

CORRELATION BETWEEN ESTROGEN HORMONE CONCENTRATION AND ESTROUS CYCLE OF RAT FED SOYBEAN FLOUR AND TEMPEH FLOUR

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ABSTRACT

The objective of this research was to examine the correlation between estrogen level and estrous cycle of rat fed with soybean flour and tempeh flour. A completely randomized design was applied in this study with 6 treatment groups and 5 replications. Rats in group 1, 2, and 3 were non-ovariectomized rats which were fed with pellet, 10 g soybean flour/100 g body weight/day, and 10 g tempeh flour/ 100 g body weight/day, respectively. The rats in group 4, 5, and 6 were ovariectomized rat which were fed with pellet, 10 g soybean flour/100 g body weight/day, and 10 g tempeh flour/100 g body weight/day, respectively. The rats were after fed with pellet, soybean and tempeh flour for 4 weeks, the estrous cycle phases of rats were examined based on the presence of vaginal epithelial cells and the number of qualitative vaginal epithelial cells, while the estrogen concentrations in serum were measured by radioimmunoassay method. The data was analyzed using analysis of variance and followed by Duncan multiple range test at 95% confidence. Non-ovariectomized rats given tempeh flour have longer estrus cycle than the non-ovariectomized rats which given soybean flour. The isoflavon contained in tempeh flour and soybean flour induced proliferation and cornification of vaginal epithelial cell. Feeding the soybean flour and tempeh flour to ovariectomized rats could optimize the estrogen hormone in initiating the estrus phase, in which of the tempeh flour addition had better effect than soybean flour. Correlation between the level of estrogen to the length of estrous cycle in both nonovariectomized rats and ovariectomized rats was positive, in which the higher the levels of estrogen the longer the length of estrous cycle.

Key words: estrogen, estrus cycle, isoflavon, ovariectomy, *Rattus norvegicus*

ABSTRAK

Penelitian ini bertujuan mengetahui hubungan antara kadar hormon estrogen dan panjang siklus estrus pada tikus yang diberi tepung kedelai dan tepung tempe. Penelitian ini menggunakan metode eksperimental dengan pola rancangan acak lengkap (RAL) yang terdiri atas 6 (enam) kelompok perlakuan dan 5 (lima) kali ulangan, kelompok perlakuan tersebut adalah kelompok 1: tikus non ovariektomi yang diberi pelet/kontrol (Non-Ov K), kelompok 2: tikus non ovariektomi yang diberi tepung kedelai 10 g berat kering/100 g bb/hari (Non-Ov Kd), kelompok 3: tikus non ovariektomi yang diberi tepung tempe 10 g berat kering/100 g bobot badan/hari (Non-Ov T), kelompok 4: tikus ovariektomi yang diberi pelet/pakan biasa (Ov K), kelompok 5: tikus ovariektomi yang diberi tepung kedelai 10 g berat kering/100 g bobot badan berat kering/hari (Ov Kd), dan kelompok 6: tikus ovariektomi yang diberi tepung tempe 10 g berat kering/100 g bobot badan/hari (Ov T). Data kadar estrogen dianalisis dengan menggunakan analysis of variance (ANOVA) dan Uji Duncan dengan selang kepercayaan 95% ($\alpha = 0.05$). Kadar hormon tikus ovariektomi yang diberi tepung tempe dan yang diberi tepung kedelai mempunyai efek pada epitel vagina yaitu terjadinya proliferasi dan kornifikasi sel epitel vagina. Pemberian tepung kedelai dan tepung tempe pada tikus ovariektomi dapat mengoptimalkan hormon estrogen dalam memunculkan fase estrus. Pemberian tepung tempe pada tikus non ovariektomi dan tikus ovariektomi lebih baik dari pada pemberian tepung kedelai. Hubungan antara kadar hormon estrogen dan panjang siklus estrus berkorelasi positif.

