NEW ZEALAND RABBIT'S SPERMATOZOA AFTER THE TREATMENT OF TANNIN EXTRACTION OF *Pluchea indica*

Eko Susetyarini[1*)], Poncojari Wahyono[2)], Roimil Latifa[3)], Endrik Nurrohman[4)]
Biology Education Department, Faculty of Teacher Training and Education, University of Muhammadiyah Malang
email: susetyorini@umm.ac.id

Abstract

This current research aimed at investigating the structure and average size of New zealand rabbit's spermatozoa. This research employed experimental design and was conducted in Chemical Engineering Laboratory of State Polytechnic of Malang and Biology Laboratory of University of Muhammadiyah Malang. This research was initiated from August to September 2019. The design of this research was specifically Completely Randomized Design, with four treatments of tannin extraction of Pluchea indica, and control with oral nasogastric with the dose of 3 ml/kgbb in four-time repetitions. A light microscope and Scanning Electron Microscope (SEM) were used for observation. One-way ANOVA was used for data analysis. As for the control group, the spermatozoa were found normal. Whilst, with reference to the three other treatments, the spermatozoa were seen to be abnormal, with apparent dilation in the neck, pseudo-droplet (bumpy neck), and dag defect (twisted tail). Based on the results, there was an influence of tannin extraction of Pluchea indica on the abnormality of New zealand rabbit's spermatozoa (with $p<0.05$). Regarding the size of the spermatozoa, the average length of spermatozoa was 8.74 µm for the head, 1.86 µm for the upper neck, and 56.48 µm for the tail. With reference to HOS (Hypo-Osmotic Swelling) test, it showed that abnormal spermatozoa was in the form of severance of the head and the tail, as well as dag defect. To sum up, this research has indicated that the condensed tannin extraction contributes a similar effect to that of the pure one on the structure of spermatozoa.

Keywords*: Tannin Extraction, New zealand Rabbit, Spermatozoa*

1. INTRODUCTION

According to the data of National Family Planning Coordinating Board (BKKBN) (2012), overpopulation is the most complex issue in Indonesia. Accordingly, a Family Planning (KB) is initiated by the government as a national program in response to the issue (Purwaningsih, 2016). One of the real practices of KB program is the provision of contraception (Muslichah, 2015). In fact, contraception is much more targeted to women due to its highly limited choice for men. Some of contraceptive methods for men encompass periodic abstinence, condom, disconnected intercourse, and vasectomy (Sumaryati, 2004). In addition, the process and program of KB are still lack of interest among men (Wilopo, 2006) due to many determining factors; one of which is knowledge about the safety measure.

Contraception safety is very essential. There are several requirements to meet by good contraceptive methods, namely non-hazardous, reliable, affordable, acknowledged by common people, safe, and easy to get (Soewolo, 2005; Albar, 2000: 27), reversible, and less risky (Prajogo *et al*, 2003; Wilopo, 2006, Herlina *et al*, 2008). Up to present, there have not been any fully ideal contraceptive methods (Bayyinatul, 2012). For that reason, the use of contraception with a very minimum side-effect totally needs further research. One of antifertility sources is in the form of an active compound existent in plants (Sitasiwi, 2016; Haris *et al*, 2017).

Generally, antifertility refers to a specific term to name a compound or substance to interfere a reproduction system (Dabhadkhar *et al*, 2015), by means of obstruction on the processes of pre-ovulation and embryonic pre-implantation (Brewis and Combie, 1999). Joshi *et al* (2011) assert that the exposure to antifertility compounds

can result in zygote destruction, prevention from ovulation, fertilization, or implantation.

