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The DNA and Spermatozoa Quality of Mice (Mus musculus albinus) after Administration Aqueous Leaves and Seeds Extract of Neem (Azadirachta indica A. Juss)

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Objective*:** This study aimed to evaluate the DNA of mice spermatozoa quality, and its effect after aqueous leaves and seeds extract of neem treatment. **Method:** Thirty six male mice were divided into nine groups. The first group was plotted as a control group and four groups were orally treated by neem leaves extract (two different doses groups) and seeds extract (two groups) in 0.5 mL/mouse/day at doses 2.5 and 5.0 mg/kg BW each extract treated for thirty six days then sacrificed. The other four groups were treated same as those in previous four groups and sacrificed at thirty six days post last treatment (day 72nd). Spermatozoa evaluated for its motility, viability, spermatozoa concentration, morphology and DNA damaged. **Results:** neem leaves extract caused a decrease of sperm motility, concentration, and morphology ($P<0.05$) but there were not differ in viability spermatozoa ($P>0.05$). Neem extract stopped for 36th days, indicated spermatozoa quality is not fully recovered. The DNA damaged evaluation using Halomax kit showed that extract were not damaged to DNA. ***Conclusion: Leaves and seeds extract of neem have a potential to control spermatozoa motility, viability and its concentration. The required time to physiologically recover from extract treatment effect was more than 36 days.

Keywords : *Neem, aqueous extract, DNA, mice, spermatozoa*

I. INTRODUCTION

Family Planning Program was first proclaimed by the Indonesian government program on June 1970, along with the establishment of National Population and Family Planning Board (BKKBN). However this program has not resulted to the optimum yet (the Ministry of Health Republic of Indonesia 2014).[1] The data of BKKBN (2015) showed that population rate in Indonesia has been quite worrying with an increase of 1.49% or 4.5 million per annum.[2] The prevalence of family planning in Indonesia has reached 75.8% in its implementation with 74.2% female acceptor and only 1.6% male acceptor respectively.

Some plant made products have been proven to effectively reduced male and female fertility rate and can be used as a contraceptive.[3] The mechanism of using male contraceptive serves as an anti-spermatogenesis agent which controls sperm production, prevents sperm maturation, and hinders sperm transport along vas deferens[4] The ideal male contraceptive has to be widely available, low cost, friendly use in addition to having no effect and impact upon libido, and being reversible in its nature. [5]

Neem is one plant that commonly used as a male contraceptive. It contains numerous components of chemical substance i.e. flavonoid, tannin, alkaloid, steroid, triterpenoids, phenol, carotenoid, steroid and ketone. [6] Compounds of substances may function as anti-fertility which principally works in two ways, by cytotoxic or cytostatic and hormonal effects that inhibit metabolism rate of genital cell by unbalancing the hormone [7].

Flavonoid results anti-spermatogenic effect by decreasing sperm quality [8]. It can inhibit a number of oxidation reactions of either enzyme or non-enzyme [9]. Such hindrance occurring in body will inhibit as well some cell development processes including the genital cell during gametogenesis. Saponins is function to decrease motility, viability and sperm morphology with the absence of toxic effect [3]. Tannin is an active substance contained in a plant with astringent taste and able to inhibit protein synthesis [9].

Neem activity as anti-spermatogenic substance has been formerly reported by some researchers, but this information has not been widely spread out. The report stated that treatment of aqueous neem leaves extract for 28 days results anti-spermatogenic activity in male mice (sperm motility, amount, and abnormality[10], (anti-androgenic [11], spermicides and anti-fertility, [7] in addition to reducing fertility rate [12]. Testis functions as a place for spermatogenesis and androgen production. Spermatogenesis occurs in a structure called seminiferous tubules and takes about 34.5-35.5 days to complete [13].

The information that neem leaves were considered as future natural medicine for male contraceptive. However, a series of studies is further required for ensuring that neem leaves has anti-spermatogenic effect on male. For safety reason, reversibility effect and the sperm DNA quality post administration of aqueous neem leaves and neem seeds extract need to be evaluate. This study aimed to evaluate neem leaves and neem extract potential to control DNA and sperm quality, and the length of reversibility effect after treatment.

