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Evaluating the activity of cowpea extract and genistein against GPx and SOD levels in a mouse model of endometriosis

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Abstract : To evaluate the effect of genistein and cowpea extract in increasing SOD (superoxide dismutase) and GPx (glutathione peroxidase) in the mouse model of endometriosis. In this study, the experimental research method was an in vivo experiment of mice (Mus musculus) with a post-test only control group design. Selection of subjects was randomly performed. Preparations of peritoneal fluid from the mouse model with endometriosis was measured with the quantitative colorimetric determination of SOD activity using the Enzychrom superoxide dismutase Assay Kit (Bioassay System), as well as the measurement of GPx using a colorimetric assay for cellular glutathione peroxidase, namely Bioxytech GPx-340 (Oxis research). SOD and GPx were analyzed with the Analysis of Variance (ANOVA). There were increased levels of GPx and SOD following treatment with genistein and cowpea extract. There was no significant difference in activity between genistein and cowpea extract in increasing SOD and GPx levels in peritoneal fluid from the mouse model of endometriosis. SOD levels following cowpea extract treatment tended to be smaller than with genistein, but it can be said that the cowpea extract has a similar ability to genistein, although slightly smaller.
Keywords: cowpea extract, genistein, glutathione peroxidase, lesions, peritoneal fluid.

Introduction

Endometriosis is a benign disease defined by the presence of endometrial glands and stroma outside the uterus, and is associated with both pelvic pain and infertility. The management of endometriosis is unsatisfactory, as many patients remain unsatisfied with the treatment received. The problem does not rely upon endometriosis lesions, but the symptoms which emerge[1].

Oxidative stress is considered a potential factor involved in the pathophysiology of endometriosis. Reactive oxygen species (ROS) or free radicals can promote the growth and attachment of endometrial cells in the peritoneal cavity, triggering endometriosis and infertility. The development of endometriosis is not solely a result of the continuous addition of endometrial tissue through regurgitation of menstruation in the peritoneum, but can also be the result of a proliferative phenotype of ectopic endometrial cells through dysregulation of endogenous ROS production. Our murine model showed that antioxidants could be used as a safe and efficient treatment for endometriosis[2-4].

One of the antioxidants that have a positive effect on endometriosis is genistein. Recent studies reported genistein to be the most potent phytoestrogen available today, due to its ability to induce the regression of endometrial cells in a mouse model of endometriosis[5]. The underlying mechanism of genistein in lowering or inhibiting endometrial cell proliferation are poorly understood. However, King suggests that genistein has

estrogenic effects, which will trigger the upregulation of SOD (superoxide dismutase) and GPx (glutathione peroxidase) antioxidant molecules at a certain dose[6].

One of the plants that is widely known to contain genistein is cowpea. This bean can be found all across Indonesia. Cowpea contains two isoflavones, which have been identified as genistein and daidzein[7]. The study aims to evaluate the effect of genistein and cowpea extract in increasing SOD (superoxide dismutase) and GPx (glutathione peroxidase) in a mouse model with endometriosis.

Materials and Methods

Animal model of endometriosis

The method used was *in vivo* experimental research on female mice (*Mus musculus*). The design of the study was post-test only with control group design. The experiment was quadruplicate for the negative control group, the positive control group, and treatment groups were given different doses of genistein and cowpea extract: 50 mg/day, 100 mg/day, 200 mg/day, 300 mg/day, 400 mg/day, and 500 mg/day. Subjects were randomly allocated to the treatment and control groups.

To create a mouse model of endometriosis, female mice (*Mus musculus*) aged ± 3 months and weighing 20-30 grams were prepared. Mice were adapted in the same cage and fed with the same food and drink. Intramuscular injection of cyclosporine A was injected at a dose of 10 mg/kg BW/day for 1 week. Furthermore, estrogen was administered intramuscularly on days 1 and 5. Ethinyl estradiol was prepared at a dose of 30 µg/kg BW. On day 14 after implantation, the mouse was ready as a mouse model of endometriosis for treatment with genistein and cowpea extract.

Administration of genistein and cowpea extract

Genistein and cowpea extract were injected orally at doses equivalent to genistein 50 mg/kg BW/day, 100 mg/kg BW/day, 200 mg/kg BW/day, 300 mg/kg BW/day, 400 mg/kg BW/day, and 500 mg/kg BW/day for 21 days and genistein orally carried out for 21 days with a dose in each group at 50 mg/kg BW/day, 100 mg/kg BW/day, 200 mg/kg BW/day, 300 mg/kg BW/day, 400 mg/kg BW/day and 500 mg/kg BW/day.

