HAYATI
Journal of Biosciences

Vol. 32 No. 4, July 2025 1003-1009
DOI:10.4308/hjb.32.4.1003-1009
ISSN: 1978-3019
EISSN: 2086-4094

Research Article

Expression of the BAX Gene, CO1 Gene, and their Relationship to the Motility and Spermatozoa Concentration of Rats Treated with *Moringa oleifera* Leaf Extract

Uswatun Hasanah[1,2*], Cut Fauziah[1], Nasihin Saud Irsyad[1], Andri Pramesyanti Pramono[2], Dinda Nuraini Hanifah Wahab[3], Wafa Nabila Nawawi[3]

[1]*Department of Molecular Biology, Faculty of Medicine, UPN Veteran Jakarta, Jakarta Selatan 12450, Indonesia*
[2]*Research and Innovation National Agency (BRIN), Jakarta, Indonesia*
[3]*Faculty of Medicine, UPN Veteran Jakarta, Jakarta Selatan 12450, Indonesia*

ARTICLE INFO

Article history:
Received May 16, 2024
Received in revised form January 24, 2025
Accepted March 10, 2025

KEYWORDS:
Antioxidants,
Gene expression,
Oxidative stress,
Sperm

ABSTRACT

Spermatogenesis is the process of spermatozoa formation. An increase in free radicals, such as in hyperglycemia conditions, causes oxidative stress, which can interfere with spermatogenesis. Oxidative stress is an inequality condition between the body's capacity to produce antioxidants and the rise of free radicals. Antioxidants such as flavonoids are contained in plants such as Moringa oleifera leaves. During spermatogenesis, an apoptosis process involves the BAX gene. BAX is a gene that functions as pro-apoptosis. There is also a CO1 gene in mitochondria, which plays a role in cellular metabolism. Gene expression examination assesses the comparative threshold cycle (CT) with RT-PCR, and spermatozoa quality examination includes assessing spermatozoa concentration and motility. This study used 30 male white Sprague Dawley rats as the test animals, which were divided into five treatment groups: treatment of moringa leaf extract dose 200 mg/kg BW, treatment dose 300 mg/kg BW, treatment dose 400 mg/kg BW, normal control group, and positive control group. The results showed a decrease in BAX gene expression and CO1 gene expression in the treatment group of test animals. The results of sperm analysis showed an increase in spermatozoa concentration in all treatment groups compared to the control group. For sperm motility in the 400 mg/KgBB dose treatment, an increase in sperm motility compared to the control group. This study concludes that administering moringa leaf extract reduced the expression of the Bax and CO1 genes. However, no significant changes were observed in sperm concentration and motility within the treatment group.

1. Introduction

Spermatozoa are germ cells in the male reproductive organs. The spermatozoa structure consists of a head that contains DNA, a midpiece that contains mitochondria, and a tail for movement. Spermatozoa are formed through spermatogenesis, which involves mitosis, meiosis, and cell differentiation (Rathke *et al.* 2014). A disturbance in the spermatogenesis process can affect motility spermatozoa (Chao *et al.* 2023) and cause a decrease in spermatozoa concentration due to excessive apoptosis. Apoptosis is programmed cell death involving a series of genes, including the Bcl-2 family, such as Bax (Young & Nelson 2001).

The Bcl-2 protein family primarily controls mitochondrial membrane permeability and the release of apoptotic agents into the cytoplasm from the intermembrane space (Zamzami & Kroemer 2001). Pro-apoptotic Bcl-2 family members stimulate the release of apoptotic factors. The pro-apoptotic protein Bax (Bcl-2-associated X protein) localizes to germ cells undergoing

* Corresponding Author
E-mail Address: uzwah2603@gmail.com

apoptosis during the early stages of spermatogenesis (Print & Loveland 2000).