II. MATERIALS AND METHODS

2.1 Extract Material:

Neem leaves and seeds (*Azadirachta indica A. Juss*) have been selected as the main materials of the research and were collected from laboratory of Ingredients and Medicine Plants Unit (BALITTRO), Bogor. The raw materials were dried by using an oven with temperature or 60°C, grinded to powder and macerated and extracted with the use of aqueous. The extract resulted was analyzed to determine the phytochemical content in relation to the function of anti- spermatogenic and anti-fertility.

2.2 Preparation of Mice:

A *completely randomized design* (CRD) was applied in this experimental research with four-time repetition. Thirty six male mice weighing 20 to 25 g (DDY strain) (from Animal Breeding Center Jakarta, Indonesia National Agency of Drug and Food Control, Laboratory Facilities of Ministry of Health) with age 12-14 weeks. The animals were fed with pellets (PUR) 512 which is certified mice food (Charoen Pokphand Indonesia) and drinking water was available *ad libitum*. Animals were acclimatized for two weeks before the experiments were started. Body weight of mice were weighed. The study was conducted after obtaining Institutional Animal Ethical Committee clearance.

2.3 Administration of neem treatment:-

Randomized classification of the male mice was conducted by dividing thirty six male mice into nine groups consisting of four mice respectively. Group I was plotted as a control group (C1-1) with the absence of treatment. Group II and III (l1-1 and l2-1) were treated by administering aqueous neem leaves extract at doses 2.5 and 5.0 mg/kg BW for 36 days. Group IV and V (s1-1 and s2-1) were orally treated by administering aqueous neem seeds extract at doses 2.5 and 5.0 mg/kg BW for 36 days.

The treatments were performed orally in 0.5 mL/mouse/day in the morning at 6-8 a.m and sacrificed in the day 36. Group VI and VII (L1-2 and L2-2) was treated by administering aqueous neem leaves extract at doses 2.5 and 5.0 mg/kg BW for 36 days followed with another 36 days with no treatment to see its reversibility. Group VIII and IX (S1-2 and S2-2) was treated by administering aqueous neem seeds extract at doses 2.5 and 5.0 mg/kg BW for 36 days and sampled on day 72, which was 36 days after last treatment. Macroscopic parameter observed are; consistency, color and pH, and microscopic are; motility, viability, concentration and DNA spermatozoa.

2.4 Sampling procedure of sacrificed male mice:

After each treatment, mice from all groups were sacrificed on 36th days and 72th days. An intraperitoneal anesthesia using ketamine (doses 1.5 mg/kg/BW) and xylazine was given to the mice at doses 0.3 mg/kg/BW continue by dislocation *os axis.* Samples from the testis and epididymis collected from all animals were prepared for macro and microscopic evaluation.

III. ETHICAL CLEARANCE

Mice were housed in a temperature (29-30°C ± humidity (62 %) controlled room in which a 12 hour light/12 hour dark cycle was maintained. All procedures using animals were reviewed and approved by the Institutional Animal Ethical Committee. The study and all procedures were carried out in accordance with the guidelines for the care and use 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).

IV. ASSESSMENT OF SEMEN QUALITY EVALUATION

4.1 Macroscopic evaluation of semen

Semen was taken from epididymis to figure out the color which was visually observed, the consistency which was observed from the viscosity of the semen sample, and pH which was measured by means of pH indicator paper.

4.2 Spermatozoa motility:

A conventional method was employed by mixing one drop of semen with 5 drops saline solution. The mixture was then made homogenous, taken out one drop and covered with a cover glass. The observation and calculation of the motile sperm were performed under light microscope (Olympus CH 20, Japan) with 400x magnification of five different fields, and the data were expressed in percentage.

4.3 Spermatozoa viability:

Sperm viability was assessed by using Eosin-Nigrosine staining (Merk Chemicals, Germany) for separated unstained (live) and red-stained (dead) sperm. 10 µL semen samples added with 40 µL Eosin-Nigrosine were made smear slide and dried on hot plate for 15 seconds. The smear slide was observed under light microscope with 400x magnification. Result of coloring indicates that live sperm does not absorb color, whereas the dead sperm absorbs red color, because of failure of plasma membrane. Percentage of sperm viability was determined based on the ratio between live sperm count and the total of live and dead sperm count. Mean ± SEM of motility and viability data of control and treated groups were determined.

