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In vivo study: black cumin extract (*Nigella sativa*) on antral follicle diameter and granulosa cell layer thickness as an effort to prevent infertility

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A B S T R A C T

Background: Infertility is a health problem that affects approximately 10-15% of couples trying to achieve pregnancy worldwide. Various studies have shown that *Nigella sativa* (*N. Sativa*) has many health benefits, including reproductive health. However, studies examining the effectiveness of *N. Sativa* in preventing folliculogenesis disorders have never been reported.

Purpose: This study aims to determine the benefits of administering *N. Sativa* in preventing folliculogenesis disorders as measured by the diameter of the antral follicles and the thickness of the granulosa cell layer.

Methods: This research design uses an experiment with a randomized posttest-only controlled group design approach. There were 27 experimental animals (mice) divided into three groups, namely the negative control group (9 animals) and the positive control group (9 animals), as well as the treatment group (9 animals). The positive control group was exposed to two cigarettes of cigarette smoke per day. The treatment group was exposed to cigarette smoke and *N. Sativa* 300 mg/KgBW/day for 28 days. After that, the mice were sacrificed to take their ovaries. Then, the ovarian organ samples were stained with Hematoxylin-eosin and examined for the diameter of the antral follicles and the thickness of the granulosa cell layer in the antral follicles with photo slides at 400x magnification in 5 fields of view, statistical analysis using the Independent T-test.

Results: There were significant differences in antral follicle diameter and granulosa cell thickness in mice exposed to cigarette smoke and *N. Sativa*. Administration of *N. Sativa* extract can increase granulosa cell thickness through estrogenic function. Administration of *N. Sativa* extract showed estrogenic activity by increasing serum estradiol levels. *N. Sativa* heterotrophic activity cannot be separated from the flavonoid and phenolic compounds contained in *N. Sativa*, which have been proven to have high estrogenic activity.

Conclusion: *N. Sativa* extract is efficacious in improving the diameter of antral follicles and the thickness of granulosa cells caused by exposure to cigarettes.

INTRODUCTION

Infertility in couples of childbearing age worldwide is estimated at around 50-80 million.[1] Data from WHO in 2013 states that the incidence of infertility in Asia is between 8-12%.[2] In Indonesia, 15-20% of the population experiences infertility.[3] Sa 'Adah and Purnomo think that two factors influence the incidence of infertility, namely internal and external. Internal factors include hormonal disorders, tumors, and ovarian cysts. External factors, including age, smoking habits, and alcohol, also affect fertility.[4] Cigarette smoke is a pollutant for humans and the surrounding environment. Many medically diagnosed diseases are caused by cigarette smoke, either directly (active smoking) or indirectly (passive smoking).[5] Indonesia ranks third in the country with the highest cigarette consumption in the world after China and India, with a smoking population of 65.2 million, including 4.8 million female smokers. The prevalence of passive smoking in Indonesia is 48.9% or 97,560,002 people. Meanwhile, the prevalence of passive smoking in women of childbearing age (15-49 years) is 66%.[6]

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Cigarette smoke containing tar, nicotine, and carbon monoxide enters the respiratory tract as solid vapor and is the primary source of exogenous prooxidants. An imbalance between prooxidants and antioxidants causes oxidative stress. Oxidative stress causes damage and malfunction of cells through DNA, proteins, and lipids. Lipid peroxidation is one of the most biologically important free radical reactions. The membrane lipid peroxidation reaction causes MDA to increase.[7] Previous research results stated that exposure to cigarette smoke could increase MDA levels in the ovaries, especially in oocytes, granulosa cells, and theca cells.[8]

High MDA levels in the ovaries cause cell damage and inhibit folliculogenesis. Folliculogenesis can proceed normally if the pre-antral and antral phases develop ideally. The gonadotropin-dependent antral phase is characterized by a rapid increase in follicle size (±25-30 mm). The thickness of the granulosa cell layer is used as a reference for cell homeostasis in maintaining the continuity of folliculogenesis because a smaller number of granulosa cell proliferation can improve oocyte quality.[9] Antral follicle size is an essential predictive marker in women undergoing fertility treatment, where women with antral follicle sizes of 6–7 mm tend to have better fertilization outcomes.[10]

Nigella sativa (*N. Sativa*) seeds are one of the medicinal components commonly used in the Ayurveda and Tibbenabvi (Prophetic Medicine) systems.[11] *N. Sativa* and all its components, especially thymoquinone, have the potential to ward off radicals and inhibit oxidative stress by increasing the production of antioxidant enzymes.[12] Based on previous research, black cumin can reduce amenorrhea and blood MDA levels in PCOS patients due to the thymoquinone content in black cumin oil as an antioxidant.[13] Other research states that giving black cumin extract, rich in essential oils, phenolics, flavonoids, and estrogenic, can increase estrogen, GDF-9, the number of primary follicles, antral follicles, and ovarian diameter.[8,13]

A study found that thymoquinone had a better effect on ovarian function in PCOS model mice.[14,15] The novelty of this research is about the benefits of administering *N. Sativa* in preventing folliculogenesis disorders. This research has an essential contribution in identifying the benefits of administering *N. Sativa* extract in preventing folliculogenesis disorders as measured by the diameter of the antral follicles and the thickness of the granulosa cell layer. Therefore, this study aims to determine the benefits of administering *N. Sativa* in preventing folliculogenesis disorders as measured by the diameter of the antral follicles and the thickness of the granulosa cell layer.

