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# The Effect of Black Pepper (*Piper nigrum* L.) Extract on Morphology and number of Mice (*Mus musculus* L.) Spermatozoa Induced by Progestin Hormone

(Pengaruh Ekstrak Lada Hitam (Piper nigrum L.) terhadap Morfologi dan Jumlah Spermatozoa Mencit (Mus musculus L.) yang diinduksi Hormon Pregestin)

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Abstrak

Hormon progestin digunakan sebagai kontrasepsi hormonal pria. Pemberian progestin pada pria normal dapat menekan libido dan fungsi testis secara efektif, serta menurunkan kadar testosteron sehingga dapat mengganggu proses spermatogenesis. Penelitian ini mengenai efektivitas ekstrak lada hitam (*Piper nigrum* L.) terhadap morfologi dan jumlah spermatozoa mencit (*Mus musculus* L.) yang diinduksi progestin. Penelitian ini menggunakan rancangan acak lengkap (RAL) dibagi menjadi empat kelompok perlakuan, masing-masing kelompok terdiri dari 5 ekor mencit dan diberi perlakuan sesuai dengan rancangan percobaan. Kelompok P0 sebagai kontrol (diberi H2O), P1 (diinduksi dengan 1,25 mg progestin), P2 (diinduksi dengan 1,25 mg progestin dan 3,33mg/Kg BB ekstrak etanol lada hitam), dan P3 (diinduksi dengan 1,25 mg progestin). 25 mg progestin dan 3,33mg/Kg BB ekstrak air suling lada hitam). Hormon Progestin diberikan dua kali (minggu ke-1 dan minggu ke-2). Ekstrak lada hitam diberikan setiap hari selama 35 hari. Data dianalisis dengan one way ANOVA dan dilanjutkan dengan uji LSD pada taraf 5%. Hasil penelitian menunjukkan bahwa pemberian ekstrak lada hitam dapat meningkatkan morfologi normal dan jumlah spermatozoa mencit setelah diinduksi hormon progestin sehingga kesuburan pada pria meningkat.

Kata kunci: ekstrak lada hitam, morfologi dan jumlah spermatozoa, kualitas spermatozoa, fertilitas.

Abstract

*The progestin hormone is used as male hormonal contraception. Giving progestin to normal men can suppress libido and testicular function effectively, and reduce testosterone levels so that it can interferer with the spermatogenesis process. This study is about the effectiveness of black pepper (Piper nigrum L.) extract on morphology and number of spermatozoa in mice (Mus musculus L.) induced by progestin. This study used a completely randomized design (CRD) which has divided into four treatment groups, each group contained 5 mice and treated according to the experimental design. Group P0 as a control (given H2O), P1 (induced by 1,25 mg progestin), P2 (induced by 1,25 mg progestin and 3.33mg/Kg BW ethanol extract of black pepper), and P3 (induced by 1,25 mg progestin and 3.33mg/Kg BW distilled water extract of black pepper). The Progestin hormone is given twice (week 1 and week 2). Black pepper extract was given daily for 35 days. Data were analyzed by one-way ANOVA and continued with the LSD test with a level of 5%. The result showed that giving of ethanol extract and distilled water extract from black pepper could improve the normal morphology and number of spermatozoa in mice after induced by progestin hormone so that fertility in men increases.*

*Keywords: black pepper extract, morphology and number of spermatozoa, quality of spermatozoa, fertility.*

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## INTRODUCTION

Quality spermatozoa are capable of fertilizing an egg or ovum. Sperm quality can be determined from several aspects including the quantity (number) of spermatozoa, motility, viability and morphology of spermatozoa. The number of quality spermatozoa is more than 20 million/ml of ejaculate. The number of spermatozoa produced by the testes is very dependent on spermatogenesis. If the spermatogenesis process goes well, the number of sperm produced will be normal [1].

Spermatogenesis occurs through the process of mitotic proliferation of spermatogonial cells, meiotic division in spermatocytes, and structural changes from spermatids to spermatozoa [2]. Spermatogenesis requires the stimulation of the two gonadotropin hormones, namely luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH plays a role in stimulating Leydig cells to produce the hormone testosterone and maintaining testosterone concentrations. Meanwhile, FSH plays a role in stimulating Sertoli cells to produce Androgen Binding Protein which functions to bind the hormone testosterone suppressing gonadotropin secretion can reduce testosterone hormone levels which results in disruption of the spermatogenesis process and decreased fertility in men, so it can be used as a method of male hormonal contraception. One of the hormones that can be used as male hormonal contraception is the hormone progestin.