Mitochondria have a role in the electron transport chain for energy production in spermatozoa movement. Mitochondria contain many genes, including the CO1 gene regulating cell respiration. Cytochrome Oxydase1 (CO1) is a gene in mitochondrial DNA that produces ATP. The CO1 gene has few mutations in its base sequence and many conserved parts (Hebert *et al.* 2003). Mutations in the mtDNA of sperm result in either functionless or malfunctioning proteins, affecting sperm motility. Several studies have analyzed the effect of mitochondrial dysfunction on spermatozoa quality (Dzudzor *et al.* 2021). Changes in mitochondrial function due to mutations in mitochondrial genes might be responsible for low sperm motility (Thangaraj *et al.* 2003).

Moringa oleifera is a plant that contains high antioxidant substances. The high content of bioactive compounds such as flavonoid, quercetin, alkaloids, saponins, and phenols is helpful as an antidote for free radicals. The antioxidants in *Moringa oleifera* leaves can ward off oxidative stress caused by hyperglycemia. Hyperglycemia is an event of high blood glucose levels, which is indicated as diabetes mellitus (Jannah *et al.* 2018). Insulin resistance affects the body's metabolism by producing reactive oxygen species (ROS). ROS can affect spermatogenesis in the male reproductive organs, decreasing spermatozoa quality (Saputri *et al.* 2021). In several studies, *Moringa oleifera* leaf extract can maintain motility, acrosome integrity, and spermatozoa morphology. Meanwhile, research on the effect of moringa leaf extract on changes in gene expression in sperm has not been widely conducted. Based on this background, research was carried out regarding the impact of Moringa oleifera leaf extract on the expression of the BAX and CO1 genes and their relationship to the concentration and motility of rat spermatozoa.

2. Materials and Methods

2.1. Study Time and Places

The research was carried out from July 2023 to February 2024. The study was conducted in the Animal Medical Education and Research Center Laboratory (MERCE), Molecular Biology Laboratory FK UPN Jakarta, and Genomic Laboratory.

2.2. Research Animals and *Moringa oleifera* Leaf Extraction

The research sample consisted of 30 Sprague Dawley strain white rats weighing 150-250 grams. The rats were separated into five groups of treatment, namely the normal control group, which were not induced by alloxan and were not treated with *Moringa oleifera* leaf extract; the positive control group of rats, which were caused by alloxan and were not given *Moringa oleifera* leaf extract treatment; and the treatment groups which were caused by alloxan and were treated with extract of *Moringa oleifera* leaf at a dose of 200 mg/kg body weight (BW), 300 mg/kg BW, and 400 mg/kg BW.

Moringa leaves are extracted using the maceration method. Moringa leaf powder is mixed with 70% ethanol at room temperature for 4 days, then filtered using filter paper. Another solvent portion is added, and the extraction process is repeated until the final extract is colorless. All extracts were combined and evaporated at 40ºC using a rotary vacuum evaporator. The concentrated extract was then further evaporated in a water bath at a temperature of 70°C until the extract weight reached a constant value. Then, the spectrophotometric method was tested to determine the flavonoid content as quercetin in moringa leaf extract and assess the antioxidant activity $IC_{50\%}$.

2.3. Preparation and Treatment of Test Animals

Male rats were adapted to the research environment for seven days. Rats were only given standard food and drinking water ad libitum. The procedure was conducted in standard facilities at a temperature of 25°C, with 12 hours of darkness and 12 hours of light. On day 8, the treatment and positive control groups were injected with alloxan at 125 mg/kg BW intraperitoneally, then monitored for three days, and blood sugar was measured. On the 11^{th} day, the rats were given the amount of *Moringa oleifera* leaf extract according to the specified dose. The extract was given once a day in the morning before feeding for 26 days by force-feeding the extract into the rats' mouths using a probe. This study has obtained ethical clearance from a committee of the Faculty of Medicine, UPN Veteran Jakarta, with the number 333/VII/2023/KEPK.

2.4. Spermatozoa Sample Collection

Experimental animals were treated from acclimatization to extract administration for 36 days. On day 37, the rats were terminated, and spermatozoa suspension was made by dissecting the rats and taking part in the epididymis. The epididymis organ was taken with tweezers and placed in a petri dish. Then, the epididymis was mixed using 1 ml of 0.9% NaCl solution and stirred until homogeneous to obtain a spermatozoa suspension.

