The effect of *Vigna unguiculata* on the estrogen receptor-α expression and the endometrial thickness in rats treated with depot medroxyprogesterone acetate (DMPA)

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Abstract

Depot medroxyprogesterone acetate (DMPA) is a contraceptive method that prevents ovulation and reduces endometrial thickness. This study aimed to investigate the influence of *Vigna unguiculata* (VU) on estrogen receptor-α expression and endometrial thickness in rats subjected to DMPA treatment. The research utilized a true experimental design involving 25 female Wistar rats divided into 5 experimental groups. The DMPA+VU experiment lasted for 4 weeks, and data were analyzed using a Complete Randomized Design. Estrogen receptor-α expression in the endometrium was assessed through immunohistochemical techniques, and endometrial thickness was determined via Hematoxylin-eosin (HE) staining, observed under dot slide microscopes (Olympus XC10) at 400× magnification. The study found that estrogen receptor-α expression and endometrial thickness were significantly higher in both the DMPA+VU2 and DMPA+VU3 groups compared to the DMPA group and the DMPA+VU1 group ($p<0.05$). DMPA treatment reduced estrogen receptor-α expression and endometrial thickness. However, the administration of *Vigna unguiculata* extracts at 2.5 mg/kg and 5 mg/kg led to an increase in estrogen receptor-α expression and endometrial thickness. The study implies that VU extract may have a positive impact on estrogen receptor-α expression and endometrial thickness in rats treated with depot medroxyprogesterone acetate.

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Conflict of interest: the authors declare no conflict of interest.

Ethics approval and consent to participate: the protocol used in this study was approved by the ethics committee for experimentation of the Brawijaya University No.307/EC/KEPK/S2/08/2019. Diets were made by following the American Institute of Nutrition recommendations. The animals were fed using ad libitum during the experimental period.

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Introduction

Progestin contraceptives offer a safe and highly effective method for regulating fertility.[1,2] Depot Medroxyprogesterone Acetate (DMPA) is considered highly effective in preventing pregnancy because it is administered through an injection every three months.[3,4] DMPA prevents ovulation and thickens cervical mucus, making it difficult for sperm to penetrate, and it also thins the uterine lining, impeding gamete transportation through the fallopian tube.[5–7] Infertility may depend on the rate at which the body metabolizes DMPA. Women who use DMPA as a contraceptive may face an increased risk of issues such as atrophy, decreased microvascular density, epithelial and vascular blood vessel depletion, and mammary gland disruption. After discontinuing DMPA injections, it can take 1-2 years for these contraceptive effects to wear off and for the woman's body to prepare for pregnancy.[8] Research conducted on rats has shown that DMPA can induce apoptosis in the ovaries, leading to a disruption in estradiol, an antioxidant gene. An enzyme known as superoxide dismutase (SOD), which resides within the mitochondria, is affected, preventing estradiol from effectively functioning as an antioxidant. This state of affairs leads to oxidative stress, marked by the production of free radicals, which can cause oxidative modifications in cellular macromolecules, impair protein function, and mediate apoptosis.[9]

Vigna unguiculata contains phytoestrogens of the isoflavone type, including genistein (0.02 mg/100 grams) and daidzein (0.01 mg/100 grams), along with a minimal fat content of 1.40 grams/100 grams.[10] Soy isoflavones have the ability to bind to estrogen receptors (ERs), showing a higher binding affinity for ER-β compared to ER-α. In contrast to ER-α, which is abundant in granulosa cells of growing follicles from postnatal day 5, ER-β deficient mice treated with genistein do not exhibit multiple oocyte follicles (MOFs), indicating that genistein inhibits oocyte nest breakdown through an ER-β mechanism.[11]

Previous research has demonstrated that DMPA significantly decreased the levels of SOD in the ovary and endothelial Nitric Oxide Synthase (eNOS) in the endometrium. The decrease in SOD levels was significantly attenuated by the highest doses of green tea extract. DMPA induces apoptosis in the ovary and endometrium, while green tea extract prevents the increase in the ovary.[12–14] Therefore, this study aimed to explore the preventive ability of *V. unguiculata* in minimizing the side effects of DMPA. *V. unguiculata* extract can increase the expression of estrogen receptor-α and enhance endometrial thickness in rats treated with depot medroxyprogesterone acetate.

Materials and Methods

Design and sample

This study was a true experimental design and utilized adult virgin female Wistar rats (*Rattus novergicus*) aged 30 weeks, with a weight ranging from 100 to 125 grams. These rats were procured from the Pharmacology Laboratory at the Faculty of Medicine, Brawijaya University, East Java, Indonesia. Subsequently, they were housed in a controlled environment with conditions maintained at 25±10°C and 65-70% relative humidity, following a 12-hour light-dark cycle. The rats were divided into distinct groups, each consisting of five rats. These groups were as follows: the control group, the DMPA-administered group, the DMPA + *V. unguiculata* (DMPA+VU1 0.5 mg/kg) group, the DMPA + *V. unguiculata* (DMPA+VU2 2.5 mg/kg) group, and the DMPA + *V. unguiculata* (DMPA+VU3 5 mg/kg) group. The treatment with *V. unguiculata* (UV) was administered for a duration of 4 weeks.

