

The Effects of Curcumin and Pentagamavunon-0 (PGV-0) on the Steroidogenesis, Proliferative Activity, and Apoptosis in Cultured Porcine Granulosa Cells at Varying Stages of Follicular Growth

Heru Nurcahyo¹, and Sri Kadarsih Soejono²

Abstract

This study was conducted to examine the effects of curcumin and PGV-0 on steroidogenesis, proliferative activity, and apoptosis in cultured porcine granulosa cell from varying stages of follicular growth (*in vitro*) that were stimulated by FSH, LH, and/or PGF_{2α}.

Granulosa cells of porcine follicle ovaries were categorized into small (1-2 mm), medium (> 2-5 mm), and large (> 5-11 mm) follicles. Concentration of progesterone (P) and 17β-estradiol (E₂) in the medium of culture were determined by EIA (enzymeimmunoassay) methods. Proliferative activity was determined by monoclonal antibody of proliferating cell nuclear antigen (MAb-PCNA) and avidin-biotin immunoperoxidase enzyme (ABIP) methods. Apoptosis was determined by *in situ* DNA 3'-end labelling method.

The conclusion are: (1) Curcumin inhibited P and E₂ production as well as the proliferative activity in cultured granulosa cell of mature follicle, and stimulated apoptosis of granulosa cells regardless the follicular maturation stage. The increasing of P and E₂ production and proliferative activity stimulated by FSH or LH was reduced by the concomitant treatments with curcumin in cultured granulosa cells from all sizes of follicle. Meanwhile, treatment with PGV-0 had no significant effect on P and E₂ production, proliferative activity, and apoptosis in cultured granulosa cells from all sizes of follicle. PGV-0 decreased P and E₂ production and proliferative activity stimulated by FSH or LH in cultured granulosa cells from all stages of follicular growth.

Key Words: Granulosa cells; curcumin; Pentagamavunon-0 (PGV-0); steroidogenesis; PCNA; apoptosis.

Introduction

Rhizome of the plant *Curcuma sp.* have been a commonly used for yellow colouring agent, spices, cosmetics, traditional medicine for treating of some diseases in indigenous people (Ammon & Wahl, 1991). Extract of the rhizome *Curcuma sp.* has anti-fertility effect in mice (Garg, 1974). The powdered dry rhizome of the plant *Curcuma sp.* is commonly called turmeric. Curcumin is the major bioactive component and yellow pigment isolated from the turmeric. Began *et al.* (1999) reported that curcumin [1,7-bis (4'-hydroxy-3'-methoxyphenyl)-1-6-heptadiene-3,5-dione] (**Fig. 1A**) has functional groups which responsible to its biological activities are: 1) central β-diketone, 2) double bond on aliphatic chain, 3) hydroxyl groups (phenol), and 4) methoxy groups in aromatic terminal ring. The functional groups of curcumin has biological activity as a free radical scavenging (Venkatesan & Rao, 2000), an inhibitor of cyclooxygenase (COX) and lipoxygenase (LOX) activities

1 Department of Biology, State University of Yogyakarta (UNY), Yogyakarta, Indonesia

2 Department of Physiology, Faculty of Medicine, GMU, Yogyakarta, Indonesia

(Huang *et al.*, 1991), inhibits activation of protein kinase C (Simon *et al.*, 1998), inhibits synthesis of proteins regulator (Lin *et al.*, 1998), and induce apoptosis (Jaruga *et al.*, 1998). Recently, curcumin's analogues has been synthesized by structure modification on terminal aromatic and active-methylene group (Sardjiman, 2000). One of the curcumin analogues is a Pentagamavunon-0 (PGV-0) [2,5-bis (4'-hydroxy-3'-methoxy-benzylidene) cyclo-pentanone] (**Fig. 1B**) with replacing the central β -diketone by cyclic monoketone (cyclopentanone) (Sardjiman, 2000). It has anti-inflammatory activity more potent and safer in compare than other analogues. The compound also known as MOLNAS (National Molecule), and it has been patented as anti-inflammatory drug (Supardjan, 2002). The functional groups of PGV-0 identical to curcumin except that they lack of central β -diketone, and it's aliphatic chain shorter than curcumin (Supardjan, 2003). Structurally, functional groups of curcumin and PGV-0 similar to functional groups of the $\text{PGF}_{2\alpha}$ (**Fig. 1C**) therefore can understand that curcumin and PGV-0 have pharmacological action similar to the $\text{PGF}_{2\alpha}$.

