Impact of *Anadara granosa L.* and *Spirulina platensis* on Sperm Morphology and Motility in *Mus musculus*

Netty Ino Ischak[1*], Loso Judijanto[2], Eddyman W Ferial[3]

Abstract

Background: Infertility, characterized by the inability to conceive, affects both men and women equally, with male infertility often linked to factors such as sperm quality and hormonal imbalances. Nutrition plays a significant role in spermatogenesis, with zinc (Zn) being crucial for sperm development. This study explored the effects of combining Anadara granosa L. (blood cockles) and Spirulina platensis on sperm morphology and motility in Mus musculus mice. Methods: Thirty male mice were divided into six treatment groups, with varying proportions of blood cockles and Spirulina platensis. Treatments were administered for 21 days, and sperm samples were collected from the cauda epididymis for morphological and motility analysis. Data were statistically analyzed using ANOVA and Kruskal-Wallis tests. Results: After 21 days, groups treated with a combination of Anadara granosa L. and Spirulina platensis showed improved sperm motility, with the group receiving a 50% mixture demonstrating the highest motility (89%). Morphological analysis revealed no significant alterations across treatment groups, though zinc (Zn) contributed to enhanced sperm quality by facilitating spermatogenesis and improving metabolic functions in sperm cells. Conclusion: The combination of Anadara granosa L. and Spirulina platensis significantly improved sperm motility, primarily due to the high zinc content. However, morphology remained unaffected, suggesting the treatment's potential as a dietary intervention for male infertility linked to motility deficiencies. Further studies are recommended to explore long-term impacts and optimal dosing.

Keywords: Anadara granosa, Spirulina platensis, Sperm motility, Sperm morphology, Preclinical testing.

Significance | This study explored nutrient effects of Anadara granosa and Spirulina platensis on improving sperm quality in preclinical Mus musculus trials.

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Introduction

Preclinical testing, in the field of pharmacology, involves conducting tests on experimental animals or other biological materials, such as tissue cultures and microorganism cultures like bacteria and fungi (Ardakani et al., 2018). This preclinical test aims to scientifically validate the efficacy and safety of a suspected medicinal substance. Once the preclinical test results confirm the medicinal properties and safety of a substance, the next step involves direct human testing, also referred to as clinical trials (Andry et al., 2023). Testing candidate drugs requires preclinical testing, a development study, as it provides insights into the pharmacological effects, pharmacokinetic profiles, and toxicity of these drugs (Delfita, 2014). According to 2011 data from the Central Statistics Agency, the fertility rate has continued to decline from 5.61 to 2.41 from 1971 to 2010. This indicates that infertility cases in Indonesia have increased from year to year (Dewi et al., 2018). According to 2011 data from the

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BKKBN, the percentage of infertility cases experienced by adult men and adult women was the same. Adult men contributed around 30-35% of cases; adult women contributed around 30-35% of cases; and a combination of men and women contributed around 30-35% of cases. Handling cases of infertility between husband and wife immediately is crucial, as it can lead to serious problems in the family environment (El-Hakim et al., 2018; Sugiantari et al., 2020).

Infertility is the inability to conceive and give birth to children. If a husband and wife have had coitus and have not used contraception for a period of two months and no pregnancy occurs, then clinically the couple is likely to experience infertility. There are two factors that contribute to infertility: internal factors, which include cellular levels, tissues, and reproductive organs, and external factors, which involve the environment and lifestyle (Farag et al., 2015).

Researchers are developing various methods to boost the fertility of male reproductive organs, particularly in humans, by enhancing internal factors such as sperm quality and hormone regulation. Maintaining the function of cells, tissues, and reproductive organs depends heavily on the intake of macro- and micronutrients, as a deficiency in these nutrients can lead to infertility, leading to irregularities in the volume, quality, and quantity of sperm. Macronutrients (carbohydrates, proteins, and fats) and micronutrients (vitamins and minerals) are two groups of nutrients that play an important role in determining sperm quality. We use volume, number of spermatozoa cells, morphology, and motility as benchmarks in determining and assessing sperm quality and quantity (Intan et al., 2014).

