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The effects of *Vigna unguiculata* on cardiac oxidative stress and aorta estrogen receptor- β expression of ovariectomized rats

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ABSTRACT

Objective: To investigate whether *Vigna unguiculata* (*V. Unguiculata*, VU) able to reduced oxidative stress in cardiac and modulate the aorta estrogen receptor- β of ovariectomized rats. **Methods:** Thirty female Wistar rats were divided into 5 groups (n=6); control (sham) group; ovariectomy group (OVX); OVX + VU_1 (0.5 mg/kg); OVX + VU_2 (2.5 mg/kg); and OVX + VU_3 (5 mg/kg). The administration VU was started 28 days after surgery following 30 days later. Cardiac malondialdehyde (MDA) and superoxide dismutase (SOD) levels were measured colorimetrically. Estrogen receptor- β in the aorta was analyzed immunohistochemically. **Results:** Level of MDA was significantly higher in the OVX group compared to the control group (P< 0.05), but the level of SOD was significantly lower. The level of MDA was significantly lower in OVX + VU compared with OVX group (P<0.05), to reach the level at a control group in OVX + VU_2. Administration of VU significantly increases the level of SOD compared with OVX group (P<0.05), to reach the level at a control group in third dose of VU (P>0.05). The level of estrogen receptor- β was significantly decreased in the OVX group compared to the control group (P<0.05). OVX + VU_3 could significantly increase the level of estrogen receptor- β compared to OVX group (P<0.05), to reach a level in the control group (P>0.05). **Conclusions:** *V. unguiculata* is an alternative therapy in decreasing cardiac oxidative stress in ovariectomized rats. Besides, high dose of *V. unguiculata* also able to increase aorta estrogen receptor- β expression in ovariectomized rats.

1. Introduction

Cardiovascular disease as one cause of death has been shown to be lower in women prior to menopause than in men and postmenopausal women[1-3]. The mechanisms underlying these lower risks in premenopausal women have not been elucidated completely, but can at least be partly explained by the beneficial effects of estrogen on vasculat [4-6]. Previous studies showed that estrogen suppressed oxidative stress and inflammation in the vascular[7-9].

At the cellular level, reactive oxygen species (ROS) may act as second messengers in various signal transduction and elicits a wide spectrum of responses ranging from proliferation of growth, or differentiation, arrest to senescence, and cell death by activating several major signaling pathways. The intensity and duration of the stress as well as the cell type involved are important factors in determining which pathways are activated, and the particular outcome[10]. Oxidative stress plays an important role in the pathogenesis of vascular disease via several mechanisms such as endothelial dysfunction, inflammation, cell migration, growth, and apoptosis[11].

Estrogen effects on the cardiovascular system are

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mediated by two different oestrogen receptor (ER) subtypes, estrogen receptor-α and -β, which are encoded by different genes, possess a similar domain structure, and are activated by the non-selective ER agonist 17b-estradiol in cardiac myocytes and vascular cells[12-15]. These receptors are expressed in endothelial cells, smooth muscle cells, and macrophages[16-19]. Several clinical studies reported the beneficial effects of estrogen replacement therapy (ERT) on the incidence of cardiovascular diseases in postmenopausal women[20]. In contrast, other clinical trials, conclude no beneficial effects of ERT[21, 22]. Thus, the efficacy of ERT on cardiovascular disease is still controversial in clinical settings.

Cowpea (*Vigna unguiculata* (L.) Walp.) (*V. unguiculata*, VU) (kacang tunggak, Indonesian name) was recognized as a potential source of good nutritional properties based on protein and other nutrients[23-25]. The whole seeds have been reported to contain about 0.18%-0.59% tannins, phenolic acids, such as p-hydroxybenzoic acid, protocatechuic acid, 2,4-dimethoxybenzoic acid, and cinnamic acid derivatives, such as p-coumaric acid, caffeic acid, cinnamic acid and ferulic acid[26-28]. Previous studies showed that the cardioprotective potency of *V. unguiculata* in preventing cardiovascular diseases and this effect is attributed to the presence of antioxidants of the flavonoid fraction of *V. unguiculata* leaves[29]. This study aimed to determine whether V. unguiculata able to reduce oxidative stress in cardiac and modulate the aorta estrogen receptor-β among ovariectomy rats.