4.4 Spermatozoa concentration:

Semen was diluted with Formol saline solution with ratio1:500. The mixed solution was then put into Neubauer chamber, and calculated with 400x magnification. The spermatozoa were counted from each sample in five fields of vision randomly and be counted with $5 \times 25 \times 10^6$ million/mL and percentage of concentration spermatozoa was recorded.

4.5 Spermatozoa morphology:

Morphological observation was conducted by making sperm smear of the mixture of semen and saline solution with ratio 1:10 and followed by Williams stained. Normal and abnormal sperm were then evaluated under light microscope with 400x magnification.

4.6 Spermatozoa DNA quality of male mice using *Kit Halomax*®:

Spermatozoa which is able to fertilize the eggs has to be a live, motile and intact DNA. [14] DNA damage in mice sperm was examined by following the protocol included with the SPERM-HALOMAX-Mus Kit (ChomaCell SL, Madrid, Spain), with slight staining modifications. Spermatozoa were grouped as with or without a halo. Kit Halomax® principally works to see sperm DNA fragmentation (SDF) which can be noticed from the chromatin coming out of the cell in which called ‘halo’ for the damaged and shows no ‘halo’ for the intact DNA. [15] The percentage of spermatozoa with a halo was recorded for each animal.

4.7 Statistical Analysis:

All treatments were run in four replicates. Data were analyzed with statistical analysis system using SAS software. All data for control and experimental groups were subjected to statistical evaluation, using Tukey-test. Statistical significance was determined by one analysis of variance (ANOVA). The differences were considered significant when $P<0.05$. The values were expressed as mean ± SD.

V. RESULTS

5.1 Phytochemical extract of aqueous neem leaves and seeds:

Phytochemical testing shows that neem leaves and seeds contain flavonoid as quercetin, tannin and saponins (Table 1). Neem leaves extract contains more flavonoid, tannin and saponins than neem seeds extract (Table 2). Quercetin has biological activities as anti-bacterial, anti-oxidant, hypolipidemia, and anti-spermatogenic. [16]

TABLE I: Phytochemical content

Analysis from powder	Aqueous extracts of neem		Testing method
	Leaves	Seeds	
Phytochemical testing			Qualitative maceration
Saponins	+	+	
Tannin	+	+	
Flavonoid	+	+	
Alkaloids	+	+	
Triterpenoid	+	+	
Steroid	+	+	
Steroid	+	+	

"+" represents Present; "-"= represent absent

TABLE II: Active materials in aqueous neem leaves and seeds extract which is assumed to have an effect on spermatogenesis

Analysis of extract	Aqueous extracts of neem		Testing method
	Leaves	Seeds	
Flavonoid as quercetin (%)	0.82	0.014	Spectrophotometri
Tannin (%)	11.18	0.48	Spectrophotometri
Saponins (%)	2.65	2.8	TLC Scanner

5.2 Semen macroscopic evaluated:

According to macroscopic consistency, the color and pH of semen (Table 3) do not differ from the control group ($P>0.05$) post administration of aqueous neem leaves and neem seeds extract as well as 36 days of neem treatment. Result of pH measurement shows that the mice semen values range normal: 6.5-6.8. Normal semen pH of mice is the range 6.0-7.0. [17]

TABLE III: Macroscopic semen after treatment and recover

Treatment	Macroscopic Semen		
	Consistency	Color	pH
C 1-1	Viscose	White	6.8±0.14
l 1-1	Viscose	White	6.6±0.17
l 2-1	Viscose	White	6.6±0.14
s 1-1	Viscose	White	6.6±0.15
s 2-1	Viscose	White	6.7±0.12
L 1-2	Viscose	White	6.6±0.13
L 2-2	Viscose	White	6.5±0.08
S 1-2	Viscose	White	6.5±0.13
S 2-2	Viscose	White	6.5±0.12

5.3 Microscopic semen evaluation:

5.3.1 Spermatozoa motility and viability

Spermatozoa motility and viability (Figure 1 and 2) shows that there were a decrease in motility when compared to the control group ($P<0.05$) post administration of neem leaves and seeds extract. The decrease of viability may be figured out in 36 days post stopped treatment when compared to the control ($P<0.05$).