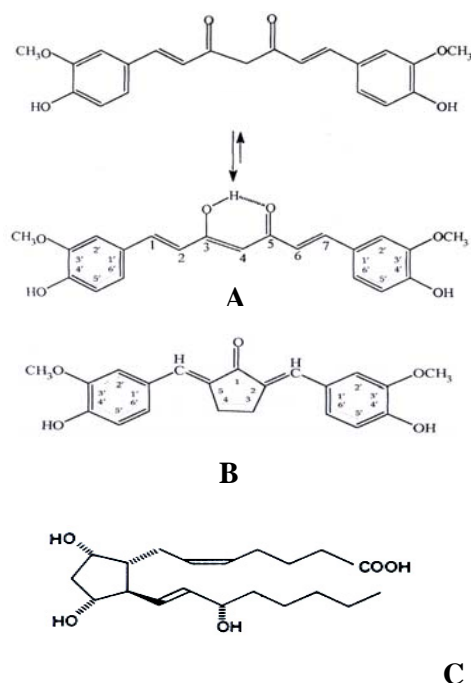


Figure 1. Structure molecule of curcumin (A), PGV-0 (B), and $\text{PGF}_{2\alpha}$ (C)

Ovarian granulosa cells are primary site of estrogens (E_2) and progesterone (P) biosynthesis and play an essential role in follicular development, maturation of developing ovum, and ovulation. Steroidogenesis and proliferation of ovarian granulosa cells are complex processes involve the action of follicle stimulating hormone (FSH), luteinizing hormone (LH), prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) and numerous growth factors. The important role of FSH regulates the steroidogenesis (Lino *et al.*, 1985), differentiation and function of granulosa cells that is indispensable for ovulation and subsequent corpus luteum formation (Hsueh *et al.*, 1989), increases the number of LH receptors in the granulosa cells (Bukovsky *et al.*, 1993). The

1 Department of Biology, State University of Yogyakarta (UNY), Yogyakarta, Indonesia

2 Department of Physiology, Faculty of Medicine, GMU, Yogyakarta, Indonesia

important role of LH stimulates the steroidogenesis (Kessel *et al.*, 1985), follicle development and luteinization (Adashi, 1991). The role of PGF_{2α} regulates the final stage of follicular growth causes rapid decreases in binding of LH to its receptor and reduction in the accumulation of 3',5'-cyclic adenosine monophosphate (cAMP) (Thomas *et al.*, 1978), and reduce the transcription of steroidogenic enzyme P-450_{sidechain cleavage} (P-450_{sc}) and 3β-hydroxysteroid dehydrogenase (3β-HSD) (Li *et al.*, 1993).

Follicle growth is thought to be the result of dynamic balance between cell proliferation and programmed cell death (apoptosis). Granulosa cells one of the most rapidly growing normal cell types (Sato *et al.*, 1994). Granulosa cells have shown proliferating cells nuclear antigen (PCNA) a cofactor of DNA polymerase δ as a sensitive marker of granulosa cells proliferation (Robker and Richards., 1998). Immunocytochemical PCNA labelling has proven useful in evaluating the proportion of proliferating cells (Maruo *et al.*, 1995). On the other hand, *in situ* 3'-end labelling with digoxigenin-dideoxy-UTP (dig-dd-UTP) has been used to evaluate the occurrence of apoptosis (Takekida *et al.*, 2000).