Kata kunci: estrogen, siklus estrus, isoflavon, *Rattus norvegicus*

INTRODUCTION

The soybean *Glycine max* (L.) Merr. (Leguminosae) is a species of legume that is native to East Asia and is considered a very important plant because of its phytochemical content. *Glycine max* L. is widely used in the food industry as a supplement and is included in the formulations of many drugs. Recently, the use of soybeans has been extensively studied for the prevention and treatment of various chronic diseases (Adlercreutz *et al.*, 2004; Kondratyuk and Pezzuto, 2004; Manach *et al.*, 2004). Isoflavones, such as genistein and daidzein, are produced in soybean seed assumed to be associated with health benefits in the human diet. Year and location had a significant effect on isoflavone concentrations.

Soybean seed isoflavone levels exhibit a location specific response, and the temporal variability experienced between years appears to influence

changes in soybean isoflavone levels more than seed location (Laurenz *et al.*, 2017).

Soy isoflavone, a soy-derived phytoestrogen, is a group of biologically active plant substances with chemical structures similar to that of an endogenous estrogen-estradiol. This might partly explain the ability of these compounds on binding to estrogen receptors (ERs) and exerting various effects (Husain *et al.*, 2015). Soy extract has a positive effect on vaginal pH in comparison to placebo, 70 mg isoflavone supplement has beneficial effects on bone formation process; however, it showed no benefit compared to the placebo on climacteric symptoms or quality of life (Hansongyi *et al.*, 2017). Soybean extract might have positive estrogenic effects on the skin through the increase of collagen by a high-dose of ethyl acetate extract, thus, benefit to postmenopausal facial skin (Belkiz *et al.*, 2014). Recently, isoflavone has been used as a potential therapeutic for postmenopausal ischemia/reperfusion

injury and even other cardiovascular diseases in clinic. This resulted in higher number of fractures incidence in the group of women consume soy diet supplements (Levis *et al.*, 2011).

Soy flour used in snack ingredient showed the level of substitution of whole wheat flour (WWF), especially levels of 15%, had a significant effect on the hardness perceived by the panelist during sensory evaluation. The texture of soy flour (TSF) at concentrations of $\geq 15\%$, favored the fracturability and crispness of the samples. It was found that the best expansion index was with the combination of 5% TSF and 15% WWF (Rodríguez-Vidal *et al.*, 2017). Kefir combination from goat milk and soy milk can be used as antidiabetic through maintaining serum triglyceride, decreasing plasma glucose, increasing glutathione peroxidase and improving pancreatic β -cells (Nurliyani *et al.*, 2015). Male rats receiving soy milk supplemented with 100 mg/kg glyphosate showed the decrease in round spermatids and increase in abnormal sperm morphology compared to control (Nardi *et al.*, 2016).

Soy isoflavone protects myocardial ischemia/reperfusion injury in ovariectomized rats through increasing PI3K/Akt/eNOS signal pathway and decreasing oxidative stress (Tang *et al.*, 2016). Female ovariectomized rodents with a diminished level of estrogen (a major female sex hormone) have been chosen as animal model to examine the effectiveness of anti-estrogens such as genistein against breast cancer and bone loss. Genistein is an active soy isoflavone which has anticancer activities however its oral bioavailability higher in female than in male rats is still unknown (Hilakivi-Clarke *et al.*, 2010; Kaustubh *et al.*, 2012). In reproductive system, reproductive hormone plays a very important role. For example the reproduction will be success depends on the balance of reproductive hormones. The main reproductive hormones in female animals is estrogen. Soy protein isolate is a selective estrogen receptor modulator (SERM) interacting with a small sub-set of 17β -estradiol/E2-regulated genes and anti-estrogenic in the presence of endogenous estrogens (Ronis *et al.*, 2016).