Shellfish are an abundant source of nutrition, especially in the waters of eastern Indonesia. The delicious taste of these shellfish makes them a popular food item among the public, particularly those who enjoy seafood. In addition to its delicious taste, it turns out that shellfish contain a lot of nutritional value and vitamins. The meat and shells of shellfish contain the same nutrients, including zinc (Zn). Zinc (Zn) is a type of micromineral that has benefits in increasing testosterone levels in both humans and other mammals (Iriandini et al., 2023). One type of shellfish that improves male reproduction quality is the bloodcockle *Anadara granosa L.*, which contains quite high levels of zinc (Zn) (Kartika et al., 2013). The high Zn content in blood cockles plays a role in the process of spermatozoa formation (spermatogenesis) and also has the ability to improve sperm quality (Khalil, 2016; Madyatmadja et al., 2021).

People have widely used *Spirulina platensis* as food, feed, and medicine. *Spirulina platensis* is a food ingredient that is very rich in vitamin B12 (Nirmalasari, 2017; Payaran et al., 2014). *Spirulina platensis* also contains several vital elements such as zinc (Zn), magnesium (Mg), manganese (Mn), calcium (Ca), and selenium (Se), as well as vitamins such as vitamin C and vitamin E. Vitamins and minerals contained in *Spirulina platensis* microalgae can increase and improve sperm quality (Lindawaty et al., 2016).

Researchers have looked into blood cockles (*Anadara granosa L.*) and *Spirulina platensis* microalgae and found that both have potential, especially when it comes to their nutritional content (Nirmalasari, 2017). However, they haven't looked into how adding *Spirulina platensis* to blood cockles' capsules might improve the quality of spermatozoa. The aforementioned description guided the conduct of this study, which aimed to assess the morphology and motility of spermatozoa in Mus musculus mice using preclinical tests.

Materials and Methods

This study used 30 male mice weighing 20–30 grams and aged around 8–11 weeks, which were divided into 6 treatment groups, with each group containing 5 mice. We acclimatized the mice in laboratory conditions for 7 days before treatment, and provided them with standard AD-2 feed and drinking water.

This study used a Completely Randomized Design (CRD) with 6 treatments and 5 replications. We collected mice that met the criteria in one container and then randomly transferred them to 6 cages, numbered 1 to 6, each containing 5 mice. We randomly administered treatments using a lottery method with picric acid marking to identify the replications of each group.

Treatment

As part of the treatment, each cage received varying dosages of *Anadara granosa L.* and *Spirulina platensis* blood cockles.

Cage 1: 70% blood cockles, 30% Spirulina.

Cage 2: 50% blood cockles, 50% Spirulina.

Cage 3: 30% blood cockles, 70% Spirulina.

Cage 4: 100% blood cockles.

Cage 5: 100% Spirulina.

Cage 6: Control (no treatment).

We administered the treatment twice a day for 21 days.

Spermatozoa Collection and Analysis

We collected spermatozoa by surgically removing the mouse testicles from the cauda epididymis, then processed and observed them for morphological and motility analysis. We observed the sperm morphology by staining them with 1% eosin and examined them using a microscope with 400x magnification, focusing on normal and abnormal shapes.

We analyzed sperm motility by chopping the cauda epididymis in 0.9% physiological NaCl, observing under a microscope with 100x magnification, and calculating based on WHO criteria.

Data Analysis

The ANOVA test analyzed quantitative data for normally distributed data, followed by the LSD test. We used the non-parametric Kruskal-Wallis test if the data was not normal, and if necessary, the Mann-Whitney test.

Results and Discussion

After 21 days of treatment, the nutrients in *Anadara granosa L.* blood cockle powder and *Spirulina platensis* microalgae tend to make mouse

spermatozoa better in terms of their shape and ability to move. The very high zinc (Zn) mineral content in *Anadara granosa L.* blood cockles play an important role in spermatogenesis and can improve sperm quality. Lower spermatozoa concentration, reduced spermatozoa movement, and abnormal spermatozoa morphology correlate with the level of zinc (Zn) in sperm. Zinc (Zn) minerals help spermatozoa cell metabolism enzymes do their job. These enzymes provide ATP for the spermatozoa to move around and can also activate androgen hormones, which improve the normal process of spermatogenesis and spermatozoa maturation. *Anadara granosa L.* contains a cholesterol group that synthesizes testosterone.