TABLE II. Spermatozoa motility after neem leaves and seeds treatment

T. NO	TREATMENTS	MOTILITY (%)
I.	C 1-1	82.5 ± 7.5^a
II.	l 1-1	57.5 ± 5.7^b
III.	l 2-1	59.5 ± 6.3^b
IV.	s 1-1	60.5 ± 2.4^b
V.	s 2-1	64.5 ± 3.1^b

VI.	L 1-2	32.5 ± 4.8^{b}
VII.	L 2-2	32.5 ± 4.8^{b}
VIII.	S 1-2	25.0 ± 6.3^{bc}
IX.	S 2-2	15.0 ± 4.0^{c}

Values were expressed as Mean ± SEM (n = 4). * Significant difference P<0.05 versus control

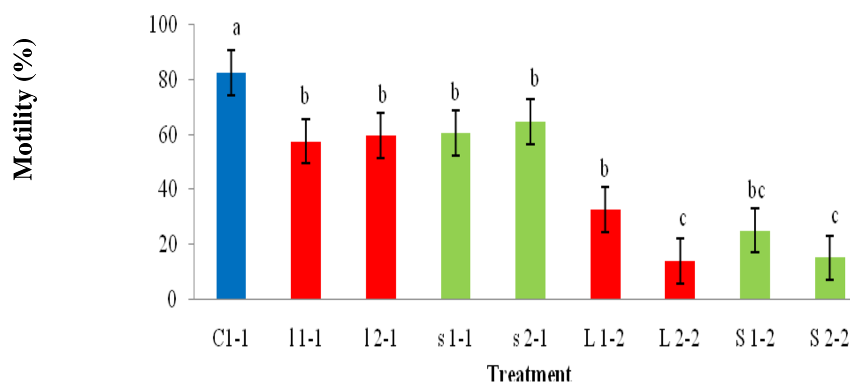


Fig 1: Spermatozoa Motility after Neem Leaves and Seeds Treatment
(C1-1 = Control, l1-1, s1-1, s2-1 = leaves (l) and seeds (s) at doses 0.25 and 0.50 mg.kg BW for 36 days; L1-2, L2-2, S1-2, S2-2 = Leaves (L) and Seeds (S) at doses 2.5 and 0.50 mg/kg for 36 days, and with no treatment for another 36 days

TABLE III. Spermatozoa viability post neem leaves and seeds treatment

T. NO	TREATMENTS	VIABILITY (%)
I.	C 1-1	83.4 ± 2.0^{a}
II.	l 1-1	74.0 ± 6.2^{a}
III.	l 2-1	74.9 ± 6.0^{a}
IV.	s 1-1	79.3 ± 2.6^{a}
V.	s 2-1	69.1 ± 6.3^{a}
VI.	L 1-2	36.2 ± 4.3^{c}
VII.	L 2-2	24.5 ± 2.5^{d}
VIII.	S 1-2	49.6 ± 3.6^{b}
IX.	S 2-2	39.7 ± 0.8^{c}

Values were expressed as Mean ± SEM (n = 4). * Significant difference P<0.05 versus control.

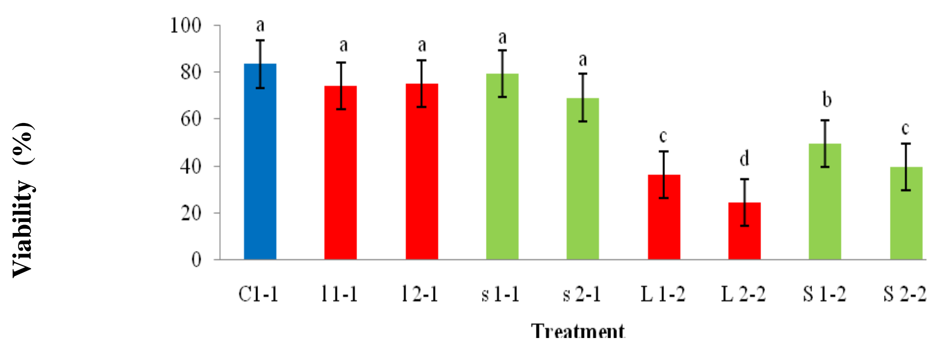


Fig 2: Viability after neem leaves and seeds treatment

(C1-1 = Control, l1-1, s1-1, s2-1 = leaves (l) and seeds (s) at doses 0.25 and 0.50 mg.kg BW for 36 days; L1-2, L2-2, S1-2, S2-2 = Leaves (L) and Seeds (S) at doses 2.5 and 0.50 mg/kg for 36 days, and with no treatment for another 36 days

