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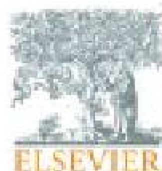

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The effects of genistein as antiinflammatory and antiangiogenesis in primary endometriosis cell culture

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ABSTRACT

Objective: To investigate whether genistein is anti-inflammation and anti-angiogenesis in endometriosis cells. **Methods:** Primary endometriosis cells were divided into seven groups, including control group and genistein-treatment group at dose 5 until 50 μmol/L. The times of incubation is 6, 24, and 48 hours. Levels of TNF-α, IL-6, IL-1β, MMP-2, and VEGF-A in supernatant cells were determined using the enzyme linked immunosorbent assay. **Results:** The level of IL-1β was significantly lower in genistein-treatment at dose 20 until 50 μM compared to the control group (6 hours of treatment) (P<0.05). In 24 hours of treatment, the level of IL-1β was significantly lower in genistein-treatment start at all doses compared to control group (P<0.05). For 48 hours of incubation, the level of IL-1β was significantly lower in of genistein-treatment group at dose 10 until 50 μM than that to control group (P<0.05). The level of TNF-α and IL-6 were significantly decreased in genistein-treatment compared to control group in all duration of treatment (P<0.05). Level of VEGF-A were significantly lower in genistein-treatment at dose 20 until 50 μM compared to control group (P<0.05). The level of MMP-2 was significantly lower in the genistein-treatment at dose 10 and 30 μM compared to control group in 6 hours of treatment (P<0.05), at dose 10, 40, and 50 μM (24 hours of treatment), and all doses (48 hours of treatment). **Conclusion:** In conclusion, genistein act as anti-inflammatory and anti-angiogenesis in primary culture cells of endometriosis.

1. Introduction

Endometriosis is one of the most frequent benign gynecological diseases that has been known for over 50 years. This disease was characterized by the implant and growth of viable endometrial tissue outside the uterine cavity. Endometriosis showed altered humoral immune response, increase macrophage and its activity which induces general inflammatory response[1-5]. The reported endometriosis prevalence in women of reproductive age ranges up to 10%[1]. In infertile women, the prevalence may rise to 50%[6]. Nevertheless, these numbers are likely to be underestimated. The most common clinical symptoms include chronic lower abdominal and pelvic pain, dysmenorrhea, dyspareunia, abnormal uterine bleeding, and infertility.

Interleukin (IL)-1β is a versatile inflammatory cytokine that may promote the disease. IL-1β stimulates endometriotic cells to produce various cytokines and

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growth factors that play roles in adhesion, growth, invasion, inflammation and angiogenesis in endometriotic tissues [7-12]. The levels of IL–1β are increased in the peritoneal fluid (PF) and peritoneal macrophages in women with endometriosis and in endometriotic tissues[13]. On the other hand, IL–1 receptor antagonist, a competitive antagonist for IL–1β, is decreased in the PF[14]. Another antagonist for IL–1β, soluble IL–1 receptor type II, is also decreased in the PF of women with endometriosis[15].

Surgical removal of ectopic lesions represents the first line intervention but is hampered by a relevant percentage of recurrences[16, 17]. In addition, a variety of medical hormonal therapies, all aimed to reduce the levels of circulating estrogens, are currently available[18]. However, these treatments are often unsatisfactory and cannot be used over long periods of time, due to the occurrence of severe adverse effects. Therefore, new and improved therapeutic solutions that can efficiently reduce lesions with limited side effects and no interference with the patient's fertility are definitely desirable.

