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Original Research Article

Administration of Ethanol Extract of Black Soybean Seeds (*Gycine max (L.) Merr.*) on the Number of Ovarian Tertiary Follicles and Serum Estradiol Levels in Wistar-Strain Rats (*Rattus novergicus*) Exposed to Cigarette Smoke

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Abstract

Background: Premature ovarian insufficiency is ovarian aging that occurs before the age of 40, with a global prevalence of 1.1%. Premature ovarian insufficiency can be induced by environmental exposures, particularly through free radicals generated by cigarette smoke.

Objective: The purpose of this study was to prove that the administration of ethanol extract of black soybean (*Glycine max (L.) Merr.*) can inhibit the decrease in ovarian tertiary follicle count and serum estradiol levels in Wistar strain rats (*Rattus norvegicus*) exposed to cigarette smoke.

Methods: This research is an experimental study with a post-test-only control group design involving 30 Wistar-strain rats (*Rattus norvegicus*) that met the inclusion criteria. The rats were divided into five groups: group N (normal), group K (control), and group P1-P3 which received black soybean seed extract (*Glycine max (L.) Merr.*) at doses of 375 mg/kgBW (P1), 750 mg/kgBW (P2), and 1500 mg/kgBW (P3) administered orally via gavage every day for 28 days. The control group and treatment groups were exposed to cigarette smoke, after the administration of the extract, for 2 hours per day, 5 days a week, for 28 days. Serum estradiol levels (ng/L) were measured using the ELISA method, and ovarian tissue samples were collected for counting the number of ovarian tertiary follicles.

Results: There was a significant difference in serum estradiol levels ($p < 0,001$), with the highest serum estradiol level observed in Group P1 (564.4 ± 74.28) and the lowest in Group K (116,7 ± 38,01). There was no significant difference in the number of ovarian tertiary follicles ($p=0,645$).

Conclusion: The ethanol extract of black soybean seeds (*Glycine max (L.) Merr.*) at a dose of 375 mg/kg BW significantly inhibits the decrease in estradiol levels but did not affect the number of ovarian tertiary follicles in Wistar strain rats (*Rattus norvegicus*) exposed to cigarette smoke.

Keywords: *Black soybean seeds extract; Premature ovarian insufficiency; Serum estradiol tertiary follicle.*
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INTRODUCTION

The diagnosis of premature ovarian insufficiency is established when a woman experiences amenorrhea for 4–6 months before the age of 40, accompanied by persistently elevated FSH levels (> 40 IU/L) and decreased E2 levels (< 50 pg/ml). Ovarian histology examination is the definitive assessment for evaluating follicular reserve in patients with premature ovarian insufficiency. Histological analysis reveals that most tertiary (antral) follicles undergo atresia. A patient is considered to have diminished ovarian reserve if the total bilateral antral follicle count is less than 7.[1]

One of the causes of premature ovarian insufficiency is exposure to free radicals, with one of the largest sources being cigarette smoke. The composition of tobacco smoke consists of a gas phase and a tar phase. The gas phase generates more than 10^{15} free radicals, while each gram of tar produces nearly 10^{17} free radicals in each inhalation.[2] Data from the Global Adult Tobacco Survey (GATS) in Indonesia shows that within 10 years, from 2011 to 2021, the percentage of female smokers in Indonesia increased by 43.75%.[3]

Apoptosis of granulosa cells is the main cellular process responsible for the reduction in follicle and oocyte function and quantity, which is a key feature of ovarian aging.[4] Previous studies have shown that oxidants such as hydrogen peroxide can cause oxidative damage to DNA and induce early apoptosis in granulosa cells.[5] Oxidative stress can lead to premature and excessive activation of primordial follicles.[4]

Black soybean (*Glycine max (L.) Merr.*) is a subtropical plant native to Southeast Asia. Phytochemical analysis of black soybean (*Glycine max (L.) Merr.*) seeds has identified the presence of alkaloids, flavonoids, tannins, triterpenoids, glycosides, and saponins. Among all soybean seed color variants, black soybean (*Glycine max (L.) Merr.*) has the highest total polyphenol, flavonoid, and anthocyanin content, with an IC50 value of 90 µg/mL, indicating strong antioxidant activity.[6]