2. Material and methods

2.1. Animal

Thirty, 12 weeks old, virgin female Wistar rats, 150-2 500 gram were obtained from LPPT, Gadjah Mada University, Yogyakarta. After acclimatization for a week, the rats were divided into five groups (n=6) including control (sham) group; ovariectomized (OVX) group; and OVX + *V. unguiculata*-treatment groups. The control rats either had bilateral ovariectomy (OVX group) under ketamine anesthesia. The rats in the test group had bilateral ovariectomy. The control rats were given 0.5 mL of distilled water. The test rats were given low dose: 0.5 mg/kg (OVX + VU_1); medium dose: 2.5 mg/kg (OVX + VU_2) and high dose; 5 mg/kg (OVX + VU_3) of *V. unguiculata* topped up to 0.5 mL of distilled water. The administration VU was started 28 days after ovariectomy. The test and control rats were oral gavaged with 0.5 mL *V. unguiculata* or water at approximately 9:00-10:00 am everyday for 21 days. Daily measurement of body weight and the total food intake was recorded.

2.2. Ethics

All the experiments were approved by the Brawijaya University Ethic Committee and the Teaching and Research Committee and followed the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.3. Ovariectomy

Animals were anesthetized (Ketamine 120 mg/kg + Xylazin 20 mg/kg), and a small abdominal incision was made. The ovaries were then located, and a silk thread was tightly tied around the oviduct, including the ovarian blood vessels. The oviduct was sectioned and the ovary removed. The skin and muscle wall were then sutured with silk thread[30].

2.4. Extraction procedure

One hundred grams of *V. unguiculata* (cowpea) varieties KT-6 were obtained from market Klungkung, Nusa Penida, Bali. *V. unguiculata* seeds dried until the moisture content is free, then ground into powder. Cowpea powder weighed up to 100 grams, then put in a glass Erlenmeyer flask (1 L). Cowpea powder soaked with 900 mL of ethanol (96%) and shaken until completely mixed. The mixture was allowed to stand for 1 night to settle. Upper layer containing ethanol and dissolved active substance repeatedly taken later moved in evaporation flask. Pumpkin evaporation mounted on the evaporator and water bath is filled to the brim. The tools were installed and given the flow of electricity. Ethanol solution was allowed to evaporate on evaporation flask and allowed to finish dripping on the reservoir flask (± 1.5 to 2 hours to 1 pumpkin). Extraction results put in plastic bottles /glass and stored in the freezer. Genistein content was measured using Liquid Chromatography Mass Spectrometry.

2.5. Malondialdehyde analysis

Levels of malondialdehyde (MDA) in whole cardiac tissue were measured by reaction with thiobarbituric acid (TBA) compound. Cardiac tissues at 100 mg were pulverized in a cold mortar. Add 2 mL of phosphate buffer, 100 μL TCA 10%, 250 μL HCl, 200 μL Na-Thio Barbiturates into the test tube and homogenized using a vortex. The test tube was then heated in a water bath at temperature of 105 ℃ and then centrifuged. The supernatant absorbance was read using a spectrophotometer at a wavelength of 532 nm.

2.6. Analysis of superoxide dismutase

The principle superoxide dismutase (SOD) analysis is the reaction between xanthine and xanthine oxidase produces superoxide radicals. Superoxide radicals will reduce NBT (nitroblue tetrazolium) into purple formazan. SOD can inhibit NBT reduction through reaction with superoxide radicals that produce O_2 and $H2O_2$. SOD concentrations were then determined using a standard curve of SOD.