2.5. Spermatozoa Concentration Assessment

The spermatozoa suspension was homogenized with physiological NaCl to take 0.01 ml of the sample. Observations were microscopically using a bright field microscope with a magnification of 40 × 10 (objective×ocular) and a Neubauer haemocytometer covered with a glass deck. Then, calculations were conducted.

2.6. Spermatozoa Motility Assessment

A total of 0.1 ml of spermatozoa suspension was transferred to an object glass and covered using a deck glass. Microscopic observation was performed using a bright field microscope with a magnification of 40 × 10 (objective×ocular). Calculations were carried out by assessing the motility percentage and counting 200 spermatozoa.

2.7. RNA Extraction

Spermatozoa were stored in the RNA Shield and centrifuged for 1 minute. The supernatant was removed, and then ethanol was added. The sample was re-centrifuged, and the supernatant was discarded. The process continued by administering DNase I, then 400 µL of RNA Wash Buffer was added to the sample, then centrifuged, and then a mixture of 5 µL of DNase I and 75 µL of DNA Digestion Buffer was added. Samples were incubated for 15 minutes at room temperature. Next, 400 µL of RNA Prep Buffer was added, then centrifuged, and the part that did not pass through the filter was taken. The sample was added to 700 µL of RNA Wash Buffer and centrifuged again. The final stage is the addition of 100 µL DNase/RNase-Free Water. RNA purity and concentration were calculated using a spectrophotometer at A_{260}/A_{280} nm.

2.8. Primer Design and Optimization

The sequences of the BAX, CO1, and housekeeping genes (β-actin genes) are listed in Table 1.

Table 1. The marker type and the sequence of primer base

Marker	forward primer (5'-3')	reverse primer (5'-3')	PCR product (bp)	Source
BAX	5'-TGGAGA TGAACT GGACA GCA-3'	5'-GATCAG CTCGGG CACTTT AG-3'	201	NM_004324
CO1	5'ATGAGCA AAAG C CCACTT TG-3'	5'CGGCCG TAAGTG AGATGA AT-3	170	JX426131.1
β-actin	5'-CGACGA GGCCCA GGCAAG AGAGG-3'	5'-TCAGGC AGCTCAT GCTCTTC TCCAGG-3'	180	M10277

2.9. cDNA Synthesis

cDNA synthesis begins by formulating RNA concentrations for various treatments adjusted to the RNA concentration. The RNA sample was put into a solution containing 4x DN Master Mix of 4 µl, and Nuclease Free-Water was added to reach a total volume of 16 µL. The mixture was incubated for 5 minutes at 37°C, and 4 µL 5x RT Master Mix II was added so that the total reaction volume reached 20 µL. The mixture was re-incubated at 37°C for 15 minutes, 50°C for 5 minutes, and heated at 98°C for 5 minutes.

2.10. Quantitative PCR (qPCR)

After cDNA was formed, the reaction was carried out in a total volume of 20 µl. The Real-time (RT)-PCR reaction in this study was carried out with a pre-denaturation cycle for 12 minutes at 95°C for 1 cycle, denaturation for 12 seconds at 95°C, annealing at 60°C for 32 seconds, and elongation for 60 seconds at 72 °C as much as 40-50 cycles. The control treatment used the β-actin gene. RT-PCR results are CT values analysed using the $2^{(-\Delta\Delta Ct)}$ method. ΔΔCT from method 2 - ΔΔCT, ΔCT was calculated with the formula:

$$\Delta CT = CT \text{ (target gene)} - CT \text{ (reference gene)}$$

2.11. Data Analysis

Data analysis begins with a normality test to see whether the data is in normal distribution and a homogeneity test to see whether the data is homogeneous. After that, the one-way ANOVA parametric test was performed with $P<0.05$ for the significant difference.

