Histomorphology of testis in male mice (Mus musculus albinus) post given the aqueous seed extract of neem (Azadirachta indica A. Juss)

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Abstract. Neem has antispermatic effect in animals and male men. The objectives of this research are to evaluate the effect of aqueous seed extract of neem on the structure of testis. Male mice of DDY strain were divided into five groups. Group I as a control group (C1-1) with the absence of treatment. Group II and III (s1-1 and s2-1) were orally treated by administered aqueous seeds extract of neem at doses 0.25 and 0.50 mg/kg body weight (bw) for 36 days. Group IV and V (S1-2 and S2-2) was treated by administering aqueous seeds extract of neem at doses 0.25 and 0.50 mg/kg bw for 36 days followed with another 36 days with no treatment. The results showed that neem seed extracts cause a decrease of spermatogenic cell (p<0.05). It can be concluded that exposure to the aqueous seed extracts of neem can decrease spermatogenic cell, and its effect more than 36 days.

1. Introduction

Neem is one of the most widely used medicinal plants in the world such as antimicrobial, antiviral, antifungal, antimalarial, antioxidation and anticarcinogenesis properties [1]. Studies have shown that the all parts of the neem have some medicinal properties [2]. Furthermore neem has been used as traditional medicines for treatment such as antimalarials, spermicides, antituberculosis agents, anti pyrretics, antiviral drugs, antiseborrheics, anti-allergic medicines, antienzymic, and antifungal agents [3]. Neem have antiseptic, anti-helminth, antibacterial, anti-diabetic and antifertility properties [4]. Therefore, the continuous investigation of neem for safety and toxicity using animal models to evaluated the responses on chemical agents varies widely [5].

Some plant made products have been proven to effectively reduced male and female fertility rate and can be used as a contraceptive [6]. Several plants such as allium sativum, aegle marmelos, garcinia cambogia, and azadirachta indica are reported to impede various stages of testicular spermatogenesis in many different animal species such as dogs, rats, humans and monkeys [7]. Its
product is cyclo phosphamide (acrolein) from *allium sativum* [8], *eagle marmelos* has been found to contain marmin and fagarine [9], *garcinia cambogia* consists of alkaloids, phenolic, carbohydrates, steroids, proteins, terpenoids, tannins [10] and containing hydroxycitric acid (HCA) which has given suppression effect in epididymal in male rats [11], component of *azadirachta indica* substance i.e. flavonoid, tannin, alkaloid, steroid, triterpenoids, phenol, carotenoid, steroid and ketone [12].

The results showed that neem seeds extract decreases sperm quality in male mice due to reduced motility, viability, concentration and morphology of normal sperm. The impact of neem treatment still lasts for 36 days post last treatment. The administration of neem seeds treatment does not damage sperm DNA quality in male mice, but only reduces the testis function [13]. So, the objective this research to evaluated the effect of aqueous seed extract of neem on the structure of testis.

In the male reproductive system, weight loss of the gonads as well as reduced sperm count and epididymal sperm motility are considered standard criteria for the characterization of toxic agents that may cause fertility problems in the treated subject [14]. Sperms morphology serves as an important and sensitive indicator of chemical toxicity on the reproductive cells. They can be used to evaluate the spermatogenic damage, fertility and heritable genetic changes, which provide a direct measure of the quality of sperm production in chemically treated animals [15-17].

Spermatogenesis is a complex process by which an interdependent population of undifferentiated germ cells undergoes multiplication and maturation to form functional haploid spermatozoa. Spermatogenesis consists of three phases: (a) the spermatogonial phase; (b) the spermatocyte phase; and (c) the spermatid phase. During the spermatogonial phase, the diploid spermatogonium undergoes mitosis to form stem cells and primary spermatocytes. This is followed by the spermatocyte phase, in which the primary spermatocytes undergo two rounds of meiosis to form haploid spermatids. The final phase, also called spermiogenesis, involves the differentiation of immature spermatids into mature spermatozoa. Spermiogenesis comprises polarization of the spermatid, formation of acrosomal cap and flagellum, cytoplasmic remodeling and elongation of the nucleus. Endocrine regulation by testosterone and the architecture of the Sertoli cells and seminiferous tubules also forms an important decisive factor in spermatogenesis [18].