Leydig cells synthesize testosterone, which the enzyme Dehydroxytestosterone (DHT) then activates. 5-α-Dihydrotestosterone then reduces testosterone into androgen, which contributes to the maturation of spermatozoa. The spermatozoa membrane is rich in unsaturated fatty acids and is at high risk of oxidative stress (ROS), which can cause lipid peroxidation. This condition can cause impaired testicular function, especially spermatogenesis. Spermatogenesis produces spermatogenic cells, such as spermatogonia, spermatocytes, spermatids, and spermatozoa. Lipid peroxidation can damage the structure of saturated fatty acids in spermatogenic cells, leading to spermatogenic cell dysfunction and decreased spermatozoa quality.

Spirulina platensis, in addition to containing zinc (Zn), also contains manganese, iron, calcium, magnesium, and selenium. *Spirulina platensis* microalgae also contains vitamin E and vitamin C and is the highest source of vitamin B12. Vitamin C binds oxygen radicals in cells, preventing the formation of lipid peroxidation, which can inhibit spermatozoa motility. Vitamin B12, which is an antioxidant, plays a role as a coenzyme in the process of methionine synthesis. In addition, vitamin B12 stimulates oxidative phosphorylation, which is a metabolic pathway to produce energy in the form of ATP (Adenosine Tri Phosphate). In mitochondria, Vitamin B12 facilitates electron transport, creating a proton concentration gradient (H+) for ATP synthesis, which spermatozoa use to migrate toward female egg cells.

Spirulina platensis has the potential to maintain normal spermatozoa related to antioxidant activity because it contains constituents in the form of C-phycocyanin, ß-carotene, vitamins, minerals, proteins, fats, and carbohydrates. There is a lot of C-phycocyanin in *Spirulina platensis*. This chemical adds one atom to free radicals and helps collect them. It can also stop cell damage in the seminiferous tubules caused by oxidative stress. ß-Carotene also has an important role in protecting cells from oxidative stress, which can transfer excess electrons from free radicals. *Anadara granosa L.* and *Spirulina platensis* contain protein content that contributes to the formation of structures during spermatogenesis and serves as a raw material for the production of hormones involved in spermatogenesis.

Sperm Motility of Mus Musculus Mice

Sperm motility is the movement of spermatozoa cells that move forward in a straight and rapid (progressive) manner. We carry out the motility examination by observing the movement of spermatozoa. Sperm movement is classified into four groups: forward, fast, and straight; forward, slow, and winding; no forward movement; vibrating in place or moving in a circle; and no movement at all. If more than 50% of the spermatozoa move, their motility is considered good and normal, and if less than 50% move, their motility is considered poor (subfertile).

The study's results indicate a tendency for an increase in the average percentage of spermatozoa motility following a 21-day treatment (Figure 1). The control group, K, which did not receive any intervention, demonstrated the lowest average motility percentage at 68%. Group P received a treatment of 70% *Anadara granosa L.* and 30% *Spirulina platensis*, resulting in an average percentage of motility of 82%. The group Q, which received an intervention of 50% *Anadara granosa L.* and 50% *Spirulina platensis*, demonstrated the highest average percentage of motility at 89%. Group R, which received an intervention of 30% *Anadara granosa L.* and 70% *Spirulina platensis*, achieved an average percentage of motility of 73%. Group S, which received an intervention of 100% *Anadara granosa L.*, achieved an average percentage of motility of 83%, while group T, which received an intervention of 100% *Spirulina platensis*, achieved an average percentage of motility of 69%.

Adding *Spirulina platensis* microalgae to *Anadara granosa L.* blood cockle powder had a significant effect or positive correlation on motility, as shown by the Kruskal-Wallis test. Based on the post-hoc Mann-Whitney test, there was a positive correlation or significant difference between the control group (K) and the P, Q, and S treatment groups. We also found significant differences between the P treatment group and the Q and T groups, as well as between the Q treatment group and the R and T groups. Meanwhile, between the control group (K) and the R and T treatment groups, there was no significant difference ($p > 0.05$). The same thing also happened between the P treatment group and the R and S treatment groups: The Q treatment group with S, the R treatment group with S and T, and the S treatment group with T.

