

## Protective effect of ethanolic extract of white oyster mushroom on morphological rat sperm damage due to cigarette smoke exposure

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### ABSTRACT

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**Background:** Cigarettes smokes are a great external source of free radicals. The negative effects of cigarette smoke exposure can be systemic and affect all body systems, including the reproductive. Male rats exposed to cigarette smoke have a risk of oxidative stress and sperm damage. This can be overcome with herbal antioxidants such as white oyster mushrooms (*Pleurotus ostreatus*).

**Objective:** This study aimed to examine the protective effect of ethanolic extract of white oyster mushroom against damage to sperm morphology of rats exposed to cigarette smoke.

**Methods:** This study is an experimental study using 40 rats which were divided into 5 groups. Group I (normal control), group II (negative control) were only given exposure of cigarette smoke, Groups III, IV and V (treatments groups 1, 2, and 3) were given exposure of cigarette smoke and ethanolic extract a dose of 125, 250, and 500 mg/Kg BW/day for 14 days. On the 15th day, the percentage normal rat sperm were calculated under a 400x magnification microscope.

**Results:** Normal sperm count in group I was 79% ± 0.79, group II was 39% ± 0.55, Grup III, IV and V were 56% ± 0.15, 65% ± 0.54 and 66% ± 0.21. The ANOVA test showed that there was a significant difference between group with 95% Confidential Interval and p ≤ 0.05. The results of the post hoc test-Tukey test showed that treatment groups 2 and 3 were in the same subset.

**Conclusion:** Ethanolic extract of white oyster mushrooms can prevent sperm morphology damage in rats exposed to cigarette smoke, with an optimal dose of 250 mg/200mg BW.

**Latar Belakang:** Asap rokok merupakan sumber radikal bebas eksternal yang besar. Efek negatif dari paparan asap rokok dapat bersifat sistemik dan mempengaruhi semua sistem tubuh, termasuk reproduksi. Tikus jantan yang terpapar asap rokok memiliki risiko stres oksidatif dan kerusakan sperma. Hal ini dapat diatasi dengan antioksidan herbal seperti jamur tiram putih (*Pleurotus ostreatus*).

**Tujuan:** Penelitian ini bertujuan untuk menilai efek protektif ekstrak etanol jamur tiram putih terhadap kerusakan morfologi sperma tikus yang diberi paparan asap rokok.

**Metode:** Penelitian ini merupakan penelitian eksperimental dengan menggunakan 40 ekor tikus yang dibagi menjadi 5 kelompok. Kelompok I (kontrol normal), kelompok II adalah kontrol negatif yang hanya diberi paparan asap rokok, kelompok III, IV dan V adalah perlakuan 1, 2, dan 3 yang diberi paparan asap rokok dan ekstrak etanol dengan dosis 125, 250, dan 500 mg/kg berat badan (BB)/hari selama 14 hari.

Pada hari ke-15, persentase spermatozoa tikus yang normal dihitung di bawah mikroskop dengan perbesaran 400 kali.

**Hasil:** Jumlah sperma normal pada kelompok I adalah  $79\% \pm 0.79$ , kelompok II  $39\% \pm 0.55$ , kelompok III, IV dan V adalah  $56\% \pm 0.15$ ,  $65\% \pm 0.54$  and  $66\% \pm 0.2$ . Hasil uji ANOVA menunjukkan bahwa terdapat perbedaan yang signifikan pada masing-masing kelompok eksperimen dengan confidential interval 95% dan  $p \leq 0,05$ . Hasil uji post hoc dengan uji Tukey menunjukkan bahwa kelompok perlakuan 2 dan 3 berada pada subset yang sama.

**Kesimpulan:** Ekstrak etanol jamur tiram putih dapat mencegah kerusakan morfologi spermatozoa pada tikus yang terpapar asap rokok, dengan dosis optimal 250 mg atau 200 mg/kg BB.

## INTRODUCTION

Cigarettes are a world health problem and it is estimated that deaths from cigarettes currently reach 3 million deaths per year. In 2030, it will reach 10 million, and 70% of these deaths occur in developing countries.<sup>1</sup> Cigarettes are dangerous for active smokers as well as passive smokers (secondhand smoke). More than 19.2% of students in Indonesia currently smoke and 66.2% are exposed to secondhand smoke in public spaces.<sup>2</sup>

Cigarette smoke contains more than 4,700 chemical components, such as carbon monoxide, carbon dioxide, tar, nicotine, nitrogen oxides, hydrogen cyanide, ammonia, and formaldehyde.<sup>1</sup> Free radicals and reactive oxygen species (ROS) in cigarette smoke are contained in gas phase and tar phase. Free radicals in cigarette smoke include quinones (Q), semiquinones (QH<sup>•</sup>), and hydroquinones (QH<sub>2</sub>). Quinone/hydroquinone polymers (Q/QH<sub>2</sub>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and hydroxyl radicals (OH) are very dangerous because they are very reactive and can cause damage to cell membranes, proteins, and deoxyribonucleic acid (DNA).<sup>4-6</sup>