The progestin hormone or Depot Medroxy Progesterone Acetate (DMPA) is an esterification of progesterone which suppress pituitary gonadotropin secretion which inhibits the production of FSH and LH, so it is used as a hormonal contraceptive in women. The working principle of DMPA is also used to suppress gonadotropin secretion in men so that it will suppress spermatogenesis and the use of progestin has been proven to suppress gonadotropin secretion (FSH and LH) and spermatogenesis so that it can be used as a male contraceptive. According to Ding et al (2015) a decrease in testosterone hormone levels can disrupt the spermatogenesis process which results in decreased fertility in men [3].

Several ways can be done to increase fertility in men. One of them is using black pepper extract. Black pepper (*Piper nigrum* L) has an active ingredient in the form of piperine. The piperine content in black pepper extract can increase the testosterone hormone by minimizing the conversion of testosterone and FSH, as well as the number and size of Leydig cells [4]. Based on research conducted by Sutyarso, et al (2016) shows that black pepper extract has a positive effect on testosterone levels and increases fertility in male mice [5]. Research by Ekaputri, et al (2018) shows that giving black pepper extract at a dose of 3.33 mg/Kg BW can increase the hormones FSH and testosterone, but at a dose of 6.66 mg/Kg BW and a dose of 13.32 mg/Kg BW can have a negative effect on the male reproductive system and has the potential to be antifertility in mice [6].

Blackpepper contains a spicy substance, namely piperine alkaloid which acts as an antioxidant [7]. Black pepper has some chemical ingredients include saponins, flavonoids, essential oils, cavicin, resin, egg white starch, piperine, piperiline, piperoleine, poperanine, piperonal, dihydrocarveol, kanyo-fillene oxide, ceriptone, tranpiocarrol, and pepper oil [8]. Study recently, piperine has a role as an antioxidant, immune modulator, anticancer, hepatoprotective, anti-inflammatory, and antimicrobial. Piperine has also been reported can inhibit oxidative stress, and is used as protection against radical free, ROS,

and inhibition of fat peroxidation [9]. Based on the background explanation above, it is necessary to observe the effect of black pepper extract on normal morphology and number of spermatozoa in the testicles of mice (Mus musculus L.) which were induced with the hormone progestin.

## MATERIALS AND METHODS

### Extract Preparation and Dosage

Black pepper ethanol extract is made using maceration techniques. Every 50 grams of black pepper powder is macerated with 500 ml of 96% ethanol solvent. The black pepper powder was soaked in one third of 500 ml of 95% ethanol using an Erlenmeyer flask for 3x24 hours at 25$^{o}$C. Then the Erlenmeyer flask was shaken using a shaker at low speed. Every 24 hours, the macerate is taken and filtered. The residue is further macerated with the same solvent and process twice. The filtrate is filtered and then evaporated at 70$^{o}$C in an oven until an extract is obtained in paste form. The paste is named ethanol extract [10].

To make black pepper water extract, 50 grams of black pepper powder were put into 500 ml of distilled water that has been heated until it boils. After that, it was stirred for 15 minutes. Then filtering is carried out. The filtrate was evaporated in an oven at 70$^{o}$C until an extract was obtained in paste form. This paste is named as water extract [10].

Determination of the dose of black pepper extract is based on research conducted by Ekaputri, et al (2018), namely a dose of 3.33 mg/Kg BW because it can increase the hormones FSH and testosterone [6]. In this study, a dose of 3.33 mg/Kg BW was used for each black pepper ethanol extract and black pepper water extract. Next, determine the dose used in mice with a body weight of 30 grams, namely 0.09 mg/mice.