### 2. Materials and methods

#### 2.1. Plant material and preparation of extract

Neem seeds (*Azadirachta indica* A. Juss) were harvested from a plantation in laboratory of Ingredients and Medicine Plants Unit (BALITTRO) in Bogor, Indonesia state. The seeds were dried, powderized, and extracted with the use of aqueous. The extract resulted was analyzed to determine the phytochemical content were carried out using the methods of Harborne [19] in relation to the function of testis histomorphology.

#### 2.2. Animals and ethical clearance

Twenty five male DDY mice (12-14 weeks old, 28-32 g) were obtained from the Animal Breeding Center Jakarta, Indonesia National Agency of Drug and Food Control, Laboratory Facilities of Ministry of Health). After acclimatization for 2 weeks, the mice were randomly divided into 5 groups (n=5) and gavaged with 0, 0.25, and 0.50 mg/kg bw/day of neem seed aqueous extract for 36 days treatment and stopped the treatment after 36 days were performed orally. The use animals and the experimental protocol were in compliance to the guidelines set by laboratory animals of Bogor Agricultural University of Animal Care and Use Committee (ACUC) Faculty of Veterinary Medicine Ethics Committee and that the National Institute of Health Guide for Care and Use of Laboratory Animals (Ethical Approval Letter ACUC Number: 038/KEH/SKE/VI/2015).
2.3. Histological examination

Animals were dissected and their testes were removed. The testis was used for histological examined and then its was fixed in Bouin’s fluid, dehydrated, cleared and embedded in paraffin wax, cut at ~ 5µm and stained with Haematoxylin and eosin.

2.4. Statistical analysis

Data were analyzed with SAS system (Version 9.0) and presented as mean ± standard deviation (SD). Statistical significance was evaluated with Least Significant Difference (LSD)-test and the difference was considered statistically significant at p<0.05.

3. Results and discussion

3.1. Results

The results obtained showed significant difference in the amount of spermatogenic cells among male mice exposed to different doses of aqueous seed extract of neem when compared to the control group (figure 1).

![Image](image1.jpg)

**Figure 1.** Morphology changes of mice testis when treated with aqueous seed neem extract with various of doses. (a) 0.25 mg/kg bw (100x) (36 days); (b) 0.25 mg/kg bw (400x) (36 days); A. (Control) (100x), B. Control (400x); C. 0.25 mg/kg bw (400x) (36 days); D. 0.50 mg/kg bw (400x) (36 days); E. 0.25 mg/kg bw (400x) (72 days); F. 0.50 mg/kg bw (400x) (72 days). Haematoxylin & Eosin staining. (400x).

Results obtained from the Figure 1 showed amounts of spermatogenic cells with numerous regular and irregular variably sized in seminiferous tubules. As presented in Figure 1 showed spermatogenic cells contain necrotic debris, irregular cells and moderately depleted amounts of spermatogenic cells in seminiferous tubules (C, D, E and F) (p<0.05). After 36 days of aqueous seed extract of neem exposure and 36 days after stop treatment spermatogenic cells damage occurred. Base on the Fig.1 (C, D) showed the increased depletion of seminiferous tubules observed in the histopathology of testes of
mice orally exposed aqueous extract of neem. Aqueous seed extract of neem responsible for the significant decrease in spermatogenic cells, damage of the cells and increase abnormal sperm cells. Its effect decrease in sperm motility, live/dead sperms and an increase in the number of abnormal sperm cells, which is an indication of decrease fertility [13]. The apparent a decrease in the number of spermatogenic cells are still happen post 36 days stop the treatment.

