Effect of Sweet Potato Anthocyanin (Ipomoea Batatas L.) on Levels of Follicle Stimulating Hormone and Folliculogenesis in Rattus Norvegicus Exposed to Cigarette Smoke

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Abstract

Cigarette smoke is one of the exogenous free radicals that can enter the blood circulation so that it can disrupt all body cells and tissues, including the reproductive organs. The anthocyanin in purple sweet potato is one of the bioactive that can counter free radicals. This study was conducted to prove the effect of anthocyanin in purple sweet potato from Gunung Kawi cultivar on follicle-stimulating hormone (FSH), and folliculogenesis levels on Wistar rats (*Rattus norvegicus*) exposed to cigarette smoke. This study was an experimental study with Randomized Post Test Only Control Group Design, using 30 female Wistar rats aged 1-2 months weighing 150-200g. The rats were divided into three groups of where they were exposed to cigarette smoke and administered with anthocyanin (each group with the doses of 20, 40 and 80 mg/kgbw/day). One positive control group was exposed to cigarette smoke without being administered with anthocyanin for 56 days, and one more group served as a negative control. The FSH levels in the serum were measured by employing the ELISA method, and the folliculogenesis (follicle amount and number of ovary follicular granulosa cells) was measured from histopathological slides with Haematoxylin-Eosin staining. The results indicated that anthocyanin in purple sweet potato significantly increased the FSH levels, the follicle amount and the number of primary follicular granulosa cell, secondary, and Graafian on female Wistar rat ovaries exposed to cigarette smoke with a p-value of less than 0.05. To ensure the anthocyanin dose that had maximum effect, more research is required with more dose variations and chronic toxicity tests for the safety of the anthocyanin in purple sweet potato of Gunung Kawi cultivar.

Keywords: *anthocyanin, purple sweet potato, Gunung Kawi cultivar, FSH, ovary, folliculogenesis*

Introduction

Cigarette smoke is one of the exogenous free radicals that can enter the bloodstream so that it can disrupt all body cells and tissues[1]. The body physiologically produces antioxidants to counter the reactivity of free radicals. This antioxidant captures the free radicals and prevents its reactivity amplification by cutting the free radical's chain oxidation reactions with cellular components, causing this antioxidant to earn its name as the free radical scavenger. If the number of free radicals exceeds the amount of antioxidant in the body, free radicals will increase the ROS in the blood. If this condition continues without any resistance from the body, oxidative stress will take place[2,3].

Oxidative stress can cause synthesis and secretion disturbances on hypothalamic GnRH. This failure will cause the pituitary gland to fail the synthesis and secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH). In addition, oxidative stress caused by cigarette smoke can affect folliculogenesis by inhibiting the follicle growth, increasing apoptosis, decreasing ovary volume and follicle count, and causing damage to the ovary, including granulosa cell degeneration[4–6]. The cigarette smoke component that causes oxidative stress can cause DNA impairment to the follicles in the ovary, which is the source of estrogen hormone[7]. Ganoon (2012) also suggests that nicotine suppresses follicle growth in the ovary, which results in the decreased levels of estrogen hormone[7,8].

One of the bioactive that can be used as antioxidants to counteract free radicals is anthocyanin. Anthocyanin is a potential antioxidant because of its capability to rapidly reduce oxygen species and turn it into a more stable aryloxyl radicals[9]. This notion is supported by the research suggesting that anthocyanin can have higher antioxidant activity than vitamin E, vitamin C and beta-carotene[10,11]. Zhao (2013) also reveals that the antioxidant effects of anthocyanin extract of sweet potato purple are higher than vitamin C[12].

Based on the background mentioned above, this study aims to prove the effect of anthocyanin in purple sweet potato (*Ipomoea batatas L.*) of Gunung Kawi cultivar on FSH serum and folliculogenesis levels on Wistar rats (*Rattus norvegicus*) exposed to cigarette smoke.

