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Original Article

Genistein modulates the estrogen receptor and suppresses angiogenesis and inflammation in the murine model of peritoneal endometriosis

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ABSTRACT

The purpose of this study was to investigate the effect of genistein administration on the modulation of the estrogen receptor, inhibition of inflammation and angiogenesis in the murine model of peritoneal endometriosis. A total of thirty-six mice (*Mus musculus*) were divided into six groups ($n = 6$), including the control group, endometriosis group, endometriosis group treated with various doses of genistein (0.78; 1.04; 1.3 mg/day), and endometriosis group treated with leuprolide acetate (0.00975 mg/day every 5 days for 15 days). Analysis of estrogen receptor-α, estrogen receptor-β, TNF-α, IL-6, VEGF, and HIF-1α were performed immunohistochemically. Expression of estrogen receptor-α, estrogen receptor-β, TNF-α, IL-6, VEGF and HIF-1α increased significantly compared with the control group ($p < 0.05$). All doses of genistein decreased the expression of estrogen receptor-α, increased estrogen receptor-β, lowered VEGF and HIF-1α significantly compared with endometriosis group ($p > 0.05$). Genistein also decreased the expression of TNF-α and IL-6 (1.04 and 1.3 mg/day) compared with the endometriosis group, reaching level comparable to that of the control group ($p > 0.05$). It was concluded that genistein is able to modulate estrogen receptor-α and estrogen receptor-β and inhibit the development of inflammation and angiogenesis in the murine model of peritoneal endometriosis. Thus, genistein can be a candidate in the treatment of endometriosis.

1. Introduction

Endometriosis is the proliferation of endometrial tissue outside the uterine cavity. Until now, endometriosis is a gynecological disorder that often occurs among women at reproductive age (10%) or infertile women (20–50%).[1–5] Peritoneal environment seems to be a very important media in the development of endometriosis pathology. Estrogen receptor-β is one of the two nuclear receptors that mediates estrogen action. In the context of endometriosis, estrogen receptor-β is significantly higher (more than 100 times) in endometrial lesion than ectopic endometrium. It is thought to be caused by changes in gene promoter. This overexpression will lead

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to a decrease in the expression of estrogen receptor-α.[6–9] Furthermore, the ratio of estrogen receptor-β/estrogen receptor-α is very high in endometrial stromal cells associated with the increased inflammation.[10]

In addition to the estrogen receptor, a peritoneal fluid of endometriosis contains macrophages and other secreted products, including growth factors, cytokines, and angiogenic factors.[11,12] Vascular endothelial growth factor (VEGF) is an angiogenic factor, and it will trigger biological effects by binding to two receptors, namely VEGFR-1 or VEGFR-2.[13,14] VEGFR-2 binds to VEGF-A, VEGF-C and VEGF-D. VEGFR-2 signal is essential for vascular dilation, endothelial cell migration and cell proliferation.[15–18] Previous researches proved that VEGF was found to increase in peritoneal fluid of patients with endometriosis.[19–21]

Genistein has common name of 5,7-dihydroxy-3-(4-hydro xyphenyl)-4H-1-benzopyran-4-one. This compound is belonging to the group of isoflavones, heterocyclic polyphenols formed in plants.[22] Genistein is one of two main isoflavone aglycone which can be found in soybeans and it gets more attentions for two decades due to its roles in inhibiting hormones-related diseases.[23] Genistein is phytoestrogen which is frequently consumed and may influence cell functions through selective interaction with estrogen-β receptor in relation to co-activator and provokes downstream signal.[24] Genistein as one of the active substances of soy has various biological effects, such as an anti-neoplastic effect in various types of tumors through various mechanisms.[25,26] Previous researches also indicated that genistein has an anti-inflammatory and anti-angiogenic effects. Anti-inflammatory action of genistein is demonstrated in inhibiting the expression and release of pro-inflammatory cytokines induced by lipopolysaccharide.[27] Anti-angiogenic action of genistein is demonstrated *in vitro* and *in vivo* through the inhibitory ability of expression/excretion of VEGF.[28] As far as we know there is still controversy about genistein action against endometriosis. Therefore, this study aimed to determine whether genistein is able to modulate the estrogen receptor, inhibit inflammation and angiogenesis in the murine model of peritoneal endometriosis.

