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# Anti-infertility Effects of Zinc (Zn) Supplements on the Spermatozoa Quality of Rats (*Rattus norvegicus* L.) Exposed to Cigarette Smoke

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**Abstract:** Cigarette smoke contains nicotine compounds ($C_{10}H_{14}N_2$), tar and carbon monoxide (CO), which cause inhibition of Leydig cells, damage to seminiferous tubules and damage to spermatozoa cells. The aim of this study was to determine the anti-infertility effect of Zinc supplements on the quality of Wistar rat (Rattus norvegicus L.) spermatozoa exposed to cigarette smoke. This research is an experimental study consisting of 4 treatments with 3 replications: Control (drinking water), P1 (14 mg/g BW), P2 (28 mg/g BW), and P3 (42 mg/g BW) Zinc. Exposure to cigarette smoke was carried out twice a day for 14 days in all treatments, and zinc was given for 14 days in treatments P1, P2 and P3. On the 29th day, mice were sacrificed by anesthesia using chloroform and dissected to observe the quality of spermatozoa with parameters of motility, viability and morphology. Data were analysed using ANOVA (Analyzis of Variance) and the BNT test with a 95% confidence level. The results showed that Zinc supplementation in treatments P1 and P2 significantly improved spermatozoa quality, whereas in P3 it decreased significantly. The conclusion of this study is that Zinc (Zn) supplements at a dose of 14 mg/g BW (P1) and a dose of 28 mg/g BW (P2) have an anti-infertility effect by improving the quality of rat spermatozoa that have been exposed to cigarette smoke. The results of this research can be important information for improving male reproduction due to exposure to free radicals from cigarette smoke.

**Keywords**: Anti-infertility; Cigarette Smoke; *Rattus norvegicus* L.; Spermatozoa Quality.

## Introduction

Cigarette smoke is a source of exogenous free radicals, which can trigger an increase in free radical levels in the body. Cigarette smoke contains three main toxic components, namely carbon monoxide (CO), nicotine ($C_{10}H_{14}N_2$) and tar [1]. These compounds cause a decrease in antioxidant levels in the body, which has a significant negative impact on human health [2]. The effects caused by exposure to cigarette smoke cause impotence, namely disorders of the male reproductive system, which is called impotence [3].

The main compound in cigarette smoke is the compound nicotine ($C_{10}H_{14}N_2$), which has addictive properties which can cause psychological dependence [4]. The effect of the nicotine compound ($C_{10}H_{14}N_2$) on reproduction can inhibit Leydig cells from producing the hormone testosterone and cause damage to the seminiferous tubules [5]. Tar compounds, which are carcinogens in cigarette smoke, can reduce testosterone levels. The carbon monoxide (CO) compound in cigarette smoke can reduce the blood's ability to carry oxygen (O2), resulting in cell death [6]. Exposure to cigarette smoke can cause damage to spermatozoa, resulting in a decrease in spermatozoa quality, and zinc supplementation has the potential to repair this damage.

Zinc administration affects the function of male reproductive organs, including effects on male sex hormones [7]. Zinc can increase libido in males by stimulating Leydig cells in the testicles to produce testosterone through the hypothalamus-anterior pituitary-testicular mechanism. Increasing testosterone levels will increase libido, so that the higher the testosterone levels, the higher the libido levels [8]. Zinc also improves spermatozoa motility by regulating spermatozoa cell metabolic enzymes that produce energy (ATP), so that spermatozoa move more actively. Zinc also acts as an antioxidant by protecting spermatozoa from oxidative damage and lipid peroxidation by inhibiting phospholipase [9]. Zinc is an important mineral that the body needs to support various functions, including reproductive health. Zinc among the public can be obtained from animal food sources such as meat, liver, shellfish, oysters, crab, lobster, salmon, eggs, egg whites, chicken meat and oysters [10]. Zinc is also found in many plant food sources, such as ground cereals, legumes such as red beans, green beans, peas, peanuts and in vegetables such as moringa, spinach and pumpkin [11].