Materials and Methods

Experiment Animals

This study utilized healthy female Wistar rats and it was acclimatized. The samples of thirty rats aged 1-2 months, weighing 150-200g were divided into five groups, each has six rats, including one negative control group (without cigarette smoke exposure with anthocyanin), one positive control group (exposed to cigarette smoke without anthocyanin) and three treatment groups. The three treatment groups were the groups administered with anthocyanin at the doses of 20 mg/kg BW, 40 mg/kg BW, and 80mg/kg BW per day given through feeding tubes for eight weeks. All animals from the five groups were fed *ad libitum*.

Before starting the cigarette smoke exposure and anthocyanin administration, vaginal swabs were performed to see the estrus cycle. The cigarette smoke exposure started when the rats were in the proestrus phase. After eight weeks of exposure, vaginal swabs were performed on day 56 to determine the proestrus phase. The dissected rats were in the proestrus phase. The experimental animals were anesthetized through intramuscular injection (IM) in the thigh using 1% ketamine at a dose of 0.2ml.

Cigarette smoke exposure

Cigarette smoke exposure was administered as much as two sticks/day, i.e. one stick in the morning and one stick in the evening for eight weeks after they were found in the proestrus phase. The Cigarette smoke exposure box was made of fiberglass sized 26x12x12. It was only filled with three rats because there were only three rooms available in the smoking pump. The brand of cigarettes used was Gudang Garam Merah clove cigarettes (*kretek* cigarettes). After every exposure, the box was always cleaned from the remaining cigarette smoke from the previous treatment.

Anthocyanin Administration

Anthocyanin was administered by calculating the prescribed doses, which were 20mg/kgbw (Anthocyanin 1), 40 mg/kgbw (Anthocyanin 2), and 80mg/kgbw (Anthocyanin 3) daily. All doses were diluted using 1 ml of aquadest and were administered for one week. The anthocyanin solution was administered by using a 1ml syringe and then put into the rats' stomach using feeding needles.

Sample Collection

After eight weeks of administration, the female Wistar rats were treated with vaginal swabs to determine the proestrus phase after dissection. Furthermore, the blood was taken intracardially through the right heart ventricle as much as 3 ml through injection syringe. Then, the blood was put in test tubes without administered with anticoagulant which was then covered with rubber plugs. Next, the left ovary organ was taken, then put into 10% formalin buffer solution.

FSH Level Examination by using ELISA method

The blood serum samples were placed into 50 µl microplates. Then, 100µl Enzyme Conjugate was added for each microplate, then shaken for 2-5 minutes. The microplates were then incubated at 37ºC for two hours. After incubation, the solution in the microplates was cleaned up. Then, the microplates were washed by using 300µl washing solution and then shaken for 3 minutes. The washing was repeated for five times. When finished, the plates were turned over, pressed firmly with absorbent paper, and dried using a tissue. Then, 100**µl TBM** substrate solution was added to each microplate in order. On the next step, the tubes were incubated for 20 minutes at a closed room temperature by using window film and then wrapped with aluminum

foil. Next, the reaction was stopped by adding 50µl stop solution into each microplate gently. Then, they were shaken for 5 seconds. The FSH level examination was conducted by inserting the microplates into the ELISA Spectrophotometer. The results were read at a wavelength of 450nm.

Making of HE Ovary Preparation

The ovary tissue was dissected to a thickness of 2-3 millimeters and then put into a 10% fixative formalin buffer. The tissue was then cut into 3-5 µm thickness for histopathological examination using Hematoxylin-Eosin (HE) staining.

Examination of Ovary Follicle Number

The number of ovary follicles was calculated from the ovary histopathological slides by using the Olympus XC 10 Dotslide Microscope for the overall cross-section and further identified with 400 magnification to calculate the primary, secondary and Graafian follicle.

Examination of Ovary Follicular Granulosa Cells

The calculation of the number of primary, secondary and Graafian follicular granulosa cells was carried out after obtaining the overall number of follicles. Then, the slides were explored to determine the examination visual fields of the primary follicular granulosa cells (4 visual fields), secondary follicular granulosa cells (5 visual fields), and Graafian follicular granulosa cells (5 visual fields) by using Olympus XC 10 Dotslide Microscope with 1,000 times magnification.

