



Original Research

Effect of Genistein on Proliferating Cell Nuclear Antigen (PCNA) Expression and Vascular Density in The Peritoneum of Endometriosis Mice Model

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ABSTRACT

Introduction: Survival of endometriosis lesions depends on the formation of blood vessels, cell proliferation, and oxygen supply. Their vascular densities can evaluate the vascularity of ectopic endometriosis tissues. Proliferating Cell Nuclear Antigen (PCNA) is one of the markers of cell proliferation that affects the growth of endometriotic lesions. Genistein is an isoflavone component that highly potential effect as an anti-angiogenic and inhibiting proliferation through the binding capacity to estrogen receptors.

Material and Methods: This experimental study uses the Stored Biological Materials of mice peritoneum tissue. The paraffin block was divided into six groups, which are the negative control group, positive control group, and four treatment groups with genistein doses of 1.3 mg/day, 1.95 mg/day, 2.6 mg/day, and 3.25 mg/day. Immunohistochemical techniques and hematoxylin-eosin for vascular densities examined PCNA expressions. Preparation analysis uses OLIVIA software. Preparation calculations are done manually with cell count software. Observations were analyzed with the ANOVA test and Dunnett T3 5% test.

Results: Based on analysis with ANOVA, there was a significant difference in PCNA expression and vascular densities because of the effect of genistein in various doses with a p-value of 0.001 ($p < 0.05$). Based on Dunnett T3 5% test increase of PCNA expression by giving genistein doses 1,3 mg/day, 1,95 mg/day, and 2,6 mg/day, a reduction in dose of 3,25 mg/day. Vascular densities reduced significantly in all treatment groups.

Conclusion: Administration of genistein in various doses significantly increases PCNA expression but reduces PCNA expression in a dose of 3.25 mg/day and significantly reduces vascular densities.

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INTRODUCTION

Recently, Endometriosis is one of the primary reproductive problems, characterized by the growth of endometriotic lesions from endometrial glands and stroma. Endometriosis generally affects 10-15% of women of reproductive age and generally occurs in 40-60% of women with pelvic pain and 30-50% in infertile women [1]. Based on World Statistics of Endometriosis data in 2014, the prevalence of Endometriosis worldwide was 176,000,000 patients [2]. Dr. Cipto Mangunkusumo Hospital Jakarta during 2006-2010 reported that Endometriosis was found in 10% of reproductive women. Dr. Muwardi Hospital Surakarta in 2010, the incidence of Endometriosis in gynecological

surgery was approximately 13.6%. RSUD Dr. Sutomo Surabaya found a higher incidence of Endometriosis was 23.8% [3].

Endometriosis and the etiopathogenesis of this disease have been extensively investigated and explained in various theories, from clinical to biomolecular [4]. Based on Sampson's implantation theory (1927), peritoneal endometriosis lesions develop from endometrial tissue, which flows retrograde through the fallopian tubes during menstruation [5]. Over the past few years, many studies have shown that the theory and the survival of endometriotic lesions are highly dependent on blood vessel formation, development, and oxygen supply [6]. Furthermore, the hypoxia condition,

as well as the stage of development of ectopic endometrial tissue, influence this disease. Therefore, Endometriosis is hypothesized to be associated with increased angiogenic factors regulation in serum and peritoneal fluid, formation of new blood vessels, Endometriosis, and peritoneum lesions [7]. These findings indicate that vascularization is a significant feature in the pathogenesis of Endometriosis, which is a potential target for the development of future diagnostic and therapeutic strategies.

Endometriosis is a benign tumor with malignant characteristics, such as its invasive nature and predisposition to metastasis and relapse [8,9]. Various studies have studied cell proliferation in Endometriosis, but little has been revealed about its exact mechanism. The high sensitivity of endometriotic lesions to cell proliferation has been considered a possible pathogenesis of this disease. A significant increase in cell proliferation in patients with Endometriosis compared to non-endometriosis. Endometriosis cell proliferation becomes the target of therapy to control the clinical progression of Endometriosis to suppress its rate of its. It is expected that the rate of endometriosis development will slow or stop. Proliferating Cell Nuclear Antigen (PCNA) is one of the potential markers of cell proliferation, which can help assess proliferating status in Endometriosis [10].