Research related to the effect of Zinc supplements on spermatozoa has been reported by [8] Giving zinc in combination with tomatoes improved the morphology and motility of spermatozoa in mice exposed to monosodium glutamate (MSG) for 30 days. The greater the dose of zinc given, the greater the increase in the quality of rat spermatozoa [12]. Giving a combination of Zinc and vitamin E to rat spermatozoa exposed to cigarette smoke can significantly increase sperm motility and morphology [13]. The novelty of this research is the use of Zinc as a supplement to improve spermatozoa quality, based on Zinc's

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antioxidant role in binding free radicals that can damage spermatozoa. It is hoped that the results of this research will provide scientific information regarding the use of zinc as a supplement to improve reproductive quality [14].

## Research Methods

This research was conducted in June-July 2024 at the FMIPA mouse cage and the Biology Laboratory of the Zoology Unit, Faculty of Mathematics and Natural Sciences, Halu Oleo University, Kendari, Southeast Sulawesi. The tools used include: 20 x 20 cm basin, wire mesh, rat drinking bottle, 2 mL volume syringe, jar, 100 mL measuring cup, surgical tools, glass object, smoking chamber, hand counter, dropper pipette, camera, stationery, analytical balance, petri dish, beaker, stir bar, and light microscope. Materials used include: rats (Rattus norvegicus L.) Wistar strain, drinking water, commercial pellets (BP11-BRAVO) produced by PT. Charoen Pokphand Indonesia, chloroform, husk, tissue paper, label paper, NaCl 0.9%, distilled water, Eosin-nigrosin dye, Giemsa dye 20%, methanol 96%, Zinc sulfate and clove cigarettes.

### Preparation of Test Animals

Two-month-old male Wistar rats (Rattus norvegicus L.) with a body weight ranging from 150-200 grams, obtained from a rat breeder, totalling 12, were acclimated for 7 days in the FMIPA UHO rat cage house, equipped with ventilation for air circulation, at a room temperature of 25-29°C. Mice were placed in clean cages with husk-lined cages, one cage per mouse; each container was labelled according to the treatment [12]. Test animals were divided into five treatments: Control (drinking water), Zinc supplement at 14 mg/g BW (P1), 28 mg/g BW (P2), and 42 mg/g BW (P3). Test animals were fasted for 12 hours before treatment to ensure comparable physiological conditions. Exposure to cigarette smoke was carried out using a smoking chamber for mice which was made using a wooden board measuring 47 cm × 28 cm × 28 cm which was equipped with a barrier between the experimental animal and the end of the burning cigarette at a distance of 18 cm and a height of the barrier 22 cm while the ventilation was 12 cm × 24 cm long.

### Treatment

Exposure to cigarette smoke was carried out in the morning and evening, with 2 cigarettes for 14 consecutive days, for 10 minutes. Cigarette smoke is exhaled repeatedly with a syringe (injection tube) until the cigarette burns out. According to [15] Exposure to cigarette smoke for 14 days causes damage to the quality of spermatozoa. Zinc is given orally on the 24th day at a dose of 2 mL/day. Zinc was given for 49 days, and one day later, observations were made on the quality of the spermatozoa in the form of motility, viability and morphology of the spermatozoa [16]. Ethical clearance was obtained from the Faculty of Natural Sciences and Mathematics under Letter Number UN.29.17.5/SK/EC/2022.

### Observation

Spermatozoa samples were taken by chopping the cauda epididymis with scissors until fine in 2 ml of 0.9% NaCl, which had been heated using a hot plate to a temperature of 32-34 °C, and stirring until a homogeneous spermatozoa suspension was obtained. The 0.9% NaCl solution at a temperature of 32-34ºC °C functions to maintain the temperature of the epididymis because at higher temperatures, denaturation of spermatozoa occurs [17]. Physiological salt solutions are able to maintain changes in the pH of spermatozoa because they have isotonic properties in cell fluid, reducing the density of spermatozoa and maintaining the viability of spermatozoa for a certain storage period [16]. Cement storage with 0.9% NaCl lasts up to 60 minutes.

