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OF BROWN RICE BRAN TO THE
NUMBER AND VIABILITY OF WHITE
RAT (*Rattus norvegicus*) SPERMATOZOA
Sprague dawley STRAINS INDUCED BY
CLOVE CIGARETTE SMOKE

By Evi Kurniawaty

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INTRODUCTION

The rate of cigarette smoking globally continues to increase every year. The World Health Organization (WHO) estimates that cigarettes will be consumed by 2 billion people worldwide in 2030. Indonesia is known to have poor performance concerning the number of smokers.¹

Indonesia ranked as the 4th of the largest number of smokers after China, Russia, and the United States.² In Southeast Asia, it became the first with 67.4% of smokers.³ As many as 80% of smokers have started to smoke at the age of <19 years. Thus Indonesia became the country with the highest number of teen smokers in the world.⁴ Nationally, the largest number of teenage smokers was in Lampung (60.9%) Most of them were men.

The number of male smokers in Indonesia was 16 times more than female. 80.4% of them consumed clove cigarettes and sucked 11.8 cigarettes every day.⁶ Smoking in Indonesia has reached the advanced stage and became habits which were defective for the health.⁷ Smoking not only caused respiratory system disorder but also affected the function of male reproductive organs leading to infertility.²

Infertility is the inability to conceive after one year of marriage. It happened to 15% of couples in the world, and 50% of cases were due to male factors.⁸ In Indonesia, the problem continues to increase annually.⁹ Besides anatomical structure disorders, 4090⁰ of cases are idiopathic. However, there was a decrease in

antioxidants and an increase in the number of free radicals in the idiopathic infertile sperm.¹⁰ Radical is derived from environmental exposure, for instance is smoking.¹¹

Cigarette smoke contains gases and particles that can be a source of free radical.¹² If an antioxidant is unable to counter free radical, oxidative stress will occur. In male sperm, oxidative stress can increase lipid peroxidation, making DNA fragmentation, causing immature apoptosis, and disrupting the hormonal system, thereby decreasing the quality of sperm, such as the number and viability of spermatozoa.^{13:14} Study of Wulandari et al. (2012)¹⁵ showed that there was a decrease in sperm viability (50.3%) in the group of rats exposed to cigarette smoke for 15 minutes compared with the unexposed group. Other studies conducted by Cui et al. (2016)¹⁶ also proved that there was a decrease in the number and viability of sperm in male smoker compared to a male who does not smoke. Antioxidants from outside the body are necessary to prevent the adverse effects of oxidative stress.¹⁷ One of them is brown rice bran.

Bran is the result of a rice mill process which is rich in phytochemical components. Components such as polyphenols, anthocyanins, anthocinidins, M-tocopherols, a-tocopherols, and **1**oryzanol are antioxidants.¹⁸ The study of ethanol extract **96% of brown rice bran** by Widarta et al. (2013)¹⁹ indicated that brown rice bran had satisfying antioxidant activity as much as 62.41%. Another study by Laili (2016)²⁰

proved that the administration of 200 mg/kg rat's body weight of bran extract was the optimal dose to increase the antioxidant rats exposed to monosodium glutamate (MSG).

Based on the description above, the author wanted to determine the effect of administration of ethanol extract 96% of brown rice bran as an antioxidant to the number and viability of spermatozoa in Sprague Dawley male rats induced by clove cigarette smoke.

RESEARCH METHODS

This was an experimental study with test only control group, total-randomised design. The animal intervention was done at Pet House Faculty of Medicine, University of Lampung (Unila) for 30 days in November-December 2017. The number and viability of spermatozoa were assessed on the 31st day at the Biomolecular Laboratory of Faculty of Medicine, University of Lampung. The sample of this study was an albino rat.

We used the albino rat (*Rattus norvegicus*) Sprague Dawley strains obtained from the Faculty of Animal Husbandry, Bogor Agricultural University. The inclusion criteria were male, 10-14 weeks old, and 200-350 gram body weight. The exclusion criteria were decreasing of > 10% body weight after the adaptation period, illness (dull-hair, falling out, and not moving actively rat), and died during the intervention. Based on the Frederer formula, 25 rats were obtained as sample and five were backup. Rats were then divided into five intervention groups, one group consisted of 6 rats. The intervention of each group can be seen in table 1.

