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Eurasian Journal of Analytical Chemistry ISSN: 1306-3057 Received 14 December 2018
January 2019 Abstract: Testes are male gonads which play an important role in reproductive function. These organs are composed of germ cells, Sertoli cells, and Leydig cells that have been formed since the fetus. Each of these cells has different main function. However each cell synergize with one another. The formation and function can be disturbed by endocrine disrupting chemicals.

The objective of this research was to reveal inhibition mechanism of spermatogonia formation of little rats by increasing dam rats estradiol level, inhibiting the formation of Sertoli cells, Leydig cells, and Androgen Receptors after the dam rats given the ethanol extract of tempeh during periconception period. The research design used was Randomized Post-test Only Control Group Design. The research was carried out at a University's Integrated Laboratory, using 32 Wistar dam rats aged 12-13 weeks, and divided into 2 groups.

The control was given aquadest; meanwhile the treatment was given ethanol extract of tempeh in 1 g / kg BW / day, contained genistein of 1.04 mg. The treatment was given 2 weeks before mating, during pregnancy, and breastfeeding. The observed data, including estradiol level of dam rats, number of Leydig Cells, Sertoli Cells, androgen receptors, and spermatogonia of little rats. The results showed that the average of Control vs Treatment Group for estradiol levels of dam rats are (49.17 ± 19.83 vs 181.19 ± 58.62), Sertoli cells are (67.69 ± 3.72 vs. 27.19 ± 7.89), Leydig Cells are (69.56 ± 3.12 vs. 31.50 ± 7.60), androgen receptors are (67.06 ± 4.54 vs. 27.75 ± 7.94), and spermatogonia are (314.25 ± 16.58 vs 234.06 ± 29.97). The data showed significant difference (p <0,05) of all variables between Control and Treatment Group.

The number of Sertoli cells has the greatest effect on the number of spermatogonia (1.780). Conclusions: the ethanol extract of Wilis soybean tempeh in 1 g / kg BW/ day, given to the Wistar dam rats during periconception period was able to inhibit the formation of spermatogonia, directly or indirectly, by increasing estradiol level of dam rats, and inhibited the formation of Leydig cells, Sertoli cells, and androgen receptors. Keywords: ethanol extract of tempeh, periconception period, Estradiol level, Sertoli cells, Leydig cells, androgen receptors, spermatogonia.

INTRODUCTION The reproductive function is determined by the testis composed of a seminiferous tubule in which there are Sertoli Cells, germ cells, Leydig Cells, and other interstitial cells. Testicular formation occurs during the fetus period. Primordial germinal cells undergo differentiation during gametogenesis periods until mature spermatozoa formed. The damage on the testes, especially in Sertoli cells and Leydig cells caused the produced spermatozoa unable to fertilize.¹ WHO estimates, 10-15% of couples in the world experienced infertile. The cause of infertility due to spermatozoa abnormalities by 25%.

The human Sertoli cells are formed at 6th to 7th weeks of pregnancy, then affiliated to forming testicular cord, where primordial germ cells are embedded. The role of Sertoli cells in fetal life is very important, Inhibition of Spermatogonia Formation on Little Wistar Rats Ni Nyoman Budiani 1, I Nyoman Mangku Karmaya 2, Ida Bagus Putra Manuaba 3, Bagus Komang Satriyasa 2, Anak Agung Ngurah Kusumajaya 4 ¹Midwifery Department, Poltekkes, The Ministry of Health, Denpasar ²The Faculty of Medical, The University of Udayana ³The Faculty of MIPA, The University of Udayana ⁴Nutrition Department, Poltekkes, The Ministry of Health, Denpasar ⁴⁷ Eurasian Journal of Analytical Chemistry especially for germ cells, which provide nutrition, differentiation, and prevent germ cells entering meiosis. The Leydig Cells differentiation is started at the 7th to 8th week induced by human Chorionic Gonadotropin hormone (hCG).²

The proliferation and maturation of fetal Leydig cells is furtherly influenced by Luteinizing hormone (LH) and testosterone. Differentiation of germ cells occurs at 14th to 15th week, followed by gonocyte transformation into fetal spermatogonia triggered by increased secretion of testosterone.³ The same developmental process also occurs in mammals, such as rats that are assumed to have similar physiological bodies with humans.⁴

Rats' embryonic sexual differentiation began on the 13th day of postcoitus (dpc), followed by Sertoli cells differentiation on the 13.5 dpc, and Leydig fetus cells on the 14.5 dpc. Leydig cells began to secrete androgens on the 15 dpc.^{5,6} The classical androgen hormones can work or act when there are receptors in their target cells. The

androgen receptors are found in the Leydig Cell nucleus, testicular Sertoli cells, and gonocytes.⁷ The lack of expression of androgen receptors in Sertoli cells and Leydig cells, can inhibit the androgen hormone activity in these cells, therefore the development of germ cells as well inhibited.