Exposure to cigarette smoke is one of the largest sources of exogenous free radicals in the body that will cause oxidative stress. This is related to the pathogenesis of several types of chronic diseases such as cancer, chronic obstructive pulmonary disease, and decreased

sperm quality which can cause infertility.<sup>3,7,8</sup> The epidemiological rate of infertility ranges from 8 -12% of couples with wives of reproductive age.<sup>9</sup> Infertility can be influenced from female factors, male factors or both. Male factors play a role more than 50% and more than 90% are caused by low sperm quantity, poor sperm quality, or both.<sup>9,10</sup> Parameters of good sperm can be assessed from the quantity and quality. Quantity is measured based on the number of sperm per ejaculate semen. Quality can be judged from the motility and/or morphology of the sperm. Many factors can interfere spermatogenesis and cause male infertility, including psychological factors, nutrition, environmental pollutants, heat stress, genetic abnormalities, excessive use of alcohol, smoking, hormone deficiency, and impotence.<sup>9,11</sup>

Infertility cases can be treated by several methods such as assisted reproductive technologies (ARTs) including in vitro fertilization (IVF), embryo transfer (ET), ovarian stimulation and surgical laparoscopy. These procedures are quite invasive, relatively expensive, and still risky.<sup>12</sup> The other alternative is to give exogenous antioxidants, either synthetic or herbal.

The previous knowledge have proven that antioxidant molecules can prevent oxidation and proinflammatory responses caused by cigarette smoke.<sup>4,13,14</sup> Several studies have shown that smokers have lower levels of vitamin E,  $\beta$ -carotene, glutathione (GSH) in plasma than nonsmokers.<sup>15,16</sup> Exogenous antioxidants can also come from plants that contain substances that are antioxidants. Indonesia has about 30.000–40.000 species of plants and many of them have medical properties. Fourty percent of Indonesian people has used traditional medicine and 70% of them are in rural areas. One of the plants that have high levels of antioxidants is white oyster mushroom (*Pleurotus ostreatus* Jacq: Fr Kumm).<sup>1,17,18</sup>

White oyster mushroom is one of food source that is currently very popular. White oyster mushrooms contain high nutrients and various secondary metabolites that

have pharmacological effects. White oyster mushroom is also the second rank mushroom that is widely cultivated.<sup>16,19,20</sup> One of the potential pharmacological effects of mushrooms is their antioxidant effect. Various previous studies have proven that white oyster mushrooms have strong antioxidant abilities both in vivo and in vitro. Jayakumara and colleagues have proven that white oyster mushroom extract has a hepatoprotective effect and can fight the hepatotoxicity caused by carbon chloride (CCl<sub>4</sub>). This protective effect is also shown in other organs such as the kidneys and brain.<sup>16,21</sup> Previous research has also proven the antioxidant effect of white oyster mushroom in preventing the decrease of lung density in mice exposed to cigarette smoke.<sup>5</sup> Components in white oyster mushrooms that are believed to have antioxidant effects include vitamin C, beta-carotene, selenium, ergothioneine and phenolic components. The phenolic component is the main component that affects its antioxidant activity.<sup>1,20,22</sup>

Macromolecular oxidative damage due to cigarette smoke can be inhibited by vitamins C and E, so that its supplementation is expected to reduce the harmful effects of smoking.<sup>3</sup> In the presented study, the researchers were interested in analyzed of protective effect of ethanolic extract of white oyster mushrooms to prevent oxidative damage caused by cigarette smoke on sperm morphology of rats exposed to cigarette smoke.

## METHODS

This research is an experimental study using a post test-only control group design. The inclusion criteria were male, healthy and active Wistar rats, with body weight of 175-250 grams and age of 2-3 months. The rats were adapted for 7 days. The exclusion criteria in this study were died rat during the adaptation period or lost more than 10% body weight. Determination of the number of samples for each group was based on Federer's formulation and obtained 8 experimental animals for 5 groups, a total of 40 rats with an estimated drop out of 20%.

The experiment was carried out in a completely randomized design. Rats were divided into five groups consisting of groups I, II, III, IV and V. Group I was a normal control, rats were only get standarized food and drink, carboxymethyl cellulose (CMC) and not induced by cigarette smoke. Group II is a negative control, given cigarette smoke without treatment. Groups III, IV and V were treatment groups 1, 2 and 3. Each group was given cigarette smoke induction 30 minutes per day/group and ethanol extract of white oyster mushroom at doses of 125 mg, 250 mg and 500 mg/kg body weight (BW)/rat/day for 14 days. On the 15<sup>th</sup> day, the rats were executed and the sperm were examined.