After reviewing the data and results, it was found that the intervention of *Anadara granosa L.* and *Spirulina platensis* increased the average percentage of mouse spermatozoa motility. The high zinc (Zn) content in *Anadara granosa L.* is crucial for the ongoing metabolism of spermatozoa cells, as zinc (Zn) is a micromineral that plays an important role in activating various enzyme functions, including the enzyme dehydroxytestosterone (DHT). Zinc (Zn) can enhance spermatozoa motility, as it contributes to the function of spermatozoa cell metabolism enzymes, which supply ATP as energy for the spermatozoa cells' movement towards female egg cells.

The protein content in *Anadara granosa L.* and *Spirulina platensis* plays a role in maintaining and preserving the structure of spermatozoa and repairing damaged tissue in the reproductive organs. A complete and normal morphological structure strongly supports spermatozoa movement. Moreover, nutrients like protein (amino acids) serve as substrates during spermatozoa maturation, causing structural changes in the spermatozoa that significantly impact their motility quality. The

production of energy in the base of the tail (midpiece), where mitochondrial organelles function to produce energy for metabolism, influences sperm motility. Spermatozoa's locomotory apparatus, an axoneme, is located in the tail. This axoneme consists of a pair of central microtubules and nine pairs of microtubules outside it. The outer microtubules are made up of subfibril A and subfibril B, which are composed of dynein protein and can hydrolyze ATP for use in spermatozoa movement.

Treatment T has the lowest average percentage of motility among all intervention-received treatment groups (groups P, Q, R, and T), and its average difference from the control treatment group (K) is very small. In addition, there was no significant effect or real difference between the control group (K) and groups R and T. This might be because *Spirulina platensis* has active compounds that stop fertility. This is why the T treatment group that got 100% *Spirulina platensis* had results that were pretty similar to those of the control group. Meanwhile, there was a significant difference between the control group and the treatment group that received either 100% *Anadara granosa L.* or an *Anadara granosa L.* intervention fortified with *Spirulina platensis*.

Previous researcher conducted a phytochemical screening test on *Spirulina platensis* extract, revealing positive results for phenolic compounds, triterpenoids, steroids, flavonoids, and saponins. Based on previous research results, that the biomass of *Spirulina platensis* contains flavonoids, steroids, phenols, and saponins. Active compounds that are antifertility in principle work in two ways, namely through cytotoxic or cytostatic effects and hormonal effects by inhibiting the rate of spermatogenic cell metabolism and disrupting the hormone system balance. Flavonoids are antiandrogens that can stop the aromaterase enzyme from doing its job, which lowers testosterone hormones. Flavonoids have a structure that is similar to the hormone estrogen (estrogenic). Compounds that have the same structure as the estrogen hormone are able to occupy reproductive organ receptors and can disrupt the axis of the hypothalamus, pituitary, and testes. The structure of mammalian spermatozoa is rich in unsaturated fatty acids in the plasma membrane, so it is at high risk of ROS attacks. In addition to lipid peroxidation, damage to mitochondria by ROS causes decreased energy availability, which affects spermatozoa motility. High levels of flavonoids as antioxidants in the body cause the formation of ROS, which have the ability to damage the plasma membrane of spermatozoa cells.

Morphology of Mus Musculus Mouse Sperm

The normal morphology of Mus musculus mouse spermatozoa is to have a head shape that resembles a hook or crescent moon with a pointed, curved, and non-circular tip, as well as a long and straight tail. The normal shape of mouse spermatozoa is a head that resembles a hook or crescent moon, with an intact and straight tail. Mouse spermatozoa have a head shape that resembles a hook at the tip of the head with a head length of between 5-8 μm, a head width of 0.5-1 μm, a body length of 13-15 μm, and a tail length of 40-45 μm. The morphological form of spermatozoa cells greatly influences fertilization; if the number of spermatozoa abnormalities is too high, it will reduce fertility. Teratozoospermia, a condition where the normal form of spermatozoa is less than 50%, significantly affects fertility, particularly in the fertilization of egg cells (ovum) in female organisms.