The present study was conducted to investigate the effects of curcumin or PGV-0 on progesterone and estrogen production, proliferative activity, and apoptosis in cultured porcine granulosa cells from varying stage of follicular growth (*in vitro*) in the stimulation of FSH, LH, and/or PGF_{2α}.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline, fetal bovine serum, penicillin-streptomycin, fungizone, (Gibco, Life Technologies, USA), monoclonal-antibody for PCNA and apoptosis (Oncogene Science, Cambridge, MA), PCNA kit (Omni Tags, Lipshaw, USA), apoptosis kit (ApopTag, Intergen, Germany), curcumin and Pentagamavunon-0 (synthesized by MOLNAS team, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia), FSH, LH/hCG, PGF_{2α} (Biogenesis, England, UK), theophylline (Sigma, USA), trypan blue stain, EIA kit for P and E₂ (Cayman, Chemical Co., USA).

Monolayers Cultures of Porcine Granulosa Cells

Sample of granulosa cells were taken from porcine follicle ovaries at a local slaughterhouse. Granulosa cells were harvested aseptically from follicular fluids and differentiated from small (1-2 mm), medium (>2-5 mm), and large (>5-11 mm) follicles from the ovaries of prepubertal pigs and cultured as previously described by Channing and Kammerman (1978). Granulosa cells were cultured at approximately 1x10⁵ cells/mL in 48-well plates and 8-chamber slides using DMEM supplemented with 10% of fetal bovine serum (FBS) for 72 h at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Immediately after confluence, the cultured cells were stepped down to serum free DMEM in the presence of porcine FSH (50 µg/mL), LH (50 ng/mL), PGF_{2α} (0.56 µM), curcumin (50 µM), and PGV-0 (50 µM) for 36 h.

Measurement of P and E₂

Concentration of P and E₂ in the medium of culture was determined by using

1 Department of Biology, State University of Yogyakarta (UNY), Yogyakarta, Indonesia

2 Department of Physiology, Faculty of Medicine, GMU, Yogyakarta, Indonesia

a progesterone and 17β -estradiol EIA (enzymeimmunoassay) kit (Cayman, Chemical Co., USA).

Immunocytochemical Staining

Proliferative activity of cultured granulosa cells were determined by immunocytochemical staining of PCNA was performed using the avidin-biotin immunoperoxidase method and a mouse monoclonal antibody against to PCNA (Mab-PCNA) was used at a dilution 1 : 80 as the primary antibody and a polyvalent immunoperoxidase as the secondary antibody as previously described by Maruo *et al.* (1995).

In situ DNA 3'-end Labelling

Apoptosis was examined using *in situ* DNA 3'-end labelling method and a kit apoptosis as done previously by Takekida *et al.* (2000).

Statistical Analysis

Data are presented as means \pm standard deviation (SD). The data was analysed by one-way ANOVA to know differences between means and when indicated significant differences continued by multiple comparison using Duncan's test. Differences between means at $P < 0.05$ were considered statistically significant.

Results and Discussion

The condition of porcine granulosa cells from small (1-2 mm), medium (>2-5 mm), and large (>5 mm) follicles in the culture medium DMEM supplemented with 10% FBS after 24 h incubation at 37°C in a humidified atmosphere of 5% CO_2 and 95% air showed that the granulosa cells were able to gradually growth, and after 72 h incubation develop approximately confluence.

