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Effect of genistein on proinflammatory cytokines and estrogen receptor-β in mice model of endometriosis

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ABSTRACT

Objective: To investigate the effect of genistein on proinflammatory cytokines, NF-κB activation, and estrogen receptor-β expression in a mice model of endometriosis. **Methods:** Forty female mice (*Mus musculus*) were divided into eight groups (n=5 each), including the control (untreated) group, endometriosis group, and the endometriosis groups were given various doses of genistein (at doses of 50; 100; 200; 300; 400; 500 mg/day). Analysis of TNF-α, IL-1β, IL-6, and IL-8 level were done by ELISA technically. Analysis of estrogen receptor-β and NF-κB were done by immunohistochemistry. **Results:** The level of TNF-α, IL-1β, IL-6, and IL-8 were significantly higher in the EM group compared to the untreated control group (P<0.05). All doses genistein significantly prevented EM-induced increase in TNF-α level (P<0.05), but only at dose of 300 mg/day reach the level in the control group (P>0.05). These increased levels of IL-1β, IL-6, adn IL-8 in the EM group were significantly reduced by all doses of genistein. There were significantly (P<0.05) increased estrogen receptor-β expression and NF-κB activation in EM group compared to untreated group. Only first and fourth dose significantly (P<0.05) decreased the estrogen receptor-β expression compared to the EM group, to reach a level in the control group (P>0.05). All doses genistein significantly prevented EM-induced increase in NF-κB activation (P<0.05), to reach the expression on control group. **Conclusion:** In conclusion, genistein prohibits the increase in proinflammatory cytokines, NF-κB, and estrogen receptor-β expression in a mice model of endometriosis.

1. Introduction

Endometriosis is one of the most frequent benign gynecological diseases that was characterized by the implant and growth of viable endometrial tissue outside the uterine cavity. Endometriosis affected humoral immune response, increase macrophage and its activity which induces general inflammatory response[1–5]. Previous studies showed that deficient methylation of the estrogen receptor-β promoter results in pathological overexpression of ERβ in endometriotic stromal cells[6]. A severely high ERβ-to-ERα ratio in endometriotic stromal cells is associated with increased cyclo-oxygenase-2 levels contributing to inflammation[7].

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A variety of medical hormonal therapies, all aimed to reduce the levels of circulating estrogens, are currently available[8]. 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 are definitely desirable. Genistein inhibited the I κ B-α phosphorylation, nuclear translocation of NF- κ B p65 subunit, the inhibits cytokine production[9–11]. Our previous *in vitro* studies showed that genistein inhibits the cytokines production driven by NF- κ B. Besides, the pharmacologic, but not physiologic concentrations of genistein can modulate sex steroid receptor expression in the rat uterus [12]. This study was aimed to investigate the effect of genistein on proinflammatory cytokines, NF- κ B activation, and estrogen receptor- β expression in a mice model of endometriosis.

2. Material and methods

2.1. Animal

Forty female mice (*Mus musculus*) were divided into eight groups, including the control (untreated) group, endometriosis group (EM), and the endometriosis groups were given various doses of genistein (at doses of 50; 100; 200; 300; 400; 500 mg/day). *Mus musculus* at aged 2-3 months and weighing 20-30 grams was obtained from the Laboratory of Reproductive Physiology Embryology, Faculty of Veterinary Medicine, Airlangga University, Surabaya. This study was conducted at the Laboratory of Reproductive Physiology Embryology Faculty of Veterinary Medicine, Airlangga University, Surabaya and Laboratory of Physiology Faculty of Medicine, University of Brawijaya, Malang.

2.2. Genistein treatment

Genistein was purchased from Tokyo Chemical Industries, Japan. Before treatment, genistein was dissolved in sesame oil (1 mL volume containing 1 gram). For treatment, genistein was administered by oral gavage for 14 days started after 14 days of induction in mice model of endometriosis[13, 14].