SOD assay

The measurement of SOD (superoxide dismutase) was performed with the Superoxide Activity Assay Kit (Bio Vision) from the supernatant of homogeneous peritoneal endometriosis lesions. Superoxide dismutase was measured in accordance with Marklund et al. [8]. In total, 20 µL of sample was placed in each well; blank solution (H_2O) was placed in two wells, each containing 20 µL. To each well, 200 µL WST (Working Solution) was added. Blank solution 2 was supplemented with 20 µL dilution buffer. Then, in each well except blank solution 2, 20 mL of the enzymes working solution was added. Subsequently, wells were incubated for 20 minutes at 37°C. Then, wells were read at a wavelength of 450 nm. Three repetitions were performed.

GPx assay

GPx level was measured referring to Rostruck. A compound reaction mixture containing 0.5 ml of 0.4 M sodium phosphate (pH 7), 0.1 ml sodium azide, 0.2 ml reduced glutathione, 0.1 ml and 0.5 ml H_2O_2 in a 1:60 enzyme extract was prepared to a final concentration of 2 ml. Samples were put into a 2 ml tube and incubated at 37°C for 3 minutes. Then, 0.5 ml of 60% TCA was added, and samples were rotated at 1500 rpm for 60 min at 4°C. The measurement is based on the remaining peroxidase glutathione reaction. Supernatant was discarded and the precipitate was treated with 4 ml 0.3 M dEKTdium hydrogen phosphate and 1 ml nitrobenzoic dithio-bis (DTNB). Colors were detected at a wavelength of 412 nm and used only as a blank phosphate solution and DTNB reagent. Enzyme activity is expressed in µg GSH/min/mg protein.

Statistical analysis

All of the results of *in vivo* studies are the means of independent quadruplicate experiments for each cellular population of each animal model. The Pearson correlation test was used for one-way ANOVA while the Kruskal-Wallis test was used for the Spearman correlation test. A level of $P < 0.05$ was accepted as significant

(*P < 0.05, **P < 0.01, ***P < 0.001). All technical computerized data were analyzed using SPSS (ver. 16 PS).

Results

Comparison of GPx and SOD levels in an animal model

The comparison results of SOD and GPx activity in the control group using the independent sample *t*-test is briefly described in Table 1.

Table 1. Comparison results of control group

Variable	Negative control mean ± stand.dev	Positive control mean ± stand.dev	*p-value*
GPx (mU/mL)	50.89 ± 0.22	47.74 ± 2.07	0.023 <∝
SOD (U/mL)	4.65 ± 1.43	1.26 ± 0.76	0.006 <∝

Description: *p-value* α < 0.05 means there is a significant difference and *p-value* > 0.05 means there is no significant difference.

The results showed that there were significant differences (p = 0.023 < ∝) in the mean of GPx between the negative control (50.89 ± 0.22 mU/mL) and positive control (a mouse model of endometriosis) (47.74 ± 2.07 mU/mL). The mean GPx level was higher in the negative control than that in the positive control. The mouse model of endometriosis is therefore assumed to result in lower GPx levels compared to healthy mice. Similarly, there was a significant difference (p < 0.006) in the mean SOD level between the negative control (mice healthy) (4.65 ± 1:43 mU/mL) and the positive control (mouse model of endometriosis) (1:26 ± 0.76 mU/mL). SOD levels shown in the negative control were greater than SOD in the positive control, indicating that the mouse model of endometriosis generates lower SOD than healthy mice.

Effect of genistein and cowpea extract on GPx levels

Effects of genistein and cowpea extract against GPx levels showed that there was a significant difference between the mean GPx level in the positive control and the treatment group (Table 2). Based on its mean value, it indicates an increase in the treatment group. The administration of 50 mg genistein and cowpea extract in a mouse model of endometriosis is suggested to affect GPx level, which increases the levels of GPx compared to the mouse model of endometriosis without genistein.

Table 2. Comparisons of GPx activity (mU/mL) in genistein and cowpea extract

Observation group	Mean ± stand.dev
Positive control	47.74 ± 2.07^{a}
Genistein 50 mg/kg BW/hr	50.36 ± 1.02^{b}
Cowpea extract 50 mg/kg BW/hr	50.70 ± 1.16^{b}

Notes: Means with the different letter are significantly different from each other (*p-value* < 0.05).

