The New Detection Method of Ovarian Follicle Development Using Digitized Wide Area Measurement

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Methods

The follicle development is one indicator in determining female reproduction condition, one of them is influenced by the growth of estradiol level inside follicle. Estradiol is one of hormones needed in the ovarian follicle development because Estradiol is part of estrogen and plays an important role in follicular mature [1]. In the female reproduction cycle, the growth of estradiol level will trigger the rise of luteinizin hormone (LH) Surge which caused ovulation or the process of mature egg is released from the follicle. The ovulation ability is very influenced by the mature follicle condition in the antral phase, in the antral phase has formed antrum which contains liquid of biochemical process result which is needed in oocyte to growth into mature [1], [2]. The signs of mature oocyte are the polar body and perfect cumulus cell expansion. The follicle liquid also is needed to stimulate around follicle who has ovulation, which begins with the LH Surge process and caused the changing of structure and biochemical of follicle wall. The presence of stimulation Prostaglandine and active collagenase caused the extrusion of egg cell from inside follicle which surrounded by cumulus oophorus and leaved follicle cell into corpus luteum [1], [3]. The increasing of estradiol can be reached by steroidogenesis process and forkhead (FKHR) in the proliferation of granulose cell through the estrogen receptor (ER) [1]. The high role of estrogen in creating LH surge can become an alternative in ovarian stimulation [1], [2].

In this research used in vitro method, follicle development signed with the addition of follicle measurement one of them is caused by the addition sum of follicle liquid. In vitro growth (IVG) is the observation method of follicle development by observing the addition of diameter measurement which is an indicator of follicle development, measured using stereo microscope [4]. The follicle development signed with the addition of liquid inside follicle. There is a result of synthesis follicle stimulating hormone, estrogen, and the receptor. Those hormones also have role to steroidogenesis luteum [1]. Estrogen becomes the key success in the research of IVG, signed with the increasing measurement of
Materials and Methods

The research method used in this research was experimental laboratory with pre- and post-test only control group design. The results of the three measurements then analyzed the relationship between estradiol levels with follicle diameter and estradiol levels with the follicle area. Correlation analysis of the two test parameters was carried out using Kendall’s Tau analysis by SPSS v.26 software.

The treatment or intervention in this research did by IVG used goat follicle cell which is given addition of pregnant mare serum gonadotropin (PMSG) + human chorionic gonadotrophin (HcG) to basic culture media [5]. The research conducted at The Central Biology Laboratory of Brawijaya University, the material used goat ovarium took from 3 units of RPH Subdistrict of Sukun, Malang Regency, took to the laboratory by inserting into water bath fulfilled with NACL physiological liquid 0.9% which has been added by antibiotic 100 IU/mL penicillin (Sigma-Aldrich, St.Louis, MO, USA) and 0.1 g/mL Streptomycin (Sigma-Aldrich) with temperature 35–37°C. Then, the ovarium is cleaned from the fatty tissue [6].

The follicle isolation Rather did by ovarium was taken by steril pinset then made early ovarium measurement and did follicle isolation used surgical blade into follicle sized of 2–3 mm or secondary follicle, entered into Petri dish which content physiological NaCL liquid 0.9% [4], [7], [8]. It was to know the secondary follicle development based on estradiol level. The follicle development is one condition where there is an addition of follicle size because of the addition of liquid follicle volume.

Maturation medium used tissue culture medium-199 (TCM-199), serum supplementation (FBS 10%), Paravin oil, and PMSG + HcG as control, while the treatment group added a dose of Calyptromyia barbata leaf extract and Coclaurine dose group [4], [8]. The number of samples in this study was indicated by the number of replications carried out on goat follicles which were used as experimental animals. Replication calculations using Federer’s formula, the sample size estimate is calculated based on the formula \((t–1) (r–1) ≥ 15\) so that the total follicle samples used for 7 groups were 28 goat follicles.

The observation used inverted microscope and stereo microscope with magnification of 200× [4], [8]. On the 7th day, follicle removed to steril water liquid. Oocyte isolation from each follicle has broken. Do washing used TCM-199 and Penstrep (antibiotics) as many as 2 times. The observation and analysis the growth of follicle used with two methods those are diameter (millimeter block) and outside area (Application of ImageJ).

The measurement in getting test parameter by three ways those are estradiol level measurement, follicle diameter measurement with conventional method, and follicle wide measurement with digitalized method used ImageJ software.

