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DESCRIPTION OF ETHICAL APPROVAL
"ETHICAL APPROVAL"

NO : 361/EA/KEPK-BUB-2020

Protokol penelitian yang diusulkan oleh
The research protocol proposed by : Luh Putu Widiastini, S.SiT., M.Kes

Peneliti utama : Luh Putu Widiastini, S.SiT., M.Kes

Nama institusi
Name of the institution : STIKES Bina Usada Bali

Dengan judul : Pengaruh Pemberian Ekstrak Etanol Daun Kelor (*Moringa Oleivera*) terhadap Jumlah spermatozoa serta Morfologi Spermatozoa Tikus Putih (*Rattus Norvegicus*) Galur Wistar Usia Tua

Tittle : *The Effect of Moringa Oleivera Leaf Ethanol Extract on the Number of Spermatozoa and the Spermatozoa Morphology of White Rats (Rattus Norvegicus) Old Age Wistar Strain*

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1. Social values, 2. Scientific values, 3. Equitable assessment and benefits, 4. Risks, 5. Persuasion/exploitation, 6. Confidentiality and privacy, and 7. Informed concent, referring to the 2016 CIOMS Guidelines.
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Pernyataan Laik Etik ini berlaku selama kurun waktu tanggal 30 Desember 2020 sampai 30 Desember 2021
This declaration of ethics applies during the period December 30th 2020 until December 30th 2021.

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NIK : 13.12.0068

PAPER NAME

ARTICLE TEXT jbbi Widiastini.doc

AUTHOR

Widiastini

WORD COUNT

4291 Words

CHARACTER COUNT

24104 Characters

PAGE COUNT

11 Pages

FILE SIZE

578.0KB

SUBMISSION DATE

SEP 20, 2022 11:15 AM GMT+8

REPORT DATE

SEP 20, 2022 11:16 AM GMT+8

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PEMBERIAN EKSTRAK ETANOL *MORINGA OLEIFERA* MENINGKATKAN JUMLAH SERTA MEMPERBAIKI *MORFOLOGI SPERMATOZOA RATTUS NORVEGICUS* TUA

ETHANOL EXTRACT OF MORINGA OLEIFERA INCREASE THE NUMBER OF SPERMATOZOA AND IMPROVE SPERM MORPHOLOGY IN AGED RATTUS NORVEGICUS

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ABSTRAK

Penuaan pada pria dapat menyebabkan penurunan volume semen, Morfologi yang abnormal dan penurunan motilitas sperma. Tujuan dari penelitian ini adalah untuk mengetahui Pengaruh Pemberian Ekstrak Etanol Daun Kelor (Moringa Oleivera) Terhadap Jumlah Serta Morfologi Spermatozoa Tikus Putih (Rattus Norvegicus) Galur Wistar Usia Tua. Penelitian ini menggunakan Tikus usia tua yang berusia 18-19 bulan dengan berat badan 200-250 g, kondisi sehat dan tidak cacat fisik sejumlah 36 dibagi menjadi 2 kelompok, yaitu kelompok perlakuan (ekstrak etanol daun kelor 50 mg/kgBB/0,5 mL CMC 0.5% per hari) dan kelompok kontrol (CMC 0.5% 0.5 mL per hari) selama 30 hari. Hasil penelitian menunjukkan ekstrak Etanol Daun Kelor meningkatkan jumlah Spermatozoa (nilai p 0,000) dan memperbaiki Morfologi Spermatozoa (nilai p 0,000). Dapat disimpulkan pemberian ekstrak etanol daun kelor secara signifikan meningkatkan jumlah dan Morfologi Spermatozoa Tikus Putih Usia Tua.

Kata Kunci: Daun Kelor, Ekstrak Etanol, Jumlah *Spermatozoa, Morfologi Spermatozoa, Rattus Norvegicus* Usia Tua

ABSTRACT

Aging in men can lead to decreased semen volume, abnormal morphology and decreased sperm motility. The purpose of this study was to determine the effect of giving of Moringa Leaf (Moringa Oleivera) Ethanol Extract to the Number and Morphology of Spermatozoa of Old Age Wistar Rats (Rattus Norvegicus). This research used rats aged 18-19 months old, body weight of 200-250 g, healthy condition and no physical disabilities were 36 divided into 2 groups, the treatment group (ethanol extract of Moringa leaves 50 mg/kgBW/ 0.5 mL CMC 0.5% every day) and the control group (0.5 mL CMC 0.5% every day) for 30 days. The results showed that Moringa Leaf Ethanol Extract increased the number of Spermatozoa (p-value 0.000) and improved Spermatozoa morphology (p-value 0.000). It can be concluded that the ethanol extract of Moringa leaves significantly increased the number and morphology of the Spermatozoa of the Aged Rats.