### Animal and Experimental Design

This research used 20 male mice (Mus musculus L.) aged 2-3 months old and weighing 30 grams from Lampung Veterinary Center, Indonesia. The animals were acclimatized for one week in laboratory conditions aimed at adapting the mice to the environment. Mice were grouped into 4 treatment groups, each group contained 5 mice and were treated according to the experimental design. Black pepper extract was given to mice orally at a dose of 3.33 mg/Kg BW every day for 35 days. Based on the Material Safety Data Sheet for Piperine, it does not exceed LD 50 when administered orally with acute toxicity of 330 mg/Kg Bb mice. Giving a dose of black pepper extract based on research conducted by Ekaputri, et al (2018) shows that giving black pepper extract at a dose of 3.33 mg/Kg BW can increase the hormones FSH and testosterone, but at a dose of 6.66 mg/Kg BW and a dose of 13.32 mg/Kg BW can have a negative effect on the male reproductive system and has the potential to be antifertility in mice [6]. This research used a Completely Randomized Design (CRD).

### Progestin Hormone

Medroxy Progesterone Acetate Depot was used to induce mice with a dose of 1.25 mg given at week 1 and week 2. The injection is carried out in the right or left thigh alternately. The aim of the injection twice is so that the progestin hormone given is effective in suppressing the secretion of the hormones FSH and LH [11].

Determination of the dose used was based on research conducted by Yurnadi, et al (2008) that the group given DMPA at a dose of 1.25 mg showed the lowest concentration and viability of spermatozoa as well as testosterone hormone levels compared to

the group given a dose of 0.625 mg and 0.313 mg in male mice [11].

**Treatment Group**

The experimental mice were grouped into 4 groups, each group had different treatments, namely:

1) Treatment group 1 as the control group.
2) Treatment group 2, male mice were induced with the hormone progestin at a dose of 1.25 mg without administering the test substance.
3) Treatment group 3, male mice were induced with the hormone progestin at a dose of 1.25 mg and given ethanol extract of black pepper at a dose of 3.33 mg/Kg BW every day for 35 days.
4) Treatment group 4, male mice were induced with the hormone progestin at a dose of 1.25 mg and given distilled extract of black pepper at a dose of 3.33 mg/Kg BW every day for 35 days.

**Sperm Analysis**

The morphology of abnormal spermatozoa can be determined by counting 100 spermatozoa. Normal mouse spermatozoa consist of a head (caput) which is bent like a hook, a short middle piece, and a very long tail (cauda) [12].

The number of spermatozoa was counted using an improved Neubauer counting chamber (hemocytometer). A 10 µL sample of spermatozoa suspension that has been diluted with 1 ml of physiological salt solution (NaCl 0.9%) is taken and then placed into a counting chamber (hemocytometer). After that, it was covered with a glass cover. Then observed under a light microscope with 400x magnification. The number of spermatozoa was counted at least 100 spermatozoa in each large box. The calculation results are then entered into the formula for determining the amount/ml of suspension using the formula [12]:

$$\text{Number of cells/ml} = \text{number of spermatozoa (n)} \times 10^4 \times \text{dilution}$$

**Statistical Analysis**

Statistics that are applied in SPSS version 17 include normality, homogeneity, and One way ANOVA. The Data presented as Mean ± SEM, was analyzed with a One-way ANOVA test. If there is significant variation, the data will be tested further using the Least Significant Difference (LSD) test at a significance level of 5%.

## RESULT AND DISCUSSION

**Morphology Spermatozoa**

Based on the graph in Figure 1, it can be seen that normal morphology of spermatozoa decreased in treatment 1 (P1) compared to the control group (P0), while in treatment 2 (P2) and treatment 3 (P3) there was an increase compared to treatment 1 (P1). In this study, the average morphology of spermatozoa in the control treatment group (P0), administration of the progestin hormone (P1), administration of the progestin hormone and black pepper ethanol extract (P2), and administration of the progestin hormone and black pepper distilled extract (P3) respectively are 70.46%, 38.46%, 67.32%, and 66.53%.

