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Submission date: 12-Aug-2020 08:33PM (UTC+0700)
Submission ID: 1368797419
File name: Inhibit_the_Ovary_Follicles_Developing_of_Little_Wistar_Rats.pdf (493.76K)
Word count: 3180
Character count: 17608

DOI Number: 10.5958/0976-5506.2018.00602.2

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ABSTRACT

Background and objective: soybean tempeh contains isoflavones that are able to bind to estrogen receptors, so they have estrogenic properties. This study aims to prove that the administration of tempeh soybean extract during periods of perception can inhibit the formation of ovarian follicles. **Method:** posttest only control group design, using female rats aged 12-13 weeks with BB 150 g, a research site in Udayana University's integrated laboratory. **Result**: the results showed that there were significant differences in the mean number of follicles in the three groups, namely primordial, primary, secondary was p <0.01; atreticfollicles wasp <0.05. **Conclusion**: the administration of ethanol extract of tempeh during the perimenopause period can inhibit the formation of primordial follicles, primary follicles, and secondary follicles. High doses can increase the average number of atretic follicles.

Keywords: *ethanol extract tempeh, perimenopause period, ovarian follicles*

INTRODUCTION

Infertility is a failure of couples gets pregnant after sexual intercourse routinely without contraception for 12 months. Basic Health Research data 2013 found 43.2% married couples do not use contraception because they want children[1]. World Health Organization (WHO) estimates that 10-15% of couples in the world experience infertile[2]. The cause of infertility due to ovulation disorders of 27%, and 25% due to spermatozoa disorders[3].

Ovulatory disorders can occur because of the lack of proper follicles to be recruited and selected so that the follicle is ready for ovulation is also less likely to be absent. Problems with follicular development may occur during the period of perception, ie before, during, and after conception. This period as a critical period is sensitive to exposure or events that interfere with the physiology of cells, tissues or organs[4].

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The primordial germ cells in the fetus, arrive in the gonads and undergo sexual differentiation at 4-6 weeks gestation. Ovum differentiation requires the activity of the Y-group sex genes (SRY) to promote ovarian development by suppressing Sox9. The germ cells in the ovaries experience mitosis rapidly so that the amount of oogonia multiplies. Oogonia is converted into a primary oocyte followed by meiosis as well as the development of its wrapping cells to form a primordial follicle and then develop into primary follicles to preovulatory follicles[2,5].

The recruitment of oocytes into primordial follicles is triggered by germ cells apoptosis, which begins at 13.5 post-coitus days in rate, due to decreased levels of estrogen and progesterone in pregnancy[6,7]. In addition to estrogen and progesterone hormone levels, genistein administration of 50 mg/kg BW/day for five days in 1 day-old rats also inhibits oocyte nest break and recruitment of primordial follicles[8].

A large number of stimulated follicles grows and subsequently develops atresia, causing fewer primordial follicle reserves[9]. The process of apoptosis is controlled

by various cell signals, such as hormones, growth factors, nitric oxide and cytokine[10]. Other factors such as nutrient intake and free radicals. Pregnancy increases oxidative stress due to high metabolic activity, characterized by increased placental lipid peroxide and decreased expression of antioxidant enzymes[11]. Soybean tempeh is one of the foods that have been consumed for generations. Soybean tempe contains protein, carbohydrates, fats, vitamins, minerals, and fiber[12]. In addition, it contains isoflavones consisting of daidzein, genistein, and glycitein[13]. The hydroxyl group possessing isoflavones is antioxidant[14]. Genistein and Daidzeincan be transferred to the fetal body. The compound is also found in the stomach of infants after suckling on its mother who gets soy isoflavone[15].This study aims to prove that, giving ethanol extract of tempeh able to influence the formation of primordial follicles, primary follicles and atresia follicles.

MATERIALS AND METHOD

Animal

Female Wistar rats aged 12-13 weeks, healthy, selected as many as 18 tails with an average body weight of 150 grams. Wistar male rats aged 16-18 weeks selected 9 tails with an average weight of 190 g. The mice were obtained from the UNUD Integrated Biomedical Laboratory. Material enclosure is a plastic box, measuring 40 cm x 15 cm x 10 cm. Each cage is equipped with a feeding and drinking place that is cleaned and replenished daily. The condition of the cage is kept clean, dry, good air circulation, stable room temperature, and calm atmosphere.