A similar effect was also found in the treatment with 100 mg, 200 mg, 300 mg, and 400 mg genistein against GPx levels in a mouse model of endometriosis (Fig 1). However, there are significant differences in the mean GPx level between the administration of 500 mg genistein treatment groups and all doses. The mean GPx level shows that the administration of 500 mg genistein generated a higher value than any other dose. Treatment with 500 mg genistein might accelerate the increase in GPx level in a mouse model of endometriosis.

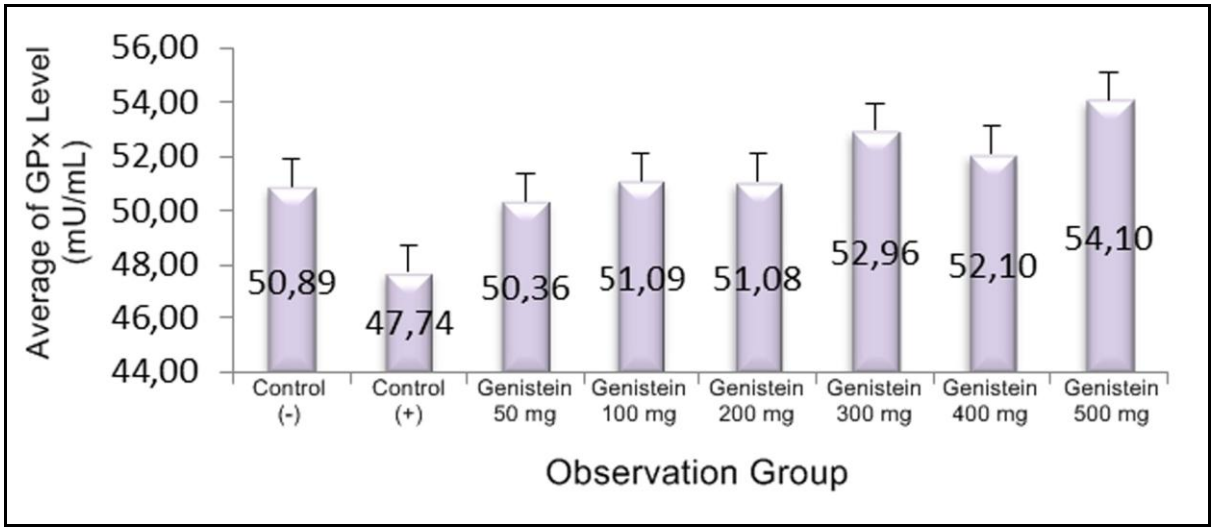

Figure 1. Effect of genistein treatment on GPx level

A similar finding was also detected in the cowpea extract treatment at a dose of 50 mg, 100 mg, 200 mg, 300 mg, and 400 mg in a mouse model of endometriosis, with the same ability to influence and increase GPx level. Although the increase was not significant, the average value of GPx levels increases following increased doses of cowpea extract, as shown in Fig 2. It shows that the administration of genistein and the extract of cowpea in a mouse model of endometriosis have the same effect, which is to increase the GPx level.