Most of the current medical treatments for Endometriosis have targeted decreasing estrogen activity. Many endometriosis therapy modalities are established nowadays, including contraceptive steroids, progestogen agents, Gonadotropin-Releasing Hormone (GnRH) agonists such as leuprolide acetate (tapers, divine), danazol, selective progestins such as dienogest (visanne), androgen, and non-steroidal anti-inflammatory agents. This treatment can only be used for a limited period because of its side effects. In addition, a high recurrence rate after treatment becomes a significant problem [11]. Further study of endometriosis treatment strategy is needed for being studied.

Anti-angiogenic agents from different groups of substances are a promising therapy for Endometriosis using phytoestrogen such as genistein. As a class of isoflavone, genistein is an inhibitor of proliferation, anti-tumor, and anti-cancer, affects cell apoptosis, and can bind to estrogen receptors (ER). Highly sources that contain genistein are soybeans and their processed products such as tofu, tempeh, sauce, and soy sauce. Genistein is also found in fruits and green tea [12]. In high estrogen levels environment such as in Endometriosis, genistein binds to estrogen β receptors, which cause inhibition of estradiol binding, thereby causing antiestrogenic effects and activating corepressors to suppress gene transcription [11].

The effects of genistein at the molecular level include inhibition of proliferation, induction of differentiation, apoptosis, and cell cycle cessation, which is associated with transforming growth factor (TGF) β 1. Genistein inhibits cell growth by modulating the TGF β 1 signal. These growth factor peptides have been identified as the main factors that regulate eukaryotic cell proliferation by weakening passage flow through the cell cycle checkpoint. The result of the genistein effect is expected to induce inhibition of endometriosis cell development [13].

The mechanism of cell proliferation and vascular density has been investigated previously in Endometriosis. Based on research conducted by Sutrisno et al. (2014) reported a significant decrease in cell proliferation of endometriosis cells culture 6 hours after administration of genistein therapy at a dose of 50 μ mol/l compared with the control group. Sutrisno et al. (2016) reported the effect of VEGF and HIF-1 expression in endometriosis vascularization. Genistein 1,3 mg/day showed a potential effect to inhibit VEGF and HIF-1 expression in Endometriosis peritoneal tissue [14].

The effect of genistein in decreasing proliferation and inhibiting the growth of endometriosis cell vascularization is still questioned. Therefore, this study is interested in the effect of giving various doses of genistein PCNA expression and vascular density in the peritoneum of endometriosis mice.

MATERIAL AND METHODS

Experimental Design

This study was experimental laboratory research with a post-test-only control group design. This study used stored biology material (BBT) peritoneal tissue of mice made paraffin blocks from experimental mouse animals in Sutrisno's (2017) study. Paraffin blocks were divided into six groups. Each group was then seen PCNA expression and vascular density after genistein administration.

Sample

The total number of samples was 31 slides from 6 treatment groups. Murine experimental animals consisted of 1 mouse negative control group (NC) from healthy mice and six mice per group of the positive control group (PC) from untreated endometriosis mice model, treatment group I (G1) with genistein dose 1.3 mg/day, treatment group II (G2) was given genistein 1.95 mg/day, treatment group III (G3) was given genistein 2.6 mg/day and the IV treatment group was given genistein 3.25 mg/day. The PCNA expression and vascular density were examined using the Nikkon

Table 1. Effect of Genistein on PCNA Expression

Groups	Mean \pm SD	p-value
PC	119.8 \pm 19.19	
G1	151.3 \pm 21.42	
G2	367 \pm 173.17	0.000*
G3	490.1 \pm 66.20	
G4	155.7 \pm 31.38	

*ANOVA test showed statistically significant

Table 2. Comparison of PCNA expression in control and treatment group with post hoc Dunnett T3 5% test.

Groups	Mean Different	p-value
PC G1	-0.236	0.027*
G2	-1.005	0.002*
G3	-1.412	0.000*
G4	-0.256	0.055

*Statistically significant

H600L brand light microscope with a 300-megapixel DSF12 camera and Nikon image system image processing software. The scanned preparations were then included in OLIVIA software after an enlargement of 400 times in 5 fields of view. The calculation of preparations was done manually with the help of cell count software.

Research Procedure

1. Histopathological Preparations

Cutting macros from the tissue made paraffin tissue embedding, deparaffinization, and HE Coloring Process, followed by increasing alcohol concentrations, clearing, and mounting.