DMPA treatment

DMPA at a dose of 2.7 mg/rat was injected every single week in the morning for 4 weeks; it was diluted with 0.2 mL of saline and injected intramuscularly. The DMPA dose was calculated according to a previous genotoxicity study in rats and previous research.[15,16]

Vigna unguiculata administration experiment

The *Vigna unguiculata* extraction process involved maceration. One hundred grams of *Vigna unguiculata* var. KT-6 were sourced from BALITKABI in Malang, East Java, Indonesia. The fruits were separated from the seeds, and the seeds were then dried in an oven at 90°C and subsequently ground into a powder. A 100-gram quantity of this powder was added to a 1-liter Erlenmeyer flask containing 900 mL of 70% ethanol. The solution was left to evaporate overnight. On the following day, the upper layers of the solution were collected and processed using an evaporation apparatus. The extracted specimen was stored at -40°C. The administration began 28 days after the injection of DMPA. Rats were orally administered with 0.5 ml of *Vigna unguiculata* daily, between 1:00 PM and 2:00 PM, for a duration of 28 days.

Analysis of expression estrogen receptor-α

Immunohistochemical (IHC) staining was performed to assess the expression of estrogen receptor-α in the endometrial tissue. We used the IHC Kit provided by SANTA CRUZ BIOTECHNOLOGY, INC (ERα(C-311):sc-787). The procedure involved deparaffinizing the slides with xylene followed by dehydration using an alcohol series. Subsequently, the slides were immersed in a citrate buffer with a pH of 6 and heated in a water bath at 95°C for 20 minutes. Afterwards, the slides underwent a blocking step using 3% H_2O_2 in methanol for 15 minutes to prevent endogenous interference. They were then washed with PBS and blocked again. The slides were incubated for 60 minutes. Next, the primary antibody (anti-estrogen receptor) was added in PBS with 0.2% BSA and incubated overnight at 4°C. The following day, the slides were washed with PBS and subsequently incubated with a biotinylated universal secondary antibody for 60 minutes at room temperature.

A 40-minute incubation of the enzyme Streptavidin-Horseradish Peroxidase (Streptavidin-HRP) was carried out at room temperature. Following this, DAB (Diaminobenzidine) was applied, with a DAB chromagen to DAB buffer ratio of 1:50, for 10-20 minutes. After a final wash with PBS and distilled water, the slides were counterstained with Mayer's Hematoxylin for 5-10 minutes at room temperature. Finally, the slides were mounted and examined. The estrogen receptor-α expression in the rat endometrial tissue was observed using a light microscope (magnification at 400×). The percentage of expression was calculated using photo dot slide and OLYMPUS software as well as immunization software.

Analysis of endometrial thickness

The analysis of endometrial thickness involved the use of Hematoxylin-Eosin staining. Sections measuring 10–30 mm on slides were subjected to the following process: they were immersed in xylene for 10 minutes (repeated twice), followed by rehydration through a series of decreasing ethanol concentrations diluted in distilled water (100%, 100%, 95%, 95%, 75%, 0%, each for 1 minute). The sections were then rinsed in deionized water, stained with hematoxylin for 45 seconds, followed by rinsing in deionized water and a 1-second staining with eosin. After the color reaction, the sections were dehydrated through an ethanol series into xylene and then mounted using Permount mounting medium (Fisher Scientific, PA). The thickness of the endometrium was observed and measured under dot slide microscopes, specifically the Olympus XC10 (at a magnification of 200×), with data automatically exported to Microsoft Excel.

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) with SPSS 17.0 statistical software (IBM, New York, USA). Post-hoc tests were performed when the analysis of variance yielded significant results. A p-value < 0.05 was considered statistically significant.

Ethical clearance

The protocol used in this study was approved by the ethics committee for experimentation of the Brawijaya University No.307/EC/KEPK/S2/08/2019. The diets were prepared following the recommendations of the American Institute of Nutrition, and the animals were provided with ad libitum access to food during the experimental period.

Results

This study investigated the levels of *Vigna unguiculata* using Liquid Chromatography-Mass Spectrometry, revealing a genistein level of 39.915 µg/gram in the ethanolic extract of *V. unguiculata* seeds. Table 1 and Figure 1 depict the levels of estrogen receptor α in the control group and treatment groups. The administration of 2.5 and 5 mg/kg of *V. unguiculata* extract significantly increased estrogen receptor α expression when compared to the DMPA group ($p<0.05$), whereas the 0.5 mg/kg dose (DMPA+VU1 group) did not show a significant increase. Estrogen receptor α expression did not significantly differ between the control group and the DMPA+VU2 and DMPA+VU3 groups. Additionally, DMPA+VU2 and DMPA+VU3 groups did not significantly differ from the control group ($p>0.05$).

