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Antioxidant Activity of Fermented Red Bean Extract on Sperm Quality of Mice Exposed to Cigarette Smoke

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ABSTRACT

Smoking has a negative effect on fertilization as it decreases sperm quality. The current research aimed to investigate the effect of fermented red bean (*Phaseolus vulgaris* L.) extract on sperm motility, viability, and plasma membrane integrity of white mice (*Mus musculus*) exposed to cigarette smoke. The red beans were subjected to a 36-hour fermentation process using *Rhizopus* spp. Then, methanol was extracted by maceration method for 24 hours until maceration was obtained. In this study, 25 male white mice aged 3 months were randomly divided into 5 groups of 5 mice. Group C (negative control) was given 0.5 mL of carboxymethyl cellulose natrium (CMC Na) 1% solution orally without unfiltered cigarette smoke exposure, and group C+ (positive control) was given 0.5 mL of CMC Na 1% solution orally and unfiltered cigarette smoke exposure. Treatment groups T1, T2, and T3 were orally given fermented red bean extract at doses of 26 mg/kg Body weight (BW), 52 mg/kg BW, and 104 mg/kg BW, respectively, and then were exposed to unfiltered cigarette smoke. For 36 days, treatment groups (except the negative control) were subjected to the inhalation of smoke from an unfiltered cigarette containing a nicotine dose of 2.2 mg. The exposure period lasted for 20 minutes each day. Each group was put into a cigarette smoke-exposing box. The sperm motility (observing the forward movement of spermatozoa), the sperm viability (examining the color of the sperm head), and the sperm plasma membrane integrity (observing the tail shape using the hypoosmotic swelling test) were then evaluated. The findings indicated significant differences in sperm motility, viability, and plasma membrane integrity of each group with positive control. A dose of 104 mg/kg BW of fermented red bean extract had the best potential to maintain sperm motility (70%), viability (82.13%), and plasma membrane integrity (61.93%) of mice exposed to unfiltered cigarette smoke.

Keywords: Plasma membrane, Red bean, Sperm motility, Sperm viability

INTRODUCTION

The smoking phenomenon is one of the biggest public health threats in the world as it causes more than 8 million deaths per year, with 1.2 million deaths resulting from exposure to cigarette smoke (He et al., 2022). Smoking is a strong cause of lung cancer and has been linked to negative effects on the male reproductive system (Morris and Channer, 2012). Research by Kim et al. (2014) indicated the negative effects of smoking on fertilization due to decreased sperm quality. Smokers showed lower plasma membrane integrity, compared to non-smokers (Taha et al., 2012). Oxidative stress caused by increased free radical activity decreases sperm viability, followed by rapid ATP loss, leading to reduced motility, axonemal damage, and changes in sperm capacitation and acrosome reaction (Oyeyipo et al., 2011; Torres-Arce et al., 2021). Toxic substances in cigarette smoke, such as nicotine, lead (Pb), carbon monoxide (CO) gas, tar, and polycyclic aromatic hydrocarbons (PAHs), can enter the mitochondria and produce reactive oxygen species (ROS) that are greater than the endogen antioxidants, causing oxidative stress (Kleemann et al., 2009). Antioxidants can prevent cell and oxidative damage in the body (Yadav et al., 2016).

Red beans have been extensively cultivated in Indonesia due to their inherent resilience, which minimizes the risk of crop failure. As a result, the production of red beans in Indonesia is relatively abundant, owing to their long-established cultivation practices. The flavonoid in red beans is isoflavone with subclasses of daidzein, glycitein, and genistein (Panche et al., 2016). Previous studies by El-Demerdash et al. (2004) in male rats showed beneficial effects of flavonoids in reducing the toxic effects of CdCl2 on the male reproductive system. In a study conducted by Suryadinata et al. (2021), it was found that providing antioxidants in the form of red mulberry juice, which contains flavonoids, led