Acclimatization is done for one week, the rats are given adjust to the light-dark cycle, covering 12 hours of light: 12 h dark. Rats were given refill drinking water in ad libitum, and standard feed 12-20 g per day. If anyone is sick, the mouse is removed from the study sample, then treated.

Chemical material

The soybean tempeh made by researchers from the local soybean varieties of Wilis, fermented for 48 hours. The tempe was extracted using 96% Ethanol and then the Freezy dryer was done. Every 100 g tempeh yields 4 grams of viscous extract, containing 1.04 mg / g of Genistein tested using thin layer chromatography (KLT) -Spektrofotodensitometri. The extract also contains Phenol 70.25 mg per 100 g GAE (Galic Acid Equivalent), antioxidant 152.31 mg / L GAEAC (Galic Acid Equivalent antioxidant capacity). Each 100 g of wet weight, containing 1.53 g water content, 0.22 g of ash, 1.94 g protein, 80.43 g fat, and 15.89 g of carbohydrate.

Research design

Female rats were randomized after acclimation, divided into 3 groups, ie control (C) given aquadest 0.3 mL; treatment 1 (T1) was given extract of tempeh 0,1 g/kg BW/day; treatment 2 (T2) was given tempeh extract 5 g/kg BW/day. Each group numbered 6 tails. Treatment is administered orally via sonde, daily from 9:00 to 10:00 AM. Determination of dosage refers to the study of Lofamia et al (2014)[16]. Treatment duration is about 56 days, covering 14 days before mating, about 21 days during pregnancy until the pup is born, and 21 days during breastfeeding.

Dam rats mated, in one cage placed 1 male versus 2 females. The dam ratwas found pregnant after a vaginal plug (+) was found.Pregnant ratsare returned to their respective enclosures until weaning. Male ratsare kept in one stable with their dam and pups siblings. The rats were separated from their dams by age 21, randomly selected each of 2 females and males little rats per dam. The females were examined for this study, while the males were used for other studies. Selected little rats, euthanasia with cervical dislocation method. Surgery to take the ovaries of female little rats, followed by histopathological examination.

Gonadal tissue preparation

The ovaries taken from the little female rats were fixed in a 10% formalin solution. The fixed tissue is processed, with the Meyer hematoxylin-eosin (HE) staining. Preparation done according to standard in a laboratory of pathobiology Faculty of Veterinary Medicine of Udayana University.

Sample histological observation

Observations Primordial follicles, primary follicles, and follicular atresia were performed using the Olympus BX 51 brand microscope, the number of cells counted at 5 fields of view. The observations were conducted in the pathobiology laboratory of the Faculty of Veterinary Medicine of Udayana University.

Statistical analysis

Statistical analysis includes descriptive analysis. Comparative analysis using Independent t-test, ANOVA, after all data has the normal distribution. Data analysis using computer assistance, using 95% confidence level ($p < 0.05$).

RESULTS

A total of 18 rats were observed, but drop out 3 tail, that is each group of 1 tail. In group K, the mother rages and wounds her child; T1, sick mother; T2, the mother refused to breastfeed. The number of female children observed per parent is 2little rats (10 little rats each group).

Comparison of the number of Primordial Follicles, Primary Follicles, Secondary Follicles, and AtreticFollicles

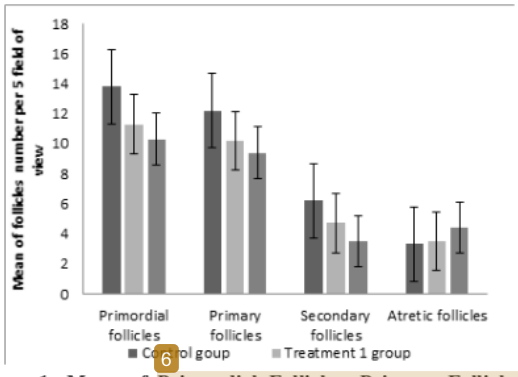

Figure 1. Mean of Primordial Follicles, Primary Follicles, Secondary Follicles, Atretic Follicles

Figure 1shows that the average number of primordial follicles, primary follicles and secondary follicles was lower in the treatment group (T1 and T2) versus control (C), while the number of follicular atresia lower in group C than treatment. To know the differences between the three groups and the differences between groups, One Way Anova analysis was performed. The results of the analysis are presented in table 1 below.