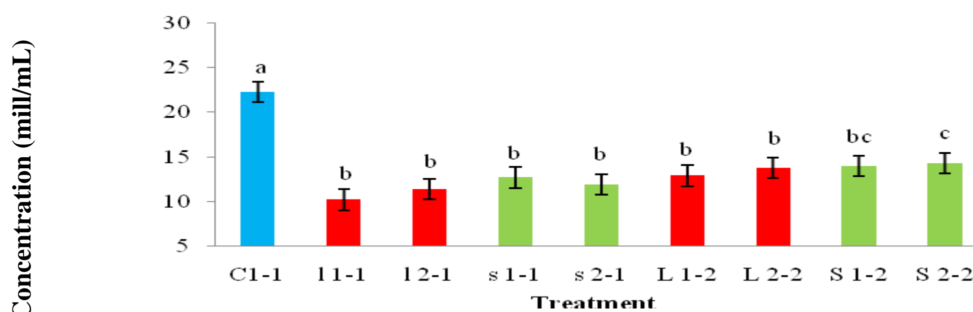
5.3.2 Spermatozoa concentration and morphology:

Spermatozoa concentration (Figure 3) shows a decrease post administration of the extract (neem leaves and seeds) and post 36 days of stopped treatment when compared to the control group (P<0.05). Spermatozoa morphology also shows a decrease normal sperm count post extract administration and this continues until day 36 post stopped treatment when compared to the control (P<0.05) (Figure 4).

TABLE IV. Spermatozoa concentration after neem leaves and seeds treatment

T. NO	TREATMENTS	CONCENTRATION (mill/mL)
I.	C 1-1	22.3 ± 1.8^{a}
II.	l 1-1	10.2 ± 2.0^{b}
III.	l 2-1	11.4 ± 1.9^{b}
IV.	s 1-1	12.7 ± 2.2^{b}
V.	s 2-1	11.9 ± 3.4^{b}
VI.	L 1-2	12.9 ± 1.6^{b}
VII.	L 2-2	13.8 ± 1.7^{b}
VIII.	S 1-2	14.0 ± 2.1^{bc}
IX.	S 2-2	14.3 ± 1.3^{c}

Values were expressed as Mean ± SEM (n = 4). * Significant difference P<0.05 versus control



Spermatozoa concentration of male mice after administration of neem leaves and seeds extract (C1-1 = Control, l1-1, s1-1, s2-1 = leaves (l) and seeds (s) at doses 0.25 and 0.50 mg.kg BW for 36 days; L1-2, L2-2, S1-2, S2-2 = Leaves (L) and Seeds (S) at doses 2.5 and 0.50 mg/kg for 36 days, and with no treatment for another 36 days

TABLE V. Spermatozoa morphology after neem leaves and seeds treatment

T. NO	TREATMENTS	MORPHOLOGY (%)
I.	C 1-1	97.8 ± 0.50^{a}
II.	l 1-1	96.0 ± 0.28^{b}
III.	l 2-1	95.0 ± 0.25^{c}
IV.	s 1-1	94.5 ± 0.28^{cd}
V.	s 2-1	94.0 ± 0.01^{d}
VI.	L 1-2	95.3 ± 0.25^{b}
VII.	L 2-2	94.5 ± 0.28^{bc}
VIII.	S 1-2	94.3 ± 0.25^{c}
IX.	S 2-2	93.8 ± 0.25^{c}

Values were expressed as Mean ± SEM (n = 4). * Significant difference P<0.05 versus control

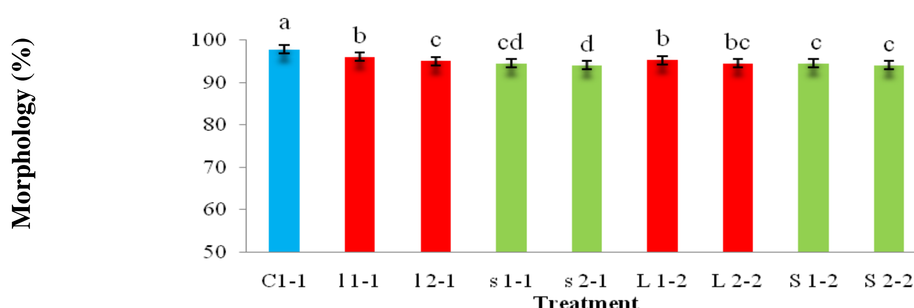


Fig 4: Spermatozoa morphology of male mice after given of neem leaves and seeds extract