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to an increase in the number of Leydig cells, spermatocytes, and spermatids in Wistar rats that were directly exposed to cigarette smoke. According to Anggraini et al. (2021), the effect of flavonoids can also enhance regeneration by detoxifying free radicals, providing competitive substrates for unsaturated lipids in membranes, and accelerating the repair mechanism of damaged cell membranes. The activity of flavonoids in guava fruit extract can significantly increase the motility and viability of white mice spermatozoa, which are decreased due to exposure to cigarette smoke by reducing lipid peroxidation and restoring antioxidant function in the testes. Some studies suggest that antioxidant supplementation (Vitamin C, Vitamin E, and selenium) may help reduce oxidative stress caused by smoking and improve sperm quality (Sadaghiani et al., 2020). The content and antioxidant activity can be increased by fermentation, producing compounds that have higher biological activity. Therefore, this study aimed to prove the effects of fermented red bean extract as an antioxidant on sperm motility, viability, and plasma membrane integrity of mice exposed to cigarette smoke.

MATERIALS AND METHODS

Ethical approval

All research methods and practices and the use of experimental animals have been approved by the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia, with the certificate 1.KEH.001.01.2023.

Extraction of fermented red bean

Red bean was fermented by *Rhizopus* spp. obtained from a market located in Batu, Malang, Indonesia. The extract was made using the maceration method. Fermented red bean powder was dissolved in a methanol solution for 24 hours. The maceration result was filtered and separated between the residue and filtrate. The residue was soaked again in methanol for 24 hours and then filtered again to obtain the filtrate. The macerate solution was evaporated using a rotary evaporator at 500°C and rotation of 120 rpm until a concentrated extract was obtained (Xu and Chang, 2007).

Experimental animals and treatment

This study was conducted from January to February 2023 on 25 male white mice aged 3 months and weighed 20-30 grams. The rats were obtained from Pusat Veteriner Farma (Farma Veterinary Center), Surabaya, Indonesia. All mice were kept in cages, at room temperature, in indirect sunlight, and in a clean-conditioned environment. They were acclimatized for a week before being subjected to the treatments. During the study, food and water were provided *ad libitum*. They were randomly divided into five groups, with 5 replicates for each group. The C- group was given carboxymethyl cellulose natrium (CMC Na) solution orally. The positive control (C+) group was given CMC Na solution orally and then exposed to cigarette smoke only. Treatments T1, T2, and T3 received 26, 52, and 104 mg/kg body weight (BW) fermented red bean extract orally, respectively, and were then exposed to cigarette smoke. Each group was put into a cigarette smoke-exposing box to be exposed to smoke from a cigarette at a nicotine dose of 2.2 mg for 20 minutes. The cigarette (Indonesia) used was obtained from the market. The CMC Na, fermented red bean extract solution, and cigarette smoke exposure were given for 36 days. Sperm samples were taken by excising the cauda epididymis tissue (Ahmadnia et al., 2007) on day 44 after being terminated by cervical dislocation and were immediately tested. The investigated parameters in this study were sperm motility, viability, and plasma membrane integrity.

Sample examination and observation

A sperm motility examination was carried out by making a suspension by mixing one drop of sperm and physiological NaCl on an object glass. A sperm viability examination was performed by smear preparation of sperm suspension with eosin-nigrosin stain on an object glass. Examination of sperm plasma membrane integrity was carried out by mixing 0.1 mL of sperm suspension with 0.9 mL of hypoosmotic solution in a microtube, then incubating it at 37°C for 30 minutes, then the suspension was examined on an object glass.

Observations were done in the Embryology Laboratory of Veterinary Medicine Faculty of Airlangga University, Indonesia, using a Nikon Eclipse E100 light microscope (Japan) with a magnification of 400x. The motility assessment was done by observing the progressive movement of spermatozoa, viability by observing the color difference of the sperm head, and the integrity of the plasma membrane by observing the shape of the sperm tail. A transparent head indicates live spermatozoa, while a purple-ish head indicates dead spermatozoa. A curved tail shape indicates an intact plasma membrane, while a straight tail indicates a damaged plasma membrane (Ramu and Jeyendran, 2013).