### Spermatozoa motility

Observation of sperm motility is aimed at determining the effect of zinc supplementation on sperm motility. The observations followed the procedure carried out by [12], cauda epididymis semen was sucked with a Haemacytometer pipette to the 0.5 line, then 0.9% NaCl was added to the 1.01 line. The mixture was then shaken for 2-3 minutes and dropped into an improved Neubauer counting chamber. Calculations were carried out on 5 boxes while the counting chamber was rotated 45 ° using a 400x microscope connected to a computer. The field of view was systematically examined and motility was calculated for 100 spermatozoa cells using a hand counter. Spermatozoa motility is divided into 4 categories based on movement type: fast progressive, slow progressive, nonprogressive, and nonmotile. The motility percentage is calculated based on the calculation formula of [16] as follows:

$$\text{Abnormal spermatozoa motility} = \frac{B + A}{A+B+C+D} \text{ x } 100\ \%$$

Description:
A = Fast progressive
B = Slow progressive
C = Nonprogressive
D = Nonmotile

### Spermatozoa viability

Spermatozoa viability is assessed to determine their ability to survive after leaving the reproductive organs. 50 µl of spermatozoa suspension was dropped onto a glass slide, 25 µl of Eosin-nigrosin dye was added, and the slide was then covered with a cover glass. Spermatozoa viability was observed using a microscope with 400x magnification connected to a computer. The visual field was checked systematically, and viability was calculated for 100 spermatozoa cells using a hand counter. Eosin dye is used because it has acidic properties, so it can detect alkaline or dead spermatozoa. If eosin is met with live sperm, eosin will not enter because the sperm membrane is the same acid as eosin, so they repel each other[17]. The percentage of spermatozoa viability is calculated using the [16] formula, as follows:

$$\text{Dead spermatozoa} = \frac{B}{A+B} \times 100\ \%$$

Description:
A = Live spermatozoa
B = Dead spermatozoa

**Spermatozoa morphology**

One drop of spermatozoa suspension is placed on a glass slide, then a smear is made by sliding another glass slide over it at a 45° angle once. After that, it was dried for 15 minutes, then fixed with 96% methanol for 5 minutes, stained with 20% Giemsa solution for 30 minutes, and rinsed with distilled water. Calculations were carried out under a microscope with 1,000X magnification connected to a computer. The field of view was systematically examined, and the morphology was counted in 100 spermatozoa cells using a hand counter. The percentage of spermatozoa morphology is calculated based on the formula according to [12] as follows:

$$\text{Abnormal spermatozoa morphology} = \frac{B}{A+B} \times 100\ \%$$

Description:
A = Normal morphology
B = Abnormal morphology

**Data analysis**

Data analysis in this study used the Statistical Package for the Social Sciences (SPSS) version 26.0. Data on abnormal prolarvae parameters were analyzed using one-way ANOVA and continued with an LSD (Least Significant Difference) test at a 95% confidence level to see which treatment gave different effects.

## Results and Discussion

Sperm quality is an indicator of a male individual's fertility. Spermatozoa quality parameters include motility, viability, and morphology.

**Spermatozoa Motility**

The motility of spermatozoa between Zinc (Zn) supplement treatments is presented in Table 1 below.

**Table 1**. Average percentage of rat spermatozoa motility between Zinc supplement treatments.

| Treatment | Spermatozoa Motility (%) Average ± SD | |
|---|---|---|
| | Normal | Abnormal |
| K | 22 ± 4.36[a] | 78 ±4.36 [a] |
| P1 | 64± 5.51[b] | 36 ±5.51[b] |
| P2 | 76± 4.36[c] | 24±4.36[c] |
| P3 | 49± 2.08[d] | 51±2.08[d] |

Note: Numbers followed by different letters indicate significant differences based on the BNT test with a 95% confidence level

Table 1 shows that giving Zinc supplements increases the normal percentage of spermatozoa quality parameters. The mean number of normal and abnormal spermatozoa motility in treatments K (control), P1, P2, and P3 was significantly different. Zinc supplements increase the percentage of normal spermatozoa motility above 70% and the percentage of abnormal spermatozoa motility below 50%. This shows that Zinc supplements can produce high-quality spermatozoa. Quality spermatozoa are spermatozoa that have a percentage of normal spermatozoa above 50% and a percentage of abnormal spermatozoa motility below 50% [17]. The percentage of spermatozoa motility in treatments P1 and P2 increased because Zinc has the ability to neutralize toxic compounds from cigarette smoke, which trigger the formation of free radicals. Zinc supplements function as antioxidants, which can increase blood testosterone levels and protect spermatozoa. According to [8]Zinc helps bind free radicals, which can damage spermatozoa. Zinc helps antioxidant enzymes, such as superoxide dismutase (SOD), convert free radicals into hydrogen peroxide. This hydrogen peroxide is then converted into water and oxygen by the catalase enzyme. This process helps maintain spermatozoa's health and increases their motility.

