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Effect of Red Fruit Oil on Ovarian Follicles Development in Rat Exposed to Cigarette Smoke

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Abstract

Red fruit oil (*Pandanus conoideus* Lam) contains active substances in the form of alpha-tocopherol, beta-carotene and unsaturated fatty acids that can potentially be antioxidants. This study aims to examine the effect of red fruit oil (*Pandanus conoideus*) on the development of ovarian follicles of rat exposed to cigarette smoke, (in increasing the number of primary follicles, secondary follicles, tertiary follicles and ovarian weight). This study used Completely Randomized Design with 20 female rats (3 months old) divided into 4 treatment groups: P0 (Positive control), P1 (negative control of exposure to cigarette smoke for 8 days), P2 (exposure to cigarette smoke for 8 days + 0.1 ml red fruit oil) and P3 (exposure of cigarette smoke for 8 days + 0.2 ml red fruit oil) with 5 time repetition and 28 days red fruit treatment for the research parameters were the number of primary follicles, secondary follicles, tertiary follicles and ovarian weight. The data obtained were analyzed using one-way ANOVA with 95% confidence level ($P < 0.05$). The results showed that the administration of red fruit oil at doses of 0.1 ml and 0.2 ml was not significantly affecting ($P > 0.05$) the number of primary follicles, secondary follicles, tertiary follicles and ovarian weight, so it can be conclusion that the administration of red fruit oil at a dose of 0.1 ml and 0.2 ml cannot increase the number of primary follicles, secondary follicles, tertiary follicles and ovarian weight in rat exposed to cigarette smoke.

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INTRODUCTION

Ovaries are a pair of primary reproductive organs in a female animal located, inside the abdominal cavity and functioning to produce an ovum and female reproductive hormone. Ovaries are the sites of follicular development from primary follicles to, secondary follicles, tertiary follicles and finally de Graaf follicles as the ovulatory ovum (Mader, 2012). The development of ovarian follicle outline is divided into two phases i.e. prenatal phase and the antral phase. Preantral or gonadotropin-independent phase is characterized by the oocyte growth, while the antral phase or gonadotropin dependent phase is characterized by a considerable increase in follicle size of approximately 25 mm. The antral phase is governed by Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) as well as other growth factors (Gannon, 2013).

The follicular phase of the pituitary ovary cycle secretes a small portion of the hormone FSH and LH in response to GnRH from the hypothalamus. Immature ovarian follicle cell is used as FSH hormone receptors. LH hormone acts on theca cells to stimulate androgen production, while the FSH hormone in granulosa cells increase the conversion of androgens into estrogen. The estrogen hormone produced by the growing follicle is released into the blood causing a continuous rise in plasma estrogen levels during the follicular phase. The remaining estrogen hormone remains inside the follicle which contributes to forming the antrum fluid and stimulates the further proliferation or granulosa cells. During the follicular phase, LH secretion is elevated and leads to an increase of estrogen concentration as well as the growth of de Graaf follicle (Sherwood, 2014).

Chemicals content in cigarettes causes various types of health problems such as disturbances in the female and male reproductive systems. According to Furlong *et al.,* (2017) there are approximately 48.5 million couples in the world experiencing reproductive fertility disorders. Reproductive disorders can be caused by the presence of free radicals, one of them comes from cigarettes. It is because in one cigarette there are 4000 chemicals such as 90% of gas and 20% of the particle. The gases contained in cigarettes are CO, CO_2, cyanide, hydrocarbons, and organic acids, while the other particles contained in cigarette are nicotine, tar and carbon monoxide (Dwirahayu *et al.,* 2016).

According to a study conducted by Bordel *et al.,* (2006), the provision of nicotine exposure for seven days in a hamster can lead to a developmental disorder of the ovary follicle up to the ovulation stage. The content of nicotine, tar, and carbon monoxide in cigarettes causes the occurrence of free radicals. Free radicals that enter the body in excessive amounts causes oxidative stress. It occurs when the number of free radicals is more than the endogenous antioxidants. Production of free radicals that exceed the ability of endogenous antioxidants can cause cell damage. An increase in oxidative stress has a negative effect on some components of the cell membrane i.e, damage to lipid membranes form malonaldehyde (MDA), damage to proteins, carbohydrates and DNA, which can disrupt all cells and tissues in the body including impaired development of ovarian follicles (Surasana *et al.,* 2013).

