Curcumin Improves the Regulation of Ovarian Folliculogenesis in Culture Media with Peritoneal Fluid from Infertile Women with Endometriosis

Hendy Hendarto[1], M Yohannes Ardianta Widyanugraha[1], Widjiati[2], Soehartono Darmosoekarto[1]

[1]Department of Obstetric and Gynecology, Faculty of Medicine, Universitas Airlangga, Surabaya (60131), Indonesia, [2]Laboratory of Embriology, Faculty of Veterinary Medicine, Universitas Airlangga Surabaya, Indonesia

Abstract

Background : Peritoneal fluid (PF) from infertile women with endometriosis contains a variety of inflammatory mediators that may interfere with folliculogenesis. The aim of the study was to evaluate effects of curcumin on the regulation of ovarian folliculogenesis by evaluating Growth Differentiation Factor (GDF)-9, Kit Ligand (KitL) and Tumor Necrosis Factor (TNF)α expressions in bovine cumulus oocyte complexes (COC)s cultured with PF from infertile women with endometriosis.

Method: COCs were aspirated from antral follicles of bovine ovaries. PF was collected from infertile women with endometriosis undergoing laparoscopy for infertility evaluation. Curcumin, a strong anti-inflammatory turmeric, was added in Tissue Culture Media (TCM)199 and PF from infertile women with endometriosis for culture media. Bovine COCs were cultured into 3 groups of media: 1) TCM199, 2) TCM199+PF from infertile women with endometriosis, and 3) TCM199+PF from infertile women with endometriosis+curcumin. GDF-9, KitL and TNFα expressions were examined using immunohistochemistry technique.

Results: GDF-9 expression of bovine COCs cultured in PF from infertile women with endometriosis with curcumin addition (2.67±0.98) was found to increase compared to those cultured without curcumin (0.50±0.67) but reduced compared to the control. This result was similar to KitL expression of bovine COCs cultured with curcumin (2.67±1.23), in which it increased compared to those without curcumin (0.33±0.49) ($p<0.05$). A significant difference in TNFα expression was noted between groups with or without curcumin ($p<0.05$).

Conclusion: In the culture of PF from infertile women with endometriosis, curcumin addition may improve the regulation of ovarian folliculogenesis through the decrease of inflammation factor.

Keywords: *endometriosis; curcumin; GDF9; kit ligand; TNFα*

Background

Endometriosis is a condition characterized by the growth of endometrial-like tissue outside the uterus that subsequently induces a chronic inflammatory reaction. Numerous symptoms are associated with endometriosis, including dysmenorrhea, pelvic pain, dyspareunia and infertility, as well as reduced quality of life [1]. The relationship between endometriosis and infertility remains unclear. One of the postulations of decreased fertility in women with endometriosis is the reduction of oocyte quality caused by abnormal folliculogenesis related to peritoneal fluid (PF) inflammation [2].

Folliculogenesis is a dynamic process marked by proliferation and differentiation of granulosa cells and maturation of oocyte. The regulation of ovarian folliculogenesis, determined by a number of growth factors and classic endocrine mechanism, provides optimal environment to produce fertilizable oocyte [3]. There are two important growth factors that contribute to the regulation of ovarian folilculogenesis including the interactions between oocyte

and granulosa cells [4].

Those growth factors are Growth Differentiation Factor (GDF)-9 produced by oocyte, which is useful for granulosa cell proliferation and differentiation; and Kit Ligand (KitL) secreted by granulosa cells, which induces oocyte maturation. In women with endometriosis, ovaries are naturally bathed in PF that is rich in inflammatory mediators. This may cause abnormal folliculogenesis and subsequently results in infertility. The PF of women with endometriosis contains a variety of inflammatory mediators, including Tumor Necrosis Factor (TNF)α [5].

Curcumin, which is derived from *Curcuma longa* (turmeric), has a strong potential anti-inflammatory activity [6]. It has been widely used both traditionally and scientifically to treat various conditions including inflammatory diseases, such as rheumatoid arthritis, chronic anterior uveitis and ulcerative colitis [7]. Treatment with curcumin can reduce implant size and cell proliferation in rat endometriosis model, but the effects of curcumin on intraovarian growth factors in endometriosis and infertility remain controversial [8]. The objectives of our study was to evaluate whether curcumin could improve the regulation of ovarian folliculogenesis in bovine cumulus oocyte complexes (COC)s by analyzing GDF-9, KitL and TNFα expressions in culture media with PF from infertile women with endometriosis[9].

Method

We evaluated the growth factors expression of folliculogenesis regulation on bovine COCs cultured in three different types of media. Tissue Culture Media (TCM) 199 only (group 1= control), TCM199 plus PF from infertile women with endometriosis (group 2=endometriosis), and TCM199 plus PF from infertile women with endometriosis added with curcumin (group 3=endometriosis+curcumin). The ethical board of Dr Sutomo Hospital approved the study [9].