Similarly with the presented in Figure 1, histopathological changes in the testis exposed aqueous extract of neem caused the damage of the spermatogenic cells such as vacuolation and necrosis of the late elongated spermatids, numerous apoptotic cells and formation of multinucleated giant cells in the seminiferous tubules [14]. So, its means the aqueous seed extracts of neem have toxic effect on spermatogenesis. Furthermore immobilization of spermatozoa caused by the content of compounds in plants can have an impact on death. Several other plants are also known to hamper fertility by targeting spermatogenesis at various stages [7]. As presented in Figure 1 (C), histopathology of mice testes exposed to aqueous seed extract of neem showed reduction in the density of the spread of spermatogenic cells with dose of 0.25 mg / kg bw. A decrease in the density of the spermatozoa is also seen in the lumen of the seminiferous tubules. The decrease was with increase in the dose concentration of extract administered, when compared to the control group. However in E and F, mice administered (0.25 and 0.50 mg/kg bw for 36 days and stopped the treatment after 36 days shows variably sized spermatogenic cells, seminiferous tubules contain necrotic debris and depleted amounts of spermatogenic cells. The effects were seen to be irreversible on withdrawal of the aqueous seed extract for 36 days. The results obtained from histomorphology changes of testis showed significant decrease in percentage spermatogenic cells such as spermatogonia, spermatocyte and spermatid with increase in dose concentration of aqueous seed extract of neem in male mice (Table 1).

### Table 1. The average of spermatogenesis post treatment with the aqueous seed extract of neem in mice for 36 days followed with another 36 days no treatment.

| Treatment | Spermatogonia | Spermatosit | Spermatid |
|-----------|---------------|-------------|-----------|
| C 1-1     | 82.50 ± 0.01a | 83.50 ± 0.01a | 177.00 ± 0.01a |
| s 1-1     | 66.75 ± 0.02b | 63.00 ± 0.02b | 64.00 ± 0.02bc |
| s 2-1     | 55.50 ± 0.02d | 63.50 ± 0.02b | 63.75 ± 0.02bc |
| S 1-2     | 61.50 ± 0.01c | 57.75 ± 0.03c | 60.30 ± 0.03c |
| S 2-2     | 47.50 ± 0.01c | 59.50 ± 0.02c | 59.00 ± 0.03bc |

*Significantly different from controls (p<0.05) by ANOVA followed by Least Significant Different (LSD) test. (C 1-1: Control, s1-1 and s2-1: seed (0.25 and 0.50 mg/kg bw (36 d)), S1-2 and S2-2 : Seed (0.25 and 0.50 (72 d)).

#### 3.2. Discussion

In this study, male mice orally administered aqueous seed extract of neem had significant decrease in spermatogenic cells. The decrease in spermatogenic cells still occurs after 36 days of treatment discontinuation. This result may also be due to the effect of this extract have toxic effect on spermatogenesis for 36 days treatment and 36 days after stop treatment. This presumption is caused by the active ingredient content of the neem seed extract. Phytochemical test showed that aqueous seed extract of neem consists of saponins, tannins, flavonoid, alkaloids, triterpenoid, and steroid [13]. The active substance could affect reduced number of spermatogenic cells in mice treated with neem seed extract, allegedly due to saponin, tannins, alkaloids content. Saponins are natural glycosides which possess cytotoxic activity [20], damaging spermatozoa ce
spermatozoa [22], tannins inhibit the sperm motility [23]. Also, the alkaloids significantly affected decreased the seminiferous tubules sperm count, motility, density, and sperm morphology [24]. The results of this study indicate that extracts of leaves and plants extracts of some medicinal plant have good potentials for use in control of birth.

The results in the present study showed that the significant increase of amount of spermatogenic cells damage in exposed mice, which increase with increase in doses. This indicates that aqueous seed extract of neem have good potentials for use in antifertility, but the effect of aqueous seed extract of neem causes testicular toxicity by decrease in spermatogenic cells. Furthermore the rat exposure to the neem seed caused the sperm immobilization activity of azadirachtin-A in rats and it has been studied that azadirachtin-A effectively inhibits sperm motility, viability and plasma membrane integrity in a dose-dependent manner [25]. Aqueous wood ash extract of A. indica might have caused damage to the premeiotic stages of spermatogenesis since during spermatogenesis, DNA synthesis occurs before pre-meiotic phase and no further DNA synthesis occurs throughout spermatogenesis in the cell cycle [14]. The other result showed effect of the neem treatment on the frequency of affected seminiferous tubules in mice testes. The affected seminiferous tubules in testes of neem treated mice showed intraepithelial vacuolation, loosening of germinal epithelium, marginal condensation of chromatin in round spermatids, occurrence of giant cells, and degeneration of germ cells [26]. In addition that there is reduced motility of sperm after reacting with neem oil for 20 min. The neem oil is possibly acting upon the enzymes in mitochondria that are responsible for production of ATP. Thus the depletion of ATP is resulting in reduction of sperm motility [27].