Lack of estrogen resulted in reproductive disorders, therefore, supplementation of soy flour and tempeh flour containing estrogen is important to be consumed. So far, research on the effects of soy isoflavone compounds are generally fed in the form of pure isoflavone isolates, which actually happens in daily life. This study aims to prove that exogenic estrogen contained in soy flour and tempeh flour can optimize the levels of estrogen in rats and to compare the levels estrogen in rats fed with soy flour and tempeh flour. The correlation between estrogen level and the estrous cycle length was also evaluate in this study.

MATERIALS AND METHODS

The study was conducted at Laboratory of Physiology and Pharmacology Faculty of Veterinary Medicine, Bogor Agricultural University. This study used 30 female white rats (*Rattus norvegicus*),

Sprague-Dawley strain parity II, with the age of twelve weeks and weighing of 200 g. The research was conducted in several stages those are soy flour and tempeh flour preparation, animal model preparation, treatment and observation phase (phase of proestrus, estrus, metestrus, and diestrus), and analysis of estrogen levels using the method of radioimmunoassay (RIA).

Preparation of Soy Flour and Tempeh Flour

Soybean and tempeh were collected from home made tempeh producer at Ciherang Bogor Indonesia. The sample of Soybean was then identified at Biology Laboratory, Bogor Agricultural University. A total of 10 kg of imported soybean seed (variety of Americana) was processed into soybean flour as follows: milling, drying in an oven at a temperature of 45° C and moisture content of 10%, and sieving (60 mesh). While the process of making tempeh was carried out using 20 kg of soybean and following the steps: soybean seeds dry cleaning, washing and soaking, first boiling, stripping the skin, soaking, second boiling, draining and cooling, fermentation, packaging and incubation. This tempeh then processed into flour.

The process of making tempeh flour was carried out as follows: tempeh was sliced into 1x2x0.5 cm³, continue with milling, drying in an oven at a temperature of 45 °C and moisture content of 10%, and sieving (60 mesh). Analysis of isoflavone content in soy flour and tempeh flour was carried out using high performance liquid chromatography (HPLC).

The isoflavone purification procedure was performed as follows. The 1.5 mL of filtrate was taken and purified in a 12 mL of chromatography column containing MN-Polyamide CC6 with a particle size of 0.05-0.16 mm. Before the filtrate was poured into the column, the MN-Polyamide CC6 was immersed with 25% methanol solution for 12 hours in chromatography. Elution was carried out in the column by the addition of 25%, 50%, 70% methanol, 50 mL each. Eluen obtained from 70% methanol elution (70% fraction) were collected and dried with a rotary evaporator at 40° C to dry. The residue (dry deposition obtained) was dissolved in 1 ml of absolute methanol and centrifuged at 4000 rpm for 5 min to separate the precipitate, then filtered. Clear filtrate is ready to be analyzed using HPLC. Identification of daidzein, glysitein and genistein with quantitative analysis was done by HPLC. HPLC used was under the following conditions: sample volume 40 μ L. Column: Li chrosorb RP-18 (250 x 4 mm, 5 μ m). Eluen: 3% acetic acid (solvent A); Acetonitrile (solvent B). Gradient: 20% in (A + B) to 60% (A + B) for 35 minutes. Detector: UV 261 nm. Flow rate: 0.8 mL/min. Temperature: 35° C.

Preparation of Animal Models

A total of 30 female rats were placed into a plastic cage with a lid made of wire and covered with chaff ram, fed with pellets and drinking water provided ad libitum. Environmental enclosure was made to avoid moisture, adequate ventilation and sufficient lighting

with the duration of 14 hours light and 10 hours dark. Each rat was placed in individual cages. All rats adapted to the environmental enclosure for 10-day trial. After adaptation, 15 rats were ovariectomized, while the others were not ovariectomized. All the rats were allotted in 6 treatment groups. Rats in group 1, 2, and 3 (non-ovariectomized rats) were fed with pellet, 10 g soybean flour/100 g body weight/day, and 10 g tempeh flour/100 g body weight/day, respectively. The rats in group 4, 5, and 6 (ovariectomized rat) were fed with pellet, 10 g soybean flour/100 g body weight/day, and 10 g tempeh flour/100 g body weight/day, respectively.