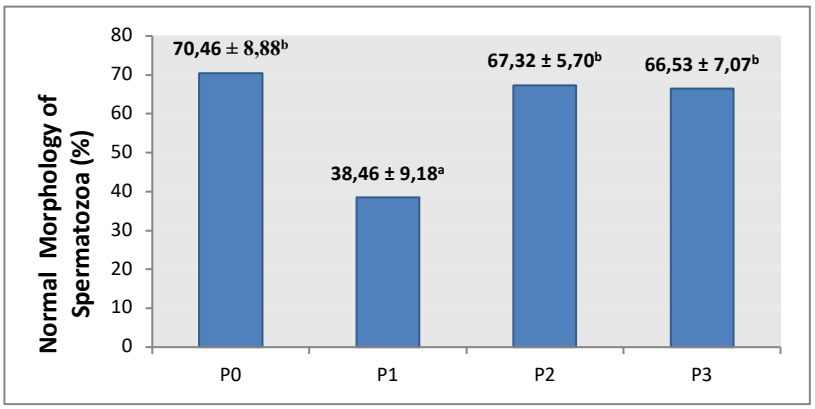
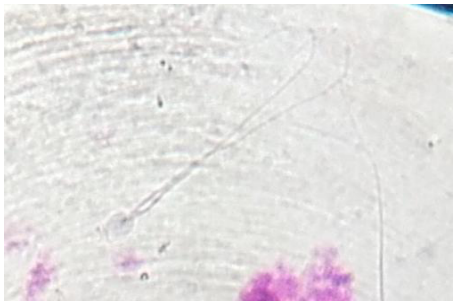



Figure 1. The result of the LSD test showed a significant difference between P1 with the Group of P0, P2, and P3. There is no significant difference between P0 with groups of P2 and P3.

a. Headless Spermatozoa

b. coiled-tailed spermatozoa



c. Double-tailed spermatozoa

d. Bent neck spermatozoa

e. Broken tailed spermatozoa

Figure 2. Abnormal morphology spermatozoa of mice.

In this study, abnormal morphology of spermatozoa was found, such as headless spermatozoa, bent tails, coiled tails and double tails. The abnormal morphology of spermatozoa can be seen in figure 2 above.

Spermatozoa abnormalities are all forms of

deviation from spermatozoa morphology. Deviations can occur in several parts of the spermatozoa, in the head the deviations include heads that are too big, too small, flat, double or even without a head, in the middle the deviations are in the form of folds or curves, while deviations in the tail are in the form of a circular tail, broken tail and double tail [13].

The results of the study also showed that administration of ethanol extract and distilled water extract from black pepper could improve the normal morphology of spermatozoa. This can happen because of the presence of flavonoid compounds which are antioxidant compounds. Content flavonoids as antioxidants can increase the durability of exposed sperm free radicals to prevent male infertility. Flavonoids play a role in increasing the amount of sperm by preventing damaged sperm membranes that cause disruption of the spermatogenic process [14].

Apart from that, black pepper also contains minerals such as magnesium and zinc which can increase testosterone hormone levels. If testosterone hormone levels increase, the spermatozoa maturation process in the epididymis will not be disturbed so that the morphology of the spermatozoa produced is perfect or normal [15]. This is in line with research conducted by Sutyarso et al (2016) which showed that there was an increase in the normal morphology of spermatozoa after administration of ethanol extract and distilled water extract from black pepper [5]. The difference in administration of ethanol extract and distilled water extract from black pepper did not provide a significant difference although the percentage results showed differences in the increase in normal morphology after administration of each extract.

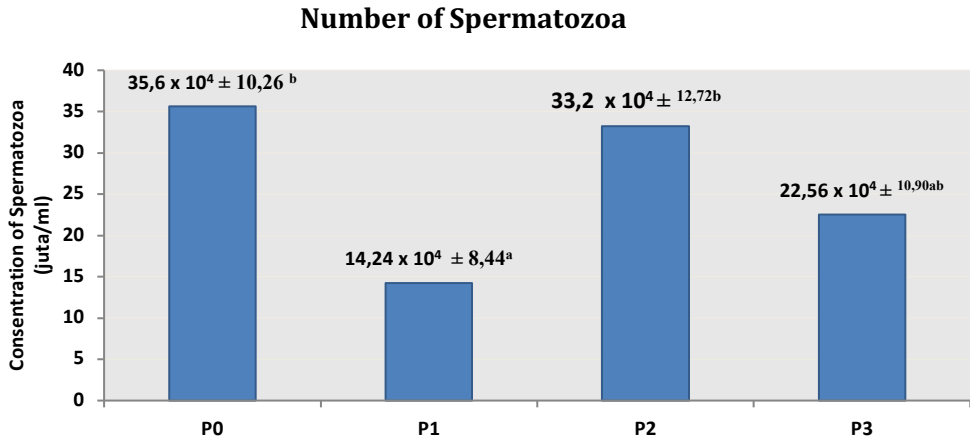



Figure 3. Calculating graph of sperm concentration (million/ml)