Table 2 presented the endometrial thickness levels of the control group and the treatment groups. The endometrial thickness was significantly higher in the DMPA+VU3 group compared to the DMPA group. Administration of 2.5 and 5 mg/kg of *V. unguiculata* extract (DMPA+VU2 and DMPA+VU3) significantly increased endometrial thickness ($p<0.05$). However, administration of 0.5 mg/kg of *V. unguiculata* (DMPA+VU1) did not significantly affect endometrial thickness. There were no significant differences in endometrial thickness between the control group, DMPA+VU2, and DMPA+VU3 groups ($p>0.05$)."

Discussion

Administering DMPA can reduce ER-α expression, making this research a valuable reference for mitigating its side effects. The data demonstrates that prolonged exposure to DMPA leads to a decrease in ER-α expression, which in turn minimizes the expected side effects associated with excessive DMPA usage. The use of DMPA induces apoptosis in the ovary, with a higher DMPA index correlating with increased ovarian apoptosis.[17] This suggests that long-term DMPA use may help mitigate side effects arising from ovarian dysfunction.[9]

Women receiving DMPA injections as a contraceptive treatment may experience side effects that lead to a reduction in estro-

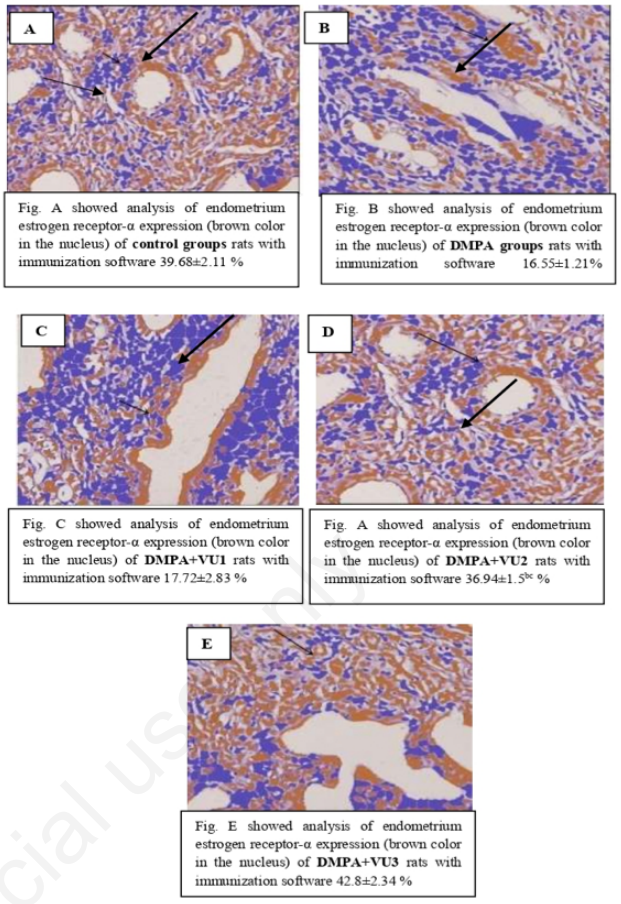

Figure 1. The levels of estrogen receptor α in the control group and treatment groups. Figure 1 showed that Immunohistochemical analysis of endometrium estrogen receptor-α expression (with immunization software) of administered groups and control groups rats. The estrogen receptor-α expression in control group(A); DMPA group (B); DMPA+VU1(C); DMPA+VU2(D); DMPA+VU3. The black arrow in the figure indicated cells expressing estrogen receptor-α (brown color in the nucleus) (M=400×).

Table 1. The estrogen receptor-α expression of endometrial of administered groups and control rats.

Parameters	Control	DMPA + *Vigna unguiculata*			
		DMPA	DMPA+VU1	DMPA+VU2	DMPA+VU3
ER-α (%)	39.68±2.11	16.55±1.21[ad]	17.72±2.83[ad]	36.94±1.5[bc]	42.8±2.34[bc]

Values are presented as mean±SD; [a]$p<0.05$; in comparison with control group; [b]$p<0.05$; in comparison with DMPA group, [c]$p<0.05$; in comparison with DMPA+VU1, [d]$p<0.05$; in comparison with DMPA+VU2; DMPA, Depot medroxyprogesterone acetate; ER-α: estrogen receptor-α; VU, *Vigna unguiculata.*

Table 2. The endometrial thickness of all experimental groups.

