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Inhibitory effect of genistein on MMP-2 and MMP-9 expression through suppressing NF- κ B activity in peritoneum of murine model of endometriosisDwi Yuliawati¹✉, Karyono Mintaroem², Sutrisno Sutrisno³¹STIKES Karya Husada Kediri, East Java, Indonesia²Laboratory of Pathology, Faculty of Medicine, University of Brawijaya, Malang, East Java, Indonesia³Division of Fertility, Endocrinology and Reproduction, Department of Obstetrics and Gynecology, Saiful Anwar Hospital, Faculty of Medicine, University of Brawijaya, Malang, East Java, Indonesia

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ABSTRACT

Objective: To analyze the inhibitory effect of genistein on matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) through inhibition of nuclear factor-kappa B (NF- κ B) activation. **Methods:** A total of 36 female mice were divided into six groups ($n=6$ in each group): control group (untreated mice), endometriosis mice group, and endometriosis mice groups administered with genistein at different doses (1.30 mg/day; 1.95 mg/day; 2.60 mg/day; and 3.25 mg/day). The genistein treatment was performed for 14 d. The expressions of NF- κ B, MMP-2 and MMP-9 on the endometriosis lesions were analyzed by the immunohistochemical technique. **Results:** The activity of NF- κ B in the endometriosis group increased significantly than that of the control group ($P<0.05$). The expression of MMP-2 or MMP-9 in the endometriosis group increased significantly than that of the control group ($P<0.05$). Administration of genistein at different doses was capable of significantly reducing the activity of NF- κ B compared to that of the endometriosis group ($P<0.05$), reaching the level comparable to that of the control group at the third highest dose ($P>0.05$). The four doses of genistein administration were capable of significantly reducing the expression of MMP-2 compared to that of the endometriosis group ($P<0.05$), reaching an expression comparable to that of the control group for the highest dose ($P>0.05$). Administration of genistein at different doses was capable of significantly reducing the expression of MMP-9 compared to that of the endometriosis group ($P<0.05$), reaching the level comparable to that of the control group at the highest dose ($P>0.05$). **Conclusions:** Genistein suppresses the expression of MMP-2 and MMP-9 through suppressing NF- κ B activity in the peritoneum of murine model of endometriosis.

1. Introduction

Endometriosis is a chronic and heterogeneous disease with the presence of the stroma and functional endometrial glands outside the uterine cavity, and it is often found in the peritoneum, ovary, or rectovaginal septum[1,2]. The etiology of most endometriosis is unknown[3]. The incidence of endometriosis in women of reproductive age is 6%-11%, with the highest incidence at 25-35

years old[4,5]. The main symptoms of endometriosis are pelvic pain and infertility[6], but it is also asymptomatic[2].

Despite the histologically benign lesion, endometriosis lesions also exhibit malignant cancer behaviors, including local invasion and aggressive spread to distant organs[5]. This behavior is due to the ability of endometriosis lesions to form an estrogenic environment

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for proliferation of cells and stimulation of proinflammatory cytokines[7,8]. Two important proinflammatory cytokines, such as tumor necrosis factor- α and interleukin-1 β , activate the transcription factors—nuclear factor-kappa B (NF- κ B) and activator protein-1. Furthermore, these transcription factors induce gene transcription and code for products, and one of them is the matrix metalloproteinases (MMPs), including MMP-1, MMP-2, MMP-3, MMP-7 and MMP-9. The MMPs trigger the degradation and remodeling of the extracellular matrix of peritoneal surfaces and other organs[9].

The growth and development of endometriosis require the activation of gelatinase-type MMPs (MMP-2/gelatinase A and MMP-9/gelatinase B)[10]. Previous findings have demonstrated an increased expression and activity of MMP-2 and MMP-9 in the stages of organ development, and a variety of pathological conditions involving tissue remodeling[11]. The expression of MMP-2 and MMP-9 is found to be higher in women with endometriosis than that in controls which modulates by the activation of NF- κ B[12–14]. The stimulator for this activity is tumor necrosis factor- α and interleukin-1 β [15].

Currently, no drug successfully cures endometriosis completely and in some cases it causes side effects of long-term use. Certain drugs can even lead to delays in conception and have not been shown to have fertility effects after treatment. Therefore, an ideal drug is currently developed with the aim of regression of the disease and its symptoms without the negative effects associated with hypoestrogenic conditions. The drug is an anti-estrogenic selective estrogen receptor modulator[16].

