the decrease in cortisol levels in P1 with a dose of 50 mg BG, P2 with a dose of 100 mg BG, and P3 with a dose of 200 mg BG shows something significant. positive when compared with normal controls who were not given any treatment. So it can be said that this happens because there is a good effect of giving black garlic extract on reducing levels of the hormone cortisol if there is no exposure to cigarette smoke.

In this case, black garlic extract is said to increase antioxidant activity and prevent cell damage. Cortisol levels help the ovulation process but inhibit ACHT secretion. Several previous studies have proven that black garlic extract should be able to reduce cortisol levels. According to research by Wiliyanarti & Wahyullah (2021), giving black garlic extract affects reducing the hormone cortisol but is not effective.

Likewise, judging from the results of the average number of secondary follicles, there is an increase in control (-) compared to normal control which is not significant,

while the increase in the number of secondary follicles in KP 2 (BG 100 mg) and KP 3 (BG 200 mg) compared to KP 1 (BG 50 mg) shows a positive thing, because the average shows a higher number of secondary follicles than normal controls that were not given any treatment. So it could be said that this happened because there was a good effect from giving black garlic extract.

Black garlic extract contains flavonoids which can increase antioxidant activity which is said to reduce oxidative stress so that it can inhibit the decline in the number of follicles (Amida et al., 2021). This is the same as the results of other studies which say that Black Garlic Extract (Allium sativum) itself contains polyphenols which are useful for antioxidants in the body (Juniantari & Susanti, 2023). So in this case, Black Garlic (Allium sativum) extract should be able to help increase the number of secondary follicles in the ovaries. If the number of follicles increases, it will affect the development of capacity in the Rattus norvegicus ovaries. No literature proves this, so this is the first proof of the effect of black garlic extract on increasing the number of follicles and cortisol levels.

A single type of black garlic was processed in a rice cooker at a temperature of 340-380 C and incubated for 15 days. The reason the researchers chose this single type of black garlic was because this product was sold and consumed by the public and CA patients, so the researchers applied it to experimental animals. The research literature states that heating black garlic is between 60-700 C because using temperatures above 700 C can damage the structure of reducing sugars, while temperatures below 600 C require a very long time in the process of making black garlic. The optimal heating time is 35 days to prevent free radicals, which produce many flavonoid compounds, tannins, sterols, and saponins (Agustina et al., 2020). Black garlic is generally processed at a temperature range of 30-900 C with relative humidity of 50-90% and an incubation period of 10-80 days. Several aspects can influence the properties and nutrition of Black garlic, including processing technology and processing variables such as time, temperature, humidity, pH, and type of pre-treatment (Ahmed & Wang, 2021).

The results of the Black garlic DPPH antioxidant test were carried out at the Batu Medika Herbal Laboratory UPT and were stated as Black garlic IC50 173 ppm. Antioxidant activity based on the IC50value is divided into three groups, namely [1] High antioxidant activity with an IC50 value <100 ppm (µg/mL), [2] Medium antioxidant activity with an IC50 value of 100-500 ppm (µg/mL), [3] Low antioxidant activity with IC50 value>500 ppm (µg/mL) (Wardhani et al., 2020). So black garlic antioxidants in this study were classified as moderate antioxidants.

Researchers suspect that exposure to cigarette smoke is less effective, causing less relevant research results. Therefore, further research on experimental animals is needed to provide a deeper understanding of the therapeutic potential of Black garlic. The limitations of this study are shown by the study findings which are not significant or do not show a relationship between the variables studied. There was no effect of exposure to cigarette smoke on cortisol levels and the number of secondary follicles. Researchers assume that the number of cigarettes consumed per day is the main role in increasing cortisol levels and ovarian conditions so the length of exposure is one of the limitations.

CONCLUSION

The results of the study showed that there was no effect due to exposure to cigarette smoke with an increase in cortisol and a decrease in the number of secondary follicles in the ovaries of female wistar rats as well as the administration of

Black Garlic (Allium sativum) extract. Black garlic extract unaffected cortisol levels and the number of secondary follicles or repair of the ovaries due to exposure to cigarette smoke. Further research is needed in dosing black garlic extract or combining it with other ingredients to provide effective results.

CONFLICT OF INTEREST

The author declares that no significant issues were found in this research.

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