Genistein, one of the active ingredients in soybean, possesses many bioactivities, including anti–neoplastic activity in multiple tumor types by several mechanisms [19, 20]. Previous studies also indicated that genistein act as antiinflammatory and antiangiogenesis agent. Genistein inhibit the increased release and expression of inflammatory cytokines, including interleukin–1β (IL–1β), tumor necrosis factor–α (TNF–α) inducing by LPS[21]. Compared with other isoflavone compounds, genistein was the most potent inhibitor of angiogenesis *in vitro* and *in vivo*. Genistein exhibited a dose–dependent inhibition of expression/excretion of vascular endothelial growth factor, platelet–derived growth factor, tissue factor, urokinase plasminogen activator, and matrix metalloprotease–2 and 9, respectively[22]. Injections of estrone or genistein (50 or 16.6 microg/g BW) significantly reduced uterine ER–alpha compared to vehicle–treated animal model of endometriosis[23]. Higher levels of urinary genistein was associated with decreased risk of advanced endometriosis[24]. Genistein caused a statistically significant regression of endometriotic implants[25]. Genistein (50 and 200 mg/kg) may not be considered for the development of antiangiogenic treatment strategies in the therapy of endometriosis[26]. As far we know, there is controversial studies to investigate the effects of genistein on endometriosis. Therefore, this study aimed to investigate whether genistein able to decrease inflammation and angiogenesis in primary culture cells of endometriosis.

2. Material and methods

2.1. Sample

The samples used in this study is a tissue of women who have endometriosis diagnosed through a physical examination to gynecologic examination via ultrasound. The informed consent was applied to patients and families, chocolate cyst wall tissue in the capture will be used as research material. The samples were collected by laparoscopy gynecologist. Endometriosis tissue that is taken is separated into two parts, a part placed in a transport medium for the culture and some other tissue undergoing pathology examination. Positive endometriosis ovarian cyst tissue will culture subsequently. Positive results were characterized by the presence of 2 or more diagnostic overview including endometrial epithelium, endometrial glands, endometrial stroma, and pigment laden macrophages [27].

2.2. Cell culture

The specimen will be aseptically processed and delivered to cold condition in a solution of L. The tissues will be placed in a sterile petri dish (diameter of 9 cm), then cut to obtain little pieces (volume $0.5 \times 2.0 \times 1$ mm^3) using a sterile scalpel. About 0.5–1.0 gram of tissue will inserted into 15 mL a sterile centrifuge tube size, containing 10 mL of solution II (enzymatic dissociation). Incubate specimen horizontally in the centrifuge tube to spread to all parts of the tube for 7 minutes until separated cell suspension showed colored turbid, homogeneous, and the separated part between the medium and the cells[28].

The cells will centrifuge at 1700 RPM for 7 minutes, then discard the dissociation solution using a sterile pipette. Resuspension cells in 10 mL culture medium then add 10 mL of complete culture medium (DMEM, FBS, penicillin, L–Glutamine, Streptomycin). Incubate specimen was vertically in the conical tube for 5 minutes in order to remove the undissociated fragments. The supernatant which contains cell suspension was inserted into the TC plate using a sterile pipette. Incubation of cells in 95% humidified incubator 37 ℃, 5% CO_2. Two days later, observation will be conducted by inverted microscope to evalute the growth of the cell. The medium will replace every 3 days or the medium become yellow, until the cells become confluent. Confluent cells characterized by cells have attached to other cells, the appearance of the cell nucleus, plasma membrane, cytoplasm and extracellular matrix, and larger cell size[28].

2.3. Treatment

When confluent cells, the cells were harvested. Count the number of cells with a microscope counting chamber using Olympus CX–22, obtained cell of 2.6×10^4. The cells were divided into seven groups, including the control group, genistein–treatment group at dose 5; 10; 20; 30; 40; and 50 μM. The duration of incubation was 6 hours, 24 hours and 48 hours.

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Four replications are applied in each group.

2.4. Analysis of IL-1 β level

The level of IL-1 β supernatant cells was measured immunoenzymatically using Specific sandwich enzyme-linked immunosorbent assays (KOMA Biotech Inc, USA, Catalog series K0331194). All procedure was done according the detail instruction in the kit.

2.5. Analysis of TNF-α level

The level of TNF-α in supernatant cells was measured immunoenzymatically using Specific sandwich enzyme-linked immunosorbent assays (KOMA Biotech Inc, USA, Catalog series K0331131). All procedure was done according kit instruction.