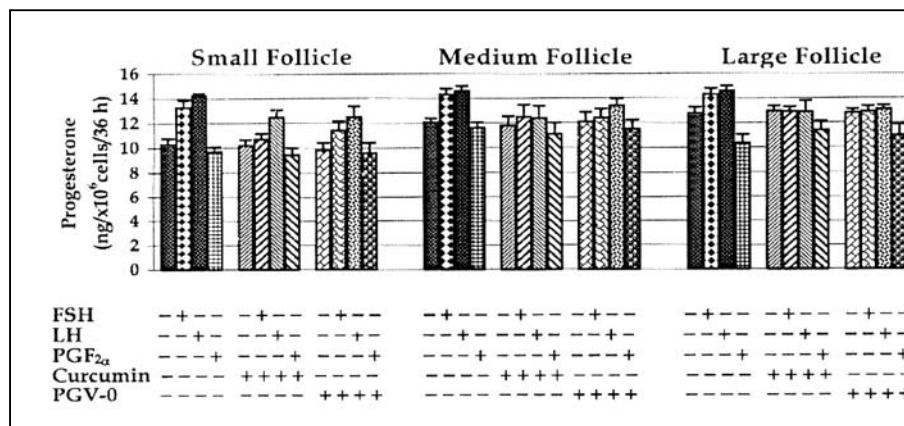


Figure 2. Effect of treatment with curcumin and PGV-0 in the absence or presence of FSH, LH, and/or $\text{PGF}_{2\alpha}$ on progesterone (P) production by cultured porcine granulosa cells from varying sizes of follicles.

Figure 2, shown the production level of progesterone (P) by cultured porcine granulosa cells in response to stimulation of the curcumin, PGV-0, FSH, LH, and/or $\text{PGF}_{2\alpha}$. Progesterone production by cultured porcine granulosa cells in response to

1 Department of Biology, State University of Yogyakarta (UNY), Yogyakarta, Indonesia

2 Department of Physiology, Faculty of Medicine, GMU, Yogyakarta, Indonesia

stimulation FSH or LH was higher in all sizes of follicles. Treatment with $\text{PGF}_{2\alpha}$ decrease in P production by large follicle granulosa cells in comparison to control untreated granulosa cells culture. Addition curcumin decreased P production by large follicle granulosa cells in comparison to control untreated granulosa cells culture, but in cultured granulosa cells of the small and the medium follicle responded no significant different in comparison to control untreated granulosa cells culture. Combined treatment curcumin and PGV-0 with $\text{PGF}_{2\alpha}$ has no significant effect on P production in cultured granulosa cells of all stages follicular growth. Coincubation LH and $\text{PGF}_{2\alpha}$ on granulosa cells culture inhibited the stimulation of P production produced by FSH or LH. Coincubation curcumin or PGV-0 supplemented with LH and $\text{PGF}_{2\alpha}$ on granulosa cells culture did not cause any significant increment in P production in comparison to control untreated granulosa cells culture of all stages of follicular growth.

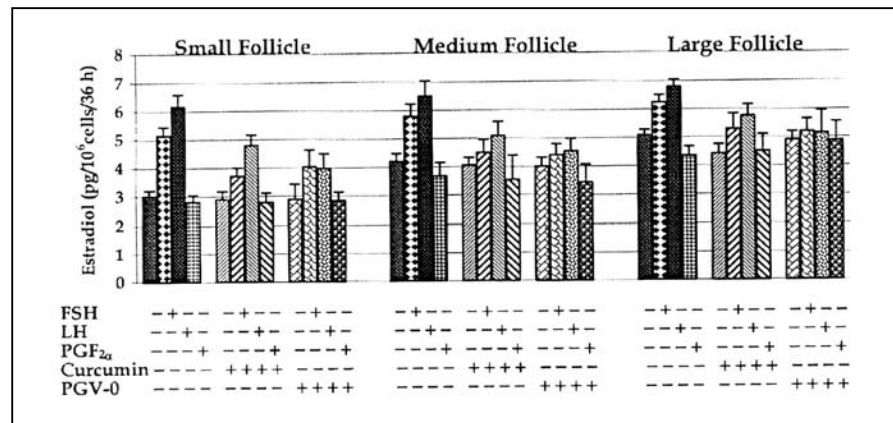


Figure 3. Effect of treatment with curcumin and PGV-0 in the absence or presence of FSH, LH, and/or $\text{PGF}_{2\alpha}$ on estradiol (E_2) production by cultured porcine granulosa cells from varying sizes of follicles.