Indonesia has vast diversity of plants. The majority of the plants are rich in benefits; one of which is for medication (Sitasiwi, 2016; Susetyarini, 2009b; Haris *et al*, 2017). Nonetheless, the use of traditional medication by means of plants to generate antifertility substance is still lack of attention and remains unexposed (Susetyarini, 2013). To specify, one of the beneficial plants is *Pluchea indica* (Susetyarini, 2009b). The compound substance in the plant has been observed to have biological and other pharmacological activities (Fitriansyah *et al*, 2018). *Pluchea indica* has various active compounds, especially in its leaves, such as alkaloid, flavonoid, and tannin (Susetyarini, 2011; Febrianta *et al*, 2015).

Those active compounds, i.e. alkaloid, flavonoid, essential oil, and tannin have made *Pluchea indica* the source of natural antifertility substance (Susetyarini, 2011). Tannin compound which is anti-spermatogenic along with its active alkaloid and tannin substances can destruct several organs that construct the target cell (Cahaya *et al*, 2017). In addition, tannin can interfere mitochondrial activities and destruct the disserving cells that can negatively affect the structure and fertility of spermatozoa (Susetyarini, 2010).

Previous research indicated that tannin extraction of *Pluchea indica* influenced the concentration of spermatozoa in white rats and the number of their tillers. Rabbits are considered highly representative for antifertility testing due to the similarity in reproduction process that involves kinds of glands and organs of reproduction (Toilehere, 1981). In addition, the reproduction system comprises two kinds, internal and external. In male rabbits, the internal organs of reproduction consist of testis, as the source of spermatozoa, and epididymis, as the channel for spermatozoa maturation; whilst the external organ only comprises penis to transfer the spermatozoa (Kastawi, 2003; Tenzer, 2003).

The referral parameters to evaluate the quality of reproduction system are based on the weight of reproduction organs, the abnormality of spermatozoa, the number of spermatozoa, morphology, and the motility of spermatozoa (Muslichah *et al*, 2015). According to Freshman (2002), evaluation on cement substance covers gross evaluation, pH (acidity) measurement, motility, morphology, the concentration of spermatozoa, cytology, cement culture, and alkaline phosphate. This current research focused on the morphological structure, size, abnormality, and plasma membrane of spermatozoa. Further, this research aimed at identifying the activities caused by various tannin extractions of *Pluchea indica* on the structure of spermatozoa, the average length and width of the spermatozoa cells, and the membrane strength of *New zealand* rabbit's spermatozoa. It is highly expected that this research can contribute potential investigation on nutritious plants for herbal or natural fertility.

2. RESEARCH METHOD

This current research employed experimental design. To execute, a Completely Randomized Design with four major treatments were applied. This research was conducted in Chemical Engineering Laboratory of State Polytechnic of Malang, Kesimma Medica Laboratory Malang, and Integrated Laboratory and Biology Laboratory of University of Muhammadiyah Malang. This research was initiated from August to September 2019. For the treatment, there were three kinds of tannin, i.e. hydrolyzed, condensed, and pure. As many as 24 male *New zealand* rabbits with the age of 14 weeks were classified randomly into four major groups of experimentation, with the addition of the hydrolyzed, condensed, and pure tannin as the treatment. As for the control group, aquades was added up by means of oral nasogastric with the dose of 3 ml/kgbb. After three months, a surgery procedure was undergone to the *New zealand* rabbits to collect the testis and manually observe the structure of spermatozoa. To examine the abnormality that occurred at the membrane, Hypo Osmotic Swelling (HOS) test, according to Susilawati (2011), was applied by adding up 1 ml hypo osmotic solution 150 m made of 7.35 grams of sodium citrate,

$2H_2O$, 13.52 grams of fructose dissolved into 1000 ml of aquades. Afterwards, the added 0.1 ml of spermatozoa was incubated under the temperature of 37°C for about 30 minutes. At last, the spermatozoa were observed, from the weakest to the strongest levels of magnification.