Treatment with Zinc supplementation at a dose of 42 mg/g BW (P3) in this study resulted in a decrease in spermatozoa motility (Table 1). This explains that, at this dose, the Zinc supplement is a high dose and can interfere with sperm motility. This is as stated by [8] Zinc, which is supposed to function as an antioxidant, will turn into a prooxidant if consumed in too much, thereby causing damage to spermatozoa. These prooxidants can cause damage to spermatozoa cell membranes and mitochondrial organelles, which function to produce energy (ATP) [18]. Damage to mitochondria reduces energy for sperm movement, so that the flexibility and stability of spermatozoa decrease, and their ability to move efficiently is reduced [19]. An illustration of the average percentage of normal and abnormal spermatozoa motility is presented in Figure 1.

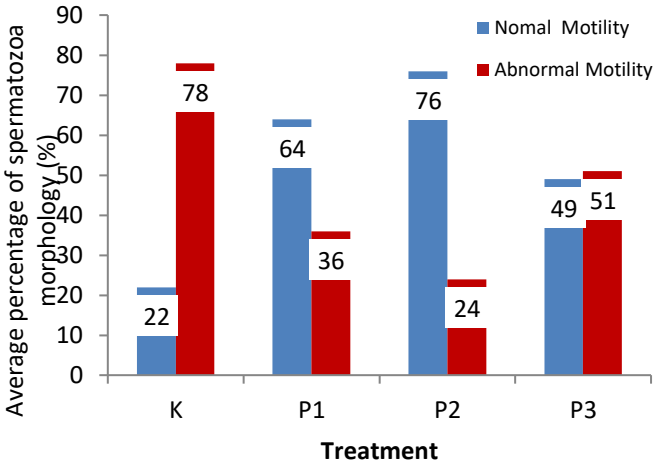


**Figure 1**. Histogram of the average percentage of spermatozoa motility between Zinc (Zn) treatments.

**Viabilitas Spermatozoa**

Spermatozoa viability between Zinc (Zn) supplement treatments is presented in Table 2. Table 2 shows that Zinc supplementation across all treatments was

significantly different from the control, indicating that Zinc supplementation at all three doses increased the percentage of live spermatozoa. The treatment dose of 14 mg/g BW (P1) and the dose of 28 mg/g BW (P2) showed a more significant increase in the percentage of live spermatozoa compared to the P3 treatment. The percentage increase in the viability of normal

**Table 2**. Average percentage of rat spermatozoa viability between Zinc supplement treatments

| Treatment | Spermatozoa Viability (%) Average ± SD | |
|---|---|---|
| | Normal | Abnormal |
| K | 9± 5.13[a] | 91±5.13[a] |
| P1 | 63±5.51[b] | 37±5.51[b] |
| P2 | 73±7.57[b] | 27±7.57[b] |
| P3 | 42±5.03[c] | 58±5.03[c] |

Note: Different letters indicate significant differences in the BNT test at a 95% confidence level

Spermatozoa in treatments P1 and P2 were 63% (P1) and 73% (P2), or an average of above 45%. This is in line with the statement, the viability of normal spermatozoa is above 45% [16]. This is because Zinc has the ability to protect spermatozoa from free radicals, and it can protect spermatozoa from free radicals by inhibiting lipid peroxidation in sperm cell membranes [8]. The percentage of spermatozoa viability in the P3 treatment decreased because the 42 mg/g BW zinc dose had a negative effect on spermatozoa. The limit for zinc consumption for adult humans is 40 mg/day, and consumption of more than that can cause toxic effects on spermatozoa. Giving excessive doses of Zinc can damage sperm cell membranes, which means it also damages organelles in cells and disrupts cell metabolism [20]. This membrane damage inhibits nutrient transport for spermatozoa, which ultimately reduces motility and causes spermatozoa death [21]. An illustration of the average percentage of viability of normal and abnormal spermatozoa is presented in Figure 2.