Keywords: Moringa Leaf, Ethanol Extract, Number of Spermatozoa, Spermatozoa Morphology, Aged Rattus Norvegicus

PRELIMINARY

In the last ten years, Indonesia's population growth rate is 1.25 percent per year. There is a slowdown in the population growth rate of 0.24 percent when compared to the period 2000–2010 which was 1.49 percent (Badan Pusat Statistik 2021). One of the reasons for this decline is the birth rate. The birth rate may decrease due to infertility or infertility.

According to the World Health Organization (WHO), infertility is a failure to achieve pregnancy after one year of active sexual intercourse without using contraception (Aziz and Agarwal 2017; Majzoub and Jr 2017). Causes of infertility in married couples can be classified into three with the proportion, female factors 45%, male factors 40%, and idiopathic factors 15% (Lestari dan Sari 2015). According to research from Kumar and Singh, (2018), male factor is responsible for 40-50% of infertility cases in couples (Aziz and Agarwal 2017).

The underlying causes of male infertility are grouped into three factors (1) pretest, (2) testicular (3) post-aesthetic (Dimitriadis et al. 2017). Four main categories of sperm damage lead to the diagnosis of male infertility including absence of sperm in the semen (azoospermia), low sperm count (oligozoospermia), malformed sperm morphology (teratozoospermia), and problems with sperm motility (asthenozoospermia). (Majzoub and Jr 2017). Spermatozoa are very susceptible to oxidative stress, because the antioxidant enzymes in Spermatozoa are low, Spermatozoa spend a long time in the reproductive tract. (Sabeti et al. 2016), and Spermatozoa plasma membranes contain high amounts of polyunsaturated fatty acids (PUFAs). This high proportion of PUFA causes Spermatozoa to undergo lipid peroxidation (Ayala et al. 2014; Lee et al. 2017; Ali et al. 2020; R. Dias et al. 2020). According to (Henkel et al. 2018), Oxidative stress is closely related to various pathologies such as infertility and the aging process (Ko et al. 2014; Bisht et al. 2017; Kumar and Singh 2018).

The reproductive aging process is characterized by a progressive decrease in physiological integrity that triggers damage to the testes, epididymis and other reproductive organs. Aging in men can cause a decrease in semen volume, abnormal morphology and a decrease in normal sperm motility (Lucio et al. 2013; Morielli and O'Flaherty 2015). Aging has a close relationship with the presence of free radicals in the body, which can induce oxidative stress. Oxidative stress, defined as an imbalance between reactive oxygen species (ROS) and antioxidant production (Luceri et al. 2018). ROS production has a central role in age-related decline in male fertility, influencing aging biomarkers (López-otín et al. 2013). The results revealed that Spermatozoa from older animals produced more free radicals compared to younger ones, as well as lower antioxidant activity (Sabeti et al. 2016).

The body needs antioxidants that function to protect, ward off free radicals and prevent chain reactions so that greater damage does not occur and repair damaged cells and tissues. The body synthesizes antioxidants called endogenous antioxidants, while antioxidants that come from outside the body or from food and drinks are called exogenous antioxidants (Widiastini et al. 2022). High quality spermatozoa require ascorbic acid, amino acids, sterols, isoquartsetin glucoside, carotene, ramentin, kaemperol, and kaemferitin, and vitamin E. One of the plants that contain all these elements is Moringa leaves (M. Oleivera) (Singh et al. 2012; Syarifuddin et al. 2017). The content of high nutritional value, properties and benefits has caused Moringa to be nicknamed the Miracle tree and Mother's Best Friend. Moringa is known to contain more than 90 types of nutrients in the form of essential vitamins, minerals, amino acids, anti-aging, and anti-inflammatory (Aminah et al. 2015). Moringa contains 539 compounds known in traditional African and Indian medicine and has been used in traditional medicine to prevent more than 300 diseases (Toripa*h et* al. 2014). The results of phytochemical tests carried out on Moringa (M. Oleifera) leaves in the South Denpasar area of Bali, it is known that the ethanolic extract of Moringa leaves (Moringa Oleifera) has antioxidant capacity, including phenolics, flavonoids, tannins, vitamin C, alkaloids and saponins.(Widiastini et al. 2021). The selection of rats (R. norvegicus) Wistar strain as subjects in this study because it had a higher average of live spermatozoa (92%) compared to the percentage of live spermatozoa in Sprague-Dawley (90.7%) (Simbolon *et al.*, 2013).

Based on this background, the purpose of this study was to determine the administration of Moringa (Moringa Oleivera) Leaf Ethanol Extract to the number of spermatozoa and the morphology of the spermatozoa of the Old Age Wistar Wistar strain.