Figure 3, shown the production level of estradiol (E_2) by cultured porcine granulosa cells in response to stimulation of the curcumin, PGV-0, FSH, LH, and/or $\text{PGF}_{2\alpha}$. Estradiol production by cultured porcine granulosa cells in response to stimulation FSH or LH was higher in all sizes of follicles. Treatment with $\text{PGF}_{2\alpha}$ decrease in E_2 production by large follicle granulosa cells in comparison to control untreated granulosa cells culture. Addition curcumin decreased E_2 production by large follicle granulosa cells in comparison to control untreated granulosa cells culture, but in cultured granulosa cells of the small and the medium follicle no significant different in comparison to control untreated granulosa cells culture. Combined treatment curcumin and PGV-0 with $\text{PGF}_{2\alpha}$ has no significant effect on E_2 production in cultured granulosa cells of all stages follicular growth. Coincubation LH and $\text{PGF}_{2\alpha}$ on granulosa cells culture inhibited the stimulation of E_2 production produced by FSH or LH. Coincubation curcumin or PGV-0 supplemented with LH and $\text{PGF}_{2\alpha}$ on granulosa cells culture did not cause any significant increment in E_2 production in comparison to control untreated granulosa cells culture of all stages of follicular growth.

1 Department of Biology, State University of Yogyakarta (UNY), Yogyakarta, Indonesia

2 Department of Physiology, Faculty of Medicine, GMU, Yogyakarta, Indonesia

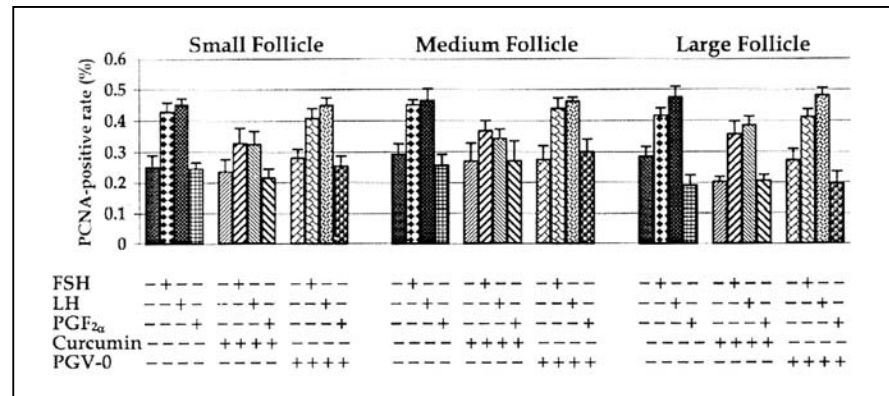


Figure 4. PCNA-positive rate of cultured porcine granulosa cells from varying sizes of follicles cultured for 72 h under serum-free conditions. in response to stimulation of the curcumin, PGV-0, FSH, LH, and/or PGF_{2α}. Results represent the mean \pm SD of four determinations.

The PCNA-positive rate on cultured granulosa cells in response to stimulation of the curcumin, PGV-0, FSH, LH, and/or PGF_{2α} are shown in **Fig. 4**. The presence of FSH or LH in all of stages of follicle granulosa cells cultured, the PCNA-positive nuclei were more abundant in comparison to control untreated granulosa cells. The presence of PGF_{2α} or curcumin in large follicle granulosa cells culture, the PCNA-positive nuclei were less abundant than those in control untreated granulosa cells. The presence of PGV-0 in all of stages of follicle granulosa cells cultured, the PCNA-positive nuclei have no significant increase compares in control untreated granulosa cells.