3. Results

The results of the extracted Moringa oleifera leaf flavonoid test as quercetin showed that 3.78% contained the flavonoid quercetin, and the IC_{50} antioxidant test results showed that it was 95.62 ppm, which shows the strong category.

3.1. RNA Extraction

RNA Extraction was performed on five treatments, and the data are presented in Table 2.

The spermatozoa RNA obtained from the extraction process had a purity value ranging from 1.55 to 1.77. The concentration of spermatozoa found ranged from 7.9 to 13.7 ng/µL.

3.2. Gene Expression

BAX gene expression was analysed using RT-PCR. The PCR reaction involves three main stages: denaturation, annealing, and elongation. The denaturation step separates the double strand of cDNA into two single strands. The annealing stage is when the primer is attached to a particular area of the cDNA. The elongation stage involves the DNA polymerase enzyme duplicating and lengthening the target DNA fragment. Analysis of the RT-PCR results obtained is shown in Figure 1.

Table 2. Purity and concentration of extracted RNA

Treatment	260/280	RNA concentration (ng/µL)
Positive control	1.73	7.9
Normal control	1.75	13.7
Moringa oleifera extract with 200 mg/kg body weight dose	1.57	11.5
Moringa oleifera extract with 300 mg/kg body weight dose	1.55	9.4
Moringa oleifera extract with 400 mg/kg body weight dose	1.77	8.4

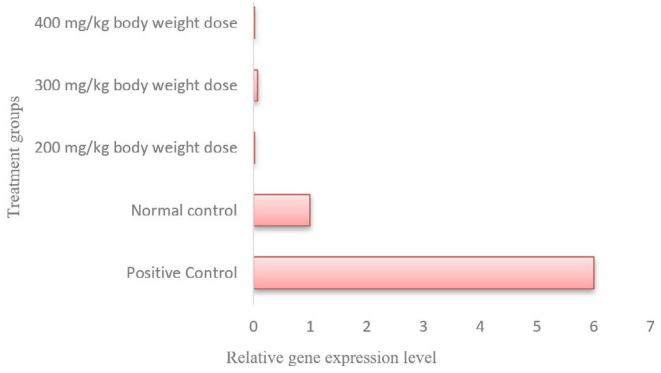

Figure 1. Bax gene expression was analysed using RT-PCR

Bax gene expression was highest in the positive control group compared to all treatment groups. In addition, the rats' group with treatment doses of 200 mg/kg BW and 400 mg/kg BW had lower Bax gene expression than the control group.

CO1 gene expression was seen in 300mg/Kg BW dose, which had the highest CO1 gene expression compared to all treatment groups. In addition, the group of rats with treatment doses of 200 mg/kg BW and 400 mg/kg BW had lower CO1 gene expression than the control group (Figure 2).

3.3. The Spermatozoa Concentration and Motility

The results showed that the highest spermatozoa concentration was found in the Moringa oleifera leaf extract treatment group at 400 mg/kgBW. The lowest concentration was found in the positive and standard control treatment. The highest spermatozoa motility was found in the *Moringa oleifera* extract treatment group at a dose of 400 mg/kg BW and normal control treatment, and the lowest motility spermatozoa was found in the positive control group (Figures 3 and 4). Sperm motility and sperm concentration data were assessed for normality and homogeneity, and the data were usually and homogeneously distributed ($p>0.05$). The ANOVA test found no significant difference in spermatozoa concentration and motility between the five treatments ($p>0.05$).