The tools were five cages, feeding boxes and drinking bottles, gavage, smoking chamber, balance scale, microscope, cover and object glass, Neubauer improved cell

counting chamber, minor set, pipette, pot, needle ICC and IOcc, hand glove, and masks. The materials were NaCl 0.9%, clove cigarette, eosin 2%, and extract of brown rice bran obtained from Serang-Banten, ethanol, and aquadest. The process of making ethanol extract 96% of brown rice bran can be seen in figure 1. The exposure to smoke was done in the smoking chamber.

The smoking chamber was a modification of Irawati's study (2015)²¹. One hole in the chamber was connected an air pump made of IOcc needle and hose. The function was to deliver smoke into the chamber. Some air holes were created to avoid hypoxia in rats during two clove cigarettes smoke exposure. After exposure of smoke, the rats were then given 96% ethanol extract of brown rice bran using gavage.

After 30 days of experimental study, the rat was terminated.

The rat termination was performed by cervical dislocation on the 31st day. Afterwards, the testis along with the epididymis of them was taken. The sperm was taken and put into the petri dish containing NaCl 0.9%. After it came homogenous, the number and viability of spermatozoa was observed in the suspension.

The number of spermatozoa was observed using the Neubauer improved hemocytometer box. It was checked under a microscope with 400x magnification. The results were inserted into the formula to determine the number of spermatozoa /ml of suspension as follows:

$$\text{Number of spermatozoa} \times \text{dilution} \times 10^6 \text{ million} \\ \text{of sperm /ml} = \text{number} \\ \text{of sperm on box A, B,} \\ \text{C, and D}$$

The spermatozoa viability (VS) was observed using the dye exclusion method with eosin staining. It was then examined under a microscope with 400x magnification. Approximately 200 spermatozoa were observed. The viable (not absorbing colour) and dead spermatozoa (absorbing colour) were calculated in the percentage. The viable spermatozoa will have a clear coloured head while the dead

spermatozoa can be detected by looking at the purple or pink spermatozoa head after being stained with eosin. To prevent sperm dehydration, the calculation was done in less than 30 minutes after removal from the testes. The formula of viability:

$$\% \text{ vs} = \frac{\text{The number of viable spermatozoa}}{\text{total of observed spermatozoa}} \times 100\%$$

No	Group	Intervention
1	Control I	Unexposed to smoke, not receive brown rice bran extract
2	Control 2	Exposed to 2 clove cigarettes smoke
3	Intervention I (P1)	Exposed to 2 clove cigarettes smoke, received brown rice bran extract at dose 100mg/KgBB
4	Intervention 2	Exposed to 2 clove cigarettes smoke, received brown rice bran extract at dose 200mgKgBB
5	Intervention 3 (P3)	Exposed to 2 clove cigarettes smoke, received brown rice bran extract at dose 400mg/KgBB