The structure of spermatozoa was observed using light microscope and Scanning Electron Microscope (SEM). To measure the length and width of spermatozoa, the cells were taken out of an efferent vase and were put on a special tip carbon of the electron microscope. In addition, observing the spermatozoa cells was targeted at four corner and middle sides, with five spermatozoa cells in each; therefore, there were 25 spermatozoa cells observed by means of SEM. The average length and width of the spermatozoa were measured as well. To probe the percentage of abnormality that occurred at the spermatozoa cells, a total of 100 sample spermatozoa cells were taken up to the three observational points using a binocular microscope. Further, a calculation on the number of normal and abnormal spermatozoa cells was conducted by referring to a guidebook of *Spermatozoatology* by Susilawati (2011) and *Reproduction in Farm Animals* by Hafez (1993). The data analysis was referred to the description of average assisted by Microsoft Excel program, and One-way ANOVA was performed by SPSS software package.

3. RESULTS AND DISCUSSION

The observation results (Table 1) indicated that the structure of spermatozoa cells found in *New zealand* rabbits consisted of head and elongated tail. In this research, the results showed that the structure of the rabbits' spermatozoa cell comprised head, neck (upper tail), middle tail, and lower tail. The spermatozoa's head was oval and round in shape, with tapering tail to the tip. Normally, spermatozoa only consists of head and tail, and the head is oval, round, and flat in shape (Garner & Hafez 2000). According to Susilawati (2011), the tail of spermatozoa is divided into four parts, i.e. neck, middle piece, core piece, and lower piece. It is also stated that the tail consists of connecting, middle, core, and tip pieces (Maulidya, 2012).

Table 1. Data of Observation on Spermatozoa's Morphology

Treatments/with Tannin	The Structure of Spermatozoa		
	Head	**Neck**	**Tail**
The Control	Normal, with oval and round shape	Normal	Normal, straight
The Condensed	Normal, with oval and round shape	*pseudo-droplet*	dag defect
The Hydrolyzed	Normal, with oval and round shape	*pseudo-droplet*	dag defect
The Pure	Normal, with oval and round shape	*pseudo-droplet*	dag defect

In the control group, it has been shown that the spermatozoa were normal; while in all treatment groups with tannin extraction, the existence of abnormalities was evident in some parts of the spermatozoa structure. In Table 1, it is indicated that the normal spermatozoa had oval and round shape of head with straight tail. Conversely, the abnormal spermatozoa were found to typify pseudo-droplet and

have dag defect (twisting tail). The abnormalities found on the structure of spermatozoa could be distinguished into two classes, primary and secondary. The former occurs during the process of spermatogenesis; while the latter occurs after the spermatogenesis to ejaculation. In details, spermatozoa cells are divided into five major categories, namely: the cells with no tail, the cells with abnormal head shape, the cells with abnormal tail shape, and the cells with abnormal tail shape along with the presence of cytoplasmic droplet in proximal piece, and the cells with abnormal tail shape in consort with distal droplet (Susilawati, 2011). To illustrate, the observation results are demonstrated in Figure 1.