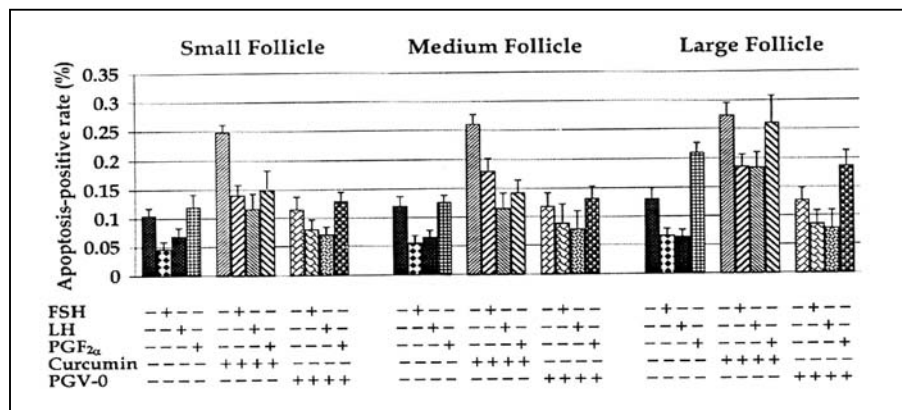


Figure 6. Apoptosis-positive rate of cultured porcine granulosa cells from varying sizes of follicles cultured for 72 h under serum-free conditions as assessed by the *in situ* DNA 3'-end labelling method in response to stimulation of the curcumin, PGV-0, FSH, LH, and/or PGF_{2α}. Results represent the mean \pm SD of four determinations.

Discussion

These results indicate that in mature follicle cultured granulosa cell, curcumin alone exerts inhibits the steroidogenic activity as well as the proliferative activity, and the other stimulates apoptosis of granulosa cells regardless of follicular maturation stage. The increased P and E₂ production and proliferative activity responded to the treatment with FSH or LH was reduced by the concomitant treatment with curcumin in cultured granulosa cells of all sizes follicle. By contrast, treatment with PGV-0 alone has no significant effect on steroidogenic activity, proliferative activity, and apoptosis in cultured granulosa cells of all sizes follicle. However, coincubation treatment PGV-0 with FSH or LH decreased FSH or LH stimulated P and E₂ production in granulosa cells culture of all stages of follicular growth. On the basis of these data it appears that curcumin and PGV-0 has an antigonadotropic action when coincubated with FSH or LH.

These results indicate that addition of PGF_{2α} caused significant decrease in P and E₂ production and proliferative activity on large follicle in comparison to control untreated granulosa cells culture. The present data are consistent with earlier reports by Henderson & Mc Natty (*cit. Behrman et al.*, 1979); PGF_{2α} attenuated the stimulation of P and E₂ production by gonadotropin in the cultured porcine granulosa cells. Li *et al.* (1993) reported that PGF_{2α} reduce the transcription of steroidogenic enzyme P-450_{sidechain cleavage} (P-450_{scc}) and 3β-hydroxysteroid dehydrogenase (3β-HSD) in the cultured porcine granulosa cells. Reported by Soejono *et al.* (2000), PGF_{2α} decreased the stimulation of P and E₂ production by gonadotropin in cultured rat luteal cells. Coincubation treatment curcumin or PGV-0 with PGF_{2α} has no significant effect on P and E₂ production in comparison with PGF_{2α}-treated granulosa cells culture of all stages of follicular growth. Coincubation treatment curcumin or PGV-0 supplemented with LH and PGF_{2α} on granulosa cells culture decrease in P and E₂ production in comparison to LH-treated granulosa cells culture of all stages of follicular growth. On the basis of these data, it appears that PGF_{2α} inhibited LH-dependent P and E₂ production by cultured granulosa cells.