Treatment and Observation

The treatment was carried out for 28 days or 4 (four) weeks. The administration of soy flour and tempeh flour were done orally three times a day in the morning (08.00 pm), at noon (12.00 GMT) and afternoon (16:00 GMT). Subsequently, the examination to determine the length of estrous cycle was performed by observing vaginal performance twice a day, in the morning (07.00 pm) and afternoon (18:00 GMT) for two weeks. The examination of vaginal preparations were carried out by fixed it with methanol for 5 minutes and stained with Giemsa 10% for 30 minutes, washed with water and air dried. Then the slides were observed under a microscope with a magnification 40x10. Determination of the estrous cycle phase was done based on the presence of vaginal epithelial cells and the number of qualitative vaginal epithelial cells; proestrus (nucleated epithelial cells), estrus (cornification cells), metestrus (pavement cells and leukocytes), diestrus (leukocytes). The examination of vagina was performed for 15 days until the diestrus phase. At the diestrus phase, rat blood was collected through intracardial for approximately 1 mL. Blood was put into a tube container, then centrifuged at 2000 rpm for 15 minutes to obtain the serum which later used for the determination of estrogen levels. In this study the estradiol measurement was represent the estrogen.

Estrogen Analysis

Estrogen concentrations in serum were measured by radioimmunoassay method (RIA) with a solid phase technique using a kit estrogen-a-count topcoat containing ^{125}I -labeled estradiol. Series of standard solution A, B, C, D, E, F, and G contain estrogen concentrations of 0, 20, 50, 150, 500, 1800, and 3600 pg/mL, respectively, obtained from Diagnostic Product Corporation (Los Angeles, CA). The recommended sample volume was 100 μL . Tubes for Non Specific Binding (NSB) and Total Count (T) were labeled and each made duplo. A total of 14 tubes were labeled A (MB), B, C, D, E, F, and G (duplo), respectively. Using a 100 μL micro pipet the standard solutions were filled into the bottom of the tube. In NSB tube was also included 100 μL of standard solution A. The other tubes were filled with 100 μL of each sample. Then, 1 mL labeled estrogen was added into each tube and mixed using vortex. The entire mixture was incubated

for 3 hours in room temperature (25° C). The remaining liquid in each tube was poured and the tube was allowed to dry for 3 minutes. The radioactivity attached to the tube was enumerated using an Automatic Gamma Counter for 1 minute. The enumeration was conducted at Physiology and Pharmacology Laboratory, Faculty of Veterinary Medicine, IPB. The percent of bound radioactivity was calculated by dividing the sample and standard CPM with the standard CPM A (MB). The standard curve equation was calculated by the linear regression equation of radioactivity bound percent as Y and the log of standard concentration as X. The estrogen sample concentration is calculated by inserting the percent value of the radioactivity bound sample to the standard curve equation.

Data Analysis

The data of estrogen level was analyzed by Analysis of Variance (ANOVA) and followed by Duncan Multiple Range Test at 95% confidence interval (5% significance level) and correlation test.

RESULTS AND DISCUSSION

Isoflavone Compound Content in Soy Flour and Tempeh Flour

The results of soy flour and tempeh flour analysis showed three kinds of major isoflavone compounds as shown in Table 1. The isoflavone compound was the sum total isoflavone content of soy isoflavones daidzein, genistein, and glycitein.

Chromatogram analysis results showed that the most dominant isoflavone compound in both soy flour and tempeh flour were daidzein and genistein. This study used 10 kg of soybeans which then processed into flour and produced 8.33 kg of soy flour, while 20 kg of soybeans were processed into tempeh and produced 34.60 kg of tempeh which then became 9.68 kg of tempeh flour. This means that 20 kg was equivalent to 9.68 kg of soybean tempeh flour. Isoflavones in tempeh flour more than the soy flour since flour-making process needs more soy tempeh. The higher the amount of soy, the higher the isoflavones content (Table 1).