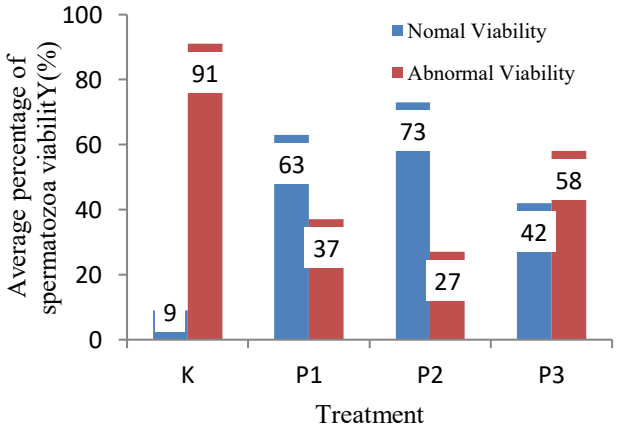


**Figure 2**. Histogram of the average percentage of spermatozoa viability between Zinc (Zn) treatments.

## Spermatozoa Morphology

The morphology of sopermatozoa between Zinc (Zn) supplement treatments is presented in Table 3. Table 3 shows that the average percentage of normal spermatozoa morphology increased after Zinc supplementation at all treatment doses. Treatments P1, P2 and P3 showed significant differences from the control. The percentage of spermatozoa morphology in treatments P1 and P2 has increased because Zinc can protect spermatozoa from free radicals and support spermatozoa maturation. [8] stated that Zinc, apart from acting as an antioxidant, also plays a role in helping the spermatozoa maturation process by increasing androgen levels in blood plasma. In Table 3, it can be seen that the mean percentage of spermatozoa morphology decreased in the P3 treatment, likely due to Zinc at a dose of 42 mg/g BW having a toxic effect on spermatozoa quality. Excess zinc intake can inhibit the absorption of copper and iron [22-23]. The effect of inhibiting the absorption of these two minerals is anaemia, which ultimately affects the spermatogenesis process and causes abnormalities in spermatozoa. An illustration of the average percentage of viability of normal and abnormal spermatozoa is presented in Figure 3.

**Table 3**. Mean percentage of mouse spermatozoa morphology between Zinc supplement treatments

| Treatment | Spermatozoa Morphology (%) Average ± SD | |
|---|---|---|
| | Normal | Abnormal |
| K | 8±6.81[a] | 92±6.81[a] |
| P1 | 59±8.54[bc] | 41±8.54[bc] |
| P2 | 66±8.33[b] | 34±8.33[b] |
| P3 | 46±8.50[c] | 54±8.50[c] |

Note: Different letters indicate significant differences in the BNT test at a 95% confidence level

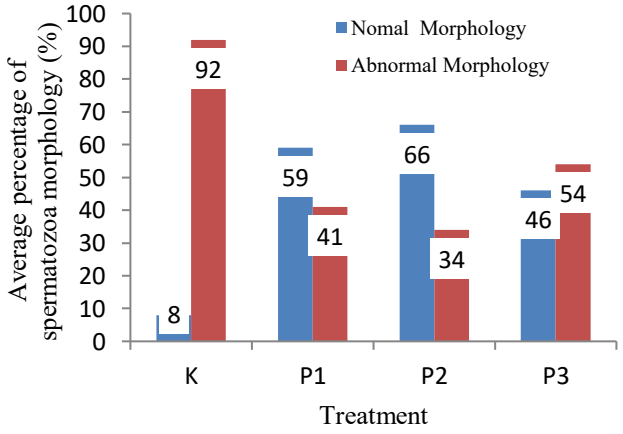


**Figure 3**. Histogram of the average percentage of spermatozoa morphology between Zinc (Zn) treatments

The morphological abnormalities found in this study were secondary abnormalities, including broken heads, broken tails, angular tails, and curved middle parts. Primary abnormalities found in this study include: double head, big head, and double tail. Various morphological abnormalities observed in this study in spermatozoa are shown in Figure 4.