The testes development during embryogenesis period can be inhibited by the estrogen hormone⁸ and exposure to endocrine disrupting chemicals (EDC).⁹ Like androgen hormones, estrogen also requires receptors in the seminiferous cord controlling gametogenesis, whereas in the Leydig cell, estrogen receptors¹⁰ Disturbance of spermatogonia formation, also caused by apoptosis that occurs since germ cell differentiation, is mediated by FSH, LH, and / or testosterone deficiencies.¹¹ Isoflavones are one of the EDCs because they have an estrogenic effect can be binding to estrogen receptors.

This compound consists of daidzein, genistein, and glycitein.^{12, 13} Isoflavones are found in soybeans and other processed products, such as tempeh. The Indonesian government recommends pregnant and lactating women to consume four servings of tempeh equivalent to 200 grams a day. High-dose isoflavone supplementation in female mice during periconception periods, give birth to a little male rats with damaged seminiferi tubule and spermatogenic cells does not develop with an unclear picture.¹⁴ Male rats aged 50 days that received genistein exposure since prenatal until 21 days, indicates spermatogenic cell overstimulation activity.¹⁵ The results show that the isoflavone effect on spermatogonia formation that received exposure to isoflavones during periconception period was still unclear.

There had been no study using the ethanol extract of Wilis soybean tempeh given to the experimental animals during the periconception period, therefore it needed further investigation. The objective of this study was to reveal the inhibition mechanism of spermatogonia formation on little rats after the dam rats given the ethanol extract of tempeh during periconception period. MATERIALS AND METHODS Animal Female Wistar rats aged 12-13 weeks, healthy, and gave birth once, selected of 40 rats with an average weight of 150 grams. Male Wistar rats aged 16-18 weeks selected of 20 rats with an average weight of 190 grams.

Those Wistar rats were obtained from Uday Further maintenance and treatment was carried out in Pharmacology Laboratory, Faculty of Medicine, Udayana University. The cage materials were plastic boxes, each measuring 40 cm x 15 cm x 10 cm. At the bottom was filled with chaffs, meanwhile at the top was covered with wire netting. Each cage was equipped with feeding and drinking places. The cage was cleaned and the chaffs were replaced every days. The feeding places along with their bottles were

cleaned always kept clean, dry, good air circulation, stable room temperature, and calm atmosphere.

Acclimatization was done for one week and the rats were given the adjustment to the light-dark cycle, covering 12 hours of light; 12 hours of dark. The rats were given refill drinking water in ad libitum, and standard feed 12-20 g per day. The rats were sick, those rats were removed from the study sample and then treated. Chemicals The soybean tempeh made by researchers from the local soybean varieties of Wilis, fermented for 48 hours. Each 100 g of Wilis soybean varieties produced 150 g of tempeh. The tempe was extracted using 96% Ethanol as a maceration material. After evaporation, Freezy dryer is done. Each 100 g of tempeh 48 Ni Nyoman Budiani et.al produced 4 grams of viscous extract.

The genistein level of viscous extract was tested by using thin layer chromatography (KLT or Kromotografi Lapis Tipis) - Spectrophotodensitometry. Each 1 mL of the preparation had a weight of 1 g, contained 1.04 mg of Genistein. Other laboratory analysis resulted that those extracts contained 70.25 mg of Phenol per 100 g GAE (Galic Acid Equivalent). Every 100 g of wet weight, contained Moisture 1.53 g, Ash 0.22 g, Protein 1.94 g, Fat 80.43 g, and Carbohydrate 15.89 g. Research Design Female Wistar rats were randomized after acclimatization, divided into 2 groups, namely Control and Treatment group with 20 rats in each group. Treatment group (T) was given ethanol extract of tempeh of 1 g / kg BW / day.