Total isoflavone content of soy flour was 206.37 mg/kg (20.637 mg/100 g), while the tempeh flour was 901.24 mg/kg (90.124 mg/100 g). In this study, the sample was given soy flour and tempeh flour as much as 10 g/100 g of body weight/day. 10 g of soy flour contains 2.0637 mg of isoflavones, while 10 g of tempeh flour contains 9.0124 mg of isoflavones. This means that the isoflavones contained in tempeh flour higher than the soy flour. According to Baú *et al.* (2015), the fermented soymilk presented 1.67 $\mu\text{mol g}^{-1}$ of daidzein, 0.28 $\mu\text{mol g}^{-1}$ of glycitein, and 1.67 $\mu\text{mol g}^{-1}$ of genistein.

Tempeh flour and soy flour containing genistein, a type of amino acid that serves as a phytoestrogen isoflavones. The content of genistein in tempeh flour

was 250.65 mg/kg while soy flour was 65.15 mg/kg, thus, the tempeh flour better than soy flour. According to Messina (2014), genistein which shares similar structure to estrogen, also acts on estrogen receptor to influence the cardiovascular system. Soy isoflavone, aglycones, are easily absorbed and found in higher amounts than glucosides in human body. Isoflavone aglycone-rich products may be more effective than glucoside-rich products in preventing chronic disease such as coronary heart disease (Izumi *et al.*, 2000).

Estrus Cycle and Estrogen Level of Nonovariectomized Rats

The results of the estrous cycle length and the average levels of estrogen in nonovariectomized rats in all treatments shown in Table 2. The length of the estrous cycle of nonovariectomized rats fed with tempeh flour were longer than nonovariectomized rats given pellets, while the length of estrous cycle of nonovariectomized rats given soy flour were shorter than nonovariectomized rats given pellets.

Furthermore, the phase of proestrus and estrus of nonovariectomized rats given soy flour and tempeh flour were longer than nonovariectomized rats fed pellet (Figure 1). The length period of time of Metestrus phase in nonovariectomized rats given soy flour were similar to nonovariectomized rats fed pellet, while the length of time metestrus phase in nonovariectomized rats given tempeh flour were longer than nonovariectomized rats fed pellet. It was assumed that the decrease of estrogen levels in metestrus phase lead to extend this phase. The length of time of diestrus

phase in nonovariectomized rats given soy flour and nonovariectomized rats given tempeh flour were shorter than the nonovariectomized rats given pellet, this is due to briefly low estrogen levels which cause the cells proliferate again. Shortening phase of diestrus in nonovariectomized rats brings benefit to individual since this phase was potential for fertility.

Isoflavones contained in tempeh flour and soy flour affects the vaginal epithelium proliferation and vaginal epithelial cells cornification. It is usually obvious by the extension phase of proestrus and estrus phase, where the phase of proestrus causes vaginal epithelial cell proliferation and estrus phase cause vaginal epithelial cells cornification. Nonovariectomized rats fed with tempeh flour caused extended estrous cycle phase which is an extension on proestrus and estrus phase. This condition is potential for fertility because it produces a long mating phase and mating incidence is probability high. According to Tou *et al.* (2003) extension of the estrous cycle in rats has important implications in reproduction because it can reduce the cumulative number of cycles and potentially in terms of fertility. Pubertal soy exposure decreases mammary ER α expression after menarche and exerts subtle effects on receptor activity and mammary gland differentiation (Dewi *et al.*, 2016).