4. Discussion

This study found that administering Moringa leaf extract to the treatment group decreased Bax gene expression. The Bax gene is expressed in certain conditions, such as oxidative stress and tissue damage, so the Bax gene is expressed unevenly. Before the treatment of Moringa leaf extract, the test animals were made in a hyperglycemia

Figure 2. CO1 gene expression was analysed using RT-PCR

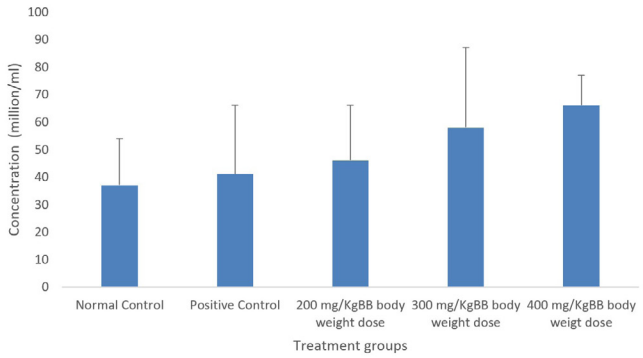

Figure 3. Mean and SD value of Sperm concentration

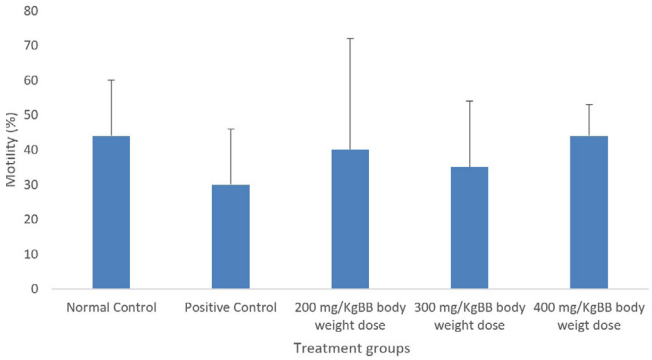

Figure 4. Mean and SD value of Sperm motility

condition that can cause oxidative stress. Oxidative stress can trigger increased apoptosis, especially in the formation of germ cells. Increased apoptosis involves changes in Bax gene expression. Bax initiates apoptosis through the cytochrome c pathway in the mitochondria (Asadi *et al.* 2021). Gene expression research using extracted RNA from sperm. The RNA obtained in this study had low concentration and low purity; this could be caused by adding RNA Shield for temporary storage during sample preparation, as the volume was too small to allow much of the RNA to be degraded. Inappropriate storage factors and the length of storage can affect RNA stability. In addition, the addition of DNase/RNase-Free Water at the final extraction stage is thought to affect the high final concentration of RNA.

The study results were obtained in the positive control group that was made in hyperglycemia conditions, and without being given moringa leaf extract treatment, high Bax gene expression was observed, indicating an increase in cell apoptosis. Different results were found in the treatment group made in hyperglycemia, where moringa leaf extract treatment was given with various doses. In all treatment groups, there was a decrease in Bax gene expression compared to the control group. BAX is a gene that is slightly expressed. Still, treatments such as making test animals hyperglycemic will cause oxidative stress and make the BAX gene express a lot to initiate apoptosis (Asadi *et al.* 2021).

Moringa oleifera leaves contain flavonoids such as quercetin, which have antioxidant and anti-inflammatory properties (Vergara-Jimenez *et al.* 2017). The flavonoid quercetin can repair cell damage and has antioxidant properties against reactive oxygen species (ROS), which can interfere with spermatogenesis (Zhang *et al.* 2023). Quercetin is instrumental in mitigating reactive oxygen species (ROS) activity in sperm cells and preserving male germ cells, primarily by enhancing the Bcl-2/Bax ratio. This ratio is crucial for assessing cell apoptosis (Dong *et al.* 2022). Quercetin offers cellular protection against free radicals by inhibiting enzymes that generate these radicals and promoting the activity of internal antioxidant systems (Liu & Guo 2015; Vergara-Jimenez *et al.* 2017). When free radicals are not excessive, cells can effectively counteract them through their endogenous antioxidant defenses. However, when these internal mechanisms are insufficient, external antioxidants become necessary. Antioxidants play a vital role in shielding cells from oxidative stress and facilitating the proliferation of spermatogonia cells (Qamar *et al.* 2023).