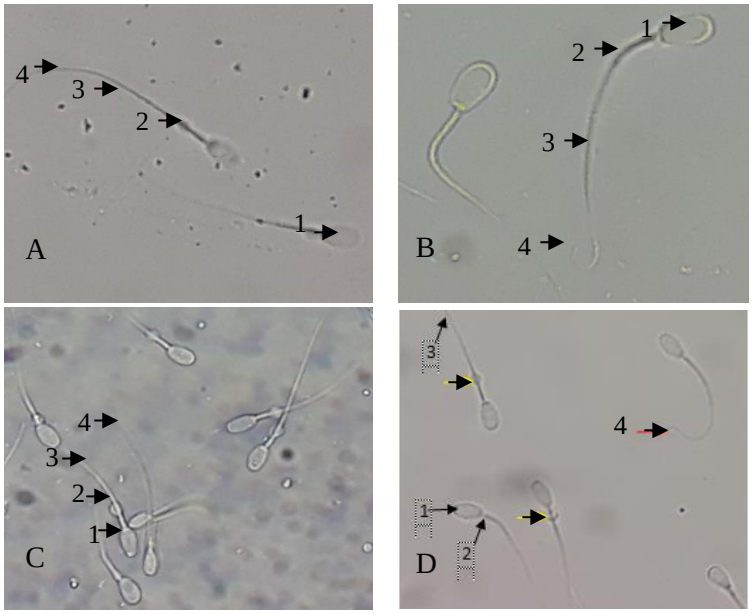

Figure 1. A. The Structure of Spermatozoa in Control group, B. The Hydrolyzed Tannin group, C. The Condensed Tannin group, D. The Pure Tannin group through a Binocular Microscope with 100x Magnification.
Note: 1. Head; 2. Upper Tail (Neck); 3. Core Tail; 4. Lower Tail

Table 2. The Percentages of Spermatozoa Abnormalities

Treatments/with Tannin	Percentages of Spermatozoa Abnormalities		
	Pseudo-droplet	Dag Defect	Normal
The Control	2%	0%	98%
The Hydrolyzed	58%	38%	4%
The Condensed	74%	24%	2%
The Pure	66%	32%	2%

Spermatozoa are produced by testis in the seminiferous tubules and are taken out through the male's reproduction channel (Yatim, 1987), which is the phase of spermatozoa construction, or commonly called as spermatogenesis (Garner & Hafez 2000; Yatim, 1987). During the spermatogenesis, the activities of spermatogenic cells are dramatically dense, in which morphological and biochemical changes appear to happen for the sake of constructing functional spermatozoa

(Muslichah & Wiratmo, 2015). Interference that happens during spermatogenesis will bring about influence on the quality of the spermatozoa being secreted (Susetyarini, 2013). Regarding the percentages of abnormalities found in spermatozoa in all treatment groups as shown in Table 2, the control group had the lowest number of pseudo-droplet cells by only having 2% in total, which means that only two out of 100 cells were abnormal, 0% dag defect, and the 98% normal. In the hydrolyzed tannin group, 58% of the cells were pseudo-droplet, 38% dag defect, and 4% normal. As for the condensed tannin group, 74% were pseudo-droplet, 24% dag defect, and 2% normal. Last, in the pure tannin group, 66% of the cells were found pseudo-droplet, 32% dag defect, and 2% normal.

The common spermatozoa abnormalities are *diadem defect knobbed sperm* (the thickening on the tip of the head), *decapitated head* (the rupture of the head), *sterilizing tail stump* (the rupture of the tail), *dag defect* (twisting tail), *pseudo-droplet* (bumpy neck), and corkscrew *defect* (serrated neck) (Susilawati, 2011). There were several factors influencing the abnormalities on the structure of spermatozoa. Based on the results of this research (as shown in Table 1 and 2), the abnormalities comprised pseudo-droplet and dag defect. The highest probability of abnormal spermatozoa was found in the treatment groups with tannin extraction of *Pluchea indica*. The secondary abnormal spermatozoa occurred at the tail due to the damage during spermatozoa penetration and chemical substances (Chenoweth, 2005). An active tannin substance is cytotoxic with a spermicide effect to spermatozoa (Choubey, 2011). According to Mughaniati (2018), an active tannin compound can result in a raise on the percentage of spermatozoa abnormalities.