The results of ANOVA test showed that estrogen levels of nonovariectomized rats were influenced by treatments ($P < 0.05$) (Table 2). Duncan test further showed that the average levels of estrogen in nonovariectomized rats given tempeh flour and soy flour were similar with nonovariectomized rats given

Table 1. Isoflavones compounds found in soy flour and tempeh flour

Components mean	Soy flour (mg/kg)	Tempeh flour (mg/kg)
Daidzein	113.63	555.55
Glycitein	27.59	95.04
Genistein	65.15	250.65
Total isoflavones	206.37	901.24

Table 2. Effect of soy flour and tempeh flour on the length of the estrous cycle and estradiol levels in nonovariectomized rats

Types of treatment	Group observations		
	Non Ov K	Non Ov Kd	Non Ov T
PSSE (hours)	108	96	180
RE $\pm$ SE (pg/mL)	21.58 $\pm$ 0.438 ^{ab}	18.294 $\pm$ 2.192 ^b	25.621 $\pm$ 1.822 ^a

PSSE= Length of the estrous cycle; RE= Average estradiol ; SE= Standard error. NonOVK= Nonovariectomized rats as control, NonOvKd= Nonovariectomized rats given soy flour, NonOvT= Nonovariectomized rats given tempeh flour.

^{a, b, ab} Different superscripts within the same row indicate significantly different ($P < 0.05$)

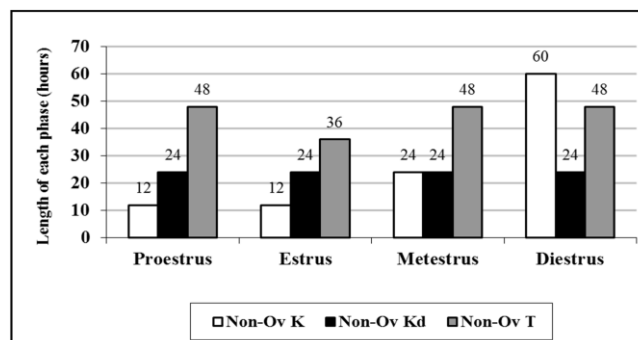


Figure 1. The average length of each phase of the estrous cycle (h) in nonovariectomized rats

pellet, however the average levels of estrogen in nonovariectomized rats only given tempeh flour was higher than nonovariectomized rats given soy flour. This means that the isoflavones contained in tempeh flour and soy flour were able to optimize the levels of estrogen, thus, hormone balance can be achieved. Levels of estrogen that is above normal levels in nonovariectomized rats given tempeh flour caused the extension of estrus phase, therefore estrus phase becomes longer. The low levels of estrogen in nonovariectomized rats given soy flour also cause the extension phase of estrus, but the length of the estrous phase of nonovariectomized rats given tempeh flour is longer than nonovariectomized rats given soy flour. It is because of the amount of isoflavones contained in tempeh flour higher than in soy flour.

Estrus Cycle and Estrogen Levels of Ovariectomized Rats

The results of estrous cycle length and the average levels of estrogen in ovariectomized rats in all treatments are shown in Table 3. The results showed that the length of estrous cycle of ovariectomized rats given tempeh flour were longer than ovariectomized rats given pellet, whereas the length of estrous cycle in ovariectomized rats given soy flour similar with the ovariectomized rats given pellet. Furthermore, the results showed that the time length of proestrus phase in ovariectomized rats given soy flour similar with ovariectomized rats given pellet, while the length of proestrus phase in ovariectomized rats given tempeh flour were longer than ovariectomized rats given pellet (Figure 2).

Ovariectomized rats given tempeh flour lengthened the proestrus phase. In this phase, levels of estrogen increase for a long period of time, resulted in the numerous proliferation of cells. The length of estrus phase in ovariectomized rats given tempeh flour similar to the ovariectomized rats given soy flour, but different

compared to ovariectomized rats given pellet which have very low estrogen levels. The length of metestrus phase in ovariectomized rats given soy flour similar to ovariectomized rats fed pellet, while the length of metestrus phase in ovariectomized rats given tempeh flour was shorter than ovariectomized rats fed pellet. The condition that estrogen levels decline very rapidly was presumed as metestrus phase. Diestrus phase in ovariectomized rats given tempeh flour was longer than ovariectomized rats fed pellet and ovariectomized rats given soy flour. This is due to at diestrus phase the estrogen levels were low because this phase is lengthened. The extension phase of diestrus in ovariectomized rats bring benefit since this phase is the resting phase and not a potential phase in the fertility.