The study's results indicated a trend in the average percentage of spermatozoa morphology following a 21-day treatment (Figure 2). The control group, K, which did not receive any intervention, exhibited the lowest average morphology percentage of 83.22%. Group P, which received an intervention consisting of 70% *Anadara granosa L.* and 30% *Spirulina platensis*, demonstrated an average morphology percentage of 89.50%. Group Q, the recipient of an intervention consisting of 50% *Anadara granosa L.* and 50% *Spirulina platensis*, demonstrated the highest average percentage of motility at 89.73%. Group R, which received an intervention of 30% *Anadara granosa L.* and 70% *Spirulina platensis*, demonstrated an average percentage of motility of 88.02%. Group S, which received an intervention of 100% *Anadara granosa L.*, achieved an average percentage of motility of 87.34%, while group T, which received an intervention of 100% *Spirulina platensis*, achieved an average percentage of motility of 86.97%.

The results of the ANOVA test yielded a p value of 0.186. In this case, the p value > 0.05 indicated that the intervention of *Anadara granosa L.* blood cockle powder fortified with *Spirulina platensis* microalgae did not significantly alter the morphology. According to the post-hoc LSD test, the treatment group that showed a significant difference or positive correlation was only in the control group (K) against the treatment groups P and Q. Meanwhile, other groups such as P, Q, R, S, and T did not show a significant difference.

Spermatogenesis involves three main stages: spermatogonia proliferation, spermatocyte meiosis, and spermiogenesis. Meanwhile, the spermatogenesis process also requires stimulation from both gonadotropin hormones, namely LH (luteinizing hormone) and FSH (follicle stimulating hormone). LH stimulates Leydig cells to produce testosterone in the testes, and FSH stimulates Sertoli cells to spur spermatogenesis. There are also hormones that can suppress spermatozoa production, including gonadothropine-releasing hormone (GnRH) analogs. The hypothalamus and pituitary produce GnRH, which in turn stimulates the pituitary to secrete LH and FSH. The presence of LH causes Leydig cells to produce testosterone, and FSH stimulates Sertoli cells to release Androgen Binding Protein (ABP).

The primary spermatocyte stage marks the beginning of the testosterone hormone's function, whereas the final spermatid stage marks the beginning of FSH's function until the formation of complete and intact spermatozoa. ABP transports testosterone to the epididymis for conversion into dihydrotestosterone (DHT), subsequently aiding in the maturation of spermatozoa to enable active movement. This aligns with the findings of previous research, which demonstrated the crucial role of testosterone in spermatogenesis and sperm maturation. Therefore, alterations in testosterone levels directly influence the morphology, motility, and viability of spermatozoa.