Based on ANOVA test, it was indicated that there was a significant influence of the addition of tannin extraction of *Pluchea indica* with various conditions on the abnormalities of *New zealand* rabbit's spermatozoa (with $p<0.05$). In addition, Susilawati (2011) states that the factors of nutrition and poisonous substances affect spermatozoa. In other words, any poison circulated throughout the body can damage the structure of spermatozoa. The use of active tannin substance should be precisely proportional and is not allowed to be overdosed since it may cause toxicity (Doostar *et al*, 2000; Kunaepah, 2008). Referring to the research carried out by Susetyarini (2005), the extraction of *Pluchea indica* affected the quality of spermatozoa in white rats. The extraction of the plant's leaves was proven to be potential for natural antifertility (Susetyarini, 2013). Kokote *et al.* (2008) postulate that the effects of exposure to antifertility substance could be evaluated in reproduction process. In fact, there are several active compounds found in *Pluchea indica*, namely: tannin, alkaloid, and flavonoid, which are all influential during spermatogenesis process. Tannin contained in the plant can result in negative effects on spermatozoa (Susetyarini, 2011b). In addition, tannin can also influence the quality of spermatozoa (with motility (5%), mortality (71.5%), abnormality (24.25%), and livability (28.5%)) (Wahyuni & Susetyarini, 2007). When the abnormality level of spermatozoa approaches a point bigger than 50% out of the total of spermatozoa, the spermatozoa are considered sterile (Nalbondov, 1990). In addition, the abnormality of spermatozoa in the level of 18-20% is considered serious due to the possibility of fertility (Barth & Oko 1989).

Any damages can cause impairment of plasma membrane; accordingly, quality decrease in spermatozoa is certainly possible to occur. Considering the importance of plasma membrane, a test on the solidity of the plasma membrane was carried out by means of a special kind of test, named *Hypo Osmotic Swelling* (HOS) test. The results of HOS test are presented in Figure 2.

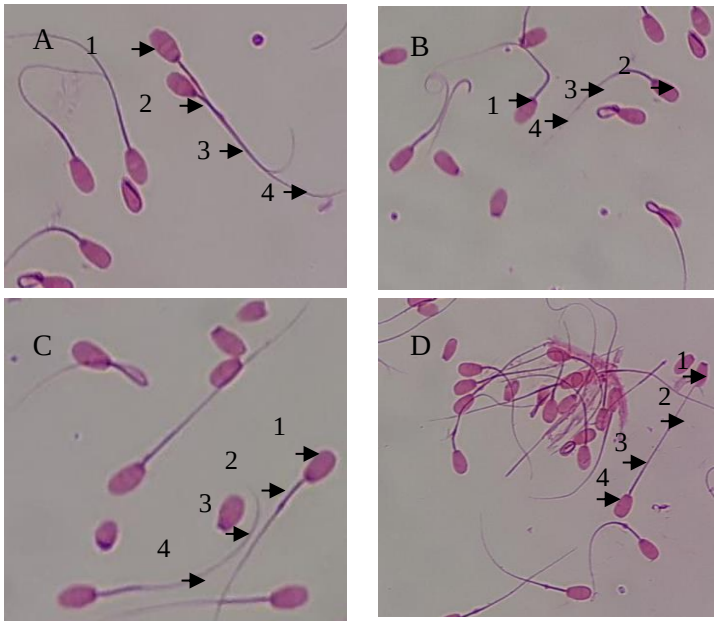

Figure 2. A. The Structure of Spermatozoa in the Control Group, B. in the Hydrolyzed Tannin Group, C. in the Condensed Tannin Group, D. in the Pure Tannin Group Binocular Microscope with 1000x Magnification

The results of observation (as shown in Figure 4) have indicated that the control group was dominated by normal spermatozoa cells shown by complete parts of the cells, such as oval and round head and tail. In addition, the other groups with tannin treatments contained spermatozoa cells that suffered from abnormalities, such as severed head, severed tail, and twisting tail. The most typical change shown by the HOS test was the swelling on the tip of spermatozoa's tail (Susilawati, 2011). The swelling occurred due to the exposure of hypo osmotic medium to spermatozoa, which let the volume flow through the cells. The whole parts of spermatozoa cells were covered by plasma membrane that functioned as transportation, from the inner to the outer points, and *vice versa* (Pinto & Kozink, 2008). Naturally, astringent tannin substance can affect permeability of membrane since it causes contraction on the cell membrane (Indeks, 1983). A number of tests on the solidity of plasma membrane of spermatozoa in animals have been widely reported; however, only a few of which tested on rabbits (Maulidya, 2012). To enlighten, the results of measurement on spermatozoa are demonstrated in Figure 3.