METHOD

Study Design

This research is an experimental study with a randomized posttest-only controlled group design.[16]

Study Site

This research was conducted at the Pharmacology Laboratory, Faculty of Medicine, Muhammadiyah Lamongan University.

Material

The materials used in this research included 1000 grams of *N. Sativa* seeds, 96% ethanol, cigarettes, and 27 female Wistar rats aged 2-3 months. Equipment consisted of rat cages, rat food, body scales, scalpels, gloves, Hematoxylin-Eosin (HE) staining and a Nikon E100 microscope.

Plant Extraction Process

N. Sativa seeds are blended until smooth. 200 mg of *N. Sativa* powder was extracted with 1 liter of 96% ethanol. After extraction, the solution was dried with an evaporator until a blackish-brown concentrate was obtained, which would be used in research.[17]

In Vivo Procedure

Animal Preparation

In this study, 27 female mice weighing 150–200 g were obtained from the mouse breeding center in Malang, Indonesia. Mice were placed at 23 ± 2 ºC for a 12-hour light-dark cycle.[18]

Experimental Procedure

The mice were divided into Negative control, Positive control, and Treatment groups (n=9 per group). The negative control group was only given 1% CMC without cigarette smoke; the positive control group was given cigarette smoke, and the treatment group was given *N. Sativa* 300 mg/KgBB for 28 days. On day 29, mice were sacrificed. Then, surgical removal of the ovaries and staining with hematoxylin-eosin were performed.[19]

Statistical Analysis

The Independent T-test was performed to determine the differences in each group's antral follicle diameter and granulosa cell thickness.

Ethical Consideration

Research this experimental design and animal handling procedures have been reviewed and approved by the guidelines of the Airlangga University animal handling ethics committee (No.134/EC/KEPK/FKUA/2022).

RESULTS

Histopathological Image of Antral Follicle Diameter

Based on Figure 1, observations at 400x magnification, the antral follicles in the mice in the negative control group appear clear and long (Figure 1A), and the antral follicles in the positive control group seem to have damage to the antral follicles. They are short in shape (Figure 1B). Diameter and antral follicles in the treatment group look clear and long (Figure 1C). Table 1 shows that the treatment group showed the highest average antral follicle diameter of 194.73±44.53 µm. In contrast, the positive control group (cigarette smoke exposure) showed the lowest average antral follicle diameter of 106.06±11.13 µm. The treatment group gave the three groups the highest average number of antral follicle diameters. The statistical analysis showed that administering *N. Sativa* extract can significantly increase the diameter of antral follicles (p<0.0001).

Histopathological Features Granulosa Cell Thickness

Based on Figure 2, observation at 400x magnification, the granulosa cells of mice in the negative group appeared clear and thick (Figure 2A), the granulosa cells in the positive group seemed to be thin (Figure 2B), and the granulosa cells in the treatment group it looks clear and thick (Figure 2C). Table 1 shows the negative control group showed the highest average granulosa cell thickness of 39.95±16 µm, then the treatment group 37.95±9.83 µm, while the positive control group (cigarette smoke exposure) showed the lowest average granulosa cell thickness of 20.64±3.54 µm. The statistical analysis showed that administering *N. Sativa* extract can significantly increase granulosa cell thickness (p<0.0001).

DISCUSSION

This study found that exposure to cigarette smoke in the positive control group caused damage to the antral follicles, characterized by a small antral diameter compared to the negative control group and the treatment group. Exposure to cigarette causes an increase in Reactive Oxygen Species (ROS) and causes cell death. The benefits of administering *N. Sativa* extract in preventing folliculogenesis disorders are measured by the diameter of the antral follicles. Cell layer, indicating that antral follicles can receive a response from Follicle-stimulating hormone (FSH) and Luteinizing Hormone (LH) to develop, thereby increasing the number of granulosa cells that produce estradiol.[15] If the diameter of the antral follicle is small, the FSH and LH response function is low, so estrogen will also be low.[20] Administration of *N. Sativa* extract can improve the diameter of antral follicles in mice exposed to cigarette smoke. *N. Sativa*, with the active ingredient thymoquinone, can ward off ROS by increasing antioxidant enzymes, namely superoxide dismutase (SOD). The decrease in ROS causes the function of the antral follicles to become routine, and the follicles can mature.[13]

Table 1. Effect of *N. Sativa* on Antral Follicle Diameter and Granulosa Cell Thickness

Variable	Negative Control	Positive Control	Treatment Group	p-value
Antral Follicle Diameter (µm)	190.94 ± 54.83	106.06 ± 11.13	194.73± 44.53	0.001
Granulosa Cell Thickness (µm)	39.95 ± 16	20.64 ± 3.54	37.95 ± 9.83	