Parameters	Control	DMPA + *Vigna unguiculata*			
		DMPA	DMPA+VU1	DMPA+VU2	DMPA+VU3
Endometrial thickness (µm)	467.40±72.15	249.80±60.05[ad]	266.20±51.64[ad]	388.00±83.46[bc]	444.00±57.75[bc]

Values are presented as mean±SD; [a]$p<0.05$; in comparison with the control group; [b]$p<0.05$; in comparison with DMPA group, [c]$p<0.05$; in comparison with DMPA+VU1, [d]$p<0.05$; in comparison with DMPA+VU2; DMPA, Depot medroxyprogesterone acetate; VU, *Vigna unguiculata.*

gen levels. In this study, a majority of the 70 women, aged 20-35 years, saw their estrogen levels decrease from <130 pg/ml to 100 pg/ml after receiving DMPA injections.[16,17] This research also suggests that rats treated with DMPA exhibit thinner endometrial tissue.[18,19] Previous studies have indicated that DMPA (a progestin-only contraceptive) reduces endometrial vascular density, resulting in atrophy and amenorrhea. DMPA primarily works by inhibiting ovulation, as evidenced by changes in levels of follicle-stimulating hormone and luteinizing hormone.[20,21]

The administration of 2.5 and 5 mg/kg of *V. unguiculata* extract significantly increased the expression of estrogen receptor α and endometrial thickness compared to the DMPA group, while 0.5 mg/kg of *V. unguiculata* did not yield the same effect. Previous studies have shown that the regulation of ER-α expression decreases, leading to endometrial depletion during menstruation. DMPA increases the rate of apoptosis in the ovary and endometrium, resulting in endometrial depletion and atrophy. This is attributed to the low activities of eNOS expression and eNOS levels in the cells and cytoplasm, which trigger apoptosis in the endometrium and ovaries[12]. A depleted endometrium typically measures between 6-9 mm, whereas a normal one ranges from 5-10 mm, varying depending on the menstrual cycle.[8] An endometrial thickness of less than 6 mm is considered indicative of an infertility disorder.[9,17]

Vigna unguiculata extract is suspected to contain phytoestrogen, specifically genistein. Phytoestrogens impact human health through both genomic and non-genomic mechanisms. Due to their low molecular weight, phytoestrogens can traverse cell membranes and interact with receptors and enzymes. Genomic mechanisms encompass estrogenic and antiestrogenic effects on the estrogen receptor (ER). Non-genomic mechanisms include the inhibition of tyrosine kinase, DNA topoisomerase inhibition, antioxidant activity, inhibition of SHBG-stimulated angiogenesis, inhibition of 5α reductase, 17β-OH-steroid-dehydrogenase, and aromatase enzymes.[22]

Phytoestrogens can bind to estrogen receptors α or β. Estrogen receptor α is more widely distributed in reproductive tissues. Genistein's action is influenced by the levels of estrogen, acting as an estrogenic compound at low estrogen levels.[23] The mechanism of genistein is similar to Selective Estrogen Receptor Modulators (SERMs) that differ chemically but can directly affect the selection of agonist or antagonist roles in estrogen-responsive tissues. It serves as an antagonist when interacting with ER-β in genes containing estrogen response elements, and as a partial agonist when acting through ER-α.[24,25] Estrogen typically enters the cell's cytoplasm before binding to an estrogen receptor. In the cytoplasm, it forms a hormone-receptor complex on an estrogen response element (ERE) and then translocates into the cell nucleus to bind with DNA. The bound estrogen receptors in DNA are involved in cell transcription to produce proteins necessary for cell division. When estrogen levels are high, genistein weakly binds to ER-α, effectively obstructing the bond between the receptors and estradiol (having an anti-estrogenic effect). Conversely, when estrogen levels are low, genistein binds to ER-α and activates a signaling cascade. This activation effect could potentially lead to increased endometrial proliferation.[26]

Phytoestrogens may serve as promising candidates for mitigating the side effects associated with DMPA use. Phytoestrogens stimulate the production of alpha estrogen receptors, and the estrogenic properties of isoflavones are key contributors to their estrogenic activity. The discovery of ERα and ERβ receptors in the endometrium, the positive effects observed with Selective Estrogen Receptor Modulators (SERMs) like raloxifene in both animals and humans, and the fact that phytoestrogens, such as genistein, share similarities with raloxifene in terms of binding to estrogen receptors, suggest that phytoestrogens can exert a selective influence on the endometrium.[27]

Conclusions

In summary, the administration of 2.5 and 5 mg/kg of *V. unguiculata* extract effectively increased ER-α expression and endometrial thickness in DMPA-injected rats. This suggests its potential application as an alternative treatment for fertility recovery following DMPA-based contraception, improving the recovery of ER-α expression and addressing endometrial depletion.

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