2.3. Endometriosis model

Mice model of endometriosis was made by myometrium and endometrium tissue implantation in immunodeficient mice. Immunodeficient mice was obtainde by Cyclosporin A (0.2 mL) injection. Before the implantation, the myometrium and endometrium of benign tumors of the uterus (adenomyosis) tissue was washed twice at 2500 RPM, supernatant was discarded and then added to PBS. Implantation was performed by injection (0.1 mL) into the peritoneal cavity. Besides, the Ethynil estradiol injections (0.1 mL) were performed intramuscularly on days one and five. Furthermore, mice were observed for 14 days to be mice model of endometriosis[15].

2.4. Analysis of TNF-α level

The level of TNF-α in peritoneum fluid was measured immunoenzymatically using mouse Specific sandwich enzyme-linked immunosorbent assays (Biolegend, USA, Catalog series 430907/430908). All procedure was done according kit instruction.

2.5. Analysis of IL-1β level

Level of IL-1β in peritoneum fluid was measured immunoenzymatically using mouse Specific sandwich enzyme-linked immunosorbent assays (Biolegend, USA, Catalog series 432604 /432605/ 432060). All procedure was done according the instruction in the kit.

2.6. Analysis of IL-6 level

Level of IL-6 in peritoneum fluid was measured immunoenzymatically using mouse Specific sandwich enzyme-linked immunosorbent assays (Biolegend, USA, Catalog series 431307). All procedure was done according the manufacturer's instruction.

2.7. Analysis of IL-8 level

Level of IL-8 in endometriosis lesion was measured immunoenzymatically using mouse Specific sandwich enzyme-linked immunosorbent assays (Bioassay Technology Laboratory, Shanghai, PRC, Catalog series E0941M). All procedure was done according the manufacturer's instruction.

2.8. Endometrial lesion homogenization

One mg of endometriosis lesions was cut into small pieces and then crushed and added RIPA buffer (10 μL PMSF, 10 mL sodium orthovanadate, and 10 μL protease inhibitor cocktail) and 500 mL PBS. Tissue suspension was homogenized by vortex for 30 minutes. This suspension was then incubated at 37 ℃ for 15 minutes, and then centrifuged 2 500 rpm for 7 minutes to form a supernatant. Supernatant was placed in a 1.5 mL microcentrifuge tube. Let the samples for 5 min at 4 ℃ (cold room and then centrifuged for 15 min and then transferred to a new tube for storage at -20 ℃.

2.9. Immunohistochemistry

The expressions of estrogen receptor- β expression and NF- κ B activation were examined according to previous protocol[16]. Immunohistochemically was performed on the peritoneum tissue. Peritoneum tissue was excises, cleaned with ice-cold normal saline and were prepared for immunohistochemistry evaluation. Peritoneum tissue sections paraffin-coated slides (Surgipath Paraplast, Leica Microsystem, Europe) were prepared by Tissue Tex Processor. Forty slides (five slides per group) were conducted for estrogen receptor- β and NF- κ B. After heating, peritoneum sections were deparaffinized in xylol (Bioss Antibodies, USA) and rehydrated in a graded alcohol (Bioss Antibodies, USA). Sodium

citrate buffer of concentration 10mM was heated until boiling in a microwave for antigen retrieval. Immunohistochemistry staining was applied following the manual of the company (Bioss Antibodies, USA). Succinctly, 3% hydrogen peroxide in 0.5% methanol was used to block the endogenous peroxidase for 5 min followed by washing the tissue sections carefully using wash buffer and then incubated with Rabbit Anti Estrogen Receptor Beta polyclonal antibody and Rabbit Anti NF- κ B Inducing Kinase NIK Polyclonal Antibody (Bioss Antibodies., inc , Massachusetts, USA) biotinylated primary antibodies for 120 minutes. After incubation, tissue sections were carefully washed with washing buffer and conserved in the buffer bath. After adding streptavidin-HRP, sections were kept for 40 minutes incubated and then washed. Diaminobenzidine substrate chromogen was applied to the sections and reincubated for over 20 min followed by careful washes and hematoxylin counterstaining for 5 seconds. Dot slide Nikon Cameran DS F12 300 megapixel (Nikon, Japan) was used to examine the immunostaining analysis. (Magnification ×400). Sections were scored semiquantitativley as follows: (0) negative; (1) weakly positive; (2) moderately positive; (3) strongly positive ; and (4) very strongly positively were determined according to the immunodetection of stain intensity and amounts of positive cells by two pathologists.