The evaluation of sperm concentration in this study revealed that all groups receiving Moringa leaf extract exhibited increased sperm concentration. An ANOVA test was conducted, which indicated that the observed increase in sperm concentration in the treatment groups did not differ significantly from that of the control group. This lack of significant difference may be attributed to the relatively short duration of Moringa leaf extract administration, which lasted only 26 days; a longer treatment period might yield more pronounced effects. Sperm concentration serves as an indicator of the quantity of sperm produced within the seminiferous tubules. The process of spermatogenesis encompasses several stages, including proliferation, differentiation, and apoptosis. Apoptosis plays a critical role in eliminating damaged cells, thereby regulating spermatogenesis and preventing these cells from progressing to subsequent stages of division (Asadi *et al.* 2021).

The study also found that in the treatment group given moringa leaf extract, there was a decrease in CO1 gene expression. CO1 is a gene in the mitochondria that encodes proteins that play an important role in cellular respiration, so it is closely related to energy availability for metabolism and cell movement (Hasanah *et al.* 2023). CO1 plays a role in cell metabolism by transferring electrons from

cytochrome c during ATP formation (Himawan 2022). Mitochondria in sperm cells are located in the midpiece. Mitochondria in sperm play a role in energy availability for cell motility. A disruption in mitochondrial function may lead to a decrease in the motility of spermatozoa (Costa *et al.* 2023). Changes in gene expression can occur if there is stimulation from the environment, such as in oxidative stress conditions. All groups given moringa leaf extract had lower CO1 gene expression than the normal control group without any treatment. The treatment group with moringa leaf extract (200 mg/kg BW and 400 mg/kg BW) found low CO1 gene expression. Gene expression occurs in response to stress to minimize oxidative damage caused by the formation of ROS. Administration of Moringa leaf extract containing antioxidants can improve oxidative stress conditions so that mitochondrial function is not disturbed. Mitochondria are organelles that are susceptible to damage due to oxidative stress. Changes in gene expression affect the process of protein synthesis and cell metabolism. In sperm motility, CO1 is an energy producer for cell movement in cellular respiration.

Sperm motility in the treatment group given moringa leaf extract increased sperm motility compared to the positive control group. To determine whether the differences in sperm motility produced in all treatment and control groups were statistically significant, an ANOVA test was conducted, and it was found that the differences in motility between all groups were not significantly different. The findings from the sperm motility assessment did not reveal any significant differences, which may be attributed to the relatively brief duration of moringa leaf extract administration, potentially insufficient to influence sperm motility. A more pronounced effect could be observed with an extended treatment period. In this investigation, sperm was harvested from the epididymis, a site where sperm maturation occurs, enabling motility. The incomplete sperm maturation at the time of collection is believed to have contributed to the lack of significant differences in motility across the treatment groups.

References

Asadi, A., Ghahremani, R., Abdolmaleki, A., Rajaei, F., 2021. Role of sperm apoptosis and oxidative stress in male infertility: a narrative review. *International journal of reproductive biomedicine*. 19, 493. https://doi.org/10.18502/ijrm.v19i6.9371

Chao, H.H., Zhang, Y., Dong, P.Y., Gurunathan, S., Zhang, X.F., 2023. Comprehensive review on the positive and negative effects of various important regulators on male spermatogenesis and fertility. *Frontiers in Nutrition*. 9, 1063510. https://doi.org/10.3389/fnut.2022.1063510

Costa, J., Braga, P.C., Rebelo, I., Oliveira, P.F., Alves, M.G., 2023. Mitochondria quality control and male fertility. *Biology*. 12, 827. https://doi.org/10.3390/biology12060827

Dong, B., Shi, Z., Dong, Y., Chen, J., Wu, Z. X., Wu, W., Han, C., 2022. Quercetin ameliorates oxidative stress-induced cell apoptosis of seminal vesicles via activating Nrf2 in type 1 diabetic rats. *Biomedicine & Pharmacotherapy*. 151, 113108. https://doi.org/10.1016/j.biopha.2022.113108

Dzudzor, B., Bimah, B., Amarh, V., Ocloo, A., 2021. Spermatozoa parameters and mitochondrial DNA sequence variants among patients at a fertility clinic in Ghana. *PloS One*. 16, e0252923. https://doi.org/10.1371/journal.pone.0252923