Table 1. Difference Count of Primordial Follicles, Primary Follicles,

Secondary Follicles, and Atretic Follicles in Three Groups (C, T1, T2)

Follicles	**F**	***p****
Primordial Follicles	20.034	0,000
Primary Follicles	12.881	0,000
Secondary Follicles	9.466	0,001
Atretic Follicles	4,03	0,029

*p** significantp<0,05

Average the three groups appear significantly different at primordial follicles ($p < 0.01$), primary follicle ($p < 0.01$), secondary follicles ($p < 0.05$), and follicle atresia ($p < 0.05$). To find out the intergroup comparison, the test is followed by Post Hoc Multiple Comparison: LSD test.

Table 2. Average Comparison of Number of Primordial Follicles, Primary Follicles, Secondary Follicles, and Atretic Follicles

Follicles	Between Group	P
Primordial	C-T1	0,000**
	C-T2	0,000**
	T1-T2	0,000**
Primary	C-T1	0,002**
	C-T2	0,000**
	T1-T2	0,171
Secondary	C-T1	0,023*
	C-T2	0,000**
	T1-T2	0,064
Atretic	C-T1	0,632
	C-T2	0,013*
	T1-T2	0,038*

C=control group; T1 = treatment 1 group given ethanol extract of tempeh 0,1 g / kgBB / day; T2 = treatment 2 group, given ethanol extract tempeh 5 g / kgBB / day.* p value <0.05 which means there is a significant difference mean the number of follicles between the two groups.**p value <0.01 which means there is a significant difference mean the number of follicles between the two groups.

Table 2 shows that the comparison of the number of primordial follicles, primary follicles, and secondary follicles between groups of C with T1 and group C with T2 is significantly different. The comparison of primordial follicular and follicular atresia between T1 and T2 groups was significantly different, while the mean of primary and secondary follicles did not differ. Comparison of mean of follicular atresia between group C with T2 and T1 with T2 was significantly different, whereas C with T1 was not different.

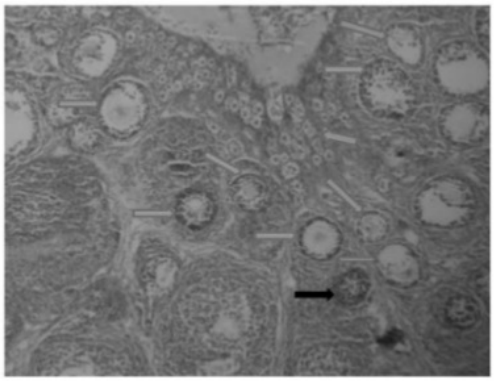

Figure 2
Overview of ovarian histology of little rats aged 21 days of control group (C), magnification 100 times. There appears to be a large number of primordial follicles (blue arrows), primary follicles (yellow arrows), secondary follicles (green arrows), and attretic follicles (black arrows).

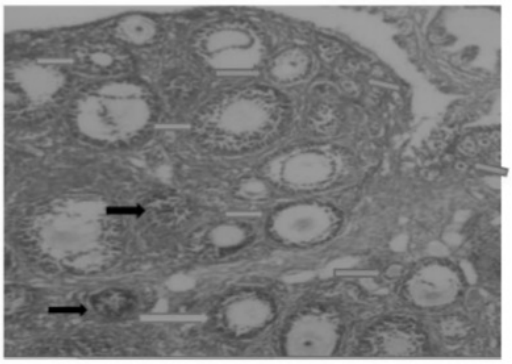

Figure 3

Overview of histology ovary of little rat 21 day treatment group 1 (T1), magnification 100 times. There appears to be a considerable number of primordial follicles (blue arrows), primary follicles (yellow arrows), secondary follicles (green arrows), and follicular atresia (black arrows)