Control group (C) was given aquadest of 0.15 ml. Treatment group was provided orally using oral gavage tube, daily from 9:00 to 10:00 AM. Dose determination referred to preliminary study results adapted from the Lofamia research, et al. (2014). The treatment duration was approximately 56-59 days, covered 14 days before mating, about 21-23 days during pregnancy until the little rats were born, and 21 days during breastfeeding. th day, at 7:00 AM after anesthesia was given for the examination of serum estradiol levels. The rats were mated, in one cage placed 1 male : 2 females rats. The rats were observed on their married behavior.

The rats were declared pregnant after vaginal plug (+) were found. The pregnant rats were returned to their cages until weaning. The little rats were treated in a clean cage together with their dams and siblings. The 21-days-old male rats were randomly selected 2 rats per dam to be examined. To the selected rats was performed euthanasia with cervical dislocation method. The surgery was performed to take the testes of male rats, followed by histopathology and immunohistochemistry examination.

ELISA Examination The examination of dam rats serum estradiol (E2) used

enzyme-linked immunosorbent assay (ELISA) E-EL-0065 produced by Elabscience Biotechnology Co., Ltd. (Elabscience). The examination procedure was in accordance to factory standard. Gonadal tissue preparation Testes taken from little male rats were fixed in a 10% formalin solution. The fixed tissue was processed, with Meyer hematoxylin and eosin (HE) staining. The preparation was carried out according to standard in pathobiology laboratory of Veterinary Faculty, Udayana University.

Sample histological observation The observations of Sertoli Cells, Leydig Cells, and spermatogonia were performed by using the Olympus CX 21 brand microscope, the number of cells counted at 5 fields of view. Documentation of observation results used Olympus BX 51 camera. The observations were carried out in the pathobiology laboratory of Veterinary Faculty, Udayana University. **Immunohistochemistry examination** The examination of androgen receptors expressed on Sertoli Cells and Leydig Cells, used indirect methods, by utilizing Histofine Kit (Nichirei Biosciences Inc.

Tokyo Japan), primary antibody of AR N-20 : sc-816 (Santa Cruz Biotechnology) and secondary antibody of Universal Immuno-peroxidase Polymer for rat tissue sections Anti-Mouse primary antibody (Nichirei Biosciences Inc. Tokyo Japan), and DAB. The staining results were observed by using a microscope. The observations included the number of expressed Androgen receptors on Sertoli Cells and Leydig Cells at 5 fields of view. **Ethics** This study, before being carried out, it has been studied by the research ethics Commission of Medical Faculty, Udayana University / Sanglah General Hospital, Bali Indonesia. **Statistic analysis** Statistic analysis included descriptive analysis.

Comparative analysis used Independent t-test, pathway analysis, after all data had normal distribution. Data analysis used computer aids, using 95% confidence level ($p < 0.05$). **RESULTS** General observation 49 *Eurasian Journal of Analytical Chemistry* The numbers of dam rats observed were 20 rats. Drop out each of 4 dam rats. In the Control group, there were 3 dam rats raged and injured their little ones, and 1 infertile rat. In the Treatment Group, there were 1 dam rat raged and ate its child, and 3 infertile rats. The numbers of little male rats observed per dam were 2 rats (32 little rats in each group).

The comparison on mean of spermatogonia **between control and treatment group** Figure 3.1 The comparison on mean of spermatogonian **between Control and Treatment group.** The mean of spermatogonia in Control group was higher than the Treatment group. *** $p < 0.01$. number of Sertoli Cells, Leydig Cells and androgen receptors. The comparison on mean of estradiol serum **levels of dam rats** Figure 3.2 The comparison on mean of dam rats estradiol serum levels of **Control and Treatment group.** The mean of the Control group was lower than the Treatment group. *** $p < 0.01$. Comparison on mean **of Sertoli Cells, Leydig Cells, and Androgen Receptors** Figure 3.3

The comparison on mean of Sertoli Cells, Leydig Cells, and Androgens Receptors of little Wistar rats between Control and Treatment groups. The mean of Control group was higher than Treatment group. *** $p < 0.01$. 50 Ni Nyoman Budiani et.al The histological figures of the little rat's testes Figure 3.4 Little Wistar rat testes histology at 100 times magnification. The Control group (A) appeared to be a dense distribution of spermatogonia (yellow arrow). Leydig cells (black arrow), Seminiferous Tubules wall (blue arrow). Treatment group (B) appeared sparse spermatogonia (yellow arrow). Leydig cells (black arrow), Tubular Seminiferous wall (blue arrow). Figure 3.5

The histology of little Wistar rats at 400 times magnification. The Control group (A) appeared solid spermatogonium distribution (yellow arrow), Sertoli cells between spermatogonium (white arrow), dense Leydig cells (black arrow). The walls of the seminiferous tubules (blue arrows). The Treatment group (B) appeared to be a sparse spermatogonium split (yellow arrow), Sertoli cells between spermatogonium (white arrow), rare Leydig Cells (black arrow). The walls of the seminiferous tubules (blue arrows). Images of immunohistochemical staining of androgen receptors magnification of 100 times.