The justification of the dosage of black soybean seed (*Glycine max* (*L.) Merr.*) extract was based on two previous studies. The first study demonstrated that administration of an aqueous extract of black soybean seeds (*Glycine max* (*L.) Merr.*) at a dose of 750 mg/kg BW for 14 days significantly reduced the oxidative stress biomarker malondialdehyde and improved pancreatic tissue histology in diabetic rats.[7] The second study showed that administration of a 70% ethanol extract of black soybean seeds (*Glycine max* (*L.) Merr.*) at a dose of 200 mg/kg BW significantly increased the number of secondary follicles, however, that study did not include any preconditioning variables.[8] Accordingly, this study employed doses of black soybean seed ethanol extract of 750 mg/kg BW as the medium dose, 375 mg/kg BW as the minimum dose, and 1500 mg/kg BW as the maximum dose.

An in vivo approach was employed to evaluate the systemic and hormonal effects of black soybean extract, as these complex physiological interactions cannot be fully replicated under in vitro conditions. Wistar-strain rats (*Rattus novergicus*) were selected because their reproductive physiology and hormonal regulation are well-characterized and comparable to those of humans, allowing reliable evaluation of ovarian response to oxidative stress and xenobiotic exposure, such as cigarette smoke. [9,10]

The duration and method of cigarette smoke exposure were based on a previous study, which demonstrated that exposure to cigarette smoke for 2 hours per day, 5 days per week, over a period of 4 weeks significantly reduced the number of primary and secondary follicles.[11]

To date, no studies have investigated the effects of cigarette smoke exposure on the number of ovarian tertiary follicles and serum estradiol levels, both of which serve as fundamental parameters for the diagnosis of premature ovarian insufficiency. Therefore, this study was conducted to prove that the administration of ethanol extract of black soybean (*Glycine max (L.) Merr.*) can inhibit the decrease in ovarian tertiary follicle count and serum estradiol levels in Wistar strain rats (*Rattus norvegicus*) exposed to cigarette smoke. The author hypothesized that administration of the extract at a dose of 750 mg/kg BW would yield the most optimal effect in inhibiting the decrease in ovarian tertiary follicle count and estradiol levels in Wistar strain rats (*Rattus norvegicus*) exposed to cigarette smoke.

MATERIALS AND METHODS

This study has been approved for ethical feasibility by the Research Ethics Committee Faculty of Medicine Universitas Udayana (Ethical Clearance No: 2462/UN14.2.2.VII.14/LT/2024).

The sample size was determined using Arifin's formula, which indicated a minimum of 3 rats and a maximum of 5 rats per group. An additional 10% of samples were included in each group to anticipate potential dropouts during the study. Accordingly, each group consisted of 6 rats, resulting in a total of 30 rats required for this study. Thirty healthy Wistar-strain rats (*Rattus norvegicus*), aged 12–14 weeks and weighing 180–220 grams, with no prior pregnancy history, were randomly selected using the systematic random sampling method, subsequently underwent a 7-day adaptation period in Laboratorium Biomedik Terpadu, Faculty of Medicine, Universitas Udayana.

On the eighth day, the rats were randomly allocated into five groups using the systematic random sampling method with an interval numbering of 5. All 30 rats were numbered from 1 to 30, and the rats were then grouped in multiples of five: rats numbered 1, 6, 11, 16, and 21 were assigned to group N; rats numbered 2, 7, 12, 17, and 22 were assigned to group K; and so on. The five groups are as follows:

a) Group N: The subjects were given only standard feed (n=6).
b) Group K: The subjects were given distilled water every morning at 8 AM for 28 days and were exposed to cigarette smoke (n=6).
c) Group P1: The subjects received oral administration of ethanol extract of black soybean (*Glycine max (L.) Merr.*) at a dose of 375 mg/kg BW every morning at 8 AM for 28 days and were exposed to cigarette smoke (n=6).
d) Group P2: The subjects received oral administration of ethanol extract of black soybean (*Glycine max (L.) Merr.*) at a dose of 750 mg/kg BW every morning at 8 AM for 28 days and were exposed to cigarette smoke (n=6).
e) Group P3: The subjects received oral administration of ethanol extract of black soybean (*Glycine max (L.) Merr.*) at a dose of 1500 mg/kg BW every morning at 8 AM for 28 days and were exposed to cigarette smoke (n=6).