MATERIALS AND METHODS

Place and time of research

This research was carried out in January-March 2021. Implementation of research at the Integrated Biomedical Laboratory, Faculty of Medicine, Udayana University.

Ingredient

Male rats (Ratus Norvegicus) Wistar strain 200-250 grams, aged 18-19 months. Moringa leaf ethanol extract 50 mg/kg BW per day for 30 days, 0.5% CMC, ketamine: xylazine, 1 set of surgical instruments, surgical board , 0.9% NaCl solution, 1% Eosin dye and 10% Nigrosin, dropper, petri dish, object glass, light microscope (Olympus brand).

Method

This study used an experimental research design, namely the randomized post-test only control group design. The sample in this study was male Wistar rat (Rattus norvegicus) old age according to the inclusion criteria, including body weight 200-250 g, rat age 18-19 months. While the exclusion criteria were rats looked sick, did not move actively. Samples that entered the drop out criteria were mice that died during the study and there was a weight loss of more than 10% after the acclimatization period in the laboratory. The sample size in this study was 36 rats which were divided into two groups, namely 18 for the treatment group and 18 for the control group. To determine the sample, the researcher used the Random Sampling technique.

Making Moringa Leaf Ethanol Extract

Moringa leaf extract was made by maceration as much as 50 grams of dry Moringa leaves, crushed using a blender, added 96% ethanol solvent, put in a container, closed and left for two days protected from sunlight. This mixture is filtered to obtain maserate. The pulp was macerated with 96% ethanol using the same procedure. Maceration was carried out until a clear maserate was obtained. The macerate was evaporated using a rotary vacuum evaporator at a temperature of 40 ^{0}C (Widiastini et al. 2021).

Research procedure

This research started from weighing the weight of all experimental animals. The dose of ethanol extract of Moringa leaves was given to the treatment group as much as 50 mg/kgBW dissolved with 0.5% CMC as much as 0.5 mL per day. The control group was given 0.5% CMC 0.5 mL per day. Giving is done through sonde, at 08.00-09.00 Wita and given for 30 days.

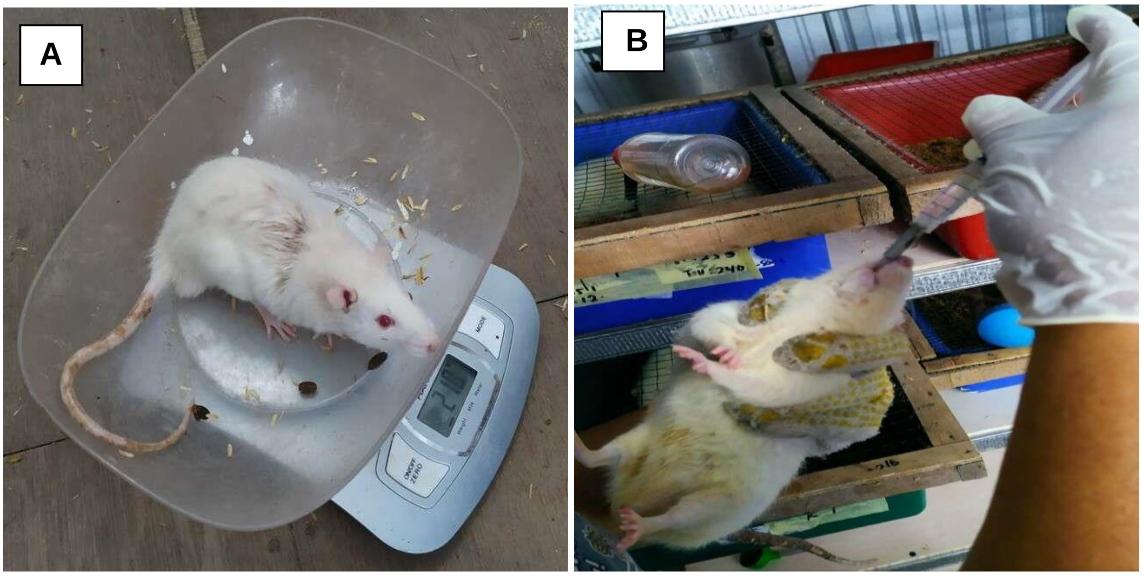

Figure 1. The treatment given to the research sample. Weighing of experimental animals (Rattus norvegicus) (A). Giving intervention to experimental animals (Rattus Norvegicus) (B).