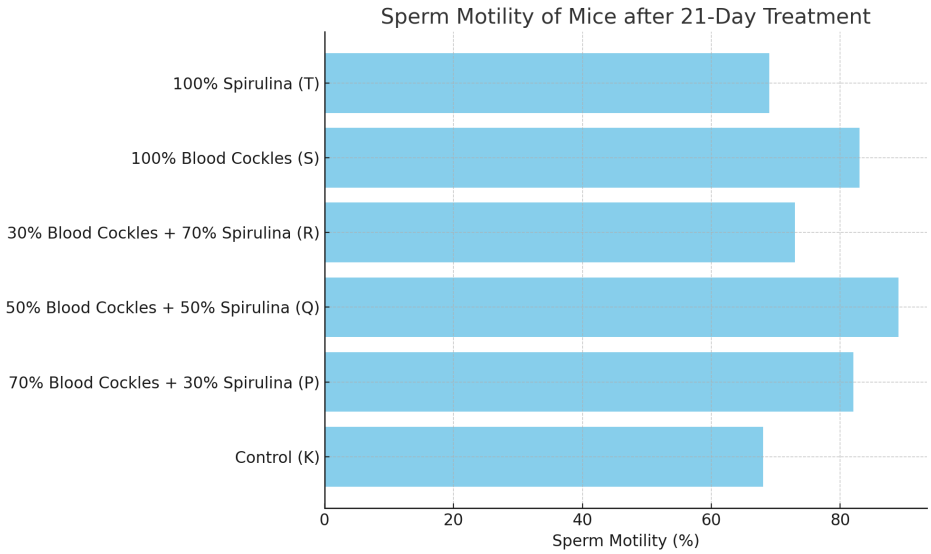

Figure 1. Sperm Motility (%) in *Mus musculus* After 21 Days of Treatment. The percentage of sperm motility in *Mus musculus* across six treatment groups

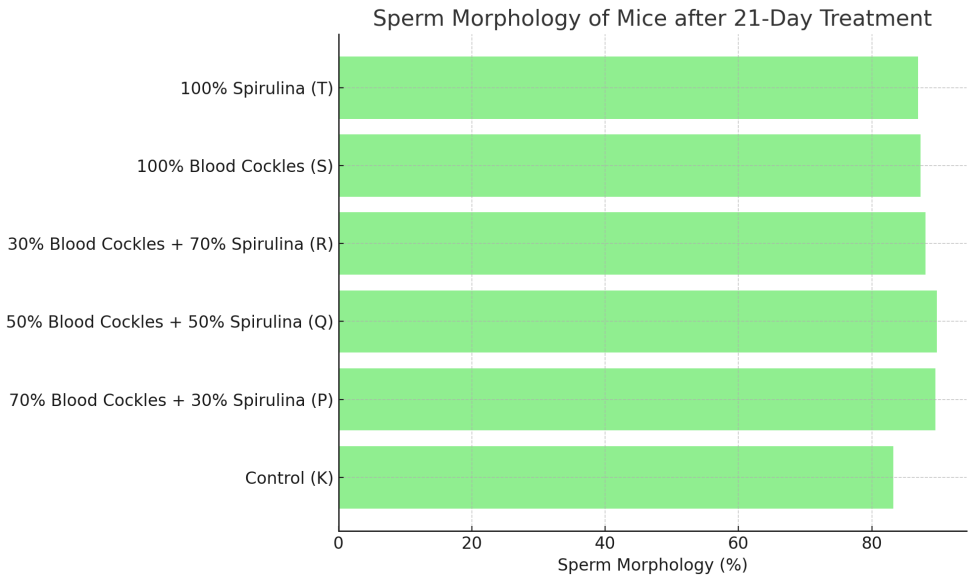

Figure 2. Sperm Morphology (%) in Mus musculus After 21 Days of Treatment. The percentage of normal sperm morphology in *Mus musculus* after treatment with various combinations of *Anadara granosa L.* and *Spirulina*.

Zinc (Zn) minerals in *Anadara granosa L.* and *Spirulina platensis* have a major role in improving spermatozoa quality. Zinc (Zn) is able to synthesize the enzyme dihydrotestosterone (DHT) to produce steroid hormones in the form of testosterone, which will later be reduced by 5-α-dihydrotestosterone into androgen in the target cells. This androgen hormone greatly influences the maturation of spermatozoa in the epididymis, so that the levels of androgen hormones required by the epididymis are higher than the testes.

The study's results revealed abnormalities in the morphology of spermatozoa in mice treated with *Anadara granosa L.* and *Spirulina platensis*. An abnormal shape in spermatozoa is classified, such as a small head, an amorphous head, and a spiral or short tail, as a primary abnormality. Moreover, secondary abnormalities manifest as spermatozoa lacking a head or tail. Spermatozoa abnormalities commonly referred to as teratozoospermia include the amorphous, large round sperm head shape and the coiled or short tail. Abnormalities in spermatozoa can be primary abnormalities and secondary abnormalities. There are two types of abnormalities: primary and secondary. Primary abnormalities happen in the seminiferous tubules during spermatogenesis, while secondary abnormalities happen in the epididymis after the spermatozoa leave the seminiferous tubules to mature.