Endometriosis peritoneal fluid

We obtained PF samples from women of 20 to 40 years old with endometriosis undergoing laparoscopy for infertility evaluation at Dr. Sutomo Hospital Surabaya. The diagnosis of endometriosis was made by visual inspection and peritoneal biopsy according to American Society for Reproductive Medicine criteria. We collected PF by aspiration from posterior cul-de-sac during the laparoscopic procedure. The samples were placed in a tube and centrifuged at 600 g for 10 min. The supernatants were stored at -80°C until analysis [10].

Curcumin

We obtained curcumin from Merck Schuchardt OHG (85662 Hohenbrunn, Germany). Curcumin (0.2 mg) was added and homogenized in 10 ml of TCM199 media and PF fluid infertile women with endometriosis (30 µl). BSA (3%) was added until the pH reached 7.4-7.8. The solution was then filtered through a 0.22-µm microfilter and 100 mL of solution was placed in a petridish for culture [11].

Bovine cumulus oocyte complex

COCs aspirated from antral follicles with a diameter of 3-8 mm were obtained from bovine ovaries in a slaughterhouse. The ovaries were washed and stored in 0.89% NaCl with penicillin-G (1000 IU/ml) and streptomycin sulfate (0.2 ug/l) at a temperature of 30-35^0 C. Before follicle aspiration, the diameter of the follicles was measured with a caliper. The COCs were aspirated using an 18-G needle connected to a 5-ml syringe containing 1 ml phosphate buffered saline (PBS) with 3 % bovine serum albumin (BSA) and 50 ug/ml gentamycin. The COCs were washed 3 times successively in PBS media and one time in TCM199, placed in TCM199 media with 50 mIU/ml FSH and 50 mIU/ml LH, divided into three groups by placing them into media groups 1, 2 and 3, and then incubated at a temperature of 38^0 C in 5% CO2 for 24 hours. Subsequently, the COCs of each groups were fixated in a glass flask and subjected to immunohistochemical staining for GDF-9 (Bioss Antibodies Inc USA, catalog no. Bs-4720R), KitL (Abcam USA, catalog no. ab52603), and TNFα (Bioss Antibodies Inc USA, catalog no. Bs-2081R) expressions.

The three expressions were semi-quantitatively assessed according to the modified Remmele method which is the result of multiplication between the percentage score of immunoreactive cells (positive cells) with the color intensity score generated on the cell [12].

Statistical Analysis

Data analysis was performed using statistical software (SPSS version 17.0 for windows) Normality of variable was tested with Shapiro-Wilk test. Non-parametric test was used to detect significant differences of all variables. $P < 0.05$ was accepted as statistically significant [13].

Results

A total of 21 bovine COCs were cultured in 3 different types of media; each group contained 7 COCs. The GDF-9 expression in bovine COC was determined by dark color of immunoreactive cells on the immunohistochemical staining result. The semi-quantitative results of GDF-9 expression in control, endometriosis and endometriosis+curcumin groups were as follows: 5.83±1.58; 0.50±0.67; and 2.67±0.98, respectively. The mean expression of GDF-9 in bovine COC cultured in PF from infertile women with endometriosis+curcumin (2.67±0.98) increased compared to those cultured without curcumin (0.50±0.67) but reduced compared to the control (5.83±1.58). (p=0.46) (Figure 1).

The semi-quantitative results of KitL expression in bovine COC cultured in control, endometriosis, and endometriosis+curcumin groups were as follows: 3.92±2.02; 0.33±0.49; and 2.67±1.23, respectively. This result was similar to those of GDF-9 expression. The mean expression of KitL in bovine COC cultured in PF from infertile women with endometriosis (0.33±0.49) reduced compared to the control (3.92±2.02) and those in endometriosis+curcumin group (2.67±1.23) (p=0.001). There was also a significant difference between KitL expression in bovine COC cultured in PF from infertile women with endometriosis + curcumin and control group (p=0.035) (Figure 2).

The semi-quantitative results of TNFα expression in bovine COC cultured in control, endometriosis and endometriosis+curcumin groups were as follows: 0.00±0.00; 8.67±3.72; and 2.17±1.69, respectively. TNFα expression in bovine COC cultured in PF from infertile women with endometriosis (8.67±3.72) increased compared to those in control group (0.00±0.00), whereas TNFα expression in bovine COC cultured in PF from infertile women with endometriosis added with curcumin (2.17±1.69) reduced compared to those cultured without curcumin; however, the level increased compared to the control (p=0.001). (Figure 3).