After passing the treatment period, namely on the 30th day, the old male Wistar strain (R. Norvegicus) was euthanized. The rats were terminated with anesthesia using ketamine: xylazine at a dose of 100 mg/kg: 10 mg/kg (comparison 10: 1) intra-muscularly (IM), then euthanized by cervical dislocation method. The testes were separated from the cauda epididymis and then put into a petri dish containing 5 mL of 0.9% NaCl, the cauda epididymis was cut as finely as possible in a petri dish and stirred until homogeneous. Buried rat body.

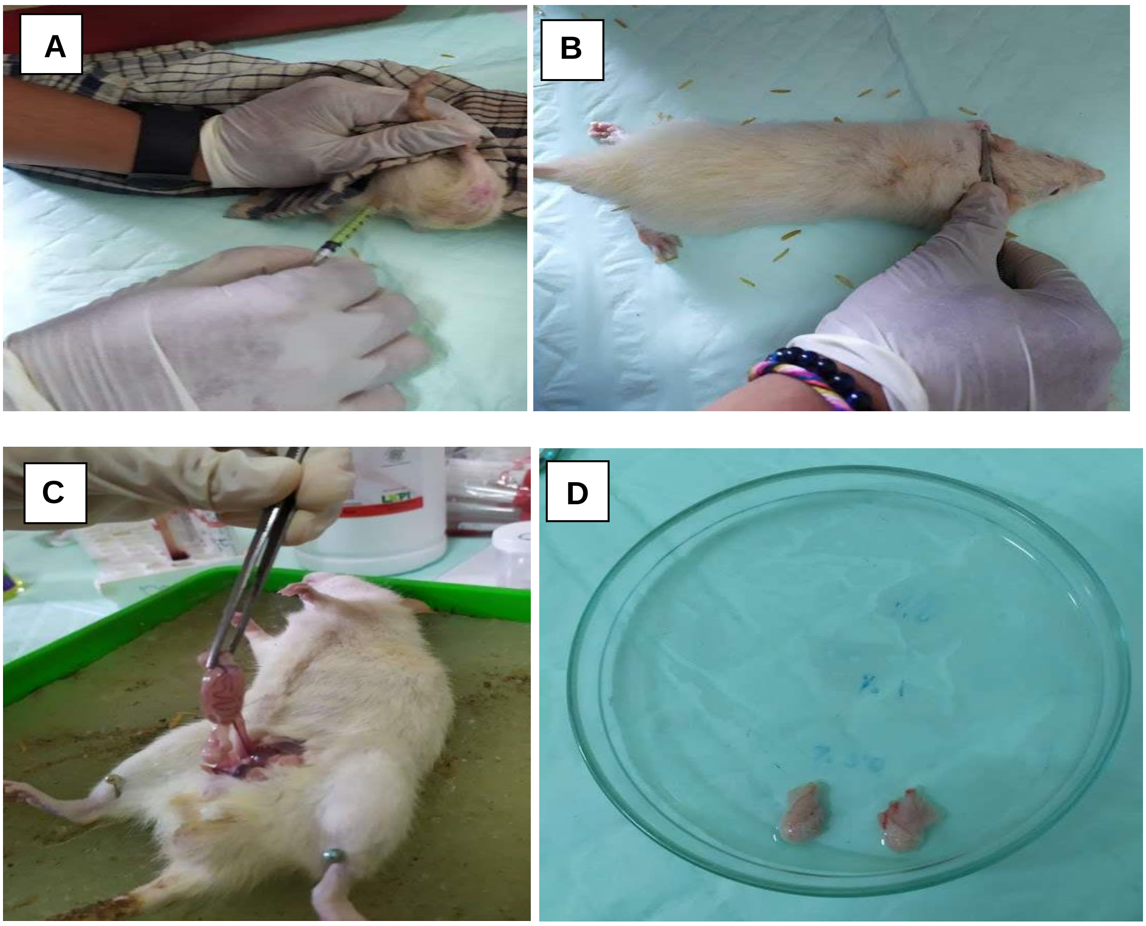

Figure 2. Actions taken after 30 days of treatment. Anesthesia of the study sample (Rattus Norvegicus) prior to euthanasia (A). The implementation of Euthanasia of the research sample using the cervical dislocation method (B). Open orchidectomy (C). Cauda epididymis study sample (D).

Counting the Number of Spermatozoa

The number of spermatozoa was observed using an improved Neubauer counting chamber (hemocytometer). The spermatozoa suspension that had been diluted with 5 mL of physiological saline solution (0.9% NaCl) was taken 10 L and then placed into a counting chamber (hemocytometer), after which it was covered with a cover glass. When covering with a cover glass no air bubbles should form. The hemocytometer containing the spermatozoa suspension was then observed under a light microscope with a magnification of 400 x which was carried out for 6 times the field of view. Spermatozoa that were counted were spermatozoa located in the middle and the edge of the chamber (top and left of the chamber), while spermatozoa located at the edge of the right and bottom chambers were not counted. The average number of spermatozoa (n) was obtained from the total number of spermatozoa present in each chamber divided into 4. The length of each chamber is 1 mm and the height is 0.1 mm, so that the volume of the chamber = 0.1 mm3 (1.0 mm2 x 0 ,1mm).