2.7. Analysis of estrogen receptor- β

Immunohistochemical (IHC) staining was carried out to see the expression of estrogen receptor- β in the aorta. Slides were deparaffinized using xylene and dehydrated using alcohol series. The slides were immersed in citrate buffer of pH 6 and heated in a waterbath at temperature of 95 ℃ for 20 minutes. After the slides were blocked using H_2O_2 3% in methanol for 15 min (endogenous blocking), they were then washed with PBS and blocked back with a sniper and incubated for 60 min. Furthermore, the primary antibody(β -estrogen receptor) was added in PBS + BSA 0.2 % and incubated overnight in 4 ℃. Once the slides were washed with PBS, they were then incubated with biotinylated universal secondary antibody for 60 minutes at room temperature. Incubation of the enzyme SA-HRP (Streptavidin Horseradish Peroxidase) was performed for 40 minutes at room temperature, and then DAB (Diaminobenzidine) was added with a ratio of DAB chromagen: DAB buffer = 1:50 for 10-20 minutes. After the slides were washed with PBS and distilled water, they were counterstained with Mayer's Hematoxilin for 5-10 minutes at room temperature. The slides were mounted and observed. Estrogen receptor- β that appears in rat aortic endothelial cells were observed with a light microscope 400x magnification and the percentage calculated using photo dotslide and OLYMPUS software.

2.8. Statistical analysis

Data are presented as mean ± SD and differences between groups were analyzed using one-way ANOVA with SPSS 17.0 statistical package. Post Hoc test was used if the ANOVA was significant. $P<0.05$ was considered statistically significant.

3. Results

In order to know the level of *V. unguiculata* we performed Liquid Chromatography Mass Spectrometry. The level of genistein in the ethanolic extract of *V. unguiculata* seed is 29.28 μg/gram.

Table 1 shows the levels of cardiac MDA and SOD in the OVX group and OXV supplemented with *V. unguiculata*. The level of cardiac MDA was significantly higher in the OVX group compared to the control group ($P<0.05$). The level of cardiac MDA was significantly lower in OVX + VU compared with OVX group ($P<0.05$), to reach the level at a control group in second dose of VU. The level of cardiac SOD was significantly lower in the OVX group compared to the control group ($P<0.05$). Administration of VU significantly increases the level of cardiac SOD compared with OVX group ($P<0.05$), to reach the level at control group in third dose of VU ($P>0.05$).

Table 1

The level of MDA and SOD of administered groups and control rats.

Parameters	Control	*Vigna unguiculata*-administered groups			
		OVX	OVX + VU_1	OVX + VU_2	OVX + VU_3
MDA (nmol/mL)	66.917±2.923	85.667±5.401[a]	58.167±3.028[ab]	63.583±2.923[bc]	69.417±1.882[abcd]
SOD (U/mL)	5.085±0.248	3.178±0.236[a]	4.169±0.209[ab]	4.493±0.451[ab]	5.178±0.601[bcd]
ER- β (%)	59.303±6.204	46.217±4.528[a]	46.689±2.976[a]	48.793±6.235[a]	56.527±8.203[bcd]

Note: values are presented as mean ± SD; [a]$P<0.05$; in comparison with control group; [b]$P<0.05$; in comparison with OVX groups; [c]$P<0.05$; in comparison with first dose administered groups; [d]$P<0.05$; in comparison with second dose administered groups; MDA: malondialdehyde; SOD: superoxide dismutase; OVX: ovariectomy; nmol/mL: nanomol/mililitter; U/mL: unit/ mililitter; VU: *Vigna unguiculata*.