Regression analysis revealed significant association among TNFα, GDF-9, and KitL expression cultured in PF from infertile women with endometriosis added with curcumin (p=0.00).

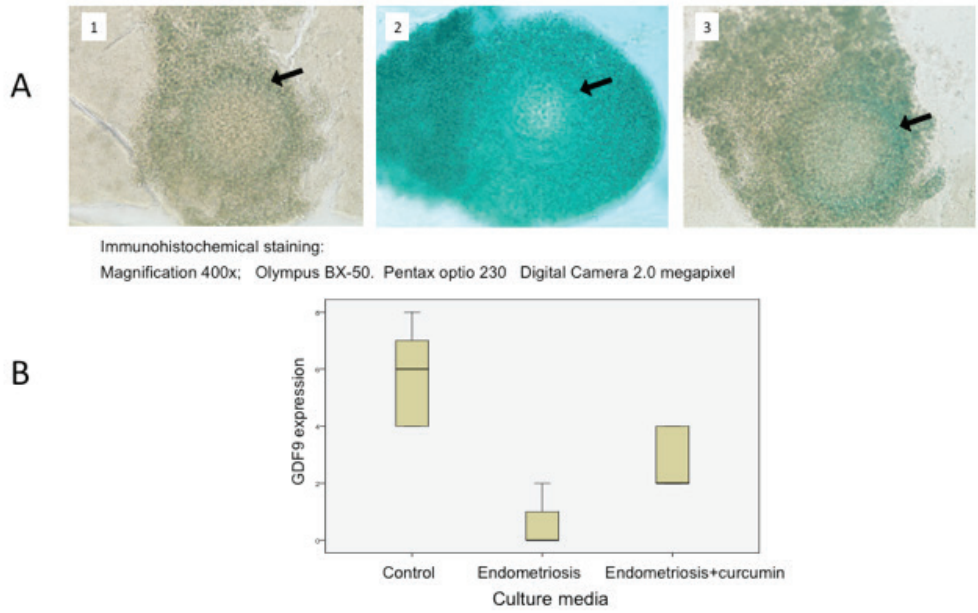

Figure 1. GDF-9 expression in bovine COC cultured in PF from infertile women with endometriosis with or without curcumin addition. Note A: Immunohistochemical staining of GDF-9 expression in 3 different types of media. Immunoreactive cells are indicated by black arrows. B: Semi-quantitative reading of GDF-9 expression in the following media: 1) control, 2) endometriosis, and 3) endometriosis + curcumin (p<0.05). GDF9=Growth Differentiated Factor-9; COC=cumulus oocyte complex; PF=peritoneal fluid

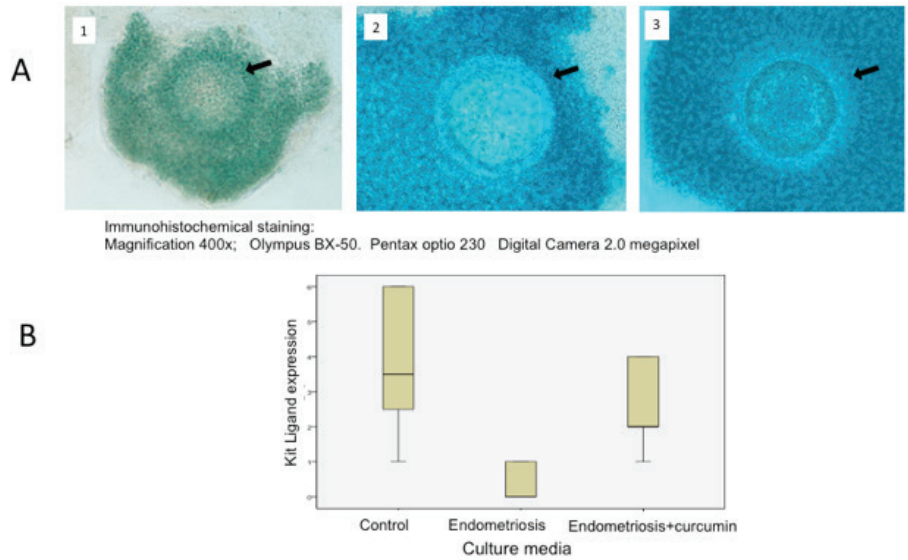

Figure 2. Kit Ligand expression in bovine COC cultured in PF from infertile women with endometriosis with or without curcumin addition. Note A: Immunohistochemical staining of Kit Ligand expression in 3 different types of media. Immunoreactive cells are indicated by the black arrows. B: Semi-quantitative assessment of Kit Ligand expression in the following media: 1) control, 2) endometriosis, and 3) endometriosis + curcumin ($p<0.05$)