Ovariectomy performed in this study was conducted on postmenopouse rat model. Ovariectomy cause the loss of ovarian and low estrogen level that resulted in proliferation and disrupted of vaginal epithelial cells cornification and cause the absence of estrus phase in ovariectomized rats given pellets . Isoflavones found in tempeh flour and soy flour can increase estrogen, it is proven that in ovariectomized rats given soy flour and ovariectomized rats given tempeh flour the estrous cycles still occur, but the higher amount of isoflavones contained in tempeh flour significantly effective to affect the estrous cycle. Results showed that the ovariectomized rats given soy flour and tempeh flour influence the proliferation of vaginal epithelium and vaginal epithelial cells cornification. This is thought to reduce the level of vaginal dryness. As reported by Winarsi (2005) that the benefits of soy isoflavones can improve immunity, reduce vaginal dryness, decrease menopausal complaints, improve memory (memory), reduce fatigue, and prevent cancer.

The results of ANOVA test showed that the levels of estrogen in ovariectomized rats influenced by treatment ($P < 0.05$) (Table 3). Duncan test further showed that the levels of estrogen in ovariectomized

Table 3. Effect of soy flour and tempeh flour on the length of the estrous cycle and estradiol levels in ovariectomized rats

Types of treatment	Group observations		
	Ov K	Ov Kd	Ov T
PSSE (hours)	72	72	108
RE±SE (pg/mL)	13.478±0.659 ^b	14.791±0.127 ^{ab}	16.288±1.127 ^a

PSSE= Length of the estrous cycle; RE= Average estradiol; SE= Standard error. OVK= Ovariectomized rats as control, OvKd= Ovariectomized rats given soy flour, OvT= Ovariectomized rats given tempeh flour.

^{a, b, ab} Different superscripts within the same row indicate significantly different ($P < 0.05$)

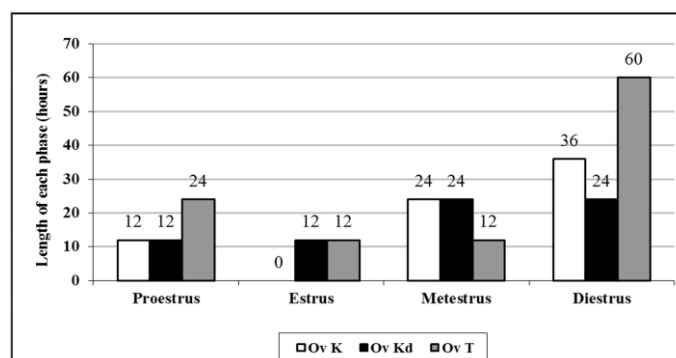


Figure 2. The average length of each phase of the estrous cycle (hours) in ovariectomized rat (hours)