2. Materials and methods

2.1. Animals

This research used female, young adults mice (*Mus musculus*) (2–3 months of age), weighing 20–30 g, and never mating. Mice were purchased from experimental animal development unit at the Laboratory of Reproductive Physiology and Embryology, Faculty of Veterinary Medicine, University of Airlangga, Surabaya, East Java, Indonesia. A total of thirty-six female mice were divided into six groups, including the control group (no treatment), endometriosis model groups, endometriosis model group treated with genistein orally at doses of 0.78; 1.04; and 1.3 mg/day, and endometriosis model group treated with leuprolide acetate (0.00975 mg/5 days) as the standard drug. This research was conducted at the Laboratory of Molecular Physiology, Faculty of Medicine, University of Brawijaya, Malang, East Java, Indonesia.

2.2. Genistein administration

Genistein (Bioword, USA) in the form of powder was dissolved in sesame oil (1 ml volume of oil containing 1 g genistein). The genistein solution was administered to the mice orally with the sonde. The administration of this solution was started after 14 days of induction of endometriosis.[29,30] The genistein solution was administered once a day in the morning according to the dosage for 15 days.

2.3. Administration of leuprolide acetate

Leuprolide acetate is a synthetic analog drug known as GnRH (Tapros, Takeda Laboratories, Japan).[31] Leuprolide acetate was administered to mice intramuscularly every 5 days at a dose of 0.00975 mg/5 days to 15 days.

2.4. Development of endometriosis model

The myometrial and endometrial tissues were implanted in immunosuppressed mice to induce endometriosis. Immunodeficiency was induced by injection of cyclosporine A (0.2 ml/mouse as a single dose) intraperitoneally. Implant tissues were derived from patients with adenomyosis. Implant tissues in size of 1 cm^3 were washed twice at 300 rpm 4 °C. Supernatant of implant tissues was then sampled, and the residue of the tissue was supplemented with phosphate buffer saline. Products of implantation were implanted in the peritoneal cavity by injection (0.1 ml). After planting the implant, ethinyl estradiol (0.1 ml) was administered intramuscularly in the first and fifth day. The development of endometriosis in mice was observed for 14 days and categorized as a model of endometriosis.[32]

2.5. Peritoneal tissue sampling

After some treatments were done completely, the mice were sectioned for the removal of peritoneal tissues. Before being sectioned, the mice were administered with anesthetic diethyl ether. Peritoneal tissue samples were stored at −80 °C until they were analyzed.

2.6. Immunohistochemistry

The expressions of estrogen receptor-α, estrogen receptor-β, TNF-α, IL-6, VEGF and HIF-1α in peritoneal tissues were analyzed using immunohistochemical techniques in accordance with previous procedures.[33]

2.7. Ethics

This research has obtained an ethical approval from Faculty of Medicine of Brawijaya University, Malang, East Java, Indonesia. All methods in this research were conducted based on the relevant manual and regulations.

2.8. Statistical analysis

The expression was presented in mean ± SD. The differences between treatment groups were analyzed by a one-way analysis of variance (ANOVA) test. The analysis was performed using SPSS 15.0 statistical package for Windows. Probability value ($p < 0.05$) is considered as significantly different and this was continued with Least Significant Difference test.

3. Results

Table 1 shows the expression of estrogen receptor-α and estrogen receptor-β in several groups. Expression of estrogen receptor-α and estrogen receptor-β in the endometriosis group was significantly higher than the control group ($p < 0.05$). The administration of three doses of genistein decreased the expression of estrogen receptor-α, reaching a significant difference with endometriosis group ($p < 0.05$), reaching level comparable to the control group ($p > 0.05$). The expression of estrogen receptor-α among different doses of genistein was not significantly different

Table 1
The expression of estrogen receptor-α and estrogen receptor-β in mice model of endometriosis treated with genistein.