(C1-1 = Control, l1-1, s1-1, s2-1 = leaves (l) and seeds (s) at doses 0.25 and 0.50 mg.kg BW for 36 days; L1-2, L2-2, S1-2, S2-2 = Leaves (L) and Seeds (S) at doses 2.5 and 0.50 mg/kg for 36 days, and with no treatment for another 36 days

5.3.2 Spermatozoa DNA quality with *Kit Halomax*®:

Result of observation shows that there is no a decrease in sperm DNA quality post administration of extract and stopped treatment when compared to the control group (P>0.05). The result shows that the administration of aqueous neem leaves and seeds extract does not decrease the sperm DNA quality of mice spermatozoa so that it's safe to use as an alternative to natural herbal contraceptive. This DNA examination means that mice DNA has high genetic quality and is in good condition as it is a complete DNA 100% (Figure 5).

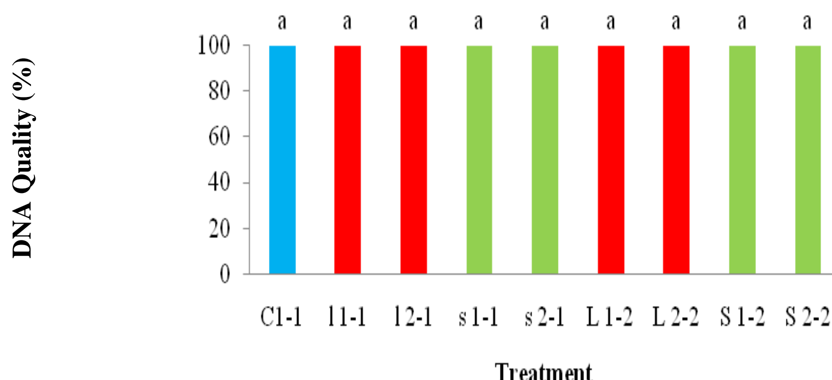
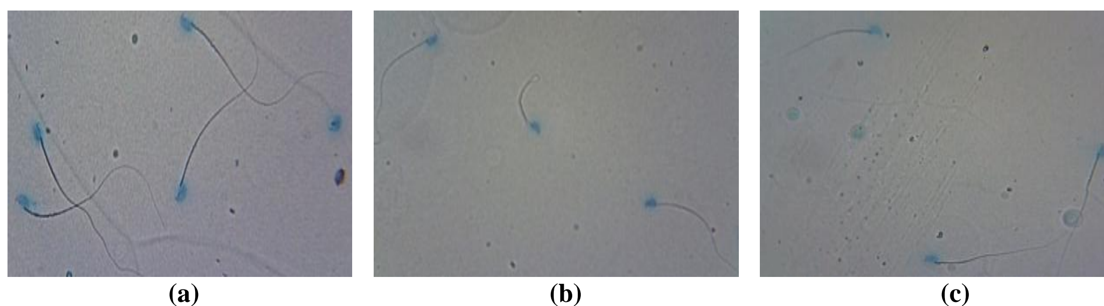


Fig 5: Spermatozoa DNA quality of mice (Kit Halomax®) after given of neem leaves and seeds extract
(C1-1 = Control, l1-1, s1-1, s2-1 = leaves (l) and seeds (s) at doses 0.25 and 0.50 mg.kg BW for 36 days; L1-2, L2-2, S1-2, S2-2 = Leaves (L) and Seeds (S) at doses 2.5 and 0.50 mg/kg for 36 days, and with no treatment for another 36 days



(a) **(b)** **(c)**

Fig 6: Spermatozoa with intact DNA and fluorescent head (Kit Halomax®
(a. Control, b. aqueous neem leave extract, c. aqueous neem seeds extract 40x magnification)