Based on Figure 4, various abnormal morphologies of spermatozoa; the most common in this study were secondary abnormalities, namely wavy tails (Figure 4C), angular tails (Figure 4E), folded tails (Figure 4G), and grooved middle parts (Figure 4H). This abnormality

occurs due to disturbances in the spermatogenesis process, decreased blood testosterone levels, or excessive zinc intake. Excess zinc can damage the mitochondrial structure in the tail of spermatozoa. Damaged mitochondria cannot produce the energy needed for sperm movement, thus disrupting tail movement and causing tail deformities, such as wavy, folded, or curved middle. According to [12] stated that damage or defects in mitochondria cause abnormalities in the morphology of spermatozoa flagella.

Primary abnormalities found in this study include small heads (Figure 4B), broken heads (Figure 4D) and taper heads (Figure 4F). This disorder is caused by mitochondrial dysfunction due to excessive zinc supplementation. Mitochondrial dysfunction reduces the energy needed to form spermatozoa heads, so that the processes of chromatin compaction and formation, as well as acrosome maturation, are disrupted [24-25]. As a result, the spermatozoa heads do not form properly and exhibit deformities such as small, broken, or flat heads. Mitochondrial dysfunction causes abnormalities in the middle part of the spermatozoa, resulting in abnormal formation of the spermatozoa head [16].

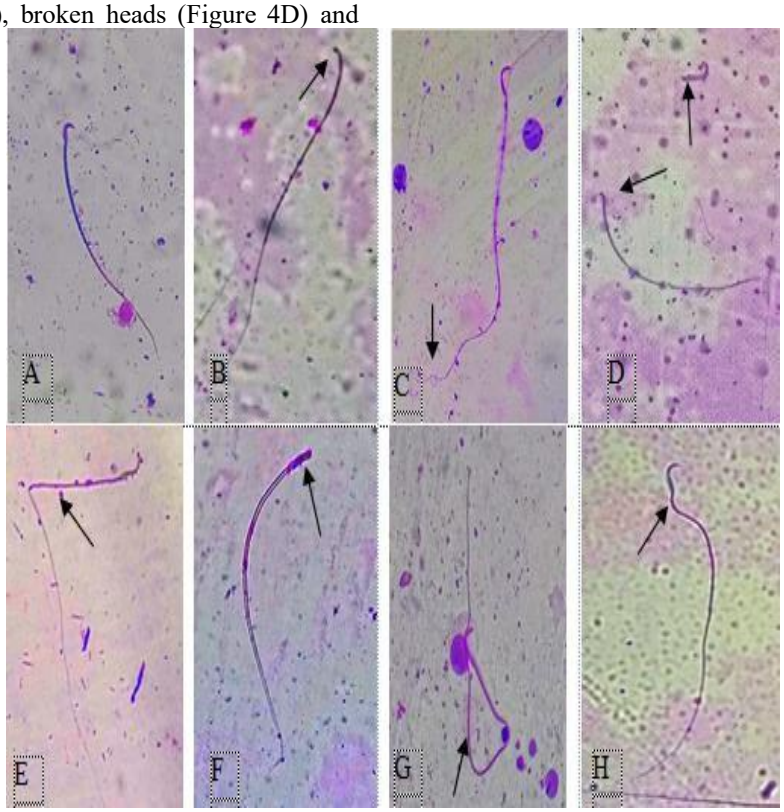


**Figure 4**. Various morphological abnormalities of spermatozoa. (A) normal; (B) small head; (C) wavy tail; (D) severed head; (E) angular tail (F) flat head; (G) folded tail (H) curved middle part.

## Conclusion

Based on the research results, it was concluded that Zinc (Zn) supplementation had anti-infertility effects on the reproductive function of male Wistar rats (Rattus norvegicus L.) exposed to cigarette smoke. This is because Zinc supplements at a treatment dose of 14 mg/g BW (P1) and 28 mg/g BW (P2) significantly increased the motility, viability, and morphology of normal spermatozoa. Zinc can be used as a supplement that can improve sperm quality due to free radicals from exposure to cigarette smoke.

### Author's Contribution

W. O. Harlis: contributed to data collection, data analysis and writing of the paper. R. Rostika: contributed data analysis and revisions to the paper draft. A. Karya: contribution is data collection and data analysis. Resman: contribution is a revision based on the review corrections.

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