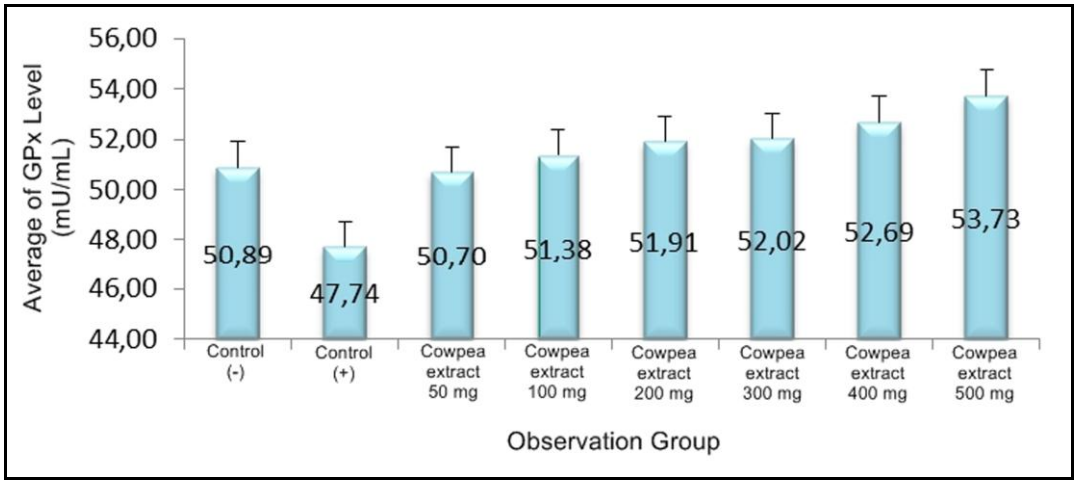

Figure 2. Effect of cowpea extract on GPx level

Effect of genistein and cowpea extract on SOD levels

Effect of genistein and cowpea extract on SOD level showed that there was a significant difference in the mean SOD level between the positive control and treatment groups (Table 3). The mean value shows an increase in the treatment group. The administration of genistein at a dose of 50 mg in the mouse model of endometriosis increased the SOD level compared to a mouse model of endometriosis without genistein.

Table 3. Comparisons of SOD activity (U/mL) genistein and cowpea treatment

Observation group	Mean ± stan.dev
Positive control	1.26 ± 0.76^{a}
Genistein 50 mg/kg BW/day	5.68 ± 1.94^{b}
Cowpea extract 50 mg/kg BW/day	4.02 ± 0.39^{b}

Description: Means with the same letter are not significantly different from each other (p-value<0.05)

The dose-based analysis in this study showed that the administration of genistein at a dose of 200 mg, 300 mg, 400 mg, and 500 mg in a mouse model of endometriosis does not affect the SOD level. The administration of genistein at a dose of 200 mg, 300 mg, 400 mg, and 500 mg of SOD showed no significant difference and had no effect, yet a dose of 50 or 100 mg influenced the SOD level (Fig 3). These results indicate that the administration of 200 mg genistein in a mouse model of endometriosis is unable to increase SOD level.

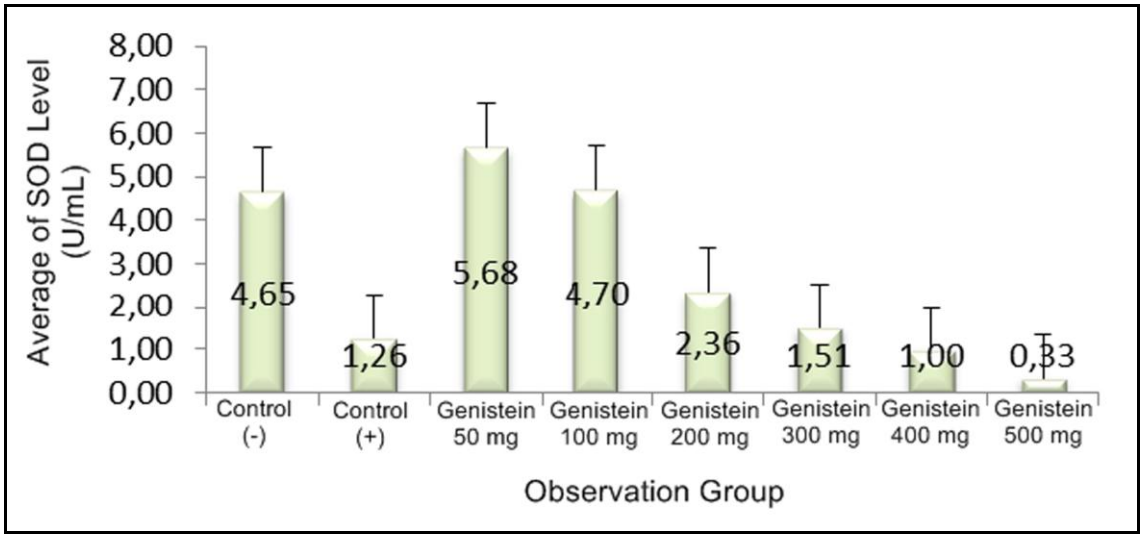

Figure 3. Effect of genistein treatment on SOD level

Similar results were obtained in the administration of genistein at a dose of 300 mg, 400 mg, and 500 mg of SOD, which showed no significant difference and has no effect at a dose of 300 mg, yet doses of 50, 100 and 200 mg influenced its activity (Fig 4). In this study, cowpea extract likely affects SOD better than genistein. However, cowpea extract is considered to have a similar ability to genistein, despite its slight difference.