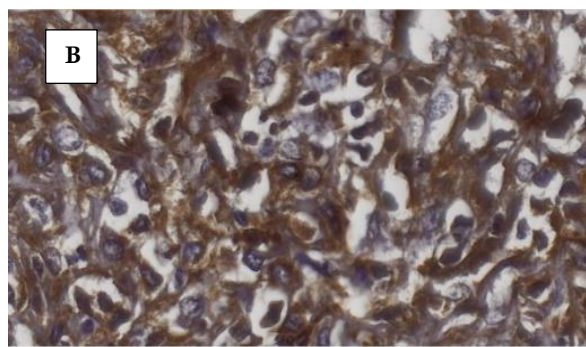
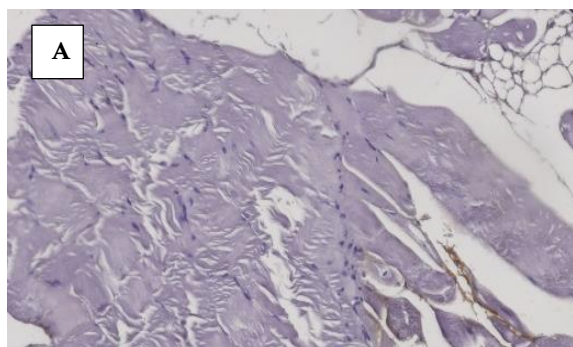


Fig. 1. Immunohistochemistry staining of PCNA expression in healthy peritoneal mice (NC) and endometriosis mice model (PC). A. NC, and B. PC. The red arrow showed PCNA expression in the peritoneal endometriosis mice model

2. Immunohistochemical examination

Slides preparation were stored in an overnight incubator and dried and heated on a hot plate for 1 hour before the coloring process, deparaffinization, rehydration, washing by running water, entered in H₂O₂ in 0.5%, washed by running water, retrieval antigen, stored at room temperature, soaked in PBS, placed the slide in the moisture chamber and gave a barrier to the surrounding of the preparation with a PAP pen, dropped the Sniper Background, drop the primary antibody, incubate 60 minutes, washed with PBS 3-5 minutes, secondary antibodies (Universal Link), incubated, washed with PBS, placed Trekavidin HRP Label, incubated, washed with PBS, dropped DAB Chromogen, washed by running water, counterstained with hematoxylin Mayers, soaked in Lithium Carbonate, washed by running water, dehydrated, and mounted with Stellan then proceed with direct observation through a microscope.

RESULTS

This study showed that the expression of PCNA was significantly different (Table 1) after various doses of Genistein oral administration ($p = 0.000$). Expression of PCNA increased in G1 (151.3 \pm 21.42), G2 (367 \pm 173.17), and G3 (490.1 \pm 66.20) groups but decreased in G4 (155.7 \pm 31.38) group. Post hoc Dunnett T3 5% test was applied to determine which groups differ (Table 2). Genistein at doses G1, G2, and G3 showed significant differences compared with the control group ($p < 0.05$). The endometriosis mice model showed positive expression of PCNA compared to healthy mice (Fig. 1). Immunohistochemistry staining showed the differential expression of PCNA with sequence from the highest G3 > G2 > G1 > G4 (Fig. 2).

Vascular Density

Oral genistein administration was shown to have a significant relationship with vascular density in endometriosis lesions (Table 3). ANOVA test showed a significant difference between oral genistein administration at various doses with vascular density in