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Extraction and Phytochemical Analysis

The extraction of black soybean (*Glycine max (L.) Merr.*) was carried out using the maceration method with 70% ethanol as the solvent. A total of 10 kg of black soybean seeds (*Glycine max (L.) Merr.*) was required to produce 150 grams of concentrated extract. The black soybean seed ethanol extract used in this study was prepared from a single batch to ensure consistency and reproducibility. The concentrated extract was then subjected to phytochemical analysis to determine the content of phenolic compounds, flavonoids, tannins, and anthocyanins, as well as an IC50 test to assess antioxidant activity.

Cigarette Smoke Exposure

Clove (kretek) cigarette smoke exposure was conducted five days a week (no exposure on Thursdays and Sundays) for 28 days. The total daily exposure duration was 2 hours, administered separately with a 6-hour rest interval. The first exposure was conducted at 9 AM for 1 hour using 2 cigarettes, while the second exposure was conducted at 3 pm for 1 hour using 2 cigarettes.

Estrous Cycle Examination

After 28 days of experimental treatment, estrous cycle examination was performed on the rats using visual inspection and vaginal smear cytology stained with 10% Giemsa for 30 minutes. The proestrus phase was characterized by the presence of nucleated epithelial cells. The estrus phase was dominated by anucleated keratinized epithelial cells. The metestrus phase was characterized by the presence of leukocytes and a few nucleated epithelial cells, while the diestrus phase was characterized by the predominance of leukocytes. If the rats were in the estrus or metestrus phase, blood and ovarian tissue sampling was carried out. If the rats were in the diestrus or proestrus phase (when estradiol levels are high), blood and ovarian tissue sampling was postponed until the rats entered the estrus phase. The maximum delay duration was five days.

Control of Confounders

The rats' body weights were matched at approximately 180–200 grams, and their ages were adjusted to 12–14 weeks before randomization to minimize variation. All rats were maintained under identical environmental conditions (12-hour light/dark cycle, temperature 25 ± 2 °C) with ad libitum access to food and water. Estrous cycles were synchronized before sample collection, and sampling was performed only during the estrus and metestrus phases to control hormonal differences. This study also required that all rats had never been pregnant and were not pregnant during the experiment.

Histological Examination of Ovarian Tertiary Follicle Count

The right and left ovaries of each experimental rat were fixed in 10% formalin for 24 hours. The samples were then dehydrated, embedded in paraffin, and **cut into** serial sections with a thickness of 5 µm using a microtome. The tissue sections were transferred to a water bath containing warm water (±60°C) until the tissue expanded, then placed onto glass slides, ensuring that each glass slide contained four serial sections of the right and left ovarian tissue. Each rat had 16 serial sections of the right and left ovarian tissue placed on 4 glass slides. The slides with the tissue samples were placed on a hot plate at 60°C for approximately 10 minutes until the paraffin melted and the tissue adhered completely. Subsequently, the samples were stained using Hematoxylin-Eosin (HE) staining.

Identification and counting of tertiary follicles were performed using a microscope at 100x magnification. The average number of tertiary follicles was calculated from four sections at every fifth interval (sections number 1, 6, 11, and 16). Subsequently, the average number of tertiary follicles was multiplied by 5 as the sampling fraction correction factor to estimate the total number of ovarian follicles, in accordance with the principles of the fractionator method. The measurement unit is expressed as the number of antral follicles per low-power field (LPF).[12]

Evaluation of Estradiol Levels

Estradiol level examination was performed using serum obtained from 1 cc of blood collected from the medial canthus of the rat's orbital sinus. The analysis was conducted using the ELISA method with the Rat Estradiol E2 BT-Lab kit. The core principle of the assay involves the use of antibodies with high specificity for estrogen molecules. This binding interaction triggers a colorimetric reaction, followed by the measurement of sample absorbance at a wavelength of 450 nm. The estradiol levels were determined by adjusting the absorbance readings to a pre-established standard curve.