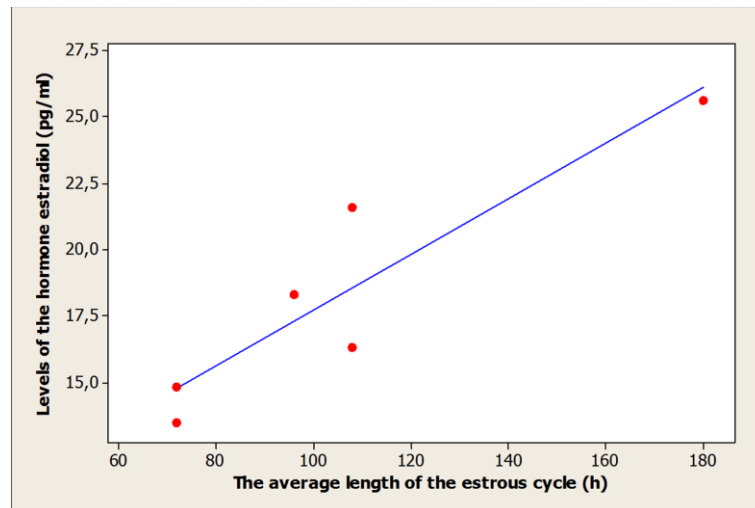


Figure 3. The relationship between levels of the hormone estrogen and the length of the estrous cycle ($y = 7.232 + 0.1048x$)

rats given tempeh flour were similar to the ovariectomy rats given soybean flour, but higher when compared to ovariectomized rats given pellet. Estrogen levels were very low in ovariectomized rats fed pellet due to lack of endogenous estrogen. This condition leads to the absence of estrus phase since this phase required high estrogen levels. Estrogen levels in ovariectomized rats given tempeh flour and soy flour affect the estrus cycle. The administration of tempeh flour and soy flour in ovariectomized rats can optimize the level of estrogen in estrus phase.

One way to overcome the decrease of estrogen levels in ovariectomized rats was by feeding tempeh flour and soy flour containing isoflavones. Isoflavones are able to influence estrogen activity that lead to increased estrogen production and stimulate the initiation of proliferation and cornification of vaginal epithelial cell. Consumption of tempeh flour and soy flour improve estrogen levels in ovariectomized rats. When an individual lack of endogenous estrogen, it is suggested to consume tempeh flour and soy flour as a natural estrogen therapy which is relatively safe. Isoflavones have high affinity for estrogen receptor beta on estrogen receptor alpha from the potential to activate estrogen signaling pathways, both genomic and nongenomic (Pilšáková *et al.*, 2010). Isoflavones are the potential trophic therapeutic which have protective effect and contribution to the development of effective therapies to decrease the symptoms of menopause (Marinho *et al.*, 2017). The ovariectomized (OVX) rat fed the isoflavone-rich diet had decreased body weight ($P < 0.05$), abdominal fat ($P < 0.05$), and serum leptin levels ($P < 0.05$) compared to those fed isoflavone-free diet (Ashley *et al.*, 2017). Soy isoflavones extract markedly alleviated the derangement of lipid metabolism, the use of this natural phytoestrogen is suggested as a strategy for relieving dyslipidemia and hepatic steatosis associated with the postmenopausal women (Panneerselvam *et al.*, 2016). Dietary daidzein has a hypocholesterolemic effect in non-ovariectomized and ovariectomized female Sprague-Dawley rats fed a cholesterol-free diet (Bhattarai *et al.*,

2017). Genistein has synergistic effects on bone formation in ovariectomized rats (Qi and Zheng, 2017). Coffee contains natural phytoestrogen for antiaging (Safrida and Sabri, 2017)

Relationship of Estrous Cycle Length with Estrogen Levels in Rats

Based on the correlation test there is a correlation between the level of estrogen and the length of estrous cycle in nonovariectomized rats and ovariectomized rats. The relationship between levels of estrogen and estrous cycle length correlated positively and significantly, the higher the levels of estrogen, the longer the length of estrous cycle. The graph showing the relationship of average levels of estrogen and the average length of estrous cycle (Figure 3).

The length of estrous cycle in ovariectomized rats related to non-reproductive ability, but depending on the phase. The extension phase of proestrus and estrus phase bring the benefit to nonovariectomized rats as this phase is the phase of potential fertility. While in the estrous cycle length in ovariectomized rats related to health. The extension phase of proestrus and estrus phase in ovariectomized rats are beneficial since it can retain moisture thus overcoming vaginal dryness problems.

CONCLUSION

Correlation between the level of estrogen to the length of estrous cycle in both nonovariectomized rats and ovariectomized rats was positive, in which the higher the levels of estrogen, the longer the length of estrous cycle.

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