Genistein is an isoflavone capable of acting as selective estrogen receptor modulators, which serves as a pure antagonist when acting *via* estrogen receptor alpha, but it can serve as a partial agonist when acting *via* estrogen receptor beta[17]. With regard to the structural similarities of 17 β -estradiol and genistein and to endometriosis with estrogenic environmental conditions to maintain cellular viability, genistein can be a pure antagonist. Previous studies have shown that genistein is capable of inhibiting the activation of NF- κ B[18]. Therefore, the purpose of the present study was to analyze the inhibitory effect of genistein on MMP-2 and MMP-9 through inhibition of NF- κ B activation.

2. Materials and methods

2.1. Animals

Mice (*Mus musculus*) were obtained from the Laboratory of Embryology, Faculty of Veterinary Medicine, Airlangga University (FKH Unair) Surabaya, East Java, Indonesia. Mice selected for the present study were healthy female mice, with 2-3 months old, 20-30 g in weight, having fine hairs and glowing eyes, and having no limp and scars. Mice were kept in plastic cages of 45.0 cm \times 35.5 cm \times 14.5 cm in size with a wire-mesh cage cover. The base of the cages was covered with sawdust of 0.5-1.0 cm in thickness and was changed

every three days. Room light was carefully controlled, with 12 h of light (06:00 to 18:00 West Indonesia Time) and 12 h of dark (18:00 to 6:00 West Indonesia Time). Room temperature ranged 27 $^{\circ}$ C to 28 $^{\circ}$ C. The mice were fed *ad libitum* twice a day with Guyofeed chicken pellets containing 12% protein.

A total of 36 mice were divided into six groups (6 animals per group): control group (untreated mice), endometriosis mice group (without genistein treatment) and endometriosis mice groups administered with genistein at different doses (1.30 mg/day; 1.95 mg/day; 2.60 mg/day; 3.25 mg/day).

2.2. Generation of murine model of endometriosis

Endometrial and myometrial tissues were taken from adenomyotic patients who were subjected to a surgery at Syaiful Anwar Hospital Malang. Tissues were removed after the patient's signature of the informed consent. It was subsequently stored in a phosphate buffer saline -containing Falcon tube. Furthermore, tissues were cut into small pieces of 0.5 cm in thickness, washed with phosphate buffer saline, and centrifuged twice at 3 000 rpm at a temperature of 4 $^{\circ}$ C for 10 min. The implant tissues were then intraperitoneally injected into the mice subjected to the immunodeficient condition by administration of cyclosporin A and to hyperestrogenic condition by injection of ethinyl estradiol. The development of endometriosis was established by means of direct evaluation of the peritoneum of mice (*Mus musculus*) model of endometriosis, and the expression of estrogen receptor alpha and estrogen receptor beta in the endometriosis lesions of the murine peritoneal tissue was measured[19,20].

2.3. Genistein

Dosage of genistein was determined based on previous studies[21], which was converted for administration in mice. The genistein used was that of genistein trademark manufactured by Bioworld, America, CAS 446-72-0, Catalog 40000007-2. Genistein purity was > 95%. Genistein was powder-shaped, purified from soybeans. Genistein was administered for 14 d by dissolving it in sesame oil to achieve the desired dose[19,20].

2.4. Collection of peritoneal tissues

Upon completion of treatment, mice were anesthetized and dissected for collection of peritoneal tissues. Prior to dissection, they were anesthetized with ketamine. Peritoneal tissue samples were stored at -80 $^{\circ}$ C until analysis.

2.5. Expression of NF- κ B

This expression was analyzed on the endometriosis lesions of the peritoneal tissue using immunohistochemical technique. The

antibody anti NF- κ B 65 was used (produced by Bioss Inc.; bs-0465R). The Remmele index using the immuno reactive score was the product of multiplication between the percentage of immunoreactive cells and color intensity score of the immunoreactive cells[22].

2.6. Expression of MMP-2

Expression of MMP-2 was the expression of MMP-2 on the endometriosis lesions of the peritoneal tissues in the murine model of endometriosis using the immunohistochemical technique. The antibody anti MMP-2 was used (produced by Bioss Inc.; bs-0412R). Expression of MMP-2 was assessed semi-quantitatively using the same technique as that of the expression of NF- κ B.

2.7. Expression of MMP-9

Expression of MMP-9 was the expression of MMP-9 on the endometriosis lesions of the peritoneal tissues of the murine model of endometriosis using the immunohistochemical technique. The antibody anti MMP-9 was used (produced by Bioss Inc.; bs-0397R). Expression of MMP-9 was assessed semi-quantitatively using the same technique as that of the expression of NF- κ B.

2.8. Ethics

The present study passed the ethics review (No. 275/EC/KEPK/07/2016) and was approved by the Health Research Ethics Committee, Faculty of Medicine, Brawijaya University, Malang, Indonesia (July 26, 2016).