Expression	Control	EM	EM + Genistein treatment (mg/day)			Leuprolide acetate (mg)
			0.78	1.04	1.30	0.00975
ER-α (%)	1.05 ± 0.60	5.80 ± 2.03[a]	2.70 ± 2.70[b]	1.10 ± 0.77[b]	2.50 ± 2.15[b]	5.00 ± 1.25[ad]
ER-β (%)	1.05 ± 0.91	5.95 ± 1.17[a]	9.65 ± 2.25[b]	10.20 ± 2.14[b]	10.05 ± 2.42[b]	7.25 ± 1.85[a]

Note: Data are presented as mean ± SD. [a]$p<0.05$ in comparison with control group. [b]$p < 0.05$ in comparison with EM group. [d]$p < 0.05$ in comparison with EM group treated by second dose of genistein; EM: endometriosis group; ER-α: estrogen receptor-alpha; ER-β: estrogen receptor-beta %: percentage; mg/day: milligram/day.

($p > 0.05$). The administration of leuprolide acetate decreased the expression of estrogen receptor-α, although it was not significantly different compared with the endometriosis group ($p > 0.05$). For estrogen receptor-β, all doses of genistein increased the expression of estrogen receptor-β significantly compared with the control group or endometriosis group ($p < 0.05$). Expressions of estrogen receptor-β among the different doses of genistein were not significantly different ($p > 0.05$).

Expression of TNF-α and IL-6 in the control group and some treatment groups is shown in Table 2. In endometriosis mice, the expressions of TNF-α and IL-6 were significantly higher than the control ($p < 0.05$). Genistein in second and third doses can lower TNF-α expression significantly compared with endometriosis group, reaching level comparable to the control group and the endometriosis group treated with leuprolide acetate ($p < 0.05$). Genistein in second and third doses can lower the expression of IL-6 significantly compared with endometriosis group, reaching level comparable to the control group and the endometriosis group treated with leuprolide acetate ($p < 0.05$). There is no significant difference between the two doses of genistein ($p > 0.05$).

Expressions of VEGF and HIF-1α in the control group and some treatment groups are presented in Table 3. In endometriosis mice, the expressions of VEGF and HIF-1α increased significantly compared with the control group ($p < 0.05$). The administration of the third dose of genistein decreased the expressions of VEGF and HIF-1α significantly compared with endometriosis group ($p < 0.05$), reaching level comparable to the control group ($p > 0.05$).

4. Discussion

After genistein was administered in certain period of time, the researcher did not find any adverse effects and toxic effects from genistein. The previous researches revealed the genistein interaction with action mechanism against chemotherapy thereby hindered the chemotherapy efficacy.[34]

Results of the research showed that the expressions of estrogen receptor-α and estrogen receptor-β in the endometriosis group were significantly higher than the control group ($p < 0.05$). This research focused on the peritoneum and extended the previous findings which found that the expression of estrogen receptor-α decreased significantly in endometrial lesions compared with normal uterine tissues.[7,35] Meanwhile, the high expression of estrogen receptor-β will suppress the expression of estrogen receptor-α.[9,36] Our results were not consistent with this ratio as both estrogen receptors were shown to increase in the endometriosis group compared with the control group. This might be caused by difference in expression patterns in the tissue.

The administration of three doses of genistein decreased the expression of estrogen receptor-α significantly compared with the control group ($p < 0.05$). This demonstrated that genistein can suppress the expression of estrogen receptor-α. This study is consistent with the previous *in silico* study that docking scores obtained from genistein were at −217.67 showing a high affinity for the estrogen receptor-α.[37] The interesting thing here is that the administration of three doses of genistein triggered a significant increase in estrogen receptor-β compared with endometriosis or control group ($p < 0.05$). We thought that the behavior of expression change of estrogen receptor-β indicated the involvement of inflammation in endometriosis. Previous findings stated that the very high ratio of estrogen receptor-β/estrogen receptor-α in endometrial stromal cells was associated with the increased cyclooxygenase-2 level which contributed to inflammation.[10] The increased estrogen receptor-β indicated its role as an anti-inflammatory in certain doses.[38] This is supported by the data of inflammatory cytokines in this study that the expressions of TNF-α and IL-6, which increased significantly in the endometriosis group compared with in control group ($p < 0.05$), can be decreased by the two highest doses of genistein (TNF-α and IL-6). The ability to reduce the pro-inflammatory cytokines is also found in the administration of leuprolide acetate. These findings are consistent with previous studies.[39]

Table 2
The expression of TNF-α and IL-6 in mice model of endometriosis treated with genistein.