The antigonadotropic action of curcumin and PGV-0 could occur at several sites i.e.: inhibition of FSH or LH binding to its receptor, or increase in cAMP degradation. Based on the response of granulosa cells to curcumin and PGV-0 supplemented with theophylline was suggest that the antigonadotropic action of curcumin and PGV-0 appears not to be due stimulation of cAMP degradation. Thus, it is conclude that the site of action of curcumin or PGV-0 in blocking FSH or LH response may occurs after the accumulation of cAMP, probably at the protein kinase C activity (Hasmeda & Polya, 1996), and/or the steroidogenic enzymes level or DNA system (Soejono *et al.*, 2001).

The block by curcumin or PGV-0 of FSH or LH dependent P production may possibly occur by a decrease in cells proliferation through stimulate programmed cell death (apoptosis). These result indicate that observation by immunocytochemistry provides evidence that the action curcumin was due to inhibit cell proliferation through stimulate apoptosis. In an earlier study Jaruga *et al.* (1998), reported that curcumin readily penetrates into the cytoplasm, and is able to accumulate in membranous structures such as plasma membrane, endoplasmic reticulum, and nuclear envelope, and cell exhibit typical features of apoptotic cell death including:

- 1 Department of Biology, State University of Yogyakarta (UNY), Yogyakarta, Indonesia
- 2 Department of Physiology, Faculty of Medicine, GMU, Yogyakarta, Indonesia

shrinkage, increased membrane permeability, and decrease in mitochondrial membrane potential. On the other hand, Simon *et al.* (1998) reported that curcumin exerts a cytostatic effect at G₂/M, which explains its anti-proliferative activity. Chen & Huang (1998) suggest that the anti-proliferative effect of curcumin may partly be mediated through inhibition of protein tyrosine kinase activity and *c-myc* mRNA expression, and the apoptotic effect may partly be mediated through inhibition of protein tyrosine kinase activity, protein kinase activity, *c-myc* mRNA expression, and *bcl-2* mRNA expression. Therefore, recently curcumin have received considerable attention as therapeutic agents for antitumor.

Based on the result of the research, efficacy of both molecule might be different, appear that curcumin more potent than PGV-0 to inhibit biosynthesis P and E₂. The particular component of the curcumin molecule that responsible for its biological activity is not known. It is possible that the hydroxyl group on the benzene rings, the double bonds in the alkenes portion of the molecule, and/or the central β -diketone maybe responsible for the high biological activity of curcumin (Huang *et al.*, 1991; Began *et al.*, 1999). The phenolic groups are important for the antioxidant activity of curcumin and its analogues (Venkatesan & Rao, 2000). The β -diketone structure of curcumin is particularly interesting because it has been shown to be highly enolized (Huang *et al.*, 1991). The presence of the β -diketone moiety in the curcumin molecule seems to be essential for the antiproliferative activity (Simon *et al.*, 1998). These finding suggested that in mature granulosa cells maintained as monolayers in medium supplemented with curcumin alone caused significant decrease in P and E₂ production as well as PCNA-positive rate in mature follicle cultured granulosa cell, and the other stimulates apoptosis of granulosa cells regardless of follicular maturation stage. The fact proven that the responses of cultured granulosa cells to curcumin or PGV-0 were influenced by the stage of follicle maturation. By contrast, treatment with PGV-0 has no significant effect on P and E₂ production, proliferative activity, and apoptosis in cultured granulosa cells of all sizes follicle. PGV-0 decreased P and E₂ production and proliferative activity stimulated by FSH or LH in cultured granulosa cells of all stages of follicular growth. Sites of action of curcumin and PGV-0 in inhibition of biosynthesis P and E₂ after degradation of cAMP by phosphodiesterase.

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1 Department of Biology, State University of Yogyakarta (UNY), Yogyakarta, Indonesia

2 Department of Physiology, Faculty of Medicine, GMU, Yogyakarta, Indonesia

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2 Department of Physiology, Faculty of Medicine, GMU, Yogyakarta, Indonesia

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2 Department of Physiology, Faculty of Medicine, GMU, Yogyakarta, Indonesia

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