Statistical Analysis

Statistical analysis was performed using SPSS version 22 at a 95% level of confidence. The research data were proven to be normally distributed and homogeneous; therefore, comparative analysis was performed using a One-Way ANOVA test. Subsequently, a Post Hoc Bonferroni test was conducted to determine specific differences between groups.

RESULTS

Phytochemical Analysis

The analysis of total tannin content in this study showed a result of 1592.636 mg TAE/100g, which is significantly higher compared to previous studies.[11,12] The results of the phytochemical analysis in this study are presented in Table 1.

Table 1. Phytochemical Analysis Results

Parameter	Result
Total Anthocyanin (mg/100g)	8.636
Total Phenol (mg GAE/100g)	216.99
Total Tannin (mg TAE/100g)	1592.636
Flavonoid (mg QE/100g)	5.99
IC 50 (ppm)	317.62

Tertiary Follicle Count

The highest average number of tertiary follicles was found in the N group, followed by the P1 group**,** while the lowest was in the K group. Statistical analysis using the One-Way ANOVA test showed a p-value of 0.645,

Table 2. Tertiary Follicle Count between Study Groups

Parameter	N Group	K Group	P1 Group	P2 Group	P3 Group
n	6	5	6	5	5
Mean ± SD	13.75±5.42	9.75±6.08	13.125±4.73	12.25±2.40	11.00±3.89
Median	12.50	11.25	11.25	12.5	10.00
Minimum	7.50	0	8.75	8.75	7.50
Maximum	20	16.25	21.25	15.00	17.50
95%CI	8.06-19.44	2.19-17.30	8.16-18.08	9.26-15.23	6.17-15.83

indicating there was no significant difference in the number of tertiary follicles among the five study groups. The F-value was 0.633**,** suggesting that the variance within groups was greater than the variance between groups. These findings are comprehensively presented in Table 2 and Figure 1.

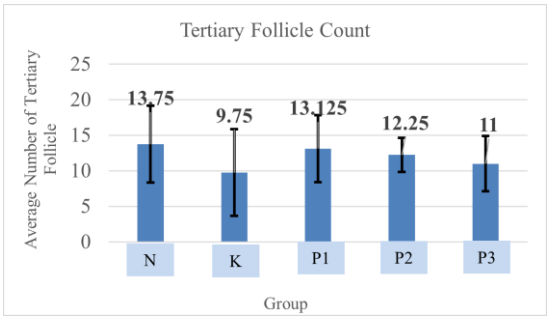

Figure 1. Mean Comparison of Tertiary Follicle Between Groups

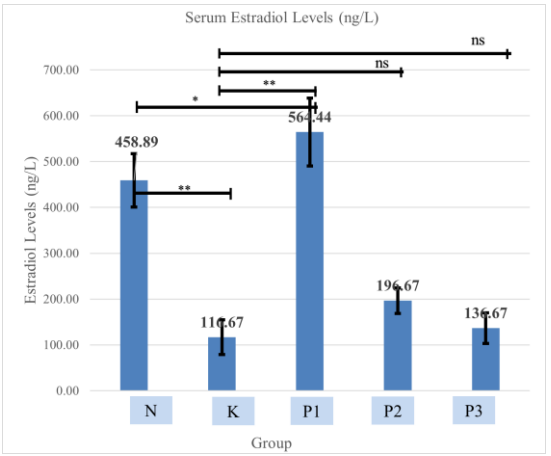

Figure 3. Mean Comparison of Serum Estradiol Levels Between Groups
* : p < 0.05; **: p < 0,001; ns : p > 0.05

Serum Estradiol Levels

The results of the One-Way ANOVA test for serum estradiol levels showed p < 0.001, indicating a statistically significant difference in serum estradiol levels among the five study groups. The highest serum estradiol level was found in group P1, while the lowest level was in group K. A Post Hoc test was then conducted to identify differences between the groups, as shown in figure 3.