2.6. Analysis of IL-6 level

Level of IL-6 supernatant cells was measured immunoenzymatically using Specific sandwich enzyme-linked immunosorbent assays (KOMA Biotech Inc, USA, Catalog series K0331194). All procedure was done according the instruction in the kit.

2.7. Analysis of MMP-2

Level of MMP-2 in supernatant cells was measured immunoenzymatically using Specific sandwich enzyme-linked immunosorbent assays (R&D System, Minneapolis, USA, Catalog series DMP2FO). All procedure was done according the manufacturer's instruction.

2.8. Analysis of VEGF-A

Level of VEGF-A in supernatant cells was measured immunoenzymatically using enzyme-linked immunosorbent assays (eBioscience, Vienna, Austria, Catalog series BMS277/2). All procedure was done according manufacturer's instruction in the kit.

2.9. Ethics

This research has been approved by the research ethics committee, Faculty of Medicine University of Brawijaya, Malang, Indonesia.

2.10. Statistical analysis

Data are presented as mean ± SD and differences between groups were analyzed using 1-way ANOVA with SPSS 15.0 statistical package. The post Hoc test was used if the ANOVA was significant. $P<0.05$ was considered statistically significant.

3. Results

The level of IL-1 β was significantly lower in genistein-treatment from 20 to 50 μM compared with control group (6 hours of treatment) ($P<0.05$). In 24 hours of treatment, the level of IL-1 β was significantly lower in genistein-treatment start at lowest dose compared to control group ($P<0.05$). For 48 hours of incubation, the level of IL-1 β was significantly lower in genistein-treatment group at dose 10 μm/L until 50 μm/L than that to control group ($P<0.05$). The optimum dose of genistein for 6 hours, 24 hours and 48 hour incubation period achieved in 30, 40, and 40 μM, respectively, as shown in Figure 1.

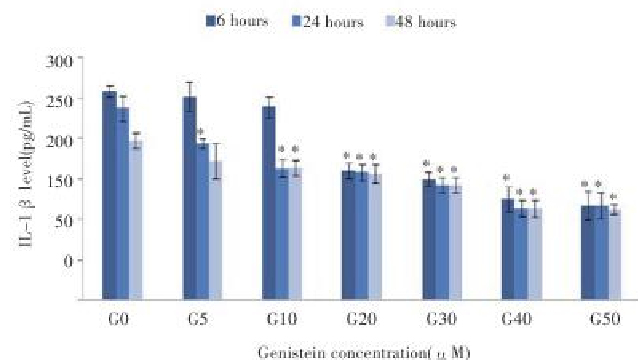

Figure 1. Changes in the IL-1 β level in the culture of endometriosis cells receiving several doses of genistein. Values (mean ± SD) were obtained from each group of 3 culture replication in each group after 6, 24, and 48 hours of the experimental period. *$P<0.05$ for comparisons with the control groups.

Figure 2 showed the level of TNF-α in control and genistein-treatment group. The level of TNF-α was significantly decreased in genistein-treatment compared to control group in all duration of treatment ($P<0.05$). The optimum dose of genistein for 6 hours, 24 hours, and 48 hour incubation period are 20, 10, and 5 μM, respectively.

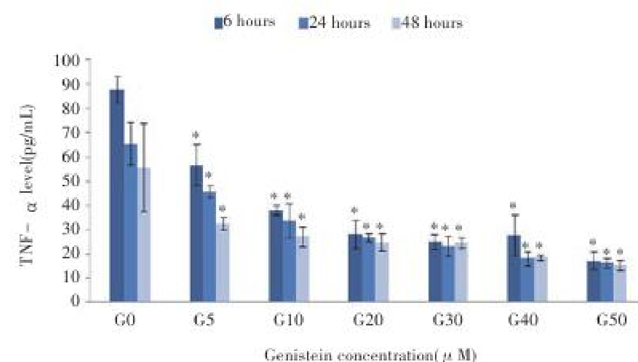

Figure 2. Changes in the TNF-α level in the culture of endometriosis cells receiving several doses of genistein. The data represent the mean ± SD (standard deviation) of three experiments each in triplicate (6, 24, and 48 hours of the experimental period). *$P<0.05$ for comparisons with the control groups.