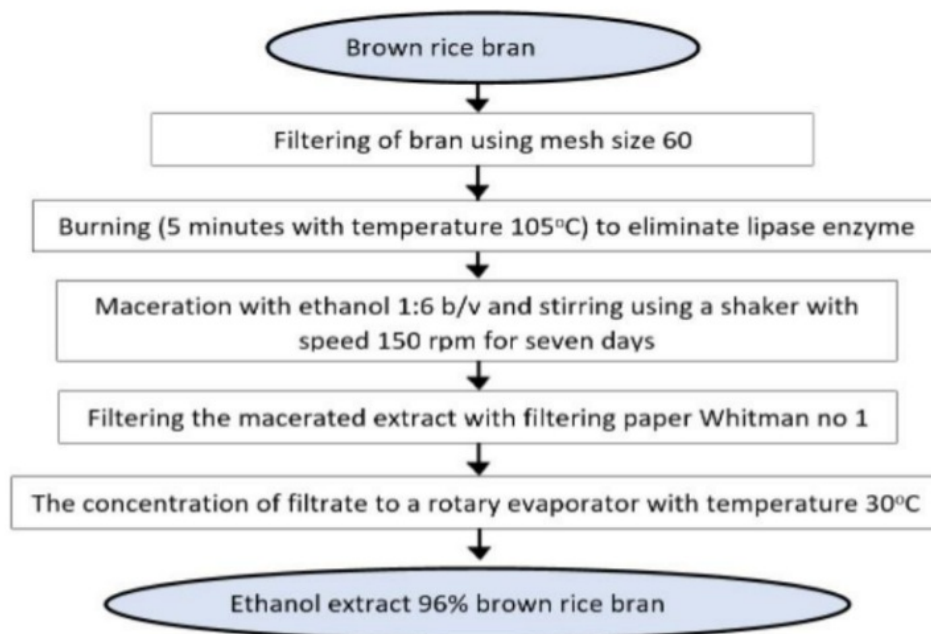


Figure 1. The Process of Making Ethanol Extract 96% Brown Rice Bran

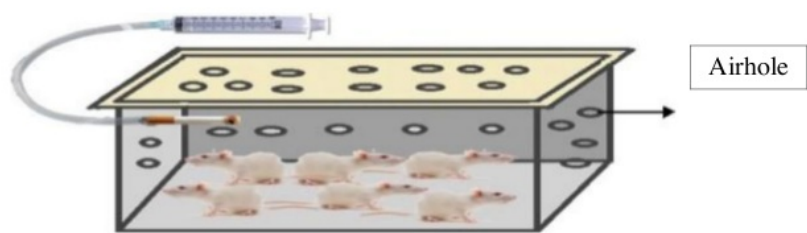


Figure 2. The Process of Clove Cigarette Smoke Exposure

RESULTS AND DISCUSSION

The result of mean calculation and One Way ANOVA test from the number of spermatozoa can be seen in table 2. The K1 group (a group of rats that were not exposed to cigarette smoke and not given brown rice

Table 2. The Mean of the Number of Rat Spermatozoa

Group	Mean+SD (Million/ml)	p
PI	91,90±7,72	0,000
	39,68±7,51	
	79,88±8,63	
	86,40±10,5	
	86,00±5,78	

Table 3. The Post the number of rat

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Group	PI	Hoc test result of spermatozoa		
PI	0.000*	0.300	1.000	1.000
	0.300	0.000*	1.000	1.000
	1.000	0.000*	1.000	1.000
	1.000	0.000*	1.000	1.000

bran extract) had the highest mean. The lowest mean was found in the K2 group (a group of rats exposed to 2 cigarettes smoke). There was a clinical increase of mean spermatozoa after administration of ethanol extract 96⁰0 of brown rice bran in multilevel doses. The result of One Way ANOVA test showed that the administration of extract

Similar results were also obtained from the spermatozoa viability test in this study. Table 4 showed that the group of rats exposed to 2 clove cigarettes smoke (K2) had the lowest viability rate, and the highest rate was in the normal group of rats (K 1). The administration of bran extract also Table 4. The Mean of the Viability of Rat Spermatozoa.

Group	Mean+SD (Million/ml)	p
PI	65.00±6.85	0,000
	29.6±5.85	
	51.4±3.50	
	60.00±6.67	
	61.00±2.91	

Table 5. The Post Hoc Test Result of the Viability of Rat Spermatozoa

Group	PI			
PI	0.008*	0.000*	0.008*	1.000
	1.000	0.000*	0.208	0.208
	1.000	0.000*	0.110	1.000
	1.000	0.000*	0.110	1.000

The illustration of spermatozoa viability examined using eosin staining in this study can be seen in Figure 3. The head of the dead or not viable spermatozoa will be pink as indicated by Y arrows. Meanwhile, the head of viable spermatozoa will not be coloured as indicated by the X arrow.

affected the mean of spermatozoa. However, there was no significant difference statistically of the mean number of spermatozoa among groups of rats fed with a multilevel dose of brown rice bran extract. The results of the Post Hoc test between the groups can be seen in table 3.

proved to affect spermatozoa viability after rats exposed to cigarette smoke (One Way ANOVA test p<0,05). Post Hoc test in table 5 showed that there was no significant difference of spermatozoa viability rate statistically in a group with multiple dosages of extract.