VI. DISCUSSION

Result of study shows that there was a decrease in sperm quality (sperm motility, concentration, morphology) after administration of aqueous neem leaves and seeds extract. The decrease in spermatozoa motility and viability caused by the presence of saponins, which has anti-fertility properties. Saponins decrease spermatozoa viability. [3] Saponins are also cytotoxic in nature. Anti-fertility material is either cytotoxic or hormonal in its effect. [9] It is cytotoxic when contributing a direct effect to the genital cell, and it is hormonal when working on a responsive organ to the related hormone. The result also indicates that there is a decrease in sperm concentration administered with aqueous neem leaves and seeds extract compared to the control group. Result of phytochemical test shows that aqueous neem leaves extract contain higher flavonoid than aqueous neem seeds extract. Flavonoid inhibits the aromatase enzyme, an enzyme catalyzing androgen conversion into estrogen and increasing testosterone hormone. An excessive increase in testosterone hormone in blood can result negative feedback at hypothalamus, therefore secretion of gonadotropin releasing hormone (GnRH) by hypothalamus was inhibited. This can fail anterior hypophysis to secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH) will lead decrease of spermatogenesis including spermiogenesis processes.[18] The decrease in spermatozoa concentration in mice after administered with neem seeds extract can also reduce spermatozoa progressive motility. [19] Tannin content in extract also able to decrease spermatozoa concentration as well. [20]The administration of aqueous neem leaves and seeds extract affects spermatozoa viability during treatment. The live spermatozoa will become transparent while the dead will be red due to the presence of coloring substance in the head by damaged cell membrane. [21] Spermatozoa viability decreases compared to the control group after treatment was stopped for 36 days. This indicates that the effect of aqueous neem leaves and seeds has not completely disappeared.

The result also shows that administration of aqueous neem leaves and seeds extract decreases normal spermatozoa morphology. The neem active materials (flavonoid, saponins, and tannin) are naturally toxic and lead to hormonal imbalance during spermatogenesis in testis. It has been concerned that motility development, transformation of spermatozoa tail structures, morphological change of acrosome, disappearance of cytoplasmic droplet, and plasma membrane transformation occur at the process of sperm maturation in epididymis. Since spermatozoa comes from the changes in spermatogenic cell in seminiferous tubules during spermatogenesis, abnormality in sperm takes place in epididymis.

The administration of aqueous neem leaves and seeds, did not effect sperm DNA. No damaged was found after treatment (36 days) as well as after the treatment were stopped. Factors assumed to contribute to the sperm DNA damaged is oxidative stress caused by free radical or ROS. Some causes of oxidative stress on sperm are testicular, idiopathic, iatrogenic, infection, and autoimmune. Oxidative stress which is a free radical can cause cross linking chromatin of oxidative basal DNA and break of DNA strand.[22] Tannin can inhibit Poly (ADP-Ribose) glychohydrolase enzyme that can damage DNA. [23]

Overall, result of the study shows that the administration of aqueous neem leaves and seeds extract have an impact on the decrease of sperm quality i.e. motility, viability and sperm morphology after 36 days of treatment stopped. The spermatozoa has not indicated an increase in sperm quality. Instead it shows a decrease or a little increase compared to the spermatozoa taken in the end of treatment. This is due to the fact that it takes more than 36 days post stopped treatment for normal spermatogenesis to occur. The sperm quality in the group with 36-days treatment and 36-days post treatment stopped is poorer than that of the control group, but shows an increase in sperm count. The administration of aqueous neem leaves and seeds extract for 28 days, followed with 42 days post stopped treatment is able to recover reproductive organs of male mice. [10]

VII. CONCLUSION

Phytochemical and pharmacological effects of neem extract have been studied. However, reversibility and the DNA quality on spermatozoa of mice as anti-spermatogenic were important to know. Administration of neem leaves and 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 leaves and seeds treatment does not damage sperm DNA quality in male mice, but only reduces the testis function.

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REFERENCES

[1] Ministry of Health Republic of Indonesia, 29 Juni Family Planning Days, 2014, Available at www.depkes.*go.id*

[2] BKKBN, Indonesia's population growth is equivalent to the population of Singapore, 2015, Available online: http://news.detik.com/berita/bkkbn (acessed on 1 March 2016).

[3] Joshi S.C, Sharma A, Chaturvedi M, Antifertility potential of some medicinal plants in males: An overview research article, *International Journal of Pharmacy and Pharmaceutical Sciences, 3(5)*, 2011, 204-217.

[4] Sharma R.S, Rajalakshmi M, Jeyaraj D.A, Current status of fertility control method in India, *Journal of Bioscience, 26(4),* 2001, 391-405.