The presence of reactive oxygen species (ROS), which affect the spermatozoa plasma membrane, causes spermatozoa abnormalities. Large amounts of phospholipids and unsaturated fatty acids make up the spermatozoa plasma membrane, and these unsaturated fatty acids are highly susceptible to ROS, particularly hydroxyl radicals. These radicals can cause lipid peroxidation, breaking down the fatty acid chain into compounds that are toxic to spermatozoa. These toxic compounds include malonadialdehyde (MDA), 9-hydroxy-noneal, ethane (C6H6), and pentane (C5H12).

Researchers believe that active antifertility compounds like flavonoids, steroids, triterpenoids, phenolics, and saponins are responsible for the abnormalities in spermatozoa morphology found in the study. Steroids, alkaloids, flavonoids, and tannins are compounds that are antifertility, especially in male organisms. Previous researchers did a phytochemical screening test of *Spirulina platensis* biomass. The tests for phenolic, triterpenoid, steroid, flavonoid, and saponin compounds all came back positive. Flavonoids, acting as antioxidants, produce reactive oxygen species (ROS) that can damage the spermatozoa cell membrane, thereby causing damage to the spermatozoa cells. Meanwhile, flavonoids are antiandrogenic due to their ability to inhibit the aromatase enzyme, which catalyzes the conversion of androgens to estrogens, thereby increasing testosterone hormones. This increase in testosterone hormones will provide a negative feedback loop to the pituitary, resulting in a decrease in FSH and LH secretion, which in turn negatively impacts spermatogenesis.

Active steroid substances have high estrogen levels, so they can cause reproductive dysfunction by inhibiting FSH secretion and having a negative effect on spermatogenesis. Another possible effect is a decrease in LH concentration, which has an effect on Leydig cells and causes testosterone production to decrease. This decrease in testosterone can interfere with the transformation process, or spermiogenesis, and affect spermatid cells. It is believed that excess steroids and triterpenoids in the body can disrupt the hypothalamus and pituitary pathways, leading to disruptions in GnRH secretion, which in turn impacts the formation, development, and maturation of follicles.

The active compounds found in *Spirulina platensis* are suspected to cause abnormal forms in mouse spermatozoa. The treatment that used *Anadara granosa L.* along with *Spirulina platensis* had a higher average percentage of morphology and motility than the treatment that used only *Spirulina platensis*. This indicates that *Anadara granosa L.* has a greater effect than *Spirulina platensis*. Furthermore, it is believed that the antifertility active compounds in *Spirulina platensis* function more effectively by upsetting the hormonal system's equilibrium, thereby influencing the male reproductive system. However, this study has certain limitations. Specifically, we didn't calculate the levels of active compounds in *Anadara granosa L.* and *Spirulina platensis*. Consequently, we don't yet know the threshold levels of these natural ingredients that mice's bodies can tolerate without causing antifertility.

Conclusion

The research concludes that the safe dose of *Anadara granosa L.* for mice is 4.16 mg/20 gr of body weight per day, while the safe dose of *Spirulina platensis* for mice is 2.6 mg/20 gr of body weight per day. We formed five treatments (P, Q, R, S, and T) from the safe dose, each with a varying dose, and compared them with the control (K). We carried out the fortification by dissolving each natural ingredient in 0.5% Na-CMC and using the same syringe to take as much as 0.2 mL of the suspension. When we mixed the blood cockle powder intervention *Anadara granosa L.* with microalgae *Spirulina platensis*, the Q treatment group (50% *Anadara granosa L.* and 50% *Spirulina platensis*) had the best average percentage for sperm shape and movement. The Kruskal-Wallis test results showed a p value of 0.006 ($p < 0.05$), which means that this intervention has a significant effect on motility. However, the ANOVA test results with a p value of 0.186 ($p > 0.05$) showed that there was no significant effect on morphology.

Author contributions

N.I.I., L.J., and E.W.F. contributed to conceptualization, fieldwork, data analysis, drafting the original manuscript, editing, and manuscript review. N.I.I. led the research design, methodology validation, supervision, and funding acquisition, while L.J. and E.W.F. were involved in data analysis, visualization, and manuscript editing. All authors have reviewed and approved the final version of the manuscript.

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Competing financial interests

The authors have no conflict of interest.

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