There was a brown patch in the interstitial area and in the seminiferous tubules showed the expression of androgen receptors (black arrow), seminiferous tubules walls (blue arrow), and sufficiently dense spermatogonia split (yellow arrow). Figure B magnification of 400 times. There were appeared expressions of androgen receptors in Sertoli cells (black arrow). Figure C magnification of 400 times. There were appeared expressions of androgen receptors in Leydig cells (black arrows). 51 Eurasian Journal of Analytical Chemistry Figure A, magnification of 100 times.

There was a brown spot in the interstitial area and in the seminiferous tubules showed expression of the androgen receptors (black arrow), seminiferous tubules wall (blue arrow), and sparse spermatogonia (yellow arrow). Figure B, magnification of 400 times. There were appeared expressions of androgen receptors in Sertoli cells (black arrow). Figure C, magnification of 400 times. There were appeared expressions of androgen receptors in Leydig cells (black arrow). The effect of ethanol extract of tempeh, dam rats estradiol levels, the amount of Sertoli Cells, Leydig Cells, and Androgen Receptor on the number of spermatogonia Based on the results of pathway analysis, was found the influence of variables on the number of spermatogonia. Table 3.1 The Effect of Ethanol Extract of Tempeh, Dam Rats Estradiol Levels, The Amount of Sertoli Cells, Leydig Cells, and Androgen Receptor on the Number of Spermatogonia.

Variables Effect Direct Indirect Total Ethanol extract of tempeh -0.260 -1.124 -0.864 Dam

rats estradiol levels -0.683 -0.154 -0.837 Sertoli Cells amount 1.780 0.000 1.780 Leydig Cells amount -1.636 2.212 0.576 Androgen Receptor amount 0.425 0.000 0.425 Table 3.1 showed that, the ethanol extract of tempeh and dams estradiol level gave negative effect to spermatogonia formation. The biggest effect is the number of Leydig cells. DISCUSSIONS The ethanol extract of tempeh contained genistein isoflavones. Isoflavones and estrogen are structurally endogenous, very similar since they have aromatic A rings, hydroxyl groups at C3 position in endogenous estrogen on equivalent position in phytoestrogens.

Isoflavones had phenolic rings, which cause them to bind to estrogen alpha receptors (R estrogen hormone).¹⁶ Genistein and daidzein were immediately absorbed after consuming soybean diets, and the peak of these compounds concentrations in serum are achieved after 4-8 hours.¹⁷ The numbers of little rats spermatogonia of Control group was found higher than the Treatment group. The mean difference between two groups was 80.188.

The numbers of spermatogonia was influenced by ethanol extract of tempeh, dam rats estradiol levels, Sertoli Cells amount, Leydig Cells amount, and Androgen Receptors amount. Simultaneously, the four variables affected the numbers of 52 Ni Nyoman Budiani et.al spermatogonia by 98.1%. The ethanol extract of tempeh and dam rats estradiol levels showed a negative effect on the numbers of spermatogonia. That is, the numbers of spermatogonia would increase when the provision of ethanol extracts of tempeh and dam rats estradiol levels were lowered. Otherwise, the number of Sertoli Cells, Leydig Cells, and Androgen Receptors showed positive effect.

The increasing in the amount of Sertoli Cells, Leydig Cells, and Androgen Receptors could increase the numbers of spermatogonia. The results of this study were consistent with findings, that the provision of genistein in pregnant rats from 12th day to 19th day in dose of 2 mg; 20 mg; and 100 mg per kg BW per day, caused the number of spermatogonia in the Treatment group lower than Control group.¹⁸ The effects in the provision of ethanol extract of tempeh in this study may be begun with the increasing in isoflavone levels that triggered the increased aromatase activity.