Red fruit oil contains beta-carotene 500 ppm, and alpha-tocopherol 700 ppm, which is indicated that red fruit oil can be used as one the source of antioxidants (Budi, 2005). Antioxidants beta-carotene in reducing oxidative stress due to exposure to cigarette smoke is to break the chain reaction of peroxidase unsaturated lipids in the brain. The content of beta-carotene has the potential to bind the activity of single oxygen that triggers the formation of free radicals. Beta-carotene can react with peroxyl radicals which are carotenoid peroxide radicals (Rizqi *et al.,* 2016).

Unsaturated fatty acids contained in red fruit oil can be used as a cholesterol forming agent. Unsaturated fatty acids will be converted to cholesterol in the liver. The fatty acids together with coenzyme form the Ko-A acyl molecule. Acyl-KoA is dehydrated in alpha and beta carbon to produce double bonds. The hydrogen atom binds to the beta carbon. Two hydrogen atoms of alpha carbon and from hydroxyl groups bind to beta carbon. The compound between alpha and beta carbon is detached. The long chain part binds to the coenzyme A molecule while the short chain present in acetyl part remains bonded to the coenzyme A forming acetylene-KoA. Mevalnoat formed from acetyl-KoA releases CO_2 and forms an isoprenoid unit. Six isoprenoid units from a steroid mother lanosterol that will be converted into cholesterol (Na'ima, 2012).

Cholesterol formed acetyl-KoA forms a steroid hormone extracted from intracell ester cholesterol stores or absorbed by cells in the form of lipoproteins that are internalized throught a process mediated by plasma membrane receptors. Biosynthesis of sex steroid in the adrenal cortex and gonads, requires four cytochrome P450 enzymes, monooxygeneses, which play a role in the transfer of electrons from NADPH via an inter-

mediate protein transfer of electrons to molecular oxygen that oxidizes (Marks, *et al.,* 2000).

The synthesis of steroid hormones will be converted to estrogen and progesterone. The steroid hormone binds to the plasma protein and estradiol binds to a globulin transport known as hoemone-wall globulin sex and binds weakly to albumin, while estrogen binds strongly to albumin. The circulation of estradiol is rapidly converted to estrone in the liver with the help of 17-hydroxysteroid dehidrogenase. Some estrone into the circulation and some will be metabolized into hydroxy estrone that is converted to estriol. The production of estradiol at the beginning of the cycle will decrease to the lowest point, but because of the influence of the hormone FSH estradiol will begin to increase. Estradiol levels will increase until ovum maturation and estradiol levels will peak until the ovulation, while the luteal phase estrogen levels will decrease. The progesterone produced by the corpus luteum together with estrogen will provide a negative feedback on the hypothalamus and anterior pituitary (Ramadhani, 2017). The purpose of this study was to analyze the effect of red fruit oil (*Pandanus conoideus*) administration on the ovarian weight as well as the number of primary follicles, secondary follicles, and tertiary follicles. The benefits of this research are, as scientific information of red fruit oil (*Pandanus conoideus*) on the development of ovarian follicles that can be used as a reference to overcoming the reproductive system disorders caused by exposure to cigarette smoke.

METHODS

Sample of Research

The method used in this study was Completely Randomized Design with four treatment groups and five replications on each group. The samples used in this study were 20 female rats (*Rattus norvegicus*) which was about 3 months old with an average weight of 150-200 g. Permission for using these animal has been granted by Ethical Committe of Health Research, Dr. Kariadi General Hospital, Faculty Of Medicine, Universitas Diponegoro, Semarang, number 39/EC/FK-RSDK/V/2018. The red fruit oil used in this study was obtained from Agro Herbal Husada Wamena Papua, Indonesia.