**Estradiol measurement level**

Estrogen level measured by enzyme-linked immunosorbent assay (ELISA) Kit from MYBIOSOURCE, cat. MBS265205, checking using microplate 96-wells. To each well inserted standard liquid that has been known the estrogen level and culture medium of research sample. Enzyme conjugate (estradiol–HRP Conjugate) added to each well incubated at room temperature for 120 min. The liquid inside the well was throw away and the well washed for 3 times with washing liquid. The liquid of substrate A and substrate B entered into each well and microplate incubated for 15 min in the temperature of 37°C. Reaction stopped by adding stop solution to each well. The reaction will result color changing from blue to yellow. Absorbance measured with the length of wave 450 nm with ELISA Reader. This examination repeated 4 times for each group of treatments. The sample of estrogen level was known by comparing the sample absorbance to standard curve made by software four parameter logistic (4-PL) Curve-Fit [9]. The level of measurement result gain ratio value in unit of pg/ml.

**Conventional diameter measurement method**

The measurement of follicle diameter ovarium did by microscopic of placing micrometer in objective lens; thus, it can be seen the measurement from above the field of ovarian follicle. The measurement result then tabulated into table with measurement unit in form of micrometer (µm). The result from the measurement of day–0 and to day of 7th then subtracted with the formula as follows:

\[
\Delta D = (D7–D0)
\]

(1)

The result of subtraction then did scoring step in which if the value \(\Delta\) maximum was 0 thus said it was
Processing image method did by changing the bright field of ovarian follicle microscopic into digital image using microscope camera used optiLab with magnification of 100×. The result from digital image then made measurement used ImageJ software. The sum of bright field followed all surface of microscopic preparations thus will no miss anything. The steps of what have done in the processing step of digital image are as follow:

a. Digitalized microscopic preparations

b. The 8th photo conversion

c. The step of “threshold” to make the picture become separated based on the level of brightness

d. Then did measurement by arranging the wide area based on “limit threshold”
e. Click analyze → measure

The result from the wide measurement in the day of 0 and the day of 7th then made a subtraction with the formula as follow:

\[
\Delta L = (L_7 - L_0)
\]

The result from the subtraction then made a scoring step in which if the score of \( \Delta \) maximum is 0 then it can be said there is no changing and resulting score of 0 and if the score of \( i \Delta \) more than 0 thus resulting score of 1.

Results

This experiment did to measure the level of estradiol which then compare with the measurement result of diameter and wide area. The result of the measurement between extradiol level, follicle diameter, and area percentage and data analysis performed in Table 1.

| Measurement                  | n  | Estradiol level | Diameter | Area percentage |
|------------------------------|----|-----------------|----------|-----------------|
|                              |    | Median          | Minimum  | Maximum         |
|                              |    | 31.03           | 26.30    | 39.40           |
|                              | 28 |                 | –        | –               |
| Sum of follicle is not added | –  | 23              | 0.86     | 17              |
| Sum of added follicle        | 5  | 5               | 0.95     | 11              |

The result is from Table 1. Above showed extradiol level which has span from 26.30 to 31.03 from 28 experimental animal measured. The result from the ovarian follicle measurement showed more diameter measurement which was not added the size compare to the measurement of area percentage, thus resulting opposite score to the sum of added measurement of follicle in which the sum of added follicle observable more to the area percentage measurement compare to diameter measurement. The result from the measurement then made a correlation test which is shown in Table 2.
leaves extract and coclaurine have included into response estrogen ER element (ERE). The recruitment transcription activity through the interaction of element gen target by 17-β Estradiol able to minimize the difference affinity of estrogen alpha and estrogen beta [9].

From Table 2 showed that the strong correlation between the areas of collagen measured digitalized to the changing of estradiol level with value was 0.453. The comparation result between estradiol level and measured diameter showed weak correlation. This case showed that the measurement of follicle diameter manually still weak toward the changing of estradiol level.

**Discussion**

Folliculogenesis is the maturation process of the follicle in the ovarian, which is started from immature follicle (primordial follicle), which develop into preovulation follicle (Follicle De Graaf) [3], [10]. The follicle development phase is needed by estrogen as steroid group hormone which has important role in the proliferation of cellular level and tissue growth related with reproduction system which covers a group of chemical hormone which consists of estron, estradiol, and estriol. Main reproduction organs which result estrogen are theca cells, granulose cells ovarian follicle, corpus luteum, and placenta. One of them is by the process of steroidogenesis/the changing process of androgen into estrogen by CYP19A1 aromatase [9], [11].

