The swiss webster mice testes structure after exposed to ethanolic neem (*Azadirachta indica*) leaf extract

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Abstract. Neems has been known as an anti-fertility effect, both in male and female mammals. This research was performed to find out the effect of ethanolic leaf extract of *A. indica* on the testes structure that indicated by testicular weight, diameter, and thickness of germinal epithelium of seminiferous tubules. Twenty Swiss Webster male mice were used, and they were divided into two groups (K and P) of ten in replicates. The mice in the K group served as a control group treated with drinking water; while mice in the P group treated with 14 mg/animal/day of ethanolic Neem leaf extract. Treatment was administered orally for 21 days. The mice were sacrificed at 22nd day under chloroform anesthesia. Testes were isolated, weighted and processed with paraffin method continued with HE staining. Diameter and thickness of germinal epithelium of semeniferous tubules were measured on five tubules in every slide of testes. Data were analyzed by Analysis of Variance (ANOVA) continued by DMRT. Our result showed that there was a significant difference (p<0.05) between treated and control group on diameter and thickness of germinal epithelium. In conclusion, spermatogenesis in mice was disrupted by ethanolic of Neem leaf extract.

1. Introduction

The main function of the testes is to produce the male germinal component (sperm) needed for reproduction and furthermore the testes are also important for the production of androgens [1, 2]. Sperms were produced at semeniferous tubules in testis which composing the testicular tissue. The seminiferous epithelium is formed by Sertoli cells that supporting successive synchronized populations of maturing germ cells. The wall of semeniferous tubule was named germinal epithelium semeniferous [1]. The Sertoli cell is a part of semeniferous tubules that regulate the spermatogenic wave by secreting Androgen Binding Protein [3]. The semeniferous tubules separated one another by interstitium containing Leydig (interstitial) cells, vasculature, macrophages, a protein, and testosterone-rich ultrafiltrate, and supporting stroma⁴. Leydig cells are the part of testes structure which produces the main testosterone, namely androgen[2].

Histopathologic evaluation of the testis is an important component of drug safety assessment and evaluation of environmental toxicants. Auta and Hasan (2016) stated that weight loss of the gonads is considered standard criteria for the characterization of a toxic agent that may disturb the animal fertility potency⁵. The exposure of the toxic agent may disturb the pituitary-hypothalamic or sex hormonal which in turn affected the spermatogenesis. Hafez and Hafez stated that the toxin compound could cause a reduction in testicular weight probably due to a reduction spermatogenesis⁶.

One of thousand plant that was proved has anti-fertility activity Neem (*Azadirachta indica*) [4]. Bansal et al. (2010) stated that Neem leaf extract has an antispermatic effect. Neem leaf extract shows an antiandrogenic that was characterized by testes histological and biochemical parameters.
changes [7]. The effectiveness of Neem as anti-fertility herb could be caused by more than 135 compounds [8] that have a cytotoxic and cytostatic effect [9]. Azadirachta in an example of the Neem compounds which supposed has anti-fertility action and Saladin that supposed as anti-spermicidal activities [9]. Flavonoid in Neem leaf extract is a compound that inhibits the aromatase enzyme [10]. The precise mechanism of Neem anti-fertility agent is not described clearly, but it predicted by two ways [10]. The first mechanism is by cytotoxic or cytostatic and the second is by disturbing hormones balance in target animals [11]. The effect of ethanolic Neem leaf extract at doses of 8.4; 11.2; and 14 mg/animal/day has a disrupting effect on female mice reproductive performance [12]. Since that male organism has gonads which homolog in structure and function of female gonads function, it could be potentially disrupted by Neem compounds also.

Base on that fact, this study was performed to find out the effect of ethanolic Neem leaf extract on Mice testes structure which characterized by testicular weight, diameter, and thickness of germinal epithelium of seminiferous tubules. The outcome of this study is offering the advantages of Neem leaf extract as an effective herb contraceptive in fertility control method.

2. Materials and Methods
2.1. Preparation of the ethanolic leaf extract of Neem
The neem leaf was collected from Diponegoro University campus area. Then the leaf was rinsed and dried at 40-50 °C for ten days. The leaf ethanolic extract was made by the maceration method using 70% ethanol as explained by Sitasiwi et al. [12]. The extracts were kept in 4 °C in a dark closed bottle until anti-fertility testing.

2.2. Laboratory Animals
Twenty male Swiss Webster mice with 25-30 g in body weight were used as test animals. The mice were obtained from the Department of Biology Laboratory of Semarang State University, Indonesia. They were acclimatized for seven days in the laboratory condition before being used for experiments. The animals were handled in a well-controlled room, with a temperature of 26 ± 1°C. The feeds and water were given ad libitum.

2.3. Experimental Design and Treatment of Animals Procedures
Twenty-eight mice were used, and they were divided into two groups (K and P) each with ten in replicates. The mice in group K served as control group treated was given only freshwater, while mice in Group P 14 mg/animal/day of Neem leaf extract. Neem extracts were dissolved in boiling water and left at room temperature for use. The treatment was administered orally by using gavage at 8.00-9.00 pm, for 21 days.