2.9. Statistical analysis

The expression of NF- κ B, MMP-2 and MMP-9 was shown in mean \pm standard deviation (mean \pm SD). Differences among treatment groups were analyzed using one-way analysis of variance test by means of SPSS 17.0 statistical package software. A *post-hoc* test was conducted in the event that the analysis of variance test found a within-group significance. A $P < 0.05$ was a statistically significant difference.

3. Results

Figure 1 showed the activity of NF- κ B on the peritoneal tissues of different groups. The activity of NF- κ B was higher significantly in the endometriosis group [$4.55 \pm 0.53\%$] compared to the control group [$0.00 \pm 0.00\%$] ($P < 0.05$). Administration of genistein

at different doses significantly reduced the activity of NF- κ B compared to that of the endometriosis group ($P < 0.05$), reaching the level comparable to that of the control group at the third highest dose [$0.00 \pm 0.00\%$] ($P > 0.05$).

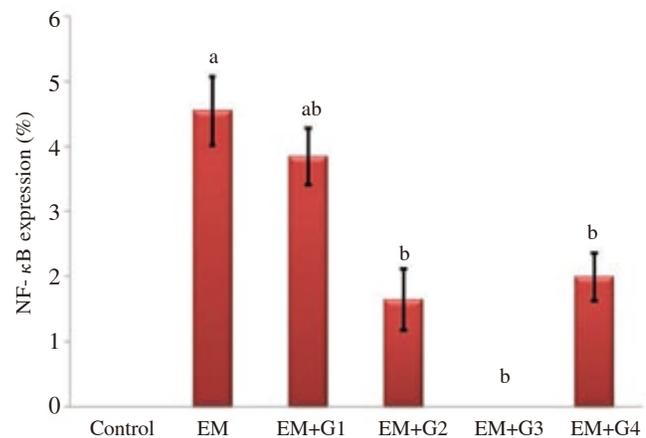


Figure 1. NF- κ B expression in peritoneal tissue of various research groups. Note: Data are presented in mean \pm SD; ^a $P < 0.05$ compared to control group; ^b $P < 0.05$ compared to endometriosis group.

Figure 2 showed the expression of MMP-2 in peritoneal tissue of different groups. MMP-2 expression significantly increased in the endometriosis group [$5.00 \pm 0.77\%$] compared to the control group [$0.00 \pm 0.00\%$] ($P < 0.05$). The four doses of genistein administration significantly reduced the expression of MMP-2 compared to that of the endometriosis group ($P < 0.05$), reaching an expression comparable to that of the control group at the highest dose [$0.45 \pm 0.38\%$] ($P > 0.05$).

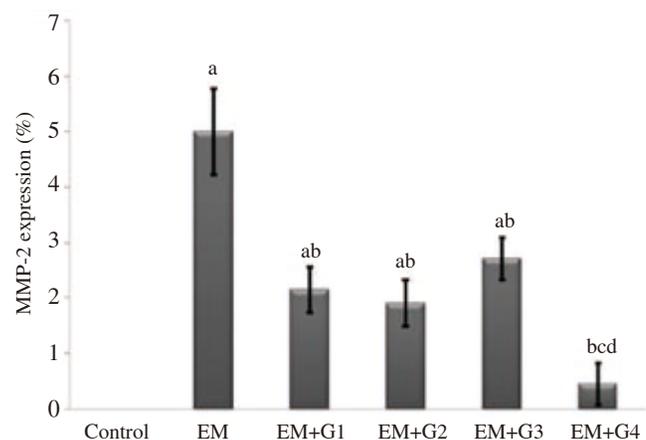


Figure 2. MMP-2 expression in peritoneal tissue of various research groups. Note: Data are presented in mean \pm SD; ^a $P < 0.05$ compared to control group; ^b $P < 0.05$ compared to endometriosis group; ^c $P < 0.05$ compared to endometriosis group received first dose of genistein; ^d $P < 0.05$ compared to endometriosis group received second dose of genistein.

Figure 3 showed the expression of MMP-9 in peritoneal tissue of different groups. MMP-9 expression significantly increased in the endometriosis group [(4.50±0.38)%] compared to that of the control group [(0.00±0.00)%] ($P<0.05$). Administration of genistein at different doses significantly decreased the expression of MMP-9 compared to that of the endometriosis group ($P<0.05$), reaching levels comparable to that of the control group at the highest dose [(0.75±0.41)%] ($P>0.05$).