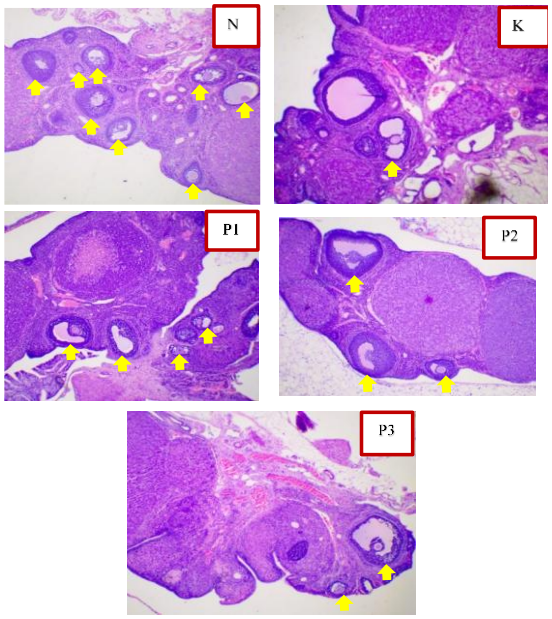

Figure 2. Representative ovarian histology of Wistar rats (H&E staining, ×400). (N) Normal group, (K) Placebo group, (P1) Dose 1 (375 mg/kg BW), (P2) Dose 2 (750 mg/kg BW), (P3) Dose 3 (1500 mg/kg BW). Yellow arrows indicate tertiary follicles.

DISCUSSION

The study results showed that group K had the lowest mean number of tertiary ovarian follicles (p = 0.645) and serum estradiol levels (p < 0.001). The Post Hoc analysis revealed a significant difference in serum estradiol levels between group K and group N (p < 0.001), indicating that cigarette smoke exposure significantly reduced serum estradiol levels.

Active and passive smoking can generate a large number of free radicals, ultimately leading to oxidative stress. Oxidative stress can result in telomere shortening and reduced telomerase enzyme activity in granulosa and cumulus cells, which are associated with oocyte quality and premature ovarian insufficiency.[2,14] Oocyte and granulosa cell apoptosis can lead to a decrease in estradiol levels, as estradiol synthesis occurs through the "2-cell-2-gonadotropin" mechanism involving granulosa and theca cells, along with the hormones FSH and LH. Additionally, cigarette smoke exposure has been shown to directly disrupt androstenedione synthesis in theca cells and hinder its conversion into estradiol by the aromatase enzyme.[15]

Statistical analysis showed that the administration of black soybean seed extract (*Glycine max* (L.) Merr.) had no significant effect on the number of tertiary follicles. This may be due to the many factors influencing the folliculogenesis process. Folliculogenesis involves various hormones, including Anti-Müllerian Hormone (AMH), FSH, LH, Inhibin B, estradiol, and progesterone, which are regulated in a coordinated, complex, and synergistic manner over time.[16] In addition to hormones, metabolic factors also play a role in the folliculogenesis process. Glucose metabolism is essential for cumulus cell proliferation and differentiation, as well as oocyte development.

Lipid metabolism is also crucial for energy production in ovarian cells. Beta-oxidation of fatty acids, which is induced by increased LH levels, plays a key role in the complex metabolism of the cumulus-oocyte cells.[17]

Similar animal studies investigating soy-derived phytoestrogen extracts have shown variable effects on ovarian folliculogenesis. One study demonstrated that administration of a medium dose of soy isoflavones (50 mg/kg BW) significantly produced the highest increase in the proportion of tertiary follicles compared to the control and other dose groups.[18] Another study reported non-significant differences in follicle counts in Wistar rats treated with tempeh extract ($p > 0.05$).[19] These differences in outcomes may be attributed to variations in extract preparation and composition, extract dosage, the presence or absence of preconditioning treatments, and the duration of the study. The lack of significant differences in tertiary follicle count might also be due to the limited duration of exposure. Folliculogenesis is a long and multi-staged process, and tertiary follicles take several estrous cycles to develop. Therefore, a 28-day treatment may not have been sufficient to produce measurable histological changes.