Statistic Analysis

The research results are expressed as average ± standard deviation (SD) of the mean. The data on FSH levels, the number of primary, secondary, and Graafian follicles, and the number of primary and secondary follicular granulosa cells were tested using One Way ANOVA difference test. Then the assessment proceeded with post-hoc LSD (Least Square Differences) test, while the number of Graafian follicular granulosa cells were calculated using Kruskal-Wallis test and further proceeded with the Mann Whitney-U test using SPSS version 20.0 (SPSS Inc., IBM). The coincidence interval was set at 95% and declared significant if the p-value was less than 0.05.

Results

The Effect of cigarette smoke exposure on FSH levels and folliculogenesis in female white rats (*Rattus Norvegicus*)

The difference test results in cigarette smoke exposure towards the FSH levels and folliculogenesis were carried out by comparing the negative control group with the positive control group.

Table 1. The Effect of cigarette smoke exposure on FSH levels and folliculogenesis in female Wistar rats (*Rattus Norvegicus*)

Variable	Negative control Avg ± stand.dev	Positive control Avg ± stand.dev	p-value
FSH level (miU/ml)	3.69±0.67	2.46±0.82	0.017<α
Primary follicles count	7.17±1.17	4.67±0.82	0.002< α
Secondary follicles count	8.33±1.21	4.50±1.05	0.000< α
Graafian follicles count	3.33±1.21	0.83±0.75	0.002< α
Primary follicular granulosa cells count	20.83±3.07	13.79±2.51	0.001< α
Secondary follicular granulosa cells count	81.37±5.12	57.17±5.89	0.000< α
Graafian follicular granulosa cells count	86.57±3.31	66.60±32.65	0.005< α

The effect of anthocyanin in purple sweet potato (*Ipomoea batatas L.*) of Gunung Kawi cultivar on FSH levels in female Wistar rats (*Rattus Norvegicus*) exposed to cigarette smoke

The histogram in Figure 1 indicates a significant difference in the FSH levels of the rats in Anthocyanin 2, and Anthocyanin 3 treatment groups in comparison to the positive control group, with a p-value of 0.020.