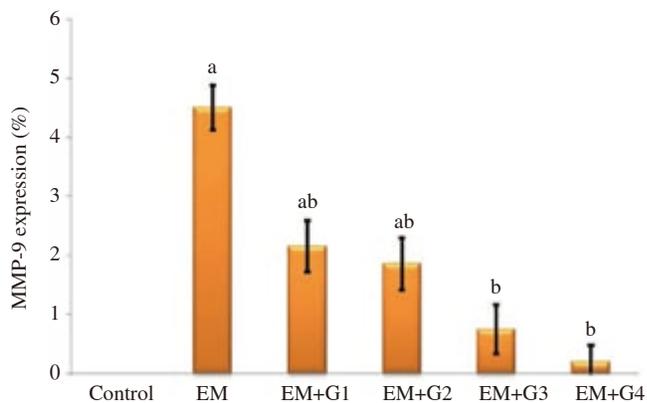


Figure 3. MMP-9 expression in peritoneal tissue of various research groups.

Note: Data are presented in mean ± SD; ^a $P<0.05$ compared to control group; ^b $P<0.05$ compared to endometriosis group.

4. Discussion

The present study showed that the activity of NF- κ B and the expression of MMP-2 and MMP-9 in the endometriosis group significantly increased than those of the control groups. This indicates that the translated protein products, namely MMP-2 and MMP-9, were upregulated at least via the increased activity of the transcription factor NF- κ B. The estrogen induces transcription factors that modulate the activity of major pro-inflammatory cytokines[9]. This finding is consistent with that of previous studies that the activation of NF- κ B is increased on endometriosis lesions which involve proliferation, apoptosis, adhesion, invasion, and angiogenesis in endometriosis[23,24]. With regard to the peritoneum, NF- κ B acts *via* regulation of the expression of MMPs in the invasion and adhesion of endometriosis cells on the peritoneal surface[15]. A previous study has shown that MMP-2 is expressed in the endometrial graft in the peritoneum[25]. Other studies have also found expression of MMP-2 and MMP-9 in peritoneal endometriosis[26]. Materials that can suppress the production of MMP-2 and MMP-9 will inhibit the adhesion and migration of endometriosis cells in the peritoneum[27].

The present study showed that administration of genistein at different doses was capable of significantly reducing the activity of

NF- κ B compared to that of the endometriosis group, reaching the level comparable to that of the control group at the third highest dose (2.60 mg/day). This suggests that genistein is capable of suppressing the activation of NF- κ B on the peritoneum of the murine model of endometriosis. This finding is consistent with the previous one that genistein is capable of inhibiting the translocation of NF- κ B and expression of genes modulated by NF- κ B. Genistein inhibits the DNA binding of NF- κ B through blocking the phosphorylation of the protein inhibitor I κ β, preventing the translocation of NF- κ B [18]. Furthermore, inhibition of this activation suppresses expression of the proteins MMP-2 and MMP-9. The present study demonstrated that all the doses of genistein were capable of significantly decreasing the expression of MMP-2 and MMP-9 compared to that of the endometriosis group. The expression of MMP-2 comparable to that of the control group was achieved at the highest dose (3.25 mg/day), while that of MMP-9 was achieved at the highest dose. This suggests that genistein is capable of suppressing the expression of MMP-2 and MMP-9 at least through suppressing the activity of NF- κ B. The differences in the optimal dose of genistein, which can return to the basal value, are thought to be caused by the presence of other regulators of transcription factors in the expression of MMP-2, namely signal transducer and activator of transcription-1 (STAT-1) and STAT-3[18]. Genistein is an activation inhibitor of NF- κ B and STAT-1[26]. Meanwhile, regulation of MMP-9 involves NF- κ B/p65, activator protein-1/c-jun, transcription factor E2F2, Smad2, STAT-1 and STAT-3. In addition, genistein can also act by means of bonding to estrogen receptors[14,28].

In the focus of endometriosis, the effects of genistein on blood flow and vascularization are inconsistent. Genistein can suppress angiogenesis in endometriosis tissue through down-regulation of vascular endothelial growth factor and hypoxia inducible factor-1 α [29]. In another study analyzing vascularization and blood flow, it was found that genistein could suppress vascularization (assessed by microvillar density) but did not affect blood flow in endometriosis graft. In this study, differences in genistein effects in MMP-2 and MMP-9 expression suppression still require continued research to assess tissue remodeling anatomy[30].

The strength of this study is the analysis of MMP-2 and MMP-9 which simultaneously perform as a product of NF- κ B transcription factor. The weakness of this study is that no other regulatory factors are assessed (especially STAT signals) and no anatomical measurements of tissue remodeling are performed. This will be the target for further study.

In conclusion, genistein suppresses the expression of MMP-2 and MMP-9 through suppressing NF- κ B activity in the peritoneum of a murine model of endometriosis.

Conflict of interest statement

All authors hereby state that there is no conflict of interest.

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