Sample Group

After the acclimatization process for one week, the rat was grouped into four groups randomly. Each group contained five rats.

Group P0: without cigarette smoke and red fruit oil.

Group P1: cigarette smoke without the administration of red fruit oil

Group P2: cigarette smoke + the administration of red fruit oil 0.1 ml BB/ day.

Group P3: cigarette smoke + the administration of red fruit oil 0.2 ml BB/ day.

The treatment of exposed by cigarette smoke were carried out after the samples try to acclimate during a week. Cigarette used with the amount of one cigarette per day. Each treatment with cigarette smoke was conducted in treatment cage. The base of cigarette takes into the plastics bottle that has been modifications before burn the tip cigarette. Then, the cigarette keeps into the treatment cage by placing in the modification in the modification place in each cage. The cage's roof was covered by gauze and glass, and also equipped with a gap for air circulation. After the last cigarette burns out, the samples were taken out from their treatment cage. Treatment with cigarette smoke was conducted every day for one week start at 10:00 PM until finish.

Surgical procedure and Weighing Ovary

Rat that have been given treatment for 28 days then carried out the process of vaginal smear which aims to identify the estrus phase. The rats dissected for ovaries, then weighed and stored in 10% BNF solution.

Histology Preparations

Histologic preparations of the ovaries are done using paraffin method and Hematoxylin Eosin (HE) staining. The first stage of histology preparation is done by fixation of ovarium with 10% BNF solution, followed by dehydration process by using stratified alcohol and clearing by using xylol. The next stage after the clearing process is done the process of infiltration of parafin and embedding in paraffin. Paraffin blocks that have been formed are attached to the holder, done trimming, and cutting using a rotary microtome with a thickness of 6 μm. Sample that have been cut are affixed to the object glass (affixing) and then staining by using hematoxylin eosin (HE). After staining is complete then the ovary tissue is covered with canada balsem and glass cover.

Observation of Number of Ovarian Follicles

Observation of histological preparations using Olympus BX51 microscope with 40x magnification to calculate the number of primary follicles, secondary follicles and tertiary follicles equipped with *DP2-BSW* computer software connected to the photomicrograph. How to calcu-

late the number of primary follicles. Secondary follicles, and tertiary follicles based on cell characteristics ie: primary follicles characterized by oocytes surrounded by a layer of granulosa cells, secondary follicles charactized by oocytes surrounded by two layers of granulosa cells began to form pellucida zone and the development of cells while tertiary follicles are characterized by oocytes surrounded by several layers of granulosa zone of the pellucida zone formed more clearly as well as theca and antrum cells begin to develop.

Data Analysis

The observational data were analyzed by *Analysis of Varian* (ANOVA) on the 95% ($P<0.05$) of confidence level (Gomez, 1995).

RESULTS AND DISCUSSION

The results of the study on the effect of red fruit oil (*Pandanus conoideus*) on the number of primary follicles, secondary follicles, tertiary follicles and rat ovarian weight (*Rattus norvegicus*) were analyzed by using ANOVA variance analysis with 95% confidence level ($\alpha = 0.05$) Table 1

The results revealed that there was no significant difference between treatment groups in terms of percentage of primary follicles. It assumed that the dose of smoke cigarete and red fruit oil did not affect the development of primary follicles. Gonadotropin realising hormone (GnRH) from the hypothalamus induces the anterior pituitary to produce and secrete FSH that stimulate follicular development. In other words, the primary follicles develop into secondary follicles due to the stimulation of FSH. Secondary follicles are growing follicles, has its antrum, cumulus oophorus and coronaradiata. Cumulus oophorus is a pile of granulosa cells surrounding the oocyte and support it in secondary follicles, whereas the corona radiata is formed by the granulosa cells surrounding the oocyte. This follicle contsists cells surrounding the oocyte. This follicle consists of a fully grown oocytes and surrounded by the zona pellucida, 5-8 layers of granulosa cells, the basal lamina, theca interna and theca externa which contains a number of small blood vessels. Under the influence of FSH, the cells of secondary follicles begin to secrete estrogen (Maheswari *et al.,* 2016)