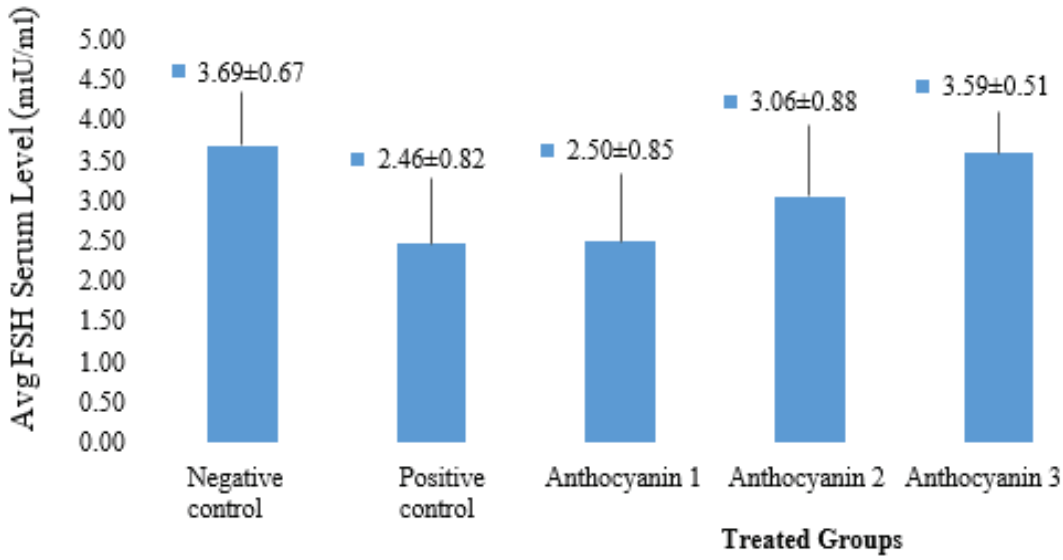

Figure 1. FSH serum level in all groups

Negative Control : rats without cigarette smoke and anthocyanin exposure

Positive controls : rats exposed to cigarette smoke but without anthocyanin

Anthocyanin 1: with a dose of 20 mg/kgbw in rats exposed to cigarette smoke

Anthocyanin 2: with a dose of 40 mg/kgbw in rats exposed to cigarette smoke

Anthocyanin 3: with a dose of 80 mg/kgbw in rats exposed to cigarette smoke

The Effect of anthocyanin in purple sweet potato (*Ipomoea batatas L. anthocyanins*) of on the ovary follicles in female Wistar rats (*Rattus Norvegicus*) exposed to cigarette smoke

The administration of anthocyanin from the purple sweet potato of Gunung Kawi cultivar significantly increased the number of primary, secondary and Graafian follicles compared to the positive control group (p-value <0.05), the mean and standard deviation of follicle counts are indicated in Table 2.

Tabel 2. The Effect of anthocyanin in purple sweet potato (*Ipomoea batatas L. anthocyanins*) of Gunung Kawi cultivar on the ovary follicles in female Wistar rats (*Rattus Norvegicus*) exposed to cigarette smoke

		Treated Groups			
Follicle counts	**Negative Control**	**Positive Control**	**Anthocyanin 1**	**Anthocyanin 2**	**Anthocyanin 3**
Primary	7.17±1.17 a	4.67±0.82 b	5.17±0.75b	6.67±1.37a	7.50±1.05a
Secondary	8.33±1.21 a	4.50±1.05 b	4.83±0.75b	6.17±1.94b	7.67±1.03a
Graafian	3.33±1.21 a	0.83±0.75 b	1.33±0.82bc	2.50±1.05ac	2.83±1.33ac

Information: the value indicates an average rate ± of SD with *p-value*<0.05

Negative Control : rats without cigarette smoke and anthocyanin exposure,

Positive controls : rats exposed to cigarette smoke but without anthocyanin

Anthocyanin 1 : with a dose of 20 mg/kgbw in rats exposed to cigarette smoke

Anthocyanin 2 : with a dose of 40 mg/kgbw in rats exposed to cigarette smoke

Anthocyanin 3 : with a dose of 80 mg/kgbw in rats exposed to cigarette smoke

The Effect of anthocyanin in purple sweet potato (*Ipomoea batatas L. anthocyanins*) of Gunung Kawi cultivar on the number of ovary follicular granulosa cells in female Wistar rats (*Rattus Norvegicus*) exposed to cigarette smoke

The administration of anthocyanin in purple sweet potato of Gunung Kawi cultivar significantly increased the number of primary, secondary and Graafian follicular granulosa cells compared to the positive control group (p-value <0.05). The mean and standard deviation of the follicular granulosa cells is presented in Table 3.

Table 3. The Effect of anthocyanin in purple sweet potato (*Ipomoea batatas L. anthocyanins*) of Gunung Kawi cultivar on the number of ovary follicular granulosa cells in female Wistar rats (*Rattus Norvegicus*) exposed to cigarette smoke

		Treated Groups			
Follicles counts	**Negative Control Group**	**Positive Control Group**	**Anthocyanin 1**	**Anthocyanin 2**	**Anthocyanin 3**
Primary	20.83±3.07a	13.79±2.51b	13.96±2.46b	18.79±1.82a	22.33±0.54a
Secondary	81.37±5.12a	57.17±5.90b	60.73±4.34b	74.20±3.88c	81.60±5.63a
Graafian	86.57±3.31a	53.27±41.35b	69.13±33.88bc	82.73±1.37ac	86.90±2.44a