[5] V. Mathew and G. Bantwal, Male contraception, *Indian Journal of Endocrinology and Metabolism, 16(6),* 2012, 910-917.

[6] Hashmat I, Hussain A, and Ajij A, Neem (*Azadirachta indica* A. Juss) - A nature's drugstore: An overview, *International Research Journal of Biological Sciences,1(6),* 2012, 76-79.

[7] M. Asif, A Review on Spermicidal Activities of Azadirachta indica, *Journal of Pharmacognosy and Phytochemistry, 1(5)*, 2013, 61-79.

[8] A. Srivastav, A. Chandra A, M. Singh, F Jamal, P. Rastogi P, and S. M. Rajendran, F.W. Bansode, V. Lakshmi, Inhibition of hyaluronidase activity of human and rat spermatozoa in vitro and antispermatogenic activity in rats *in vivo* by *Terminalia chebula*, a flavonoid rich plant, *Reproductive Toxicology, 29(2),* 2010, 214–224.

[9] Robinson T, *Ingredients Organic Compounds Plant High* (Translated by Prof. Kosasih Padmawinata, ITB. Bandung, 2003).

[10] R.K. Mishra and S.K Singh, Effect of aqueous leaf extract of *Azadirachta indica* on the reproductive organs in male mice, *Indian Journal of Experimental Biology, 43,* 2005, 1093-1103.

[11] R.H Aladakatti, and R.N Ahamed, Effect of *Azadirachta indica* leaves on rat spermatozoa, *Indian Journal of Experimental Biology,* 37, 1999, 1251-1254.

[12] R. Tiwari, A.K Verma, S. Chakraborty, K. Dhama and S.V. Singh, Neem (*Azadirachta indica*) and its potential for safeguarding health of animals and humans: A Review. *Journal of Biological Sciences, 14:* 2014, 110-123.

[13] R. Rugh, *The Mouse Its Reproduction and Development* (Burgess, Publ Company, 1976).

[14] J.M Morrell, H Rodriguez-Martinez, Biomimetic techniques *for* improving sperm quality *in* animal breeding*: a* review. *The Open Andrology Journal 1*, 2009, 1-9.

[15] Langdon W.C, *A comparative study on equine sperm chromatin using the sperm chromatin structure assay and the sperm Halomax kit*®, doctoral diss., Texas Technology University, Texas, USA, 2012.

[16] M. Daniyal, and M. Akram M, Antifertility activity of medicinal plants, Review Article, *Journal of the Chinese Medical Association, 78,* 2015, 382-388.

[17] P. Lishko, Y. Kirichok, D. Ren, B. Navarro, J. Chung, and D.E. Clapham, The control of male fertility by spermatozoan ion channels, *The Annual Review of Physiology, 74,* 2012,1-23.

[18] T.M. Salman, I.A. Alagbonsi, L.A. Olayaki, S.A. Biliaminu, H.M. Salahdeen, O.A. Olowu, Honey increases sperm count in male albino rats by enhancing testosterone production, Original Article, *Biokemistri An International Journal of the Nigerian Society for Experimental Biology*, *25 (2),* 2013, 39–44.

[19] M.H. Dehghan, T. Martin, R. Dehghanan, Antifertility effect of Iranian neem seed alcoholic extract on epididymal sperm of mice, *Iranian Journal of Reproductive Medicine, 3(2)*, 2005, 83-89.

[20] Akanji A.M, Ogung B.A.M, Emiola I.A, Toxicological effects of raw jack beans, bambara groundnuts and benne seeds on semen and sperm quality of cockerels, *Journal of Agriculture and Environmental Sciences, 4(1),* 2015, 204-211.

[21] N. Singla and M. Garg, Effect of crude cottonseed oil containing gossypol on fertility of male and estrous cycle of female Bandicota bengalensis Gray and Hardwicke, *Journal of Applied Animal Research*, *41(2)*, 2013, 156-165.

[22] S.E.M Lewis, and R.J Aitiken, DNA damage to spermatozoa has impacts on fertilization and pregnancy, *Cell* and *Tissue Research, 322(1),* 2005, 33-41.

[23] C. Blenn, P. Wyrsch, F.R Althaus, The ups and downs of tannins as inhibitors of Poly(ADP-Ribose)glycohydrolase, *Molecules 16,* 2011, 1854-1877.