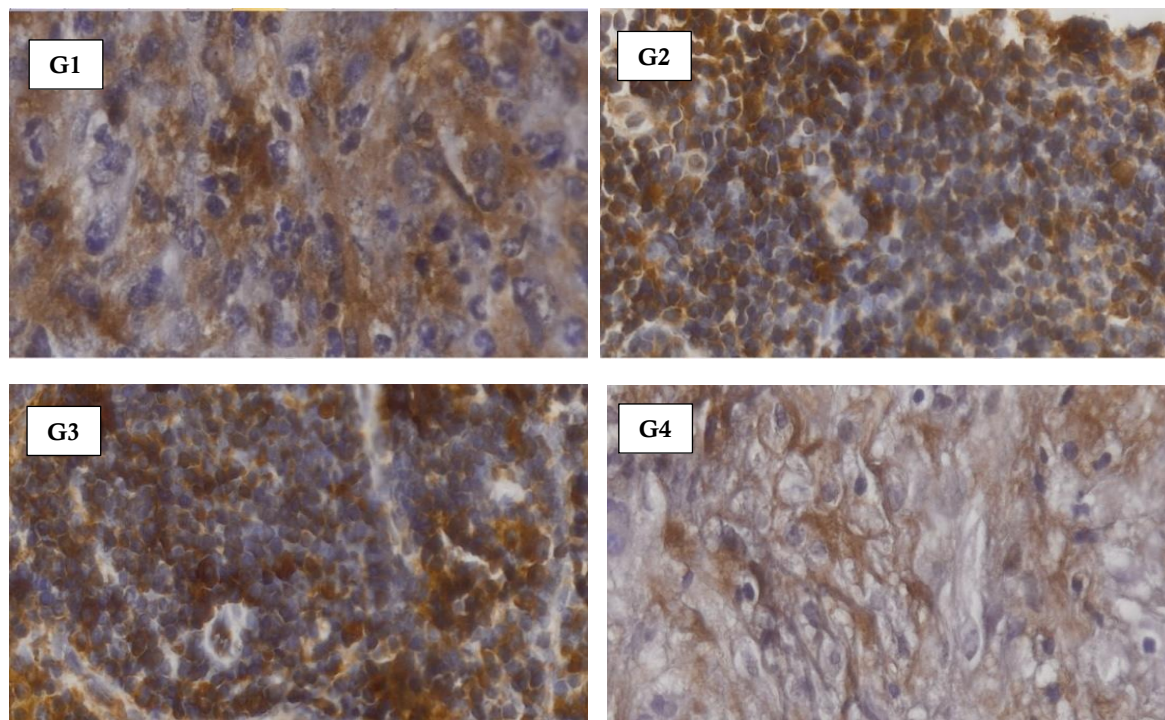


Fig. 2. Immunohistochemistry staining of PCNA Expression in Mice Peritoneum Endometriosis Model (400x optical enlargement of Nikon H600L microscope, camera DS f12300 megapixel)

peritoneal tissue of mice model endometriosis ($p = 0.000$). Vascular density decreased in G1 (6.1 ± 3.87), G2 (2.9 ± 1.60), G3 (2.0 ± 1.56), and G4 (0.2 ± 0.42) after oral genistein administration. Post hoc Dunnett T3 5% test was applied to determine which groups differ. All genistein groups differed significantly from the

control group (Table 4). The endometriosis mice model showed an increased vascular profile compared to healthy mice (Fig. 3). Immunohistochemistry staining showed a significant decrease in vascular density in the endometriosis mice model after oral genistein administration with sequence from the highest $G3 > G2 > G1 > G4$ (Fig. 4).

Table 3. Effect of Genistein on Vascular Density

Groups	Mean \pm SD	p-value
PC	12.2 ± 8.84	
G1	6.1 ± 3.87	
G2	2.9 ± 1.60	0.000*
G3	2.0 ± 1.56	
G4	0.2 ± 0.42	

*ANOVA test showed statistically significant

Table 4. Comparison of Vascular Density in Control and Treatment Group with Post Hoc Dunnett T3 5% test.

Groups	Mean Different	p-value
PC G1	6.10	0.004*
G2	9.30	0.000*
G3	10.20	0.000*
G4	12.00	0.000*

*Statistically significant

DISCUSSION

Effect of Genistein on PCNA Expression in Endometriosis Mice

Genistein is a potent inhibitor of the protein tyrosine kinase and topoisomerase II, essential for cell proliferation. Genistein also plays a role as an angiogenesis inhibitor and several steroid metabolic enzymes, such as aromatase and 5 α -reductase. The structure of genistein is similar to estradiol and has an estrogenic and antiestrogenic effect, depending on the concentration of circulating endogenous estrogen and ER. Increased PCNA expression with higher genistein doses in this study can not be excluded from endogenous estrogen influence [15].

There is a decrease in cell proliferation in administering genistein doses because genistein works as SERMs, which are antiestrogenic at high estrogen levels. The structure of genistein has similarities with the structure of 17 β -estradiol in the body; this causes genistein to be able to bind ER. Genistein has an ER- β affinity about 20-30 times higher than ER- α but has a lower activity than 17 β -estradiol. The high affinity of