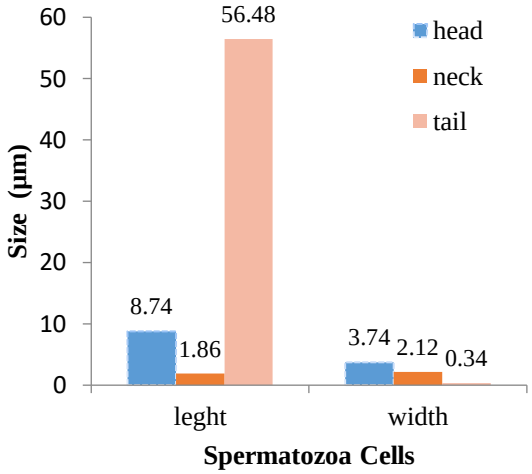

Figure 3. The Measurement Results of Morphological Length and Width of Spermatozoa

The morphology and size of spermatozoa can transform in the epididymis (Johnson & Evererit, 1988). The measurement of spermatozoa cells was done by means of SEM (as shown in Figure 3). In terms of length, it was shown that, averagely, the head was 8.74 µm, the middle piece of the neck was 1.86 µm, and the tail was 56.48 µm. Meanwhile, with reference to the width, the head typically signified 3.74 µm, the neck was 2.12 µm, and the tail was 0.34 µm. In each species, spermatozoa have different sizes, but are almost similar in shape. The differences are evident in the size and shape of spermatozoa of particular animals (Susilawati, 2011). The illustration of the measurement results on the size of spermatozoa is presented in Figure 4.

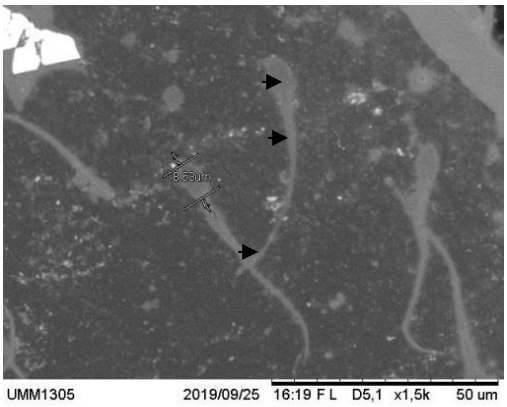

Figure 4. The Observation on the Length and Width of Spermatozoa Cells Using SEM Note: 1. Head, Neck (upper tail), 2. Middle Tail, 3and Lower Tail.

4. CONCLUSION

This current research has found that, in the control group, the structure of spermatozoa was considered normal, indicated by the parts of spermatozoa containing head, neck, and tail. Meanwhile, referring to the treatment groups, i.e. the hydrolyzed, the condensed, and the pure tannin, the spermatozoa cells were found abnormal, particularly beneath the neck piece. In addition, it was shown that the neck piece was wider than that of the normal

spermatozoa cells, and there was the swelling beneath the neck and tail twisting. There were several types of abnormality, namely pseudo-droplet and dag defect.

On the basis of the HOS test, the tannin treatment groups consisted of spermatozoa cells that were found to have severed tail, severed head, and twisting tail. Averagely, with reference to the length, the head of spermatozoa cells was 8.74 µm, the middle neck was 1.86 µm, and the tail was 56.48 µm. Regarding the width, the head signified 3.74 µm, the neck was 2.12 µm, and the tail was 0.34 µm.

The ultimate finding of this current research was that the condensed tannin showed similarity to the pure one in terms of its effects on the structure of spermatozoa. Notwithstanding, further research is required to carry out, especially in the investigation of side effects, long-termed effects, and the most ideal duration of exposure to tannin extraction of *Pluchea indica* as a natural contraceptive method under physiological and biochemical scopes of spermatozoa.

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