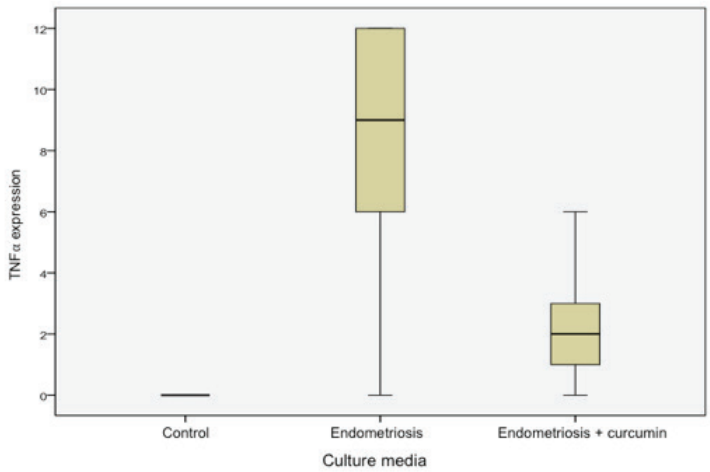

Figure 3. Semi-quantitative assessment of TNF□ expression in bovine COC cultured in the following media: 1) control, 2) endometriosis, and 3) endometriosis + curcumin ($p<0.05$).

Discussion

The PF of infertile women with endometriosis contains a variety of inflammatory mediators and the PF component plays an essential role in the process of folliculogenesis, ovulation and fertilization [14]. Communication between oocytes and granulosa cells occurs during folliculogenesis to produce mature and fertilizable oocytes. This process is impaired in infertile women with endometriosis. We previously reported abnormal GDF-9 and KitL expression in endometriosis. In our study GDF-9 and KitL expressions in bovine COC cultured with PF from infertile women with endometriosis were significantly reduced compared to control. It suggests that numerous inflammatory mediators in the PF of infertile women with endometriosis enters the bovine COC and further inhibits oocytes and granulosa cells activity. This results in abnormal folliculogenesis and subsequently infertility [15].

GDF-9 is an oocyte-specific member of the TGFβ superfamily. GDF-9-deficient female mice demonstrate a block in follicular development beyond the primary one-layer follicle stage which leads to complete infertility. In the transition to the antral stage, GDF-9 promotes follicular survival by suppressing granulosa cell apoptosis and follicular atresia. In this study, the addition of curcumin to culture media of PF from infertile women with endometriosis resulted in more improved GDF-9 expression than those without curcumin. It is possible that the anti-inflammatory effects of curcumin release oocyte suppression. By taking into account the fact that the expression of GDF-9 was still lower than the control, it indicates that it is necessary to adjust the dosage of curcumin. These findings indicate that the dose of curcumin plays a role in GDF-9 expression [16].

KitL produced by the granulosa cells in the oocyte acts by binding to c-Kit and may activate different signaling pathways. The KitL/c-Kit system regulates follicular viability, the initiation of primordial follicle growth, and oocyte and follicle development. In our study, KitL expression in bovine COC cultured in PF from infertile women with endometriosis added with curcumin significantly increased from those cultured without curcumin [17]. It indicates that curcumin has positive effect on KitL expression. The changes on two growth factor expressions, GDF-9 and KitL suggest that curcumin has a repair effect on oocyte-granulosa cell interactions and the regulation of ovarian folliculogenesis[18].

In order to evaluate that curcumin has repair effect on oocyte-granulosa cell interactions and the regulation of ovarian folliculogensis, we assessed the effect of curcumin on bovine COC inflammation via TNFα expression [14]. An increase in TNFα expression was noted in the PF from infertile women with endometriosis compared to that from normal women [19]. In addition, we found a correlation between TNFα concentration and the degree of endometriosis severity. In fact, it has been reported that TNFα plays a role in the inflammatory process and angiogenesis, triggers follicular atresia and impairs oocyte maturation [20].

The findings of increased TNFα expressions in bovine COC cultured in PF from infertile women with endometriosis suggest that TNFα from PF enters the COC and is active. The addition of curcumin to the culture media results in an improvement as indicated by the reduced TNFα expression [16]. Curcumin exhibits anti endometriosis activities by affecting MMP2 and TIMP2. It works by inhibiting cyclooxygenase2 and lipoxygenase and suppressing the activity of NFkB and pro-inflammatory gene expressions [21]. Based on its mechanism of action, curcumin can suppress an inflammatory process in bovine COC cultured in PF from infertile women with endometriosis [15].

Ethical Clearance: This research process involves participants in the survey using a questionnaire that was accordant with the ethical research principle based on the regulation of research ethic committee. The present study was carried out in accordance with the research principles. This study implemented the basic principle ethics of respect, beneficence, nonmaleficence, and justice.

Conflict of Interest: The authors declare that we have no conflicts of interest.

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