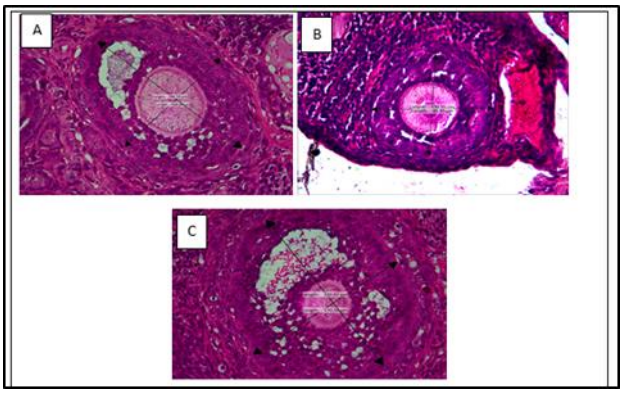

Figure 1. Antral Follicle Diameter. Black arrows explain the morphology of antral follicle diameter in the Negative control group (A), Positive control group (B), and Treatment group (C)—400x magnification.

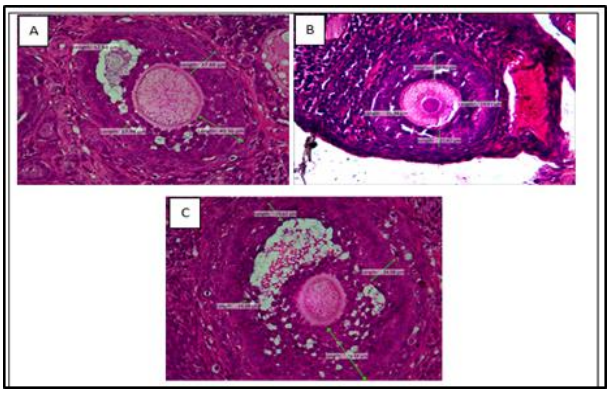

Figure 2. Granulosa Cell Thickness. The green arrow Explains the morphology of granulosa cell thickness in the Negative control group (A), Positive control group (B), and Treatment group—400X magnification.

The hormone estrogen is produced in granulosa cells. The thickness of granulosa cells greatly influences estrogen hormone levels. Based on this research shows that exposure to cigarette smoke can cause a decrease in

granulosa cell thickness compared to the negative and treatment groups. This is due to increased oxidative stress due to exposure to cigarette smoke. Granulosa cell thickness is essential in communicating granulosa cells and oocytes for follicular growth and fertility, considering that oocytes can complete maturation and reach full size only with the support of granulosa cells.[10]

Components of cigarettes, such as nicotine, CO2, Lead, Cadmium, TAR, and other components, cause infertility through damage or apoptosis of ovarian follicles. A Previous study found that exposure to cigarette smoke once a day in mice caused an increase in ROS in the body.[7] Systemically, an increase in ROS in the body causes apoptosis of granulosa cells, oocytes and theca cells in ovarian follicles.[21] The size of antral follicles is influenced by follicular apoptosis in the ovaries. It was stated that the small size of antral follicles causes low gonadotropin levels so that follicle growth is disrupted.[22]

Lead poisoning can reduce the diameter of antral follicles. Infertility is associated with decreased number and diameter of antral follicles.[23] Antral follicle diameter significantly reduced granulosa cell thickness.[24] Ovarian granulosa cells are follicular somatic cells whose origin, development, and function are closely related to the differentiation of female gametes, the formation of ovaries, and the normal development of the female reproductive cycle. Changes in the granulosa cell cycle are thought to be responsible for disrupting follicle growth and oocyte maturation.[25] Administration of *N. Sativa* extract can increase granulosa cell thickness through estrogenic function. Administration of *N. Sativa* extract showed estrogenic activity by increasing serum estradiol levels.[23] *N. Sativa* heterotrophic activity cannot be separated from the flavonoid and phenolic compounds contained in it, which have been proven to have high estrogenic activity.[26]

It is known that the highest average diameter of antral follicles was in the treatment group with a dose of 300 mg/KgBW of black cumin, and the lowest was in the positive control group given cigarette smoke ($p<0.0001$), which means that statistically, there is a significant difference in the administration of the extract. *N. Sativa* on antral follicle diameter.[25] Likewise, the average granulosa cell thickness was highest in the treatment group with a dose of 300 mg/KgBW of *N. Sativa* and lowest in the positive control group given cigarette smoke ($p<0.0001$), which means that statistically, there was a significant difference in the administration of the extract. *N. Sativa* on estrogen cell thickness.[26] *N. Sativa*, rich in phenolics, flavonoids and estrogens, can increase estrogen levels. The estrogenic effect of *N. Sativa* can increase the thickness of granulosa cells; if the granulosa cells become thick, then the diameter of the antral follicles becomes longer and develops better. [22,26,27]

CONCLUSIONS AND RECOMMENDATION

N. Sativa extract can improve the diameter of the antral follicles and granulosa cell thickness. The active ingredients thymoquinone, flavonoid and phenolic in *N. Sativa* can ward off ROS, and by increasing serum estradiol levels, follicles can mature. Clinical trial studies are needed to see the effectiveness of *N. Sativa* extract in increasing fertility.

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