Statistical analysis

All test results were expressed as the mean ± standard deviation (SD). The data were analyzed using SPSS version 20 (USA), using analysis of variance and post-hoc analysis with Duncan multiple range test to determine the significance

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of differences between groups. The cut-off $p < 0.05$ was used to indicate the significance.

RESULTS AND DISCUSSION

In this study, C+ showed the lowest motility, viability, and plasma membrane integrity (Figures 1, 2, 3, and Table 1), compared to the other groups, while C- indicated the highest on all parameters. The treatment groups T1, T2, and T3 showed significant differences between each group and the control groups ($p < 0.05$). The result is consistent with the mechanism of damage caused by exposure to unfiltered cigarette smoke exposure. This exposure tends to elevate the levels of free radicals in the body, resulting in oxidative stress, which is known to significantly diminish sperm quality (Kim et al., 2014).

Table 1. The sperm motility, viability, and plasma membrane integrity in mice exposed to cigarette smoke

Group	Mean ± SD		
	Motility (%)	Viability (%)	Plasma membrane integrity (%)
C-	$75^{e} \pm 1.67$	$85^{e} \pm 2.39$	$65.20^{e} \pm 2.33$
C+	$28.20^{a} \pm 1.83$	$40.60^{a} \pm 1.26$	$24.80^{a} \pm 2.61$
T1	$50.20^{b} \pm 2.36$	$62.40^{b} \pm 1.98$	$40.80^{b} \pm 0.8$
T2	$65.80^{c} \pm 3.21$	$79.40^{c} \pm 2.04$	$55.80^{c} \pm 2.55$
T3	$70.20^{d} \pm 2.04$	$82.20^{d} \pm 1.86$	$61.80^{d} \pm 1.86$

^{abcde} Superscripts in the same column showed significantly different results ($p < 0.05$); C-: Group of 5 mice given CMC Na 1% suspension orally, C+: Group of 5 mice given CMC Na 1% suspension orally and exposed to cigarette smoke, T1: Group of 5 mice given 26 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke, T2: Group of 5 mice given 52 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke, T3: Group of 5 mice given 104 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke

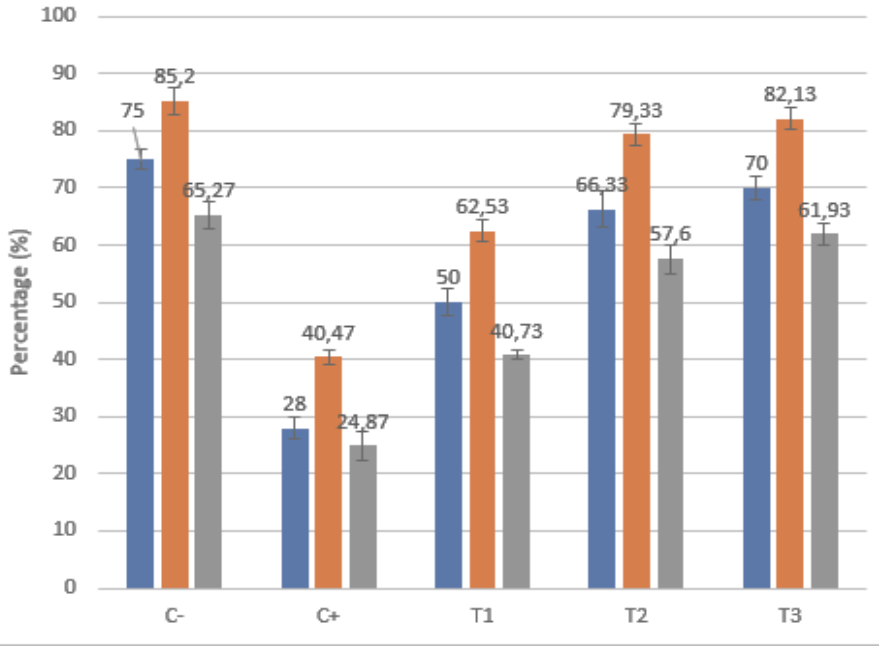

Figure 1. The effects of red bean extract in white mice sperm exposed to cigarette smoke. Blue: Sperm motility, Orange: Sperm viability, Gray: Sperm plasma membrane integrity, C-: Group of 5 mice given CMC Na 1% suspension orally, C+: Group of 5 mice given CMC Na 1% suspension orally and exposed to cigarette smoke, T1: Group of 5 mice given 26 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke, T2: Group of 5 mice given 52 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke, T3: Group of 5 mice given 104 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke

The substances in cigarettes are proven to be toxic due to the increase in free radicals and ROS production (Ahmed, 2019). Damage to spermatozoa begins with the loss of plasma membrane integrity. The plasma membrane of sperm is very vulnerable to ROS because it consists of Polyunsaturated Fatty Acid (PUFA), which has hydrocarbons with two or more double bonds. The peroxidation of lipid-containing cells initiates when free radicals attack the fatty acids within the cell membrane, causing the hydrogen atoms on the side chain to be pulled away. Fatty acids with a higher number of double bonds are more susceptible to releasing their hydrogen atoms, making the process of peroxidation easier for such fatty acids (Dutta et al., 2022). The sperm plasma membrane contains unsaturated fatty acids that can be targeted by ROS, resulting in a chemical reaction known as lipid peroxidation. This process diminishes the activity of membrane enzymes, ion channels, and the fluidity of the membrane. As a result, the required mechanisms of sperm production and fertilization are inhibited (Ahmed, 2019).

The membrane permeability is closely related to the transport of nutrients, which plays an important role in cell metabolism. Disruption of membrane permeability will result in disrupted nutrient requirements and ultimately lead to spermatozoa death. The chemical content in cigarette smoke can inhibit the process of spermatogenesis, resulting in

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lower sperm viability (Ahmadnia et al., 2007). Nicotine in cigarette smoke inhibits the performance of Gonadotropin-Releasing Hormone (GnRH), which in turn inhibits the Leydig cells in the testes from synthesizing and secreting testosterone hormone that functions in the process of spermatogenesis (Halmenschlager et al. 2009). Exposure to cigarette smoke causes significant DNA damage to spermatozoa, as evidenced by increased DNA fragmentation. Cigarette smoke exposure can increase the expression of genes involved in apoptosis, such as *Bax* and *Caspase-3*, indicating that cigarette smoke-induced DNA damage triggers apoptosis in spermatozoa (Donnelly et al., 2000).