2.10. Statistical analysis

Data are presented as mean ± SD and the differences between groups were analyzed using one-way analysis of variance (ANOVA) with SPSS 15.0 statistical package for Windows. Only probability values of $P<0.05$ were considered statistically significant and later subjected to Tukey's post hoc test.

3. Results

Table 1 presents the TNF- α , IL-1 β , IL-6, and IL-9 levels from each experimental group. The level of TNF- α , IL-1 β , IL-6, and IL-8 were significantly higher in the EM group compared to the untreated control group ($P<0.05$). All doses genistein significantly prevented EM-induced increase in TNF- α level ($P<0.05$), but only at dose of 300 mg/day reach the level in the control group ($P>0.05$). These increased levels of IL-1 β in the EM group were significantly reduced by all doses of genistein. Indeed, administration of all doses to the EM group reduced IL-1 β levels to those can not comparable to the untreated group ($P>0.05$). All doses genistein significantly prevented EM-induced increase in IL-6 and IL-8 level ($P<0.05$) to reach the level in the control group ($P>0.05$).

The exposure of endometrium and uterus implant to rat peritoneum affected the estrogen receptor- β expression and NF- κ B activation, as shown in Table 2. There were significantly ($P<0.05$) increased estrogen receptor- β and NF- κ B activation in EM group compared to untreated group. Out of all doses genistein treatment, only first and fourth dose significantly ($P<0.05$) decreased the estrogen receptor- β expression compared to the EM group, to reach a level in the control group ($P>0.05$). All doses genistein significantly prevented EM-induced increase in NF- κ B activation ($P<0.05$), to reach the expression on control group. There was no significant different between the effects of these doses.

4. Discussion

The deficient methylation of the estrogen receptor- β promoter results in pathological overexpression of ER β in endometriotic stromal cells[6]. In addition, high levels of estrogen receptor- β suppress estrogen receptor- β expression[10, 17,18]. A severely high ER β -to-ER α ratio in endometriotic stromal cells is associated with inflammation[10]. In this study, the expression of estrogen receptor- β levels were significantly greater in the EM group

Table 1
The levels of pro-inflammatory cytokines in peritoneal fluid of mice model endometriosis treated by genistein.

Level (pg/mL)	Control	EM	EM+ Genistein treatment (mg/day)					
			50	100	200	300	400	500
TNF- α	169.50 ± 9.11	2283.75 ± 863.35[a]	519.75 ± 330.16[ab]	148.25 ± 7.89[abc]	191.25 ± 29.94[abcd]	201.50 ± 49.17[bcd]	950.75 ± 427.80[abcdef]	508.50 ± 106.81[abcdeg]
IL-1 β	3600.00± 250.33	26890.00± 22455.96[a]	4160.00 ± 678.63[ab]	3325.00 ± 316.39[abc]	3885.00 ± 463.43[abcd]	7395.00 ± 2598.28[abcde]	3235.00 ± 175.40[abcef]	4000.00 ± 173.59[abdeg]
IL-6	0.47 ± 0.03	2.42 ± 1.06[a]	0.76 ± 0.27[b]	0.47 ± 0.07[b]	0.53 ± 0.03[b]	1.03 ± 0.28[b]	0.46 ± 0.07[b]	0.61 ± 0.08[b]
IL-8	1.03 ± 0.12	2.13 ± 0.87[a]	0.99 ± 0.11[b]	1.00 ± 0.10[b]	0.92 ± 0.12[b]	1.00 ± 0.13[b]	0.92 ± 0.09[b]	0.73 ± 0.16[b]

Note: values 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 the first dose administered group; [d]$P<0.05$; in comparison with the second dose administered group; [e]$P<0.05$; in comparison with the third dose administered group; [f]$P<0.05$; in comparison with the fourth dose administered group; [g]$P<0.05$; in comparison with the fifth dose administered group; TNF- α : tumor necrosis factor- α ; IL-1 β : interleukin-1 beta; pg/mL: picogram/milliliter; ng/mL: nanogram/milliliter.