4. Conclusions

Aqueous seed extract of neem caused decrease in number of spermatogenic cells (spermatogonia, spermatosit and spermatic), which is an indication of infertility. Histopathological changes in the testis such as vacuolation and necrosis of the late elongated spermatids, numerous apoptotic cells and formation of multinucleated giant cells in the seminiferous tubules means the extracts have toxic effect on spermatogenesis for 36 days treatment and stopped the treatment after 36 days. Therefore, neem was given orally not can’t be safely, by acting as a spermatoxic agent on spermatogenesis.

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References

[1] P Kupradinun, A Tepsuwan, N Tanhasri, N Meesiripan, S Tunsakul, W Tompat, Y Jarratwisarutporn and W R Kusamran 2010 The Thai J. Vet. Med 40(1)
[2] Biswas K, Chattopadhyay I, Banerjee R K and Bandyopadhyay U 2002 Current Sci, 82(10)
[3] Sharma A and Bhattacharyya K G 2005 J. Hazard. Mater 125 (1) 11
[4] Habila N, Humphrey NC and Abel A S 2011 Vet. Parasitol 180 (3-4) 6
[5] A O T Ashafa, L O Orekoya and M T Yakubu 2012 Asian J. Trop. Biomed 2(10)
[6] Joshi SC, Sharma A and Chaturvedi M 2011 Int. J. Pharm. Pharm. Sci. 3(5) 14
[7] S C D’Cruz, S Vaithinathan, R Jubendradass and P P Mathur 2010 Asian J. Androl 12(12)
[8] M Mirfardi and H Zahedan J. Res. Med. Sci. 15(5)
[9] S Rahman and R Parvin 2014 Asian Pac. J. Trop. Dis 4(1)
[10] M George, L Joseph and Ashitha KS 2015 Int. J. Univ. Pharm. Bio. Sci. 4(6) 18
[11] Saito M, Ueno M, Ogino S, Kubo K, Nagata J and Takeuchi M 2005 Food. Chem. Toxicol 43(3)
[12] Hashmat I, Hussain A, and Ajji A 2012 Int. Res. J. Biol. Sci. 1(6) 4
[13] E Lisanti, D Sajuthi, M Agil, R I Arifiantini and A Winarto 2016 IOSR. J. Pharm. 10
[14] T Auta and A T Hassan 2016 Asian Pac. J. Reprod 5(2) 5
[15] Dev K R, Yadamma K and Reddy K D 2013 Int. J. Pharm. Biol. Sci. 4(1)
[16] Gautam D, Sharma G and Goyal R.P 2010 Pharm online 1 (8)
[17] Bakare A A, Okunola A A, Adetunji A O and Jenmi B H 2009 Gen. Mol. Biol. 32(2)
[18] Saez J M, Avallet O, Lejeune H and Chatelain P G 1991 Horm. Res., 36(12)
[19] Harborne J B 1987 PhysDescr 2 (Imprint: London, NY, Chapman and Hall 1987)
[20] Podolak I, Galanty A and Sobolewska D 2010 Phytochem. Rev. 9(3) 49
[21] Z Lu, L Wang, R Zhou, Y Qiu, L Yang, C Zhang, M Cai and M MiH 2013 Plos One 8 (11) 11
[22] S B Kumbar, U C Jadaramkunti and R H Aladakatti 2012 J. Phytother. Pharmacol 1 13
[23] B Zhou, Z Qiu, G Liu, C Liu and J Zhang 2012 J. Med. Plants. Res. 6 (7) 6
[24] M T Yakubu 2012 J. Andro. 133 (6) 9
[25] R H Aladakatti and U C Jadaramkunti 2015 Int. J. Gen. Chem 1(1) 4
[26] R K Mishra and S K Singh 2005 Indian J. Exp. Biol. 43(11)
[27] Patil P and Gaikwad R D 2009 Sawane M V and Waghmare V S Online J. Health. Allied.Sci. 8(4)