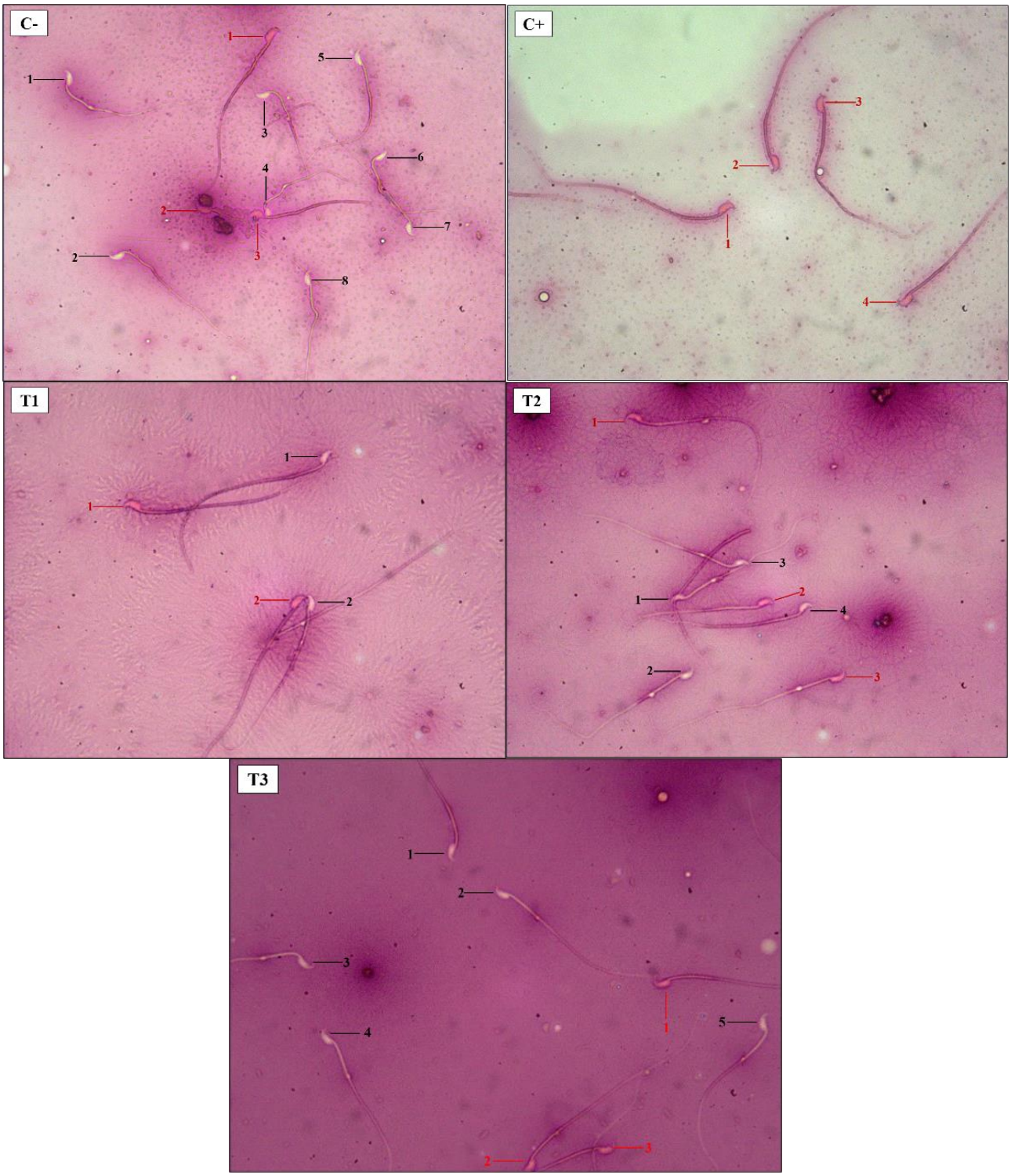

Figure 2. The mice sperm viability under a microscope at 400x magnification. Black lines with numbers show live spermatozoa. Red lines with numbers show dead spermatozoa. C-: Group of 5 mice given CMC Na 1% suspension orally, C+: Group of 5 mice given CMC Na 1% suspension orally and exposed to cigarette smoke, T1: Group of 5 mice given 26 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke, T2: Group of 5 mice given 52 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke, T3: Group of 5 mice given 104 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke

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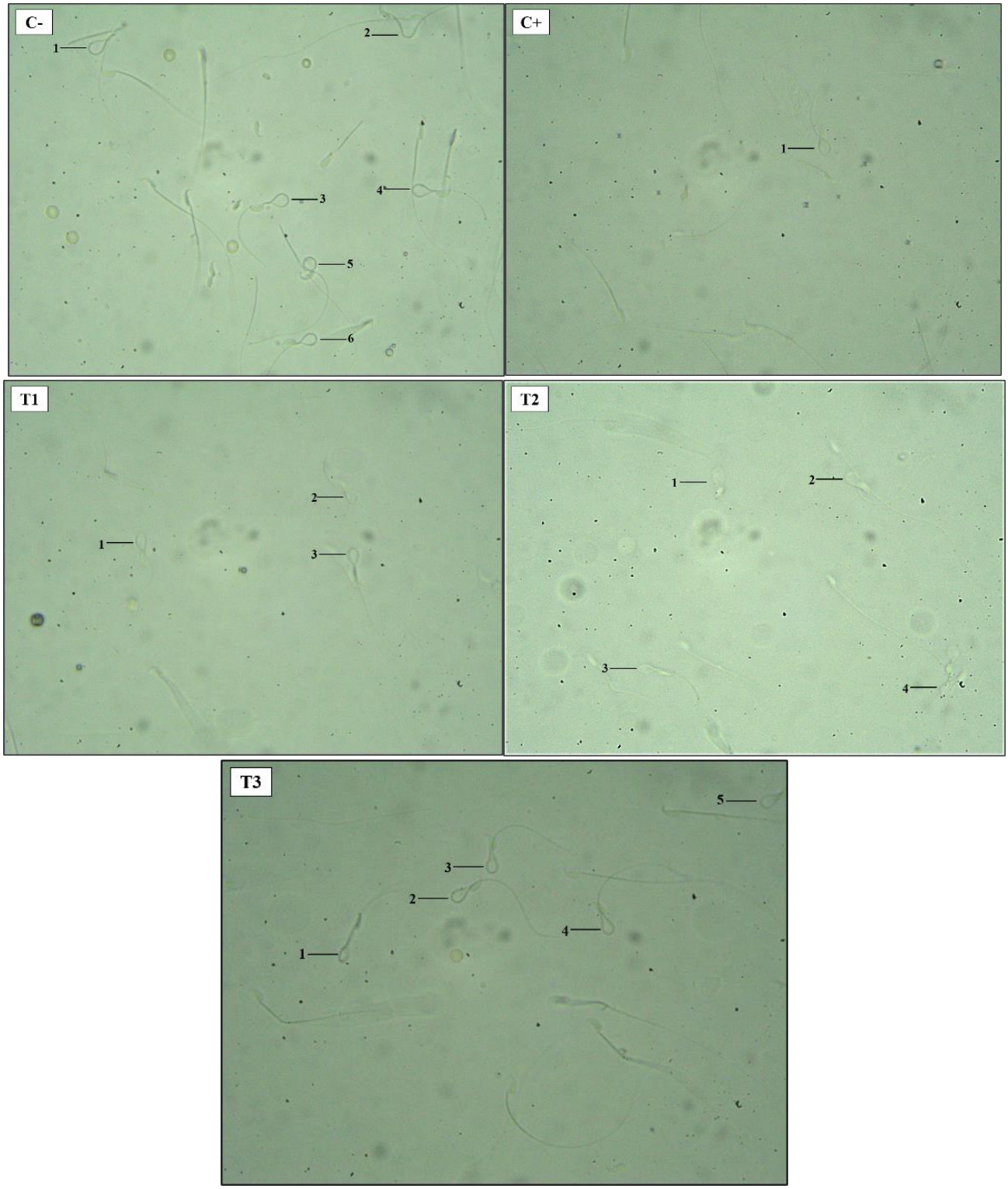

Figure 3. The mice sperm plasma membrane integrity under a microscope at 400x magnification. Black lines with numbers show intact spermatozoa plasma membrane, C-: Group of 5 mice given CMC Na 1% suspension orally, C+: Group of 5 mice given CMC Na 1% suspension orally and exposed to cigarette smoke, T1: Group of 5 mice given 26 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke, T2: Group of 5 mice given 52 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke, T3: Group of 5 mice given 104 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke.

The decrease in spermatozoa motility also begins with damage to the plasma membrane integrity of the spermatozoa. In the midpiece, which is the main part of the tail, some mitochondria function in the process of spermatozoa metabolism in producing energy in the form of ATP, which will then be used for the movement of spermatozoa (Durairajanayagam et al., 2014). Oxidative damage to mitochondria can increase the production of free radicals such as superoxide and hydrogen peroxide. These radicals can damage proteins in the plasma membrane. The electron transport chain (ETC) process will be disrupted if proteins are damaged by reducing mitochondrial activity. The ETC will decrease the rate of electrons, mitochondrial membrane damage, and a decrease in the expression of proteins required in the ETC process. Damage from this process will lead to a decrease in ATP and an increase in ROS production. The lack of energy will disrupt morphology and flagellar movement, resulting in decreased sperm motility (Yeung et al., 2009). A

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decrease in ATP as the energy for spermatozoa movement can also occur through another pathway. The chemical components of cigarette smoke will spread throughout the body via the aorta after passing through the respiratory system. The integrity of the plasma membrane can be compromised, allowing components of cigarette smoke to gain access to intracellular organelles, including mitochondria (Ahmed, 2019). The presence of carbon monoxide (CO) in the blood can reduce the amount of oxygen needed for ATP production in the mitochondria, and it results in disrupting sperm motility (Almeida et al., 2015).