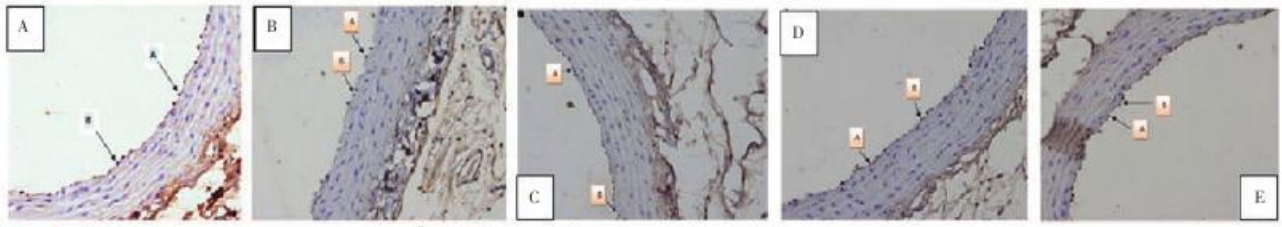

Figure 1. The expression of estrogen receptor- β of administered groups and control rats.

The expression of estrogen receptor- β in control (sham) group (A); ovariectomized (OVX) group (B); low dose (C); medium dose (D); and high dose (E). A and arrow in the figure indicated endothelial cells that express of estrogen receptor- β (brown color in nucleus). B and arrow in the figure indicated endothelial cells that not express of estrogen receptor- β .

Table 1 and Figure1 show the level of estrogen receptor-β in the control group and treatment groups. The level of β-estrogen receptor was significantly decreased in the OVX group compared to the control group ($P<0.05$). Treatment to VU (third dose) could significantly increase the level of estrogen receptor-β compared to OVX group ($P<0.05$), to reach a level in the control group ($P>0.05$).

4. Discussion

MDA is one of the main products of lipid peroxidation which can reflect the degree of lipid peroxidation and indirectly reflect the degree of oxidative stress in cells. The level of cardiac MDA was significantly higher in the OVX group compared to the control group. The level of cardiac SOD was significantly lower in OVX + VU compared with OVX group, to reach the level at a control group in second dose of VU. This finding clearly show that ovariectomy increase oxidative stress and reduced enzymatic antioxidant activity. In ovariectomized rats there was an increase in superoxide anion production and the increased protein expression of NADPH oxidase subunits, as gp91phox and p22phox. Superoxide radical, is relatively less damaging themselves, but they can form other species such as hydroxyl radical that can initiate lipid peroxidation[31]. SOD can remove superoxide radical anions and protect cells from damage. SOD activity can indirectly reflect the ability of the cell to remove superoxide radical.

The level of cardiac MDA was significantly lower in OVX + VU compared with OVX group, to reach the level at a control group in second dose of VU. Administration of VU significantly increases the level of cardiac SOD compared with OVX group, to reach the level at a control group in third dose of VU. This finding indicated that *V. unguiculata* act as antioxidant by modulation of SOD activity. Our study showed that genistein level in VU was 29.28 μg/gram. Most previous studies indicate that treatment with genistein improves the antioxidant status in tissues, as reflected by increases or prevention of loss of intracellular GSH levels, by decreased oxidized glutathione to GSH ratios, or by restoring levels of lipid peroxidation. This role of genistein is also confirmed in soy-deficient diets, which cause mitochondrial levels of glutathione to decrease as a result of increased production of reactive oxygen species [32-36].

Estrogen receptor-β has been implicated in the vascular muscle cells antiproliferative effects of estradiol during the repair response to vascular injury in both genders[37]. In this study, the level of β-estrogen receptor was significantly decreased in the OVX group compared to the control group. This finding indicated that hypoestrogen due to ovariectomy inhibit expression of estrogen receptor-β in the aorta. Treatment to VU (third dose) could significantly increase the level of estrogen receptor-β compared to OVX group, to reach a level in the control group. Genistein in *V. unguiculata* will bind to estrogen receptors, thereby exerting estrogenic effects. Evidence suggests that genistein exhibits a potency similar to that of 17 β-estradiol, acting via an estrogen receptor dependent mechanism[38, 39]. Genistein has a higher affinity for estrogen receptor-β that is highly localized in the vascular tree[40, 41].

In conclusion, the present data suggesting that *V. unguiculata* is an alternative therapy in decreasing cardiac oxidative stress in ovariectomized rats. Besides, high dose of *V. Unguiculata* also able to increase aorta estrogen receptor-β expression in ovariectomized rats.

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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