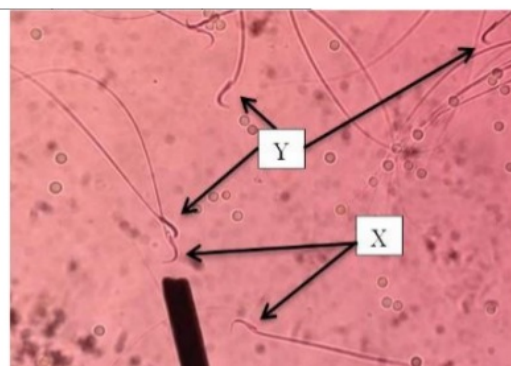


Figure 3. The Viability of White Rat Spermatozoa with Eosin Stain

The results of this study showed that exposure to 2 clove cigarettes smoke for 30 days would reduce the number and viability of spermatozoa of the rat significantly. This study was in accordance with Batubara et al. (2013)²² which stated that the number of spermatozoa in experimental animals that had been exposed to 2 clove cigarettes smoke for 30 days would decrease. Similar results were also found in Rula et al. (2014)²³ which revealed a 41% decrease in the number of spermatozoa in rats exposed to cigarette smoke for 28 days. The results of the viability in this study were in accordance with Wulandari et al. (2012)¹⁵ which stated that the viability of spermatozoa of rats exposed to cigarette smoke would decrease. Another study by Cui et al. (2016)¹⁶ on human confirmed that there was a decrease in the number and viability of spermatozoa in men who smoked compared with those not. The decline was caused by the inability of sperm to cope with oxidative stress due to free radicals found in cigarette smoke.

Sperm is a type of cell reportedly susceptible to oxidative damage caused by free radicals.²⁴ Free radicals are molecules that have one or more unpaired electrons in their outer layer that are unstable.¹⁷ To achieve stability, the molecule will always try to attract electrons from molecules or another cell, which can lead to cell structure changes.²⁵ The most damaging free radicals for the body are Reactive Oxygen Species (ROS) and their groups.²⁶ In normal sperm, some ROS such as superoxide ($\bullet\text{O}_2^-$), hydrogen peroxide (H_2O_2) and nitric oxide ($\text{NO}\bullet$). The compound is needed in the sperm process to become fertile because it will trigger phosphorylation in the capacitating process, thereby facilitating the reaction of breaking sperm acrosomes. However, the ROS produced by the sperm is at a low level, so it does not damage its

components and is still able to be overcome by the enzymatic antioxidant system in the sperm's body.²⁴ Damage of sperm will occur when the amount of ROS is excessive and not overcome by the antioxidant.^{24,25} In this study, cigarette smoke caused the formation of excessive ROS.

Cigarette smoke is known to form ROS and Reactive Nitrogen Species (RNS). The compound may be formed directly or through interaction between the chemical compounds in the gas phase. Tar on cigarettes is known to form several free radicals such as carbon monoxide (CO) and semiquinone radicals. Those are formed from the hydroquinone autoxidation assisted by tar which formed superoxide radicals ($\bullet\text{O}_2^-$).¹² Besides, cigarette smoke also contains cadmium (Cd) which produces superoxide radicals ($\bullet\text{O}_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals. All of these elements and compounds are considered as ROS. Meanwhile, nitrogen oxide (NO), a nonreactive free radical produced by tar, can interact with oxygen in the air and form nitrogen dioxide (NO_2), dinitrogen trioxide (NO_3), and dinitrogen tetraoxide (N_2O_4). Peroxynitrite radicals (ONOO^-) can also be formed due to NO interaction with superoxide radicals. The whole compound is part of the RNS.¹² The excessive of ROS and RNS cannot be overcome by the enzymatic antioxidants in the sperm. Flaherty (2014)²⁴ stated that the antioxidants produced by sperm were very sensitive to excessive ROS. These antioxidants were highly susceptible to inactivation. Sperm was unable to synthesize more antioxidants if the amount of ROS was excessive. This led to oxidative stress.

Oxidative stress experienced by sperm can lead to decreased number and viability of sperm. The decline is due to structural changes in nucleic acids, proteins, lipoproteins and lipids in the plasma membrane of sperm cells.²³ Sperm cell membranes are known to be rich in lipid components in the form of unsaturated fatty acids or polyunsaturated fatty acids (PUFAs). The bond between free radicals and PUFAs can result in the formation of lipid peroxidation as seen in Figure 4. The formation of LPO will alter the structure, integrity, permeability, loss of function of

The viability of spermatozoa is a measure of the ability or survival of sperm outside the testes. The living sperm will have an intact plasma membrane. The damage of the plasma membrane becomes the sign of sperm death due to the importance of the role of the plasma membrane to keep the sperm alive.³² The plasma membrane surrounds all sperm cells, protecting and holding organelles and intracellular components. The membrane will also defend the chemical gradients of ions and other soluble compounds. If the membrane is not intact, the organelle and its intracellular component may be disrupted, resulting in sperm death. Dead sperm is considered not fertile.³³ In dye exclusion eosin staining, dead sperm with non-intact membrane will absorb eosin colour.