2.4. Testis structure evaluation
The day after the last treatment, mice were sacrificed in 22nd day under chloroform anesthesia. The testing animals were dissected from the lower of the abdominal part and continued by isolating the left testis. After that, testes were rinsed in buffered saline solution and weighed. Testes fixed in 10% phosphate buffered saline fixative and then processed by paraffin method with Hematoxylin-Eosin staining in eight microns thickness section. The tubular diameter (DST) and the germinal epithelium thickness (GET) of seminiferous tubules were measured with a 100X magnification. The diameter of seminiferous tubules was determined by counting the mean of the largest and smallest size in five seminiferous tubules on every testes slide. The germinal epithelium thickness was measured from the basal membrane to the lumen of seminiferous tubules [12].

2.5. Statistical analysis
The data were expressed as the mean ± standard deviation, each with ten replicates. The collected data were analyzed by One-way Analysis of Variance (ANOVA) and continued by Duncan Mean Range Test. P-values differences at p<0.05 were considered significant. SPSS was applied to analyze the data.
3. Results

The result shows that the ethanolic Neem leaf extract interferes the testes structure of testing animals (Table 1.). The testicular weight (TW), the diameter of seminiferous tubules (DST) and germinal epithelium thickness of seminiferous tubules (GET) of treated animals show a significant difference (p<0.05) with the control group.

Table 1. Testicular weight, the diameter of seminiferous tubules, and germinal epithelium thickness of mice exposed to ethanolic Neem leaf extract for 21 days

|       | TW (g)   | DST Mm | GET μm          |
|-------|----------|--------|-----------------|
| K (n=10) | 1.249² ± 0.061 | 179.38² ± 3.04 | 57.59² ± 0.063 |
| P (n=10) | 0.942² ± 0.073 | 153.14³ ± 2.15 | 34.58² ± 1.029 |

Note: Values are expressed as Means ± SD. Means in the same column with different superscript letters are significantly different (P>0.05). TW= Testicular weight; DST= Diameter of Seminiferous Tubules; GET= Germinal Epithelium Thickness

The comparison of testes histological structure between the control and the treated group was presented in Figure 1. The structure of seminiferous tubules in the control group shows a normal structure that has an intact basal membrane (Figure 1.A.). The seminiferous structure of control group also shows a sticky structure one another. The germinal epithelium is strongly attached to the basal membrane. Contrary with that normal structure, the seminiferous tubules structure of the treated have an abnormal structure that indicated by damage in the basal membrane (Figure 1.B, in a red arrow). The germinal epithelium detaches from the basal membrane (Figure 1.B. in the blue arrow). The damage of testes structure of treated group also shows in the interstitial space (Figure 1.B, in the black arrow) that made seminiferous tubules apart one another.

Figure 1. Comparison of mice semeniferous tubules structure after treated with ethanolic Neem leaf extract for 21 days (HE staining; 100x magnification)

Note A: Control group; B: Treated group.

4. Discussion

Testis is a male reproductive organ that comprises of seminiferous tubules. The seminiferous tubules composed of the germinal epithelium in varies development stage. By that reason, testicular weight expressing the sperm production process that occurs in seminiferous tubules. In this present study, the testicular weights decrease could be a consequence of changes not only in diameter (DST) but also in the germinal epithelium thickness (GET) in seminiferous tubules. Gomendio et al. and Ekaluo et al. stated that toxicant exposure, like the ethanolic Neem extract, could interfere the animal testes weight¹³, ¹⁴. The testes weight reduction in this research showed that the Neem compound interferes the spermatogenesis and then decreasing the seminiferous tubules diameter and testes weight.

As the data were analyzed by ANOVA, the p-value was found the significant difference (p<0.05) between control and treated group. This result was due to the anti-fertility effect of Neem which was administered. The decreasing number or size of all parameters in this research (Table 1) proves that the anti-fertility effect of ethanolic Neem extract acts on the testicular structure through a series of mechanisms. These data illustrate that the decrease in testicular weight could be caused by
the decrease in the size of germinal epithelium thickness (GET). The size of getting express the spermatogenesis process rate of testing animals in the treated group. The arrest of mice spermatogenesis process is one of many anti-fertility effects, as stated by Assif et al.15.

Since the testicular mass was composed of seminiferous tubules and interstitial tissue, the lower testicular weight of treated group could because by the damage in the interstitial tissue, especially Leydig cells. The neem extract was proved that have decreasing effect in the number of Leydig cells1. This opinion was in line with the testes histological section (Figure 2.A and B) that shows the damage of the interstitial space (red arrow) of the testes in the treated group. As Leydig cells are responsible for the manufacturing of testosterone1, this obtained study shows that testosterone level in the treated group was predicted in the low level than the control group. According to Silva et al., exposure to the extracts could have pituitary-hypothalamic feedback effects which in turn affected testosterone synthesis in testes16. The active component of Neem extract that has an effect in disturbing hypothalamic-hypophysis axis by disturbing gonadotrophin synthesis and secretion are flavonoid, triterpenoids, and saponin17, 18. It could be concluded that antifertility compounds in Neem act by the hormonal mechanism, so the spermatogenic wave was disturbed. Ultimately, spermatogenesis disorders cause low values in germinal epithelial thickness in seminiferous tubules.

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