Hasanah, U., Abinawanto, A., Boediono, A., Kusrini, E., 2023. The effect of cryopreservation on cytochrome oxidase1 (CO1) gene and the relationship with spermatozoa motility of albino pangasius catfish (*Pangasionodon hypophthalmus*). *HAYATI Journal of Biosciences*. 30, 825-833. https://doi.org/10.4308/hjb.30.5.825-833

Hebert, P.D., Ratnasingham, S., de Waard, J.R., 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London B: Biological Sciences*. 270, 96-99. https://doi.org/10.1098/rsbl.2003.0025

Himawan, Y., Alimuddin, A., Nirmala, K., Imron, I., Suharyanto, S., 2022. Frekuensi ventilasi dan ekspresi gen CO1 ikan mas (*Cyprinus carpio*) populasi sintettik kondisi ammonia tinggi. *Media Akuakultur*. 17, 1-7. https://doi.org/10.15578/ma.17.1.2022.1-7

Jannah, R., Setiasih, N.L.E., Suastika, P., 2018. Histopatologi testis tikus penderita diabetes mellitus pasca pemberian ekstrak daun kelor. *Buletin Veteriner Udayana*. 10, 176-182. https://doi.org/10.24843/bulvet.2018.v10.i02.p11

Liu, Y.Z., Guo, M.Q., 2015. Studies on transition metal quercetin complexes using electrospray ionization tandem mass spectrometry. *Molecules*. 20, 8583-859. https://doi.org/10.3390/molecules20058583

Print, C.G., Loveland, K.L., 2000. Germ cell suicide: new insights into apoptosis during spermatogenesis. *Bioessays*. 22, 423-430. https://doi.org/10.1002/(SICI)1521-1878(200005)22:5<423::AID-BIES4>3.0.CO;2-0

Rathke, C., Baarends, W. M., Awe, S., Renkawitz-Pohl, R., 2014. Chromatin dynamics during spermiogenesis. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*. 1839, 155-168. https://doi.org/10.1016/j.bbagrm.2013.08.004

Saputri, D.A., Christijanti, W., Lisdiana, L., Iswari, R.S., 2021. Pengaruh ekstrak daun kelor (*Moringa oleifera*) terhadap kualitas sperma tikus hiperglikemia. *Prosiding Seminar Nasional Biologi.* 9, 167-171.

Thangaraj, K., Joshi, M.B., Reddy, A.G., Rasalkar, A.A., Singh, L., 2003. Sperm mitochondrial mutations as a cause of low sperm motility. *Journal of andrology*. 24, 388-392. https://doi.org/10.1002/j.1939-4640.2003.tb02687.x

Vergara-Jimenez, M., Almatrafi, M.M., Fernandez, M.L., 2017. Bioactive components in *Moringa oleifera* leaves protect against chronic disease. *Antioxidants*. 6, 91. https://doi.org/10.3390/antiox6040091

Qamar, A.Y., Naveed, M.I., Raza, S., Fang, X., Roy, P.K., Bang, S., Tanga, B.M., Saadeldin, I.M., Lee, S. and Cho, J., 2023. Role of antioxidants in fertility preservation of sperm-A narrative review. *Animal Bioscience*. 36, 385. https://doi.org/10.5713/ab.22.0325

Young, K.A., Nelson, R.J., 2001. Mediation of seasonal testicular regression by apoptosis. *Reproduction*. 122, 677-685. https://doi.org/10.1530/rep.0.1220677

Zamzami, N., Kroemer, G., 2001. The mitochondrion in apoptosis: how Pandora's box opens. *Nature Reviews Molecular Cell Biology*. 2, 67-71. https://doi.org/10.1038/35048073

Zhang, X., Tang, Y., Lu, G., Gu, J., 2023. Pharmacological activity of flavonoid quercetin and its therapeutic potential in testicular injury. *Nutrients*. 15, 2231. https://doi.org/10.3390/nu15092231