The research result shows that the treatment using C. barbata leaves extract and coclaurine have potency to increase estradiol level. This case because the extract inside nutrient components such as water, energy, proteins, fats, fiber, carbohydrate, chlorofil, calories, Vitamin A, Vitamin B, Vitamin C, calcium, phosphor, iron, and magnesium. In the previous research in analyzing green Barbata leaves to screening phytochemical, there were compounds of flavonoid, alkaloid, saponin, tannin, and steroid [12].

Estradiol is one of the parts from estrogen and holds an important role in the maturation of follicle and the ovulation process. The estrogen biological action through the receptor of estrogen which synthesized by some type of cells in two isoforms is estrogen alpha and estrogen beta with different effect, the difference caused by amino acid and tie ligand. The presence of 17-β Estradiol able to minimize the difference affinity of estrogen alpha and estrogen beta [9].

**Table 2: The correlation analysis result for test parameter**

| Measurement parameters | Estradiol level versus follicle diameter | Estradiol level versus % collagen area |
|------------------------|----------------------------------------|---------------------------------------|
| p-value                | 0.269                                  | 0.009*                                |
| Correlation coefficient | 0.093                                  | 0.453                                 |

The mechanism of estrogen signaling mediated through nucleus of receptor protein ERα and ERβ which results direct cascade through second messenger conventional including Adenylate cyclase, cAMP, phospholipase C, protein kinases C, and mitogen activated protein kinase (MAPK) which resulted fast estrogen response. On the other side, the presence of receptor nucleus estrogen alpha often localized to membrane cell such as GPR30. Receptor of epidermal growth factor (EGF) and insulin like growth factor (IGF)–1 stimulated cascade kinase which impact to agonic and antagonic function of IGF-1 [9], [11].

Estrogen effect mediated by 2 receptor those are ERα and ERβ, which equally able to bind agonic or antagonic however with different affinity [2]. ER formed from separated gen thus the structure and function is different. The receptor of alpha estrogen mediated proliferation effect, while receptor estrogen alpha has anti-proliferation characteristics. Complex receptor estrogen then binding with ERE, the latching will induct the transcription mRNA process which then translated into protein which will result estrogenic response to target cell [1].

The mechanism of receptor estrogen involved estrogen binding receptor in cytosol. This receptor binding estradiol in ligand binding domain Receptor estrogen acted by two mechanisms those are genomic action and non-genomic action. Genomic action involved ER which located in the nucleus. Estrogen send into the tissue in form of binding with protein and difuted immediately into cells as free estrogen, then the receptor estrogen has dimerization and binding with EREs which located in the target gen promotor and inducted gens transcription which correlated with proliferation cell [1].

Non-genomic action involved receptor estrogen which located in the membrane plasma and cytoplasm. This action involved complex protein and signaling molecule that is MAPK and Akt path. Estrogen can be act directly with other growth hormone such as EGF.

Estrogen related with receptor estrogen alpha which then activated parakrin factor to induce mitosis cell of epitel. Parakrin factor in form of EGF will activated by receptor binding of tiroisnt kinase which located in the epitel and then activated kinase proteins inside cytoplasmcell. Kinase protein activated assumed in form of MAPK which is main signal to activate transcription and translation,
the synthesis of protein occurs which is needed in the mitosis process of epithel cells and caused epithel to proliferate to the optimum level [13].

Based on the research result shows that C. barbata leaves and cochlaurine have influence in increasing estradiol level. Based on the affinity test result, cochlaurine has steroid structure which able to bind receptor estrogen alpha. This case will impact to the increasing of estradiol level. There are some paths of increasing the estradiol level become estrogen or known as steroidogenesis theory and FKHR through the process of granulosa cell proliferation [12].

The method used in knowing the follicle development based on the increasing of estradiol level one of them is by measuring the ovarian follicle [14]. The using of diameter scale measurement which nowadays still use to know the follicle development still have many lacks in which the follicle is not in the form of circle but poligonal. This poligonal form of course will result different diameter value however there is the nearest and the farest range from the length of diameter itself.

The difference in group of measurement using diameter shows coefficient of variation (CV) score is bigger than measurement group used wide area. Higher CV score thus will have bigger range of measurement between one and another. Those will become the prove that the wide area measurement become better with the measurement using diameter. If it is observed from the level of estradiol, it will shows high correlation to the group of measurement using wide area compare to follicle diameter.

Conclusion

Based on the analysis result that there is a strong correlation between wide area follicles measured with the application of ImageJ toward the changing level of estradiol compare to the measurement of follicle diameter.

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