The negative control group (C-) showed the highest results for all parameters compared to the other groups (Table 1). This could happen because, under normal conditions, free radicals in the body can be neutralized by endogenous antioxidants that protect cells from free radicals. The administration of fermented red bean extract in treatment groups T1, T2, and T3 showed a significant increase in motility, viability, and plasma membrane integrity compared to the C+ group (Table 1). The increase in all parameters across the groups may be attributed to a common factor, namely, the reduction in spermatozoa motility and viability. This reduction typically initiates damage to the plasma membrane surrounding and enveloping the spermatozoa. In the event of damage to the plasma membrane of spermatozoa, their viability will correspondingly decline. Living spermatozoa can be damaged by free radicals produced from cigarette smoke, which can affect spermatozoa motility. Likewise, when there is an increase in the plasma membrane, it results in an enhancement of the viability and motility of spermatozoa.

Group T3 showed the highest improvement in all parameters compared to T1 and T2, and the difference was significant. This result indicates that the optimal dose for preventing the decline in sperm quality in this study is 104 mg/kg BW ($p < 0.05$). The improvement in sperm quality in group T3 may be due to the antioxidant content in fermented red bean extract. Red beans are a good source of polyphenols. Polyphenols are a type of antioxidant that helps protect cells from free radical damage (Yadav et al., 2016). Red beans contain various types of polyphenols, including phenolic acids, flavonoids, and anthocyanins. Anthocyanins, a subclass of flavonoids, are responsible for imparting red, purple, and blue hues to various fruits and vegetables (Huang et al., 2010). The color provided by anthocyanins is formed from a long-conjugated double-bond system, so anthocyanins can act as antioxidants by capturing free radicals. Anthocyanins prevent or inhibit oxidation by scavenging free radicals and reducing oxidative stress. On a regular basis, anthocyanins act as H-atom donors or as single electron transfers. The antioxidant activity of these compounds depends on their total concentration, structure, and environment (Tena et al., 2020). The polyphenolic compounds that create flavonoids can act as scavengers of hydroxyl free radicals, thereby preventing the oxidation of lipids, proteins, and DNA in cells (Roychoudhury et al., 2017). Thus, by preventing lipid peroxidation, the risk of compromising the integrity of the sperm plasma membrane is mitigated.

During fermentation, isoflavones are broken down into forms that are more easily absorbed by the body and have higher biological activity, namely daidzein, genistein, and glycitein (Piao and Eun, 2020). Genistein is the most potent antioxidant among isoflavones. The antioxidant activity of genistein is mediated through the activation of intracellular signaling pathways that lead to the regulation of the expression of manganese superoxide dismutase (MnSOD). MnSOD is an important antioxidant enzyme that neutralizes superoxide radicals generated during cellular metabolism. Genistein enhances the antioxidant capacity of cells, helps protect cells from oxidative damage, and maintains their proper function by increasing the expression of MnSOD (Borrás et al., 2006).

CONCLUSION

The fermented red bean extract at a dosage of 104mg/kg BW has the best potential to maintain sperm motility (70%), viability (82.13%), and plasma membrane integrity (61.93%) of mice exposed to unfiltered cigarette smoke. Further research is suggested to be conducted with a higher dose of fermented red bean extract to determine the effective doses to increase and protect sperm after exposure to cigarette smoke.

DECLARATIONS

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Availability of data and materials

The data of the current study are available.

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Authors' contribution

Farah Fadhilah wrote the manuscript and conducted the research, Imam Mustofa conceptualized the research, Ratna Damayanti, Nove Hidajati, and Budi Utomo supervised the research, and Tita Damayanti Lestari revised the final form of the manuscript. All authors read and approved the final draft of the mauscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical consideration

Ethical issues, such as data fabrication, double publication and submission, redundancy, plagiarism, consent to publish, and misconduct, have been checked by all the authors before publication in this journal.

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