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Figure 3 presents the level of IL-6 in control and genistein-treatment group. The level of IL-6 was significantly lower in genistein-treatment compared to control group in all duration of treatment (P<0.05). The optimum dose of genistein for 6 hours, 24 hours, and 48 hour incubation period are 40, 50, and 5 μM, respectively.

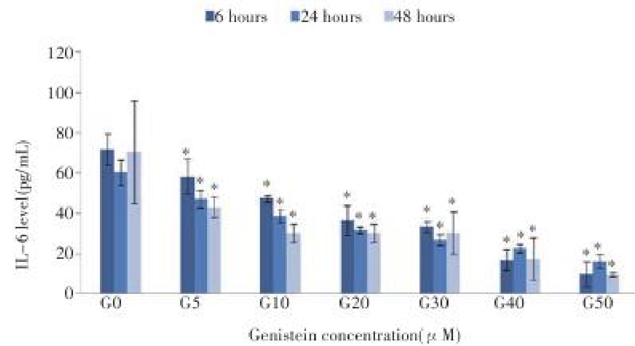

Figure 3. Changes in the IL-6 level in the culture of endometriosis cells receiving several doses of genistein. Values (mean ± SD) were obtained from each group of 3 culture replication in each group after 6, 24, and 48 hours of the experimental period. *P<0.05 for comparisons with the control groups.

Level of VEGF-A were significantly lower in genistein-treated at dose 20 to 50 μM than control group (P<0.05). The optimum dose of genistein for 6 hours, 24 hours, and 48 hour treatment period is 40 μM, as seen in Figure 4.

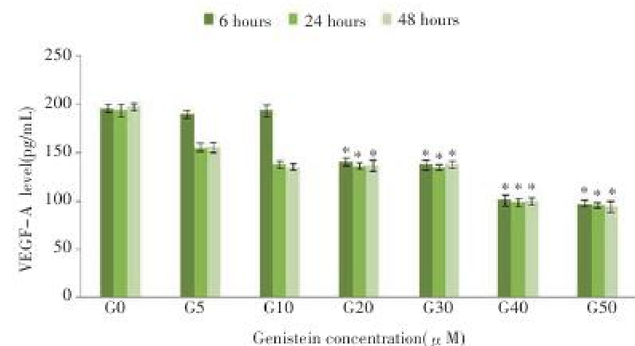

Figure 4. Changes in the VEGF-A level in the culture of endometriosis cells receiving several doses of genistein. Values (mean ± SD) were obtained from each group of 3 culture replication in each group after 6, 24, and 48 hours of the experimental period. *P<0.05 for comparisons with the control groups.

The level of MMP-2 was significantly lower in genistein-treatment at dose 10 and 30 μM compared to control group in 6 hours of treatment (P<0.05). In 24 hours of treatment, the level of MMP-2 was significantly lower in genistein-treatment at dose 10, 40, and 50 μM compared to control group (P<0.05). For 48 hours of incubation, the level of MMP-2 was significantly lower in all doses of genistein-treatment than that in control group (P<0.05). The optimum dose of genistein from 24 hours and 48 hour incubation period achieved in 10 and 30 μM, respectively, as presented in Figure 5.

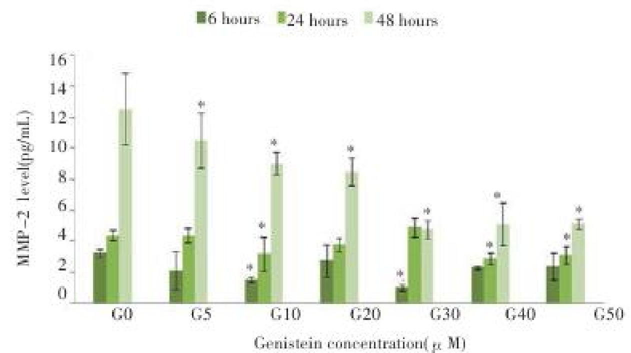

Figure 5. Changes in the MMP-2 level in the culture of endometriosis cells receiving several doses of genistein. Values (mean ± SD) were obtained from each group of 3 culture replication in each group after 6, 24, and 48 hours of the experimental period. *P<0.05 for comparisons with the control groups.