Table 2
The expression of estrogen receptor- β and NF- κ B expression in mice model endometriosis treated by genistein.

Expression	Control	EM	EM+ Genistein treatment (mg/day)					
			50	100	200	300	400	500
ER- β	0.50 ± 0.20	6.15 ± 2.16[a]	1.95 ± 1.18[ab]	1.05 ± 0.38[b]	0.45 ± 0.53[bc]	2.75 ± 0.10[abde]	1.20 ± 0.16[b]	1.40 ± 0.28[b]
NF- κ B	0.35 ± 0.30	5.00 ± 0.75[a]	0.90 ± 0.35[b]	1.05 ± 0.41[b]	0.85 ± 0.44[b]	1.10 ± 0.30[b]	0.65 ± 0.57[b]	0.60 ± 0.37[b]

Note: values 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 the first dose administered group; [d]$P<0.05$; in comparison with the second dose administered group; [e]$P<0.05$; in comparison with the third dose administered group; [f]$P<0.05$; in comparison with the fourth dose administered group; [g]$P<0.05$; in comparison with the fifth dose administered group; ER- β : estrogen receptor- β ; NF- κ B: nuclear factor-kappa beta.

compared to the untreated group. This finding indicate that the estrogen receptor- β promoter was upregulated, maybe due to of deficient methlyation. This finding confirmed extended our previous studies that estrogen receptor- β levels was exist in endometriosis cell culture. These estrogen receptor- β expression were accompanied by elevated levels of IL-1 β , TNF- α , IL-6, IL-8, and NF- κ B activation. This finding indicates that our mice model endometriosis upregulated the NF- κ B. signalling pathway to produces pro-inflammatory cytokines.

The estrogen receptor plays an important role by mediating oestrogen action and eutopic or ectopic endometrium development. In this study, genistein at first and fourth dose significantly ($P< 0.05$) decreased the estrogen receptor- β expression compared to the EM group, to reach a level in the control group. This *in vivo* study confirmed by our previous *in vitro* study, that genistein able to inhibit the expression of estrogen receptor- β . One mechanisms of its inhibition maybe due to modification of aberrant DNA methyl ation status [19]. In addition, the genistein also inhibits NF- κ B activation and TNF- α , IL-1 β , IL-6 and IL-6 levels. This finding showed that genistein attenuated I κ B- phosphorylation, nuclear translocation of NF- κ B p65, and its cytokine production. Several previous studies showed that genistein administration decreased the levels of TNF- α and IL-6 in serum and liver, as well as inhibited I κ B- phosphorylation, nuclear translocation of NF- κ B p65 subunit, and activation of c-Jun N-terminal kinase (JNK) [9-11]. Interestingly, the reversal effect of cytokine production of genistein treatment is different between cytokine. For TNF- α level, only at dose of 300 mg/day reach the level in the control group. Indeed, administration of all doses to the EM group reduced IL-1 β levels but can not reach similar levels with the untreated group. All doses genistein significantly prevented EM-induced increase in IL-6 and IL-8 level to reach the level in the control group. We hypothesized that this cytokines produces by several cells in peritoneum fluid and tissue. The affinity of each cell to genistein is different.

In conclusion, genistein prohibits the increase in proinflammatory cytokines, NF- κ B, activation and estrogen receptor- β expression in a mice model of endometriosis. Therefore this may provide a natural therapy for attenuating the inflammation and estrogen receptor alteration in endometriosis disease.

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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