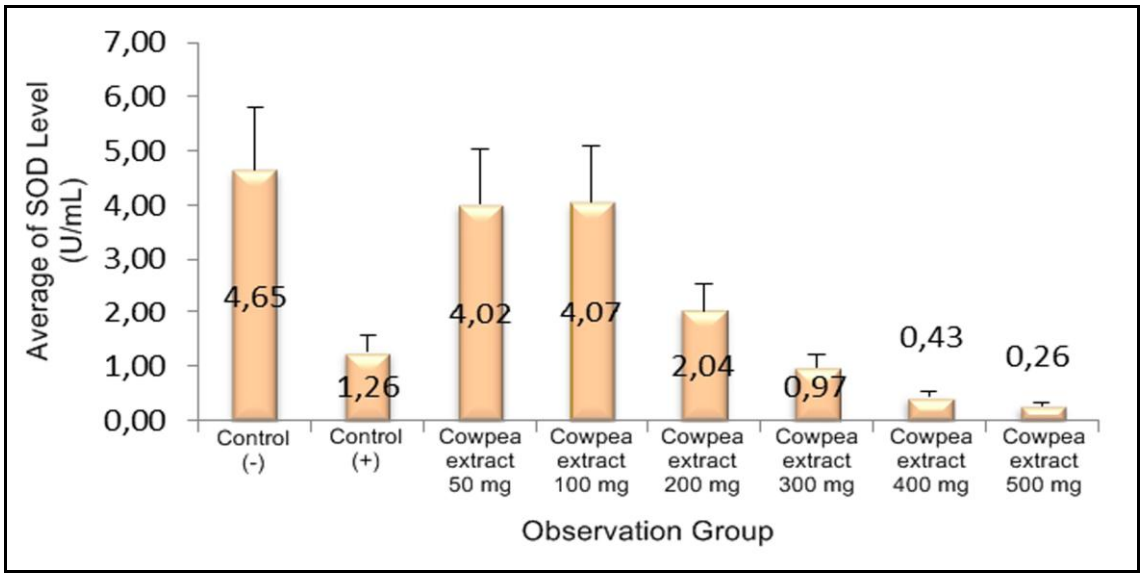

Figure 4. Effect of cowpea extract on SOD level

Discussion

In this study, endometriosis was induced using endometrial biopsy tissue, which allegedly activates macrophages to produce pro-inflammatory cytokines, resulting in a change in peritoneal cellular immunity. The condition induces oxidative stress and reduces the activity of the antioxidant enzyme SOD. Subsequent activation of transcription factors such as NF-κβ eventually causes the cells to undergo invasion, adhesion and

differentiation, tissue remodeling and cell survival, and is involved in endometriosis tissue formation[9].

Antioxidant systems are already known to protect tissues from adverse effects of free radicals. SOD and GPx are the enzyme antioxidants that prevent destructive biological effects of oxidative stress. Both enzymes bind to ROS to inhibit the production of hydroxyl radicals[10]. The previous study has also shown that women with endometriosis had higher levels of SOD and GPx in peritoneal fluid than in healthy women without endometriosis[11].

Genistein is a natural component that belongs to the family of isoflavones. Isoflavones are widespread in plants, and found in the legume group, and especially soybeans. Genistein in soybeans and cowpea in legumes (*leguminaceae*) are the main sources of isoflavones in nature[12,13]. Total isoflavone content in cowpea is 197,600 μg/100gr dry weight of cowpea, which is higher than the level in soybean[14].

Antioxidant systems are already known to protect tissues from the adverse effects of free radicals. Superoxide dismutase (SOD) and glutathione peroxidase (GPx) are antioxidant enzymes that prevent destructive biological effects of oxidative stress. Both of these enzymes bind ROS and inhibit the production of hydroxyl radicals. The cellular antioxidant capacity and inhibition of the genistein effect through activation of NADPH, as well as disorders of the mitochondrial transport chain, are clearly suppressed by genistein and the induction of phase II enzyme antioxidants, such as superoxide dismutase 1 (SOD1) and heme oxygenase-1 (HO-1), which is increased by genistein[10].

Elevated levels of GPx and SOD were present in the treatment with genistein and cowpea extract in a mouse model of endometriosis; these changes occur in a dose-dependent manner. SOD level in cowpea extract treatment tended to be smaller than genistein, yet cowpea extract is still assumed to have a similar ability to genistein, although slightly smaller.

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