The ethanol extract 96% of brown rice bran in this study was able to increase the number and viability of rat spermatozoa that had been exposed to 2 clove cigarettes

the cell membrane and may lead to sperm cell death. Secondary products of lipid peroxidation can also damage some essential molecules such as proteins and cellular DNA bases. This is appropriate with the study of Ghaffari and Rostami (2012)²⁹, which stated that there was an increase in LPO level in male smoker followed by a decrease in the number of sperm. Tafuri et al. (2015)³¹ also demonstrated that damage to the structure of the DNA might lead to apoptosis in sperm cells which reduced the number. Lipid peroxidation in sperm can also reduce its viability.

smoke. The increase occurred because the bran extract contained nonenzymatic antioxidant compounds. Nonenzymatic antioxidant compounds are antioxidant compounds that come from outside the body. Antioxidants are needed to break the free radical chain reaction. Thus, oxidative stress can stop and not cause damage to the cell. Nonenzymatic antioxidants work by being scavenger for ROS.³⁴

The brown rice bran extract contains nonenzymatic antioxidant compounds, such as α -tocopherol, γ -tocopherol, M-oryzanol, phenol, anthocyanin and anthocyanidin. α -tocopherol and γ -tocopherol are part of vitamin E. These antioxidants act as ROS scavengers in lipid peroxidation reactions. The α -tocopherol bond with peroxy lipid radical (LOO•) will form a tocopheryl radical. The radicals are relatively more stable to stop the LPO reaction due to ROS. Other compounds, yoryzanol, also have a mechanism similar to vitamin E. M-oryzanol

will donate hydrogen to neutralise free radicals in ROS.³⁷ Meanwhile, phenol

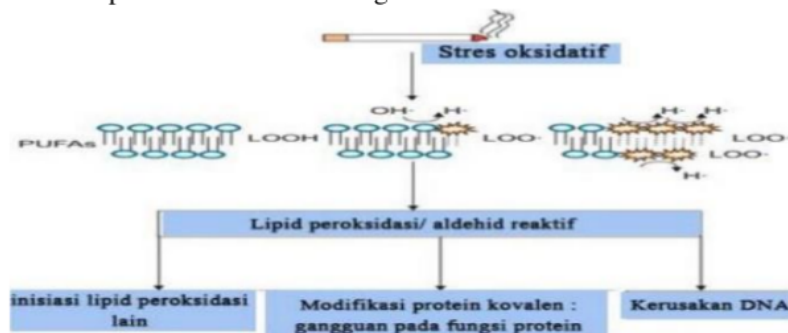


Figure 4. The Mechanism of Cigarette Smoke Causing Lipid Peroxide²⁸

compounds have better in-vitro antioxidant effects than tocopherol and ascorbic acid or vitamin C.³⁸ Other antioxidant compounds considered as flavonoid group are anthocyanin and anthocyanidin. Those also act as a ROS scavenger by donating their hydrogen. Also, those are also colour pigments in brown rice bran, which are not found in white rice. This makes brown rice bran has a better antioxidant content than white rice bran.¹⁵ The effect of sperm number improvement by brown rice bran antioxidant in this study was strengthened with the study by Amelia (2018) which stated that ethanol extract 96% of brown rice bran would increase sperm motility and morphology.³⁹ The results were also in accordance with Dwizella et al. (2018)⁴⁰ where rat spermatocytes exposed to cigarette smoke improved after administration of the extract.

There was no statistically significant difference in the rate of spermatozoa number and viability in the multilevel dose group. This result was appropriate with Amelia et al. (2018)³⁹. This was because the dose range of bran extracts too narrow. Therefore, the increase rate in number and viability of spermatozoa was not significant. Besides, it

improvement could already be found at a dose of 100mg/kg and best at a dose of 400mg /kg.

could be affected by the quality of the extract.

The brown rice bran extraction process in this study also did not use an additional of HCl 37%. According to Widarta et al. (2013)¹⁹ the addition of HCl could better maintain the quality of antioxidants from rice bran extract. HCl compounds also decomposed plant cells so that the antioxidants in plant cells could be well expressed.⁴¹ Based on this study, the dose of 100 mg/KgBB provided improved number and viability of spermatozoa compared to groups that did not receive bran extract. This was consistent with a study conducted by Heikal et al. (2015)⁴². It stated that the dose of bran extract of 100 mg / KgBB using ethanol 96% was a safe dose for long-term consumption, while a dose of 500 mg KgBB would cause toxicity in the study of the white rat.

CONCLUSION 1

Administration of ethanol extract 96% brown rice bran was able to increase the number and viability of spermatozoa of white rat (*Rattus norvegicus*) Sprague dawley strain induced by clove cigarette smoke. Clinical

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