Spermatozoa Morphological Observations

Spermatozoa morphology was observed using the Eosin 1% and Nigrosin 10% staining method, by making a smear of spermatozoa on an object glass, then 1 drop of Eosin 1% and 1 drop of Nigrosin 10% were given, then homogenized and air-dried for 5 minutes. Then observed under a light microscope with a magnification of 400x. The repetition is done 6 times. The normal morphology of spermatozoa is when the head and neck are intact and the tail is straight. Spermatozoa are said to be normal if the head is curved like a hook, the neck is straight and the tail is single and free. While the abnormal morphology if the head is small or too large, the neck is broken or branched, the tail is branched, curled and broken, there are cytoplasmic droplets on the head, neck or tail (Majzoub and Jr 2017).

The data analysis is descriptive analysis, namely by displaying the distribution of the frequency and mean of the number of spermatozoa and morphology of spermatozoa. Data normality analysis was performed using the Saphiro Wilk test, the data was normally distributed if the p value> 0.05. After obtaining the data before and after treatment, both in the treatment and control groups. After obtaining the results of the data normality test, a comparative analysis was performed. If the data is normally distributed, the analytical test used is the independent sample t test at a significance level of = 0.05 to determine the difference between the treatment and control groups. Data analysis used a 95% confidence level (95% CI/Convidence interval is another parameter to measure how accurately the Mean of a sample represents (includes) the true Population Mean value) or is stated if p<0.05. Meanwhile, if the data are not normally distributed, the analytical test used is the Mann Whitney test, to determine the difference between the treatment and control groups, data analysis uses a 95% confidence level or is declared different if p <0.05.

RESULTS AND DISCUSSION

1. Characteristics of Research Subjects

A total of 36 rats met the eligibility criteria, namely white rats (Rattus norvegicus) Wistar strain with male sex, age 18-19 months, body weight 200-250 g and healthy. The average body weight of the mice used was 229.35gr ±11.4%.

2. Number of Sperm in Rats (Rattus Norvegicus) Male Wistar Strain After Giving Moringa Leaf Ethanol Extract

Live spermatozoa were characterized by no color absorption (clear) while dead spermatozoa were indicated by the absorption of 1% Eosin and 10% Nigrosine dyes (Figure 3).

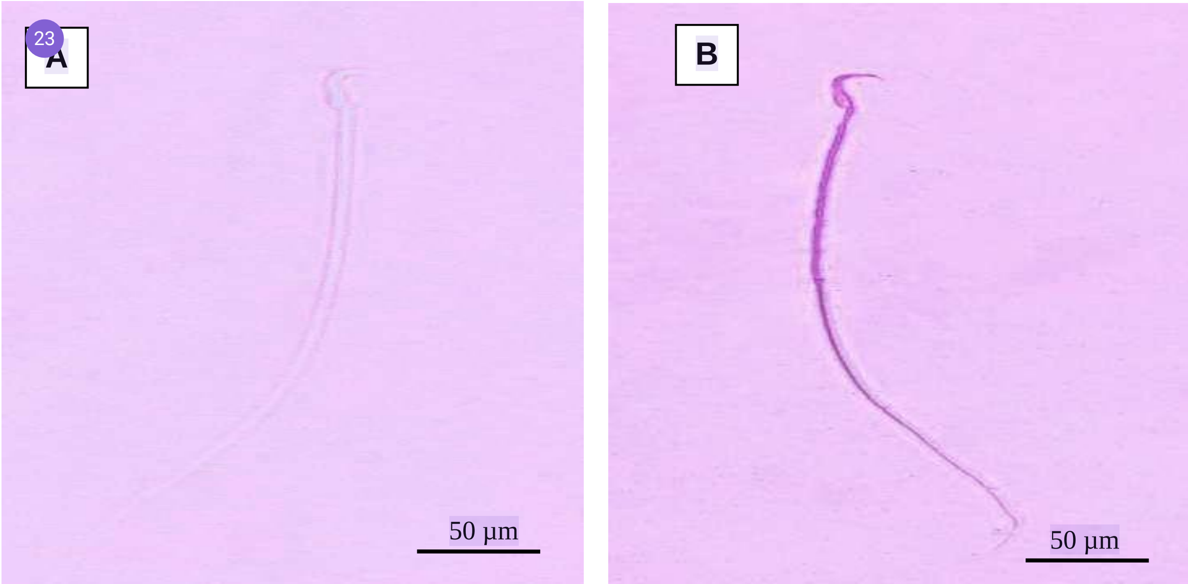

Figure 3. *Rat sperm after being given Moringa Leaf Ethanol Extract. Live spermatozoa (A). Dead spermatozoa (B) were observed under a microscope with a magnification of 400x.*

From Figure 4 shows the number of Spermatozoa in Rats (Rattus Norvegicus) Male Wistar Strain After Giving Moringa Leaf Ethanol Extract in the treatment group showed a higher density than the control group.