Information: the value indicates an average rate ± of SD with *p-value*<0.05

Negative Control : rats without cigarette smoke and anthocyanin exposure

Positive controls : rats exposed to cigarette smoke but without anthocyanin

Anthocyanin 1 : with a dose of 20 mg/kgbw in rats exposed to cigarette smoke

Anthocyanin 2 : with a dose of 40 mg/kgbw in rats exposed to cigarette smoke

Anthocyanin 3 : with a dose of 80 mg/kgbw in rats exposed to cigarette smoke

Discussion

Cigarette smoke is an exogenous free radical that can accumulate in female reproductive organs[13]. The effects of free radicals on the reproductive organs include disorders of reproductive hormones, ovary maturation, ovulation, follicle development, fertilization and implantation.

In this study, it was found that cigarette smoke exposure on female Wistar rats significantly decreased the FSH levels and folliculogenesis in comparison to the control group (p-value <0.05). This indicates that cigarette smoke contains free radicals which can cause an increase in oxidative stress, either directly or indirectly[14]. Because of the increased oxidative stress, lipid peroxide occurs which can cause damage to the arcuate nucleus and ventromedial nucleus in the hypothalamus, resulting in disruption of hypothalamic GnRH synthesis and secretion. This failure will cause pituitary failure to synthesize and secrete FSH and LH. The excessive amount of cigarette smoke entering the body through inhalation will cause oxidative stress in the brain, resulting in brain cell degeneration which will cause damage to the hypothalamus. If this stage occurs, the levels of GnRH (gonadotropin releasing hormone) will decrease and will have an impact on the decreasing levels of FSH (follicle stimulating hormone) and LH (luteinizing hormone). This level decrease of FSH and LH hormones will interfere with the development of granulosa cells and the number of ovary follicles. This will disrupt ovary function and can resultin infertility[4,14].

The administration of anthocyanin from the purple sweet potato of Gunung Kawi cultivar with several dose levels in this study has indicated that there was an increase in FSH levels compared to the positive control group. At 80mg/kgbw anthocyanin dose, the highest FSH level was obtained compared to the other doses. From the study results, it was found that the fastest dose of anthocyanin in purple sweet potato from Gunung Kawi to increase FSH serum levels, follicle counts and the number of primary, secondary and Graafian follicular granulosa cells in female Wistar rats exposed to cigarette smoke was 80mg/kgbw (A3 treatment) in comparison to the anthocyanin doses of 20 mg/kgbw and 40 mg/kgbw. This proves the theory stating that anthocyanin can act as antioxidants. Anthocyanin is neuroprotective because it can capture ROS and prevent the occurrence of lipid peroxidation processes in the brain. The absence of oxidative stress in the brain will have an impact on increasing sex hormones (FSH and LH)[15]. Anthocyanin functions as an antioxidant by reducing free radicals, donating electrons to free radicals[16,17]. In addition, the anthocyanin from purple sweet potato can increase the Total Antioxidation Capacity (T-AOC), i.e. the enzymatic antioxidants in the body (SOD and GSH-PX) and reduce MDA levels so that the oxidative stress does not occur in body organs, including the ovary. This condition will affect the process of follicle and granulosa cell development[12].

This study concludes that the anthocyanin in purple sweet potato of Gunung Kawi cultivar can increase FSH levels and folliculogenesis in female white rats exposed to cigarette smoke. However, this study cannot be generalized to humans because there has not been a chronic toxicity test and the dose of anthocyanin from purple sweet potato has not been found to be truly optimal for all organs instead of only the reproductive organs.

Conclusion

The results indicated that anthocyanin in purple sweet potato significantly increased the FSH levels, the follicle amount and the number of primary follicular granulosa cell, secondary, and Graafian on female Wistar rat ovaries exposed to cigarette smoke.

Ethical Celarance

The ethics research from Ethic Committee of the Faculty of Medicine, University of Brawijaya, Malang

with number No. 274/EC/KEPK/07/2016.

Conflict of Interest: None

Source of Funding: The research was funded by the authors

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