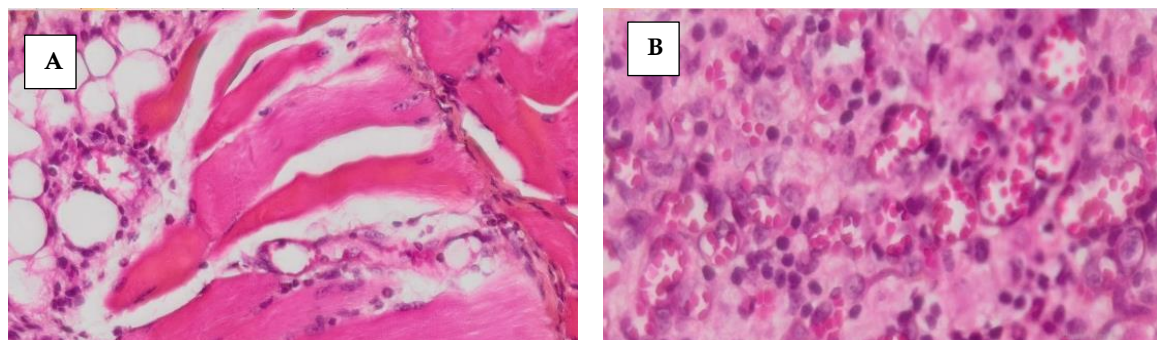


Fig. 3. Immunohistochemistry staining of vascular density in healthy peritoneal mice (NC) and endometriosis mice model (PC). A. NC and B. PC. The red arrow showed vascular density in the peritoneal endometriosis mice model

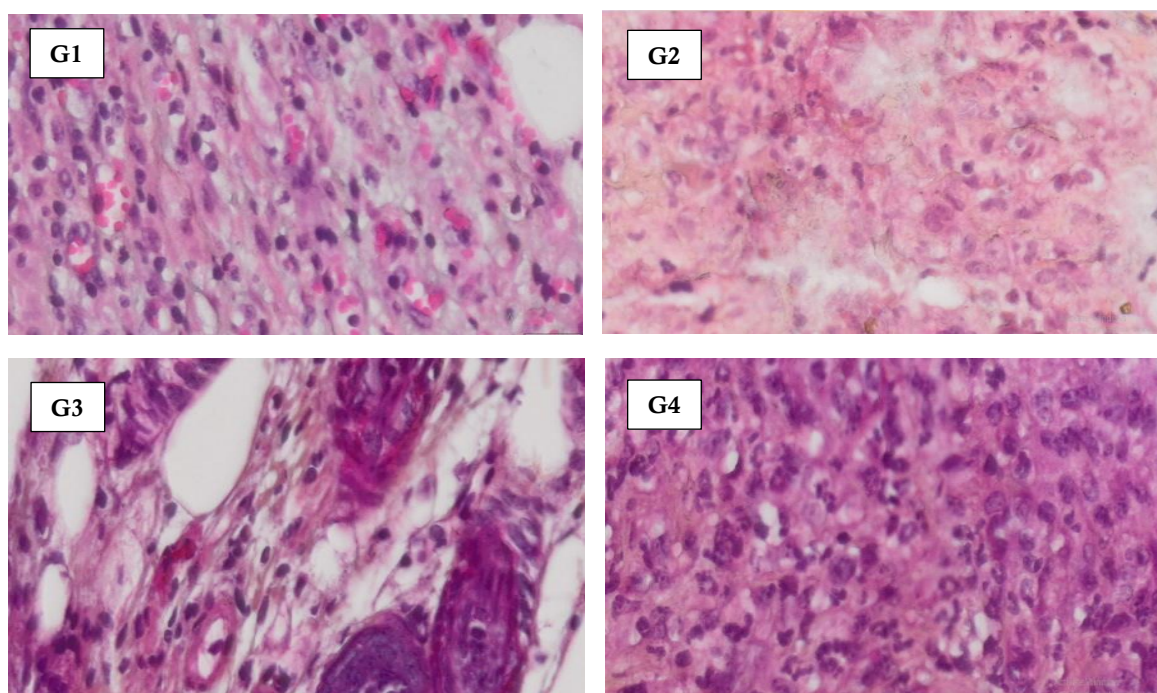


Fig. 4. Immunohistochemistry staining of vascular density in Mice Peritoneum Endometriosis Model (400x optical enlargement of Nikon H600L microscope, camera DS f12300 megapixel)

ER- β can suppress the activity of endogenous estrogen-bound ER- α by forming heterodimers. Genistein can compete in occupying ER as an ER antagonist through this mechanism. Under antiestrogenic conditions, the binding with activated co-regulator proteins is corepressor so that the transcription process is inhibited as well as mRNA and protein synthesis, which increases major inflammatory cytokines (IL6, IL8), angiogenesis factors (HIF-1 α , VEGF-A), Matrix metalloproteinase (MMP-2 and MMP-9), anti-apoptotic gene (Bcl2) and, increased protein apoptosis (Caspase 3) and cell adhesion molecules are inhibited, and pro-apoptotic protein (Bax) increases, and consequently, growth and development of Endometriosis also inhibited [13].