Based on the data analysis table 1 shows that the number of secondary follicles did not differ significantly in all treatment groups. This is thought to be due to the decrease in the number of developing secondary follicles. Oxidative stress from cigarette smoke causes mitochondrial damage triggers a continuous autophagy process within the cell and ends in granulosa cell death. Deeply affected granulosa cells have an impact on the reduced number of follicles that develop into secondary follicles wich later become tertiary follicles (Gannon, 2013). Results of observations of rat ovarian histological samples (*Rattus norvegicus*) with Hematoxylin-Eosin (HE) staining can be seen in (Figure 1).

The average value of the number of tertiary follicles showed that the administration of red fruit oil after exposure to cigarette smoke did not diffet significantly to the increase in the number of tertiary follicles. This is thought to be due to the number of secondary follicles that develop slightly so that there is a decrease in the number of follicles that develop into tertiary follicle. According to Camara *et al.,* (2015), the decrease in follicle count may also be due to the number of follicles undergoing atresia so that few follicles develop. Tertiary follicles was surrounded by two layers of tissue, the theca interna and theca externa. Theca interna is the inner layer that produces estrogen and rich in blood vessels, while the theca externa is the outer layer that will gradually merge with the ovarian stroma. Antral follicular will continue to grow in line with the development of tertiary follicles. Follicular antrum is filled with a clear fluid (liquor foliculi) that is rich in protein and estrogen. The development of this follicle is under the regulation of FSH from the anterior pituitary gland (Young *et al.,* 2010).

The content of alpha-tocopherol and beta-carotene contained in red fruit oil can be used

Table 1. Average number of primary follicles, secondary follicles, tertiary follicles and ovarian weight

Variabel	Treatment			
	P0	P1	P2	P3
Primary follicles	3.67	3.75	4.20	8.20
Secondary follicles	3.00	2.00	3.80	3.20
Tertiary follicles	4.50	3.67	5.00	3.33
Ovarian weight (g)	0.072	0.064	0.074	0.072

Description: The effect of red fruit oil administration to the number of primary follicles, secondary follicles, tertiary follicles and ovarian weight showed no significant change ($P>0.05$)