In this study was found dam ra'sesra evsin e reamegwe gher n oolgrou(0.01)Tnser isoflavone from dam rats to fetus through the placenta, caused the increasing of endogenous estrogen levels of the fetus. High levels of isoflavones could trigger the activity of aromatase enzymes that could increase the endogenous estradiol levels.¹⁹ High endogenous estrogen levels were able to provide negative feedback to the hypophyse so that levels of FSH and LH decreased. Low LH could decrease the testosterone secretion. The results of this study mRNA 20 The increased estradiol

concentrations might provide negative feedback on gonadotropin so that FSH secretion decreased.²¹ to lower LH concentrations in the Control group.²² On the other hand, isoflavones were able to inhibit the activity of enzymes involved in the administration of 10 nM genistein in fetus testis cultures of rats, was able in inhibiting Androgen secretion in early development (12.5-day).²³ The mRNA expressions of StAR, P450scc, P450c17, Insl-3 enzymes, the testosterone levels tended to be lower in the Treatment group than in Control group, on the rats given genistein 1 mg / kg BW / day by using sonde on the fifth day of pregnancy until third day of postnatal.²⁴ The amount of Sertoli Cells in the Treatment group was found less than Control group. This result was probably due to low levels of FSH and testosterone.

This condition was in accordance with the description that estrogen might inhibit the differentiation or proliferation of Sertoli Cells, therefore the amount of Sertoli Cells decreased.^{10, 25} The proliferation stimulation of Sertoli Cells was influenced by the mutual ability of FSH and testosterone by activating the MAP (Mitogen-activated protein) kinase path in Sertoli Cells.²⁶ Low testosterone levels could trigger AMH secretion. Low levels of testosterone and high levels of AMH, inhibited the proliferation of Leydig Cells, and therefore in this study were found the amount of Leydig Cells in the Treatment group was lower than Control group.

The results of this study supported a study that found the amount of Leydig Cells tended to be lower on the rats given genistein 1 mg / kg BW / day by using sonde from the fifth day of pregnancy to third day of postnatal.²⁴ Sertoli Cells and Leydig Cells were expression places of androgen receptors. In this study was found the amount of androgen receptors in the Treatment group less than Control group. The results of this study reinforced the results of a study found that giving 1 mg / rat / day injection of genistein for 5 days in rats could decrease the expression of androgen receptor mRNA.²⁷ The small amount of androgen receptors caused the androgen hormones could not act on Sertoli Cells or Leydig Cells. The role of Sertoli Cells was essential for the development of germ cells.

In this study was found that the amount of Sertoli Cells had the greatest effect on the numbers of spermatogonia (1.78). Lack of FSH, testosterone, and androgen receptors could inhibit Sertoli Cells function in maintaining germ cells, therefore the spermatogonia formation were reduced. The decreasing of FSH and testosterone caused Sertoli Cells proliferation to be inhibited and disrupt the function of these cells.

Sertoli Cells functions were to maintain and protect germ cells, secrete inhibin, ABP, AMH / MIS, prevent germ cells from entering meiosis. The disruption of Sertoli Cells function might inhibit the development of spermatogenic cells.²¹ The Increasing

amount of Sertoli Cells correlated with the increasing in spermatogenic capacity. The androgen overexpression model on Sertoli Cells (tgSCAR) in early development led to early maturation of Sertoli Cells and reduced the amount of final Sertoli Cells, therefore the amount of final germ cells was also reduced.28 53 Eurasian Journal of Analytical Chemistry The conclusions could be drawn as well as novelty of this study was the formation of spermatogonia can be inhibited with the provision of ethanol extract of tempeh as much as 1 g / kg BW / day during the periconception period, directly or indirectly, through the increasing dam rats estradiol levels, the formation inhibition of Sertoli Cells, Leydig Cells, and androgen receptors. Conflict of Interest The author declare that we did not have conflict of interest.

Acknowledgments The author would like to convey gratitude to Prof. Dr. dr. Ketut Suastika, Sp.PD-KEMD., Prof. Dr. dr. Putu Astawa, Sp.OT (K), M. Kes., Prof. Dr. dr. A. A. Raka Sudewi, Sp.S (K), and Dr. dr. Komang Bagus Satriyasa, M. Repro for the opportunities and facilities given to the author. REFERENCES [1] Barbieri, R.L. 2014. Female Infertility. In: Strauss III, J.F. dan Barbieri, R.L. editors. Yen & Jaffe Reproductive Endocrinology: Physiology, Pathophysiology, and Clinical Management. 7th Edition. Philadelphia: Elsevier Saunder, p.512-537. [2] Huff, D.S. 2011. Testis. In: Ernst, L.M., Ruchelli, E.D., Huff, D.S. Editors.

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