4. Discussion

In order to confirm endometriosis cells, we measured the expression of estrogen receptor-β and estrogen receptor-α. We found that the expression of estrogen receptor-β was higher (55.18%) compared with the estrogen receptor-α (30.40%). This finding indicates that the tissue in our culture confirmed as endometriosis. Previous studies showed that deficient methylation of the estrogen receptor-β promoter results in pathological overexpression of ERβ in endometriotic stromal cells[29]. In addition, high levels of estrogen receptor-β suppress estrogen receptor-α expression[30-31]. A severely high ERβ-to-ERα ratio in endometriotic stromal cells is associated with increased cyclo-oxygenase-2 levels contributing to inflammation[32].

The level of TNF-α and IL-6 was significantly decreased in genistein-treatment compared to control group in all duration of treatment. The optimum dose for TNF-α lowering effect of genistein were 20 μM (6 hours), 10 μM (24 hours), and 5 μM (48 hours), respectively. In addition, the optimum dose for IL-6 lowering effect of genistein are 40 μM (6 hours), 50 μM (24 hours), and 5 μM (48 hours), respectively. Genistein may attenuate the initiation of intracellular signaling cascades by LPS through inhibiting

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NF-κB activation by inhibiting the binding of LPS to TLR-4 on microglial cells[21]. Genistein administration decreased the levels of TNF-α and IL-6 in serum and liver, as well as inhibited IκB-α phosphorylation, nuclear translocation of NF-κB p65 subunit, and activation of c-Jun N-terminal kinase (JNK)[32]. Besides, genistein decreased TNF-α production by macrophages stimulated with *Candida albicans*[33].

The level of IL-1β was significantly lower in genistein-treatment at dose 20 to 50 μM compared to control group (6 hours of treatment). In 24 hours of treatment, the level of IL-1β was significantly lower in genistein-treatment start at lowest dose compared to control group. For 48 hours of incubation, the level of IL-1β was significantly lower in of genistein-treatment group at dose 10 to 50 μM than that to control group. The optimum dose of genistein from 6 hours, 24 hours and 48 hour incubation period achieved in 30, 40, and 40 μM, respectively, as shown in Figure 3. This finding indicates that genistein able to inhibit the effects of IL-1β as a versatile inflammatory cytokine that may promote the endometriosis. IL-1β stimulates endometriotic cells to produce various cytokines and growth factors that play roles in adhesion, growth, invasion, inflammation and angiogenesis in endometriotic tissues[7-12].

In this study, the level of VEGF-A were significantly lower in genistein-treatment at dose 20 μM until 50 μM compared to control group. The optimum dose of genistein in 6 hours, 24 hours, and 48 hour treatment period were 40 μM. The level of MMP-2 was significantly lower in genistein-treatment at dose 10 and 30 μM compared to control group in 6 hours of treatment. In 24 hours of treatment, the level of MMP-2 was significantly lower in genistein-treatment at dose 10, 40, and 50 μM compared to control group. For 48 hours of incubation, the level of MMP-2 was significantly lower in all doses of genistein-treatment than that to control group. Previous studies showed that genistein exhibited a dose-dependent inhibition of expression of angiogenesis marker, including vascular endothelial growth factor, and matrix metalloprotease-2 due to down-regulates the transcription and translation of genes[22, 34]. Besides genistein at the concentration of 200 μmol/L can sufficiently inhibit the proliferation of human endometrial endothelial cells (HEECs) and endometrial glandular epithelium simultaneously *in vitro*[35].

In conclusion, our data suggested that genistein act as anti-inflammatory and anti-angiogenesis in primary culture cells of endometriosis.

Conflict of interest statement

The author(s) declare(s) that there is no conflict of interests regarding the publication of this article.

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