In this study, the serum estradiol level in group P1 was significantly higher than in group N ($p = 0.017$), suggesting that the administration of black soybean seed ethanol extract (*Glycine max (L.) Merr.*) at a dose of 375 mg/kg BW not only exerted an antioxidant effect against free radicals but also involved other mechanisms contributing to the increase in serum estradiol levels. This finding is further supported by the IC50 analysis results, which were 317.62 ppm, indicating weak antioxidant activity. The phytochemical analysis in this study revealed that black soybean seeds (*Glycine max (L.) Merr.*) contain flavonoids and anthocyanins, which act as phytoestrogens. Besides binding to estrogen receptors, phytoestrogens can also influence the synthesis and metabolism of steroid hormones through cytochrome P450 aromatase, which catalyzes the conversion of androstenedione and testosterone into estrone and. At high doses (>1 µM), phytoestrogens can modulate aromatase enzyme activity.[20]

This study demonstrates that black soybean seed extract has a high tannin content. Ellagic acid is a hydrolyzable form of tannin known for its ability to act as a selective estrogen receptor modulator. Previous study had proven that the administration of ellagic acid was as effective as clomiphene citrate in increasing estradiol levels in experimental rats with a precondition of premature ovarian insufficiency.[21] This proves that ellagic acid, like clomiphene citrate, has estrogen antagonist activity by occupying estrogen receptors in the hypothalamus and inhibiting the normal negative feedback of estradiol. This condition stimulates the hypothalamus and pituitary to secrete GnRH and FSH, leading to estradiol secretion. Estradiol levels within the physiological range can directly exert negative feedback on the pituitary, with a stronger inhibitory effect on FSH than LH, causing FSH levels to decrease rapidly.[22]

Serum estradiol levels were significantly lowest in group K, followed by groups P2 and P3, which received black soybean seed extract (*Glycine max (L.) Merr.*) at doses of 750 mg/kg BW and 1500 mg/kg BW, respectively. In addition to group K, one rat also died in both groups P2 and P3. Based on these results, it can be concluded that excessively high doses of black soybean seed extract may have adverse consequences. Antioxidants are reducing agents. Excessive antioxidant supplementation can increase the levels of reductants, such as NADH and NADPH, leading to reductive stress. This condition can disrupt the electron transport chain, thus increasing electron leakage and ultimately resulting in higher ROS production.[23] Additionally, tannins, anthocyanins, and flavonoids are examples of phenolic antioxidants, which, at high concentrations, can trigger pro-oxidant activity.[24]

This finding is supported by previous in vivo study which showed that high-dose ellagic acid (0.6%) resulted in a lower number of tertiary follicles and lower serum estradiol levels compared to low-dose ellagic acid (0.3%).[25] Another in vitro study demonstrated that high-dose ellagic acid (100 mcg/mL) led to the lowest Cumulus Expansion Index (CEI), expression of CEI-related genes, and mRNA expression of genes associated with oocyte maturation compared to low (1 mcg/mL) and moderate (10 mcg/mL) doses.[26]

The limitation of this study is the lack of specific phytochemical analysis of flavonoid and anthocyanin phytoestrogens due to the unavailability of phytoestrogen testing parameters. This study also did not analyze intrafollicular apoptosis parameters, which are a fundamental pathomechanism of premature ovarian insufficiency. Future studies are recommended to analyze the phytochemical content of phytoestrogens to assess the phytoestrogenic role of black soybean seeds (*Glycine max (L.) Merr.)* and to evaluate other parameters that can support the findings of this study, such as analyzing the intrafollicular fluid Bcl-2/Bax ratio to assess intrafollicular apoptosis activity.

CONCLUSION

The administration of ethanol extract of black soybean seed (*Glycine max (L.) Merr.*) at 375 mg/kg BW significantly attenuated the reduction in serum estradiol levels, while no significant effect was observed on tertiary follicle count. Higher doses (750 and 1500 mg/kg BW) did not produce a significant inhibition of the estradiol decrease, indicating that dose escalation beyond 375 mg/kg BW did not confer additional measurable benefit.

Although direct extrapolation from animal models to humans is limited, these findings suggest that, if similar mechanisms occur in humans, a comparable moderate dose may offer a protective effect against environmentally induced declines in estradiol levels. Nevertheless, clinical studies are required to confirm this potential relevance.

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