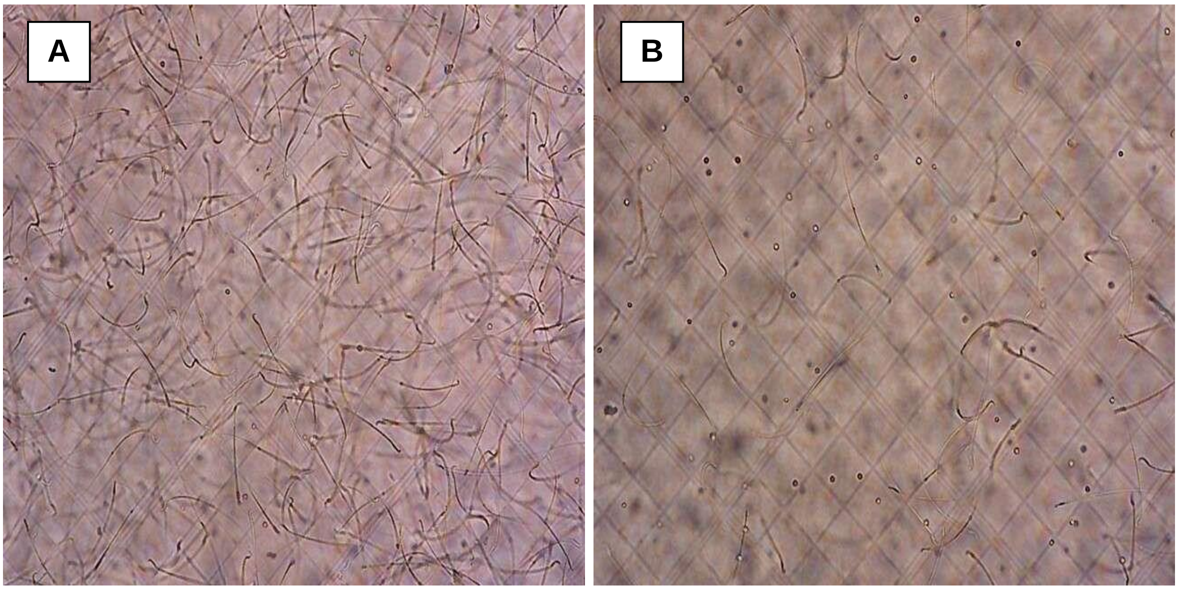

Figure 4. Number of Spermatozoa in Rats (Rattus Norvegicus) Male Wistar Strain After Giving Moringa Leaf Ethanol Extract. Treatment group (A). The control group (B) was observed under a microscope with a magnification of 400x

Tablel 1. Number of Sperm in Rats (Rattus Norvegicus) Male Wistar Strain After Giving Moringa Leaf Ethanol Extract

Group	*Mean*	95% CI	Minimum	Maximum	Normality test	P
Treatment	87,70% ± 5,82	87,11 – 88,29	86%	89,67%	0,632	0,000
Control	74,56% ± 1,18	71,66 – 77,46	65,67%	85,17%	0,172	

Description: P <0.05, there is a significant effect using the independent sample t test

From the table above, it can be seen that the treatment group has an average number (mean) of 87.70%, with a 95% CI value of 87.11 - 88.29, while the control group is 74.56%, with a 95% CI value. 71.66 – 77.46. Sperm count data in the treatment and control groups were normally distributed with a p-value of 0.632 for the treatment group and 0.172 for the control group, because the data were normally distributed, independent t-test analysis was performed and the p-value was 0.000 (p-value <0.05), which means that there is a significant difference in sperm count between the group given Moringa leaf ethanol extract and the control group, so it can be interpreted that the Moringa leaf ethanol extract significantly increases sperm count.