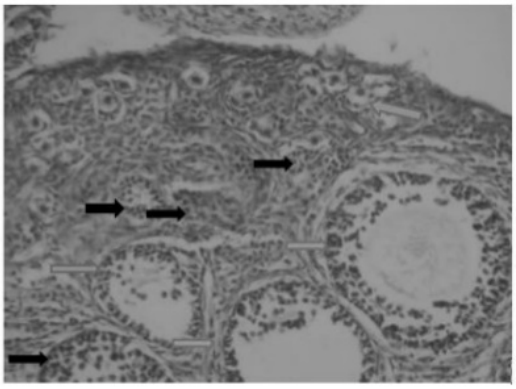

Figure 4

Overview of histology ovary of little rats age 21 day treatment group 2 (T2), magnification 100 times. The number of small primordial follicles (blue arrows), primary follicles (yellow arrows), secondary follicles (green arrow), and more atresia follicles (black arrows)

DISCUSSION

In this study, there appears to be a inhibit of recruitment of primordial follicl which causes the number of follicles to be less than the number of primordial follicles of control group of little rats. In this study, tempeh ethanol extract given containing isoflavones (genistein 1.04 mg/g extract) and flavonoids may cause estrogen levels remain high until late pregnancy and postpartum, so as to inhibit the breaking of the oocyte nest. In addition, the antioxidant properties of isoflavones may also be capable of inhibiting germ cell apoptosis. Both of these events (inhibit of oocyte nest breakdown and apoptosis) lead to the recruitment of primordial follicles to be inhibited as well, resulting in the number of primordial follicles becoming slight.

The results of this study are in line with the opinion, that the effect of genistein exposure o ovarian development of little rats, is unfavorable. At birth, rats have large oocyte nests, and during the first week of life, these oocyte nests dissociate into individual oocytes surrounded by granulosa cells. This rocess of ovarian differentiation requires decreased estrogen and progesterone postpartum. Neonatal treatment with estrogens such as 17β-estradiol and genistein interf s with this process[6,7,17]. Giving genistein injection 50 mg/kg BW/day for five days in 1 day-old rats inhibited

the breakdown of the oocyte nest and the recruitment of primordial follicles[8]. Administration ofgenistein subcutaneous injections of 50 mg/kg BW/day for three days in 18-21 day-old rats caused the number of primordial follicles and fewer primary follicles than the control group[18].

Primary follicles are a further development of primordial follicles characterized by the change of pregranulosa cells into granulosa surrounding the oocyte. Under the influence of growth factors and other factors, primary follicles develop into secondary follicles, characterized by oocytes surrounded by several layers of granulosa cells[19]. Once the primordial follicle is formed, the oocyte begins to meiosis. Oocytes develop through meiosis I to the diplotene stage of prophase I[20,21].

The number of follicles of atresia was highest in the high-dose treatment group (T2). The number of follicular atresia in the group was significantly different with the low-dose treatment group and the control group. The results of this study support the finding that the administration of subcutaneous genistein increases the number of follicular atresia in large follicles and small follicles[18]. Giving 100 mg of soy isoflavone/ kg BW/day increases the number of cells in the antral follicle having atresia in mice. Increased incidence of atresia may be associated with increased apoptosis in the follicle due to low levels of FSH. Isoflavone administration may increase serum estradiol levels. This may provide negative feedback to the pituitary so that FSH levels become low and inhibit the expression of FSH receptors[22-24]. This phenomenon occurs because of increased levels of protein factor apoptosis caspase3, FAS, BAX, combined decreased levels of protein factor antiapoptosis BcL_2[25].

This study is consistent with the findings of Budiani et al., that the administration of Genistein during the periconception period results in inhibition of leydig cell, sertoli and spermatogonia cells formation in male little rats[26]

CONCLUTION

This Study conclude that the formation of primordial, primary, and secondary follicles is inhibited in Wistar rats who received exposure to tempehethanol extract (high and low doses) since the preconception period. However, follicular formation of atresia is triggered by high doses.

Conflict of Interest: All author declare that there is no any conflict of interest within this research and publication including the financial agency.

Ethics: The research ethics from committee of Udayana University Medical Faculty / Sanglah Bali General Hospital.

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