Figure 3. shows that the spermatozoa concentration in treatment 1 (P1) decreased compared to the control (P0), in treatment 2 (P2) and treatment 3 (P3) it increased compared to treatment 1. In this study the average spermatozoa concentration in the group control treatment (P0), administration of the progestin hormone (P1), administration of the progestin hormone and black pepper ethanol extract (P2), and administration of the progestin hormone and black pepper distilled extract (P3) respectively were 35.6x104 million/ml, 14, 24x104 million/ml, 33.2x104 million/ml, and 22.56x104 million/ml.

Then, a homogeneity test was carried out and it was found that all distributions were homogeneous ($p>0.05$) and the average

number of spermatozoa after carrying out ANOVA analysis with a significance level of 5% showed significant results. Then, to find out which groups have significant differences, continue with the LSD further test at a level of 5%. The results of the LSD test show that there is a real difference between P1 and PO and P2. There are no real differences between P0 and P2 and P3, between P2 and P3, and between P1 and P3.

Spermatozoa concentration is an indicator to determine the quality of the spermatozoa produced. Spermatozoa concentration shows the number of spermatozoa obtained in one collection [16]. Based on the research results, there was a decrease in spermatozoa concentration due to administration of the hormone progestin (P1). The Depot Medroxyprogesterone Acetate (DMPA) injection contraceptive is a hormonal contraceptive that contains the hormone progesterone [17]. This hormone is used as a hormonal contraceptive in men. When used, hormonal contraception suppresses sperm production by reducing male hormone levels.

The progesterone hormone provides feedback to the pituitary gland through the hypothalamus, thereby stimulating the pituitary gland to inhibit the release of luteinizing hormone (LH) which acts on the testicular Leydig cells to produce testosterone. Inhibition of LH secretion will cause inhibition of testosterone secretion which functions in the spermatogenesis process so that it can reduce the number of spermatozoa. The number of spermatozoa produced is very dependent on the spermatogenesis process that occurs in the seminiferous tubules. If disturbances occur during the spermatogenesis process, the development of spermatogonium cells will affect the number of spermatozoa formed [18].

The research results also showed that administration of black pepper ethanol extract (P2) and black pepper distilled extract (P3) at a dose of 3.33 mg/Kg BW each could increase the concentration of spermatozoa. The research results also showed that there was no real difference between administering black pepper ethanol extract and black pepper water extract. This can happen because ethanol extract and distilled extract of black pepper have strong antioxidant activity. This is in line with research conducted by Sutyarso *et al,* (5) that the administration of black pepper ethanol extract and black pepper distilled extract can increase the concentration of spermatozoa [5].

The effect of increasing the concentration of spermatozoa given black pepper ethanol extract and black pepper distilled extract can occur due to the antioxidant compounds contained in black pepper. The antioxidant compounds contained in black pepper are flavonoids, phenolic acids and phenolic amides. Ethanol extract and distilled water from black pepper have strong antioxidant activity. Antioxidants work by donating hydrogen atoms or by donating electrons to radical oxygenated species (Aminah & Mursiti, 2021). Piperine has antioxidant activity and reduce thiobarbituric acid reactive substances and maintain levels of superoxide dismutase, catalase, glutathione-S-transferase, and glutathione, this can reduce oxidative stress induced by a high-fat diet in cells. Apart from that, antioxidant compounds are also able to increase the concentration of spermatozoa in vivo in infertile men (Gorgani, 2016).

## CONCLUSION

Based on the results of research that has been carried out, it can be concluded that giving black pepper extract (*Piper nigrum* L.) to mice (*Mus musculus* L.) which is induced by the hormone progestin can increase male fertility as shown by increasing the number of normal morphological spermatozoa and concentration of spermatozoa.

## CONFLICT OF INTEREST

I declare that there is no conflict of interest in writing this scientific work.

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