The antioxidant content in Moringa leaves works to neutralize free radicals thereby preventing oxidative damage to most biomolecules and provides significant protection against oxidative damage, has the ability to protect lipo proteins from peroxyl radicals thereby increasing the number of spermatozoa, this is in line with research conducted by (Bebas et al. 2015), showed that the administration of vitamin C 0.1 mg/ml in, can maintain the viability of spermatozoa and increase the motility of spermatozoa. Research conducted by (Diao et al. 2019), showed that supplementation with Quercetin (Flavonoid) could protect Spermatozoa from sperm damage mediated by H2O2 (hydrogen peroxide). Study (Manhal M. Dafaalla et al. 2016), It was found that there was a significant difference in sperm count in white rats between the group given Moringa leaf ethanol extract and the control group that was not given, so it can be concluded that Moringa leaf ethanol extract can have a significant effect on sperm count. Study (Priyadarshani and Varma 2014) juga menunjukkan hasil yang sama yaitu jumlah sperma meningkat secara signifikan pada tikus hiperglikemik yang diberikan bubuk kelor sebanyak 200 mg/kg berat badan dengan nilai (P < 0,05). Reaserach (Fatoba et al. 2013) get a value (P < 0.05), which means Moringa oleifera can significantly increase mass activity, progressive motility and sperm concentration in mice.

1. Sperm Morphology in Rats (Rattus Norvegicus) Male Wistar Strain After Giving Moringa Leaf Ethanol Extract

Spermatozoa morphology calculations were carried out using a light microscope with a magnification of 400x on 100 spermatozoa cells. (Figure 5).

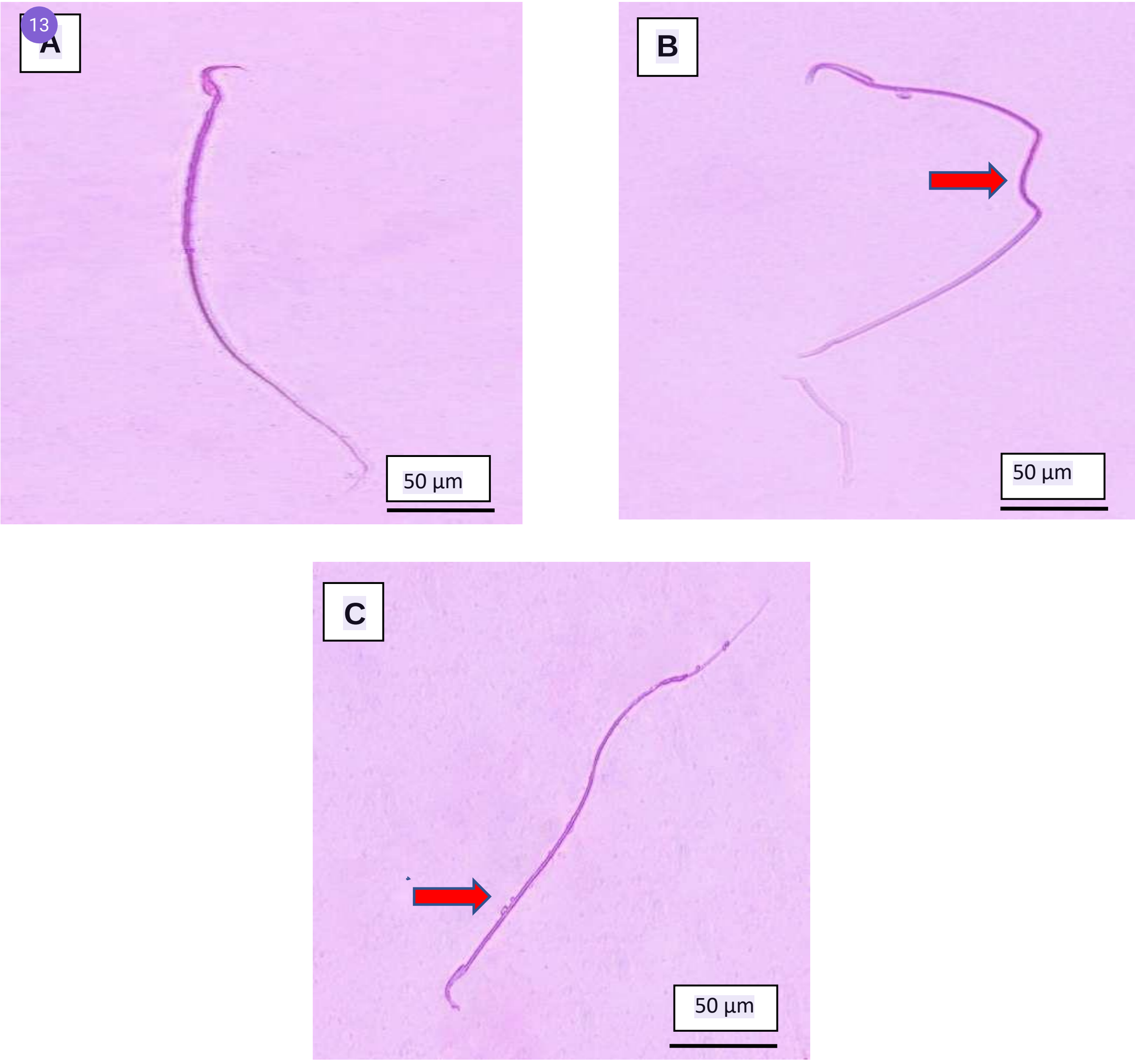

Figure 5. *Mouse spermatozoa with normal morphology (A), abnormal tail (B), and cytoplasmic droplets (C)*