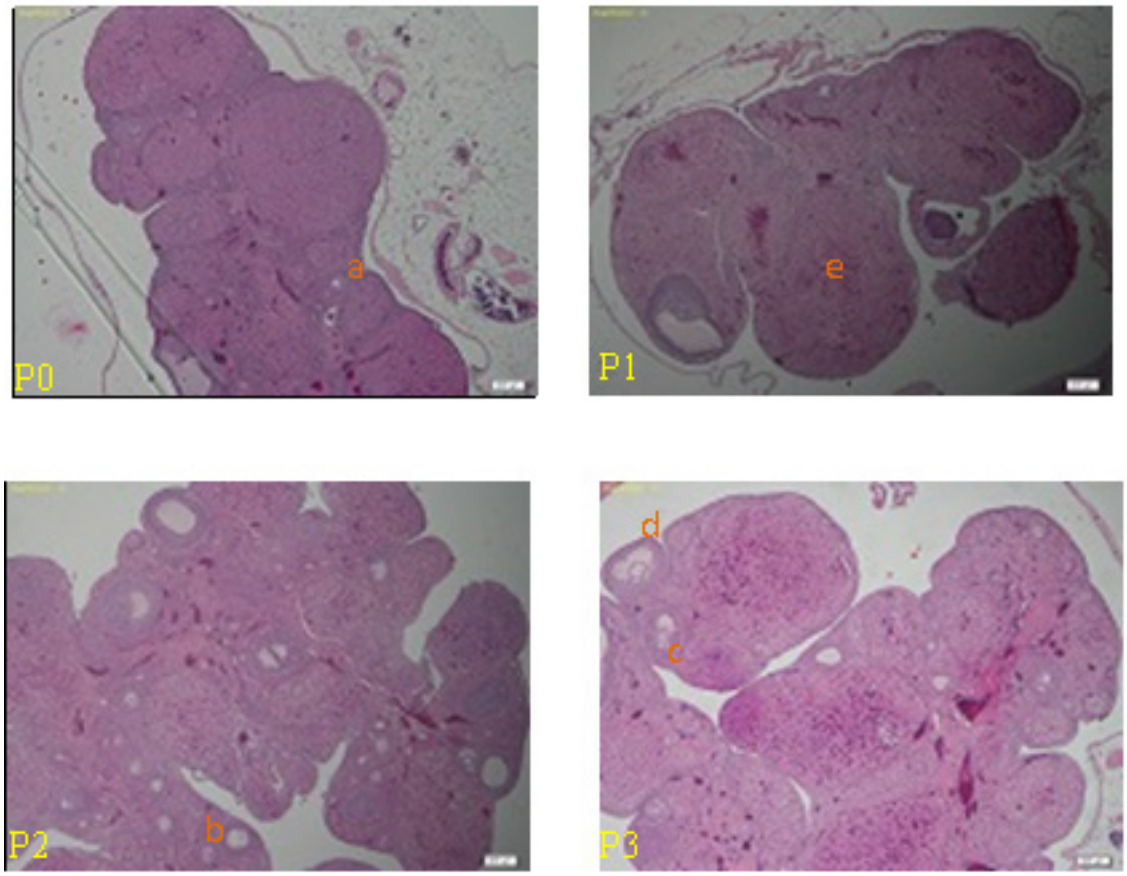

Figure 1. Ovarian follicles with Hematoxylin-eosin (HE) staining observed by using 40x, P0: Positive control; P1: Negative control; P2: Red fruit oil 0.1 ml; P3: Red fruit oil 0.2 ml; (a) primary follicles; (b) secondary follicles; (c) Tertiary follicles; (d) de graaf follicle; (e) corpus luteum.

as an antioxidant to reduce the effects of free radicals from cigarette smoke. The beta-carotene mechanism in reducing oxidative stress due to exposure to cigarette smoke is by breaking the chain reaction of unsaturated lipid peroxidation in the brain (Chunmei *et al.*, 2013). The content of beta-carotene has the potential to bind unpaired free electrons that can trigger free radical formation. Beta-carotene can react with peroxyl radicals which are oxidant substances from cigarette smoke by forming carotenoid peroxide radicals (Rizqi *et al.,* 2016). Carotenoids are able to conjugate the double structures of unpaired electrons and degrade the double structures of unpaired electrons and degrade the electrons directly, whereas the tocopherol antioxidants work by oxidizinf free radicals, the H-ions from tocopherol given to free radicals so that free radical forms become stable (Sibarani *et al.,* 2016).

The results of observation it is showed that giving of the red fruit oil did not give any effect on the development of ovarian follicle of rat. This matter will cause the decrease in the weight of ovarian. According to Satyaningtijas *et al.,* (2014), ovarian weight is influenced by high levels of estrogen. In addition, suppression of FSH levels can lead to inhibition of the development of ovarian follicles. Suherman (2007) states the weight of the ovaries will decrease if there is a suppression of the number of ovarian follicles. Ovarian weight can also be affected by granulosa cell differentiation into the luteal. Research conducted by Gunawan *et al.,* (2007) stated that when adult female rats have reached their functional size they will continue to grow until an adult body is reached, so there is no increase in ovarian mass.

Based on the results of the study can be concluded that red fruit oil can be used as one the antioxidants that can improve fertility. Although the study data showed no significant in all treatment groups.

CONCLUSION

Based on the result of the research, it can be concluded that the exposure of cigarette smoke for eight days has not been able to show any toxicity in rat, so that the administration of red fruit oil (*Pandanus conoideus*) with dose of 0.1 ml

and 0.2 ml did not give any significant influence to the increase of the number of primary follicle, secondary follicle, tertiary follicle as well as the rat's ovarian weight.

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