Expression	Control	EM	EM + Genistein treatment (mg/day)			Leuprolide acetate (mg)
			0.78	1.04	1.30	0.00975
TNF-α (%)	1.65 ± 1.14	5.45 ± 1.69[a]	4.65 ± 1.42[a]	2.20 ± 0.63[b]	1.05 ± 0.57[bc]	1.85 ± 1.36[bc]
IL-6 (%)	0.30 ± 0.60	3.70 ± 1.25[a]	3.25 ± 1.14[a]	0.45 ± 0.57[bc]	0.30 ± 0.60[bc]	1.50 ± 0.70[bc]

Note: Data are presented as mean ± SD. [a]$p<0.05$ in comparison with control group. [b]$p < 0.05$ in comparison with EM group. [c]$p < 0.05$ in comparison with EM group treated by first dose of genistein; EM: endometriosis group; TNF-α: tumor necrosis factor-α; IL: interleukin; %: percentage; mg/day: milligram/day.

Table 3
The expression of VEGF and HIF-1α in mice model of endometriosis treated with genistein.

Expression	Control	EM	EM + Genistein treatment (mg/day)			Leuprolide acetate (mg)
			0.78	1.04	1.30	0.00975
VEGF (%)	1.15 ± 1.15	7.80 ± 3.57[a]	3.25 ± 1.73[ab]	2.00 ± 1.61[b]	3.10 ± 1.62[b]	6.05 ± 2.82[ad]
HIF-1α (%)	0.60 ± 0.49	9.25 ± 2.79[a]	4.80 ± 2.73[ab]	2.15 ± 1.69[b]	3.45 ± 2.68[b]	6.95 ± 2.33[ad]

Note: Data are presented as mean ± SD. [a]$p<0.05$ in comparison with control group. [b]$p < 0.05$ in comparison with EM group. [d]$p < 0.05$ in comparison with EM group treated by second dose of genistein; EM: endometriosis group; VEGF: vascular endothelial growth factor; HIF-1α: hypoxia inducible factor-1α; %: percentage; mg/day: milligram/day.

In this study, we found that the expressions of VEGF and HIF-1α in peritoneal tissues of endometriosis group are significantly higher than the control group ($p < 0.05$). This suggested that angiogenic signals through VEGF and HIF-1α were upregulated. Previous researches proved that the pro-angiogenic factors were upregulated in peritoneal fluid of patients with endometriosis.[40–42] The results of this study were consistent with those of previous researches. The increased VEGF was intended to mobilize progenitor cells from the bone marrow. This increase is evidenced by the increase in HIF-1α.[43,44] Our research also proved that all three doses of genistein decreased the expressions of VEGF and HIF-1α significantly compared with endometriosis group ($p < 0.05$). It was not found in the administration of leuprolide acetate. These findings indicated that genistein inhibited the mitogenic activity and maintenance of endometriosis that were controlled by VEGF. Genistein activity is better than leuprolide acetate. These results are consistent with previous studies, saying that genistein inhibited neo-angiogenesis through the inhibition of HIF-1α.[43,32]

However, this research has a limitation where it does not carry out the analysis of the groups treated with combination of genistein and standard drugs so that the synergistic or antagonistic effects can be proved. This case will be the focus for the next researches.

It is concluded that genistein is able to modulate estrogen receptor-α and estrogen receptor-β and inhibits the development of inflammation and angiogenesis in the murine peritoneal of endometriosis model. Thus, genistein can be a candidate in the treatment of endometriosis.

Conflict of interest

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

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