Table 2. Sperm Morphology in Rats (Rattus Norvegicus) Male Wistar Strain After Giving Moringa Leaf Ethanol Extract

Group	*Mean* ± SD	95% CI	Minimum	Maximum	Normality test	P
Treatment	82,28% ± 6,64	78,97- 85,58	68,83%	89,33%	0,023	0,000
Control	61,89% ± 9,78	57,02 – 66,75	38,83%	78%	0,015	

Description: P <0.05, there is a significant effect using the Mann Whitney test

From the table above, it can be seen that the treatment group had good sperm morphology with an average (mean) of 82.28%, 95% CI 78.97–85.58, while in the control group the average (mean) namely 61.89% with a 95% CI value of 57.02-66.75. The data on the number of sperm in the treatment and control groups were not normally distributed with a p value of 0.023 for the treatment group and 0.015 for the control group, then the analysis was carried out using the Mann Whitney test and the results obtained p value of 0.000 (p value <0.05), which means there are There were significant differences in sperm morphology between the group given Moringa leaf ethanol extract and the control group, so it can be interpreted that Moringa leaf extract can have a significant effect on sperm morphology.

This is in accordance with previous research (Wahjuningsih et al. 2019), showed that the addition of Moringa leaf extract could improve cement quality. Study

(Carrera-Chávez et al. 2020) The results obtained that semen supplementation with Moringa oleifera seeds increased antioxidant activity, sperm membrane integrity, viability and progressive motility. Moringa leaf ethanol extract containing Ascorbic acid holds a much stronger antioxidant than others by supplying up to 65% of seminal plasma. (Sahu CR 2016). Research (Wahjuningsih et al. 2019; Carrera-Chávez et al. 2020), Moringa leaf extract which contains antioxidants is able to maintain spermatozoa membranes and protect sperm damage caused by free radicals, thereby increasing density and improving spermatozoa morphology. Administration of antioxidant compounds can increase sperm parameters, such as morphology, motility, concentration, and reduce DNA damage with a value ($P < 0.05$) (Martin-Hidalgo et al. 2019; R. Dias et al. 2020). However, it should be noted, excessive use of antioxidants can cause pro-oxidants called antioxidant paradoxes, this can reduce endogenous oxidants that are important for the induction of physiological pathways, thereby inhibiting sperm capacitation, hyperactivation and acrosomal reactions of spermatozoa which can increase various pathological conditions such as aging. and infertility in men (Henkel et al. 2018; Ali et al. 2020). So that giving Moringa leaf ethanol extract 50 mg/kgBW can increase sperm count and improve sperm morphology in Wistar strain Rattus norvegicus in old age.

CONCLUSION

This study showed that the Spermatozoa Number of Old Age White Rats (Rattus Norvegicus) Wistar Strain given Moringa Leaf Ethanol Extract (Moringa Oleivera) was higher than the control group. The treatment group (White Rats/Rattus Norvegicus Wistar strain) given Moringa Leaf Ethanol Extract (Moringa Oleivera) also showed more normal Spermatozoa Morphology than the control group. In this study, there was a significant effect of giving Moringa Leaf Ethanol Extract (Moringa Oleivera) in increasing the number of spermatozoa and improving the sperm morphology of the white rat (Rattus norvegicus) Wistar strain of old age. Suggestions that can be given in this study are further research on the provision of Moringa leaf ethanol extract by paying attention to the reproductive cycle to more deeply see the effect of Moringa leaf ethanol extract on male reproductive health.

THANK-YOU NOTE

The researcher would like to thank STIKES Bina Usada Bali which has provided grant funds for the implementation of this research. The researcher also thanks the integrated Biomedical Laboratory of the Faculty of Medicine, Udayana University which has facilitated and assisted in the process of carrying out this research.

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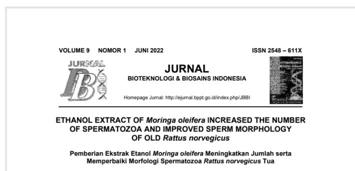

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