Based on the results of the Dunnett T3 test of 5%, there was a significant effect of genistein administration on the increase in PCNA expression between the PC and

the treatment groups G1, G2, and G3 ($p < 0.05$). Statistical analysis showed a significant increase in PCNA expression occurred in the genistein administration group at a dose of 1.30 mg/day (G1), 1.95 mg/day (G2), and 2.6 mg/day (G3). Statistical analysis showed no significant difference in the effect of genistein administration on PCNA expression between the PC and G4 ($p > 0.05$). The possible reason for this finding was that the genistein dose of 3.25 mg/day, equivalent to 1250 mg of genistein in humans, expressed an antiestrogenic effect, suppressing endometriosis cell proliferation.

Genistein as an antiproliferation agent is also reported from research conducted by Malloy K et al. Genistein inhibits cancer cell growth and induces cell cycle cessation in G2. The antiproliferation mechanism is related to its molecular profile, which potentially

inhibits tyrosine-protein kinase (EGFR/VEGFR/Her2) and modulation of ER α / β . Genistein effect in apoptosis and termination of the cell cycle in cancer cells may result from targeting this signaling pathway. The results suggest that the antiproliferative effect of genistein can be associated with the induction of the G2/M cell cycle and apoptosis in ECC-1 and RL-95-2 cells. Genistein administration significantly increased the expression of annexin V and cleaved caspase-3 activity in RL-95-2 cells but not in ECC-1 cells. These results indicate that genistein induces the G2 cell cycle as the primary mechanism in proliferation inhibition [16].

Effect of Genistein on Vascular Density in Endometriosis Mice Model

Dunnett T3 5% test between PC and the treatment group showed a significant decrease in vascular density in all groups, including G1, G2, G3, and G4 ($p < 0.05$). Decreased vascular density occurs in the genistein administration group at all dose levels. Vascular density in the PC group was highest compared to all treatment groups. The mean vascular density in P4 expressed the lowest density compared to all treatments. This study reported a significant association between a decrease in vascular density and genistein administration compared to the control group.

Genistein administration in various doses shows its role in decreasing ER expression. This is supported by high genistein docking scores indicating a high affinity for ER- α . Increased expression of ER- α . Indicates an inflammatory reaction that contributes to the formation of endometriotic lesions. Angiogenic factors are known to increase the condition of Endometriosis, causing uncontrolled vascular proliferation [15]. This study showed that administering various doses of genistein was associated with decreased vascular density in endometriotic lesions ($p < 0.05$). Increased vascular density in early endometriosis lesions causes endometriosis red lesions, which are influenced by an increase in angiogenesis due to VEGF activity. Based on the literature review, the decrease in vascular density in this study could be due to genistein's effect, which can down-regulate VEGF activity [17]. Further research is needed to prove the pathway for the genistein effect against vascular density via the VEGF pathway.

Vitro HEEC culture can be used as a model for endometriosis lesions. Genistein is an inhibitor of HEECs and endometrial gland epithelium proliferation in vitro. Increased doses of genistein in combination or without 17 α -Estradiol have been shown to inhibit the proliferation of HEECs. The effect of genistein on endothelial cell proliferation and angiogenesis is dose-dependent, and its inhibition effect increases gradually in response to dose elevation with a peak at 200 $\mu\text{mol/L}$. Genistein at low doses is known to have the estrogen-like effect that supports proliferation at doses of 1-50 $\mu\text{mol L}$. Genistein is a specific tyrosinase inhibitor that can inhibit endothelial cell angiogenesis from several organs in vitro. Another potential genistein effect is suppressing growth and tumor metastasis in vivo. The in vitro effect of genistein on the human endometrium has an anti-estrogen effect on the stroma and glandular cells. Genistein shows the potential for inhibition in angiogenesis and the proliferation of glandular epithelium in the endometrium.

Regarding this potential, genistein can be used as an alternative to synthetic progestin. Genistein's effect as an inhibitor of endometrial proliferation and hyperplasia is the potential for Endometriosis and endometrial cancer treatment. Both in vivo and in vitro studies of genistein show that genistein has a high potential as a therapeutic agent in Endometriosis [18].

CONCLUSION

Administration of genistein in various doses significantly increases PCNA expression but reduces PCNA expression in a dose of 3.25 mg/day and significantly reduces vascular densities.

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CONFLICT OF INTEREST

There is no conflict of interest in this research.

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