The research materials used were 70% ethanolic extract of white oyster mushroom, standard food, 0.5% CMC Na aquadest, 0.9% NaCl solution and eosin. The research tools used were as follows: cages and food containers for rats, digital scales, plastic bottles, oral syringe, gloves, a set of sterile surgical instruments, object glass, 1 ml disposable syringe, microscope and smoking pump.

## Research Variables

The dependent variable in this study was the number of normal sperm morphology. The independent variable was the concentration of white oyster mushroom ethanol extract.

## Preparation of Ethanolic Extract of White Oyster Mushroom

Twenty (20) kg of fresh white oyster mushrooms taken from oyster mushroom cultivation in Panandaan oyster mushroom cultivation, Jambudipa Village, Cisarua District, West Bandung Regency. Mushrooms are sliced into thin strips and dried in an oven at 500 C for 2-3 days. The dried mushrooms are then mashed by a grinding tool. The dried mushroom powder was macerated with 70% ethanol and maceration for 24 hours with occasional stirring. Maceration was repeated five times and this solution formed a dilute extract. The aqueous extract was then concentrated using a rotary evaporator until it was concentrated.<sup>23</sup>

Extraction results obtained 41.8 grams of extract from 20 kg of fresh mushroom in the form of a paste. Previous research showed that a dose of 250 mg/kg BW showed good results as an antioxidant in the lungs, so in this study the doses of 125, 250, and 500 mg/kg BW were used.<sup>5</sup> Determination of the dose for each rat, adjusted for the average body weight of rats per group.

### **Cigarette Smoke Induction**

The cigarettes used kretek cigarettes with 38 tar content. Exposure to cigarette smoke uses a smoking pump in the smoking room. The smoking room is specially made so that it is transparent, can accommodate a group of experimental animals and has ventilation holes at the top of the cage that can be opened and closed. Induction of cigarette smoke will be carried out for 30 minutes for each group of experimental animals per day.<sup>4,5</sup>

### **Sperm Morphology Examination**

Rat sperm specimens were taken from the vas deferens of rats which were chopped. Sperm morphology examination was carried out by assessing the sperm smear on a glass object stained with eosin. Count the number of normal and abnormal morphological spermatozoa in a zig zag manner with 400x magnification.<sup>24-26</sup>

The sperm morphology observation method is similar to the different count calculation, namely by zigzagging in each field of view how many normal or abnormal sperm are found in each field of view. Sperm were counted up to 100 sperm, and a percentage was made between normal sperm per 100 sperm. The morphology of the rat sperm was seen from the structure of the sperm under a microscope. Normal rat spermatozoa are divided into a hook-shaped head, a short middle piece, and a very long tail. The length of the head is approximately 0.0080 mm while the total length of the spermatozoa is about 0.1226 mm (122.6 microns). The shape of abnormal spermatozoa can be classified based on the shape of the head and tail. Abnormal sperm shape in rat is basically divided into two groups, namely sperm with abnormalities at the

head and the tail. Sperm with head abnormalities include sperm heads without hooks, abnormal hook shapes, or pin heads / isolated forms, namely sperm heads that do not have a tail. Morphological abnormalities in the tail, including a broken or split tail, isolated or not having a head, and a tail that turns or forms a spiral.<sup>24-26</sup>

### **Data Analysis**

Test for normality and homogeneity of the data distribution using the Shapiro Wilk test and Levene test. The normally distributed data were then analyzed using the parametric analysis of variance (ANOVA) test. The significant value at the ANOVA continued with the posthoc test (Tukey test) to find out which group showed the most significant results.

### **Research Place and Time**

The production of white oyster mushroom ethanol extract was carried out at Pusat Antar Universitas (PAU) laboratory of the Bandung Institute of Technology (Institut Teknologi Bandung). Furthermore, the research was carried out at the Laboratory of the Faculty of Medicine, Bandung Islamic University. This study was carried out from January to April 2021.

### **Research Ethical Aspects**

Research that uses experimental animals must apply the 3R principles, namely replacement, refinement, and reduction. This research has received ethical approval from Komite Etik Penelitian Universitas Islam Bandung with letter number 112/KEPK-Unisba/XI/2020.

## **RESULTS**

All groups in the treatment had normal and abnormal rat sperm morphology, but with different comparisons between the two. The following is a histopathological description of the vas deferens tissue of rat to see the normal and abnormal sperm count (Figure 1).

Figure 1A shows that the normal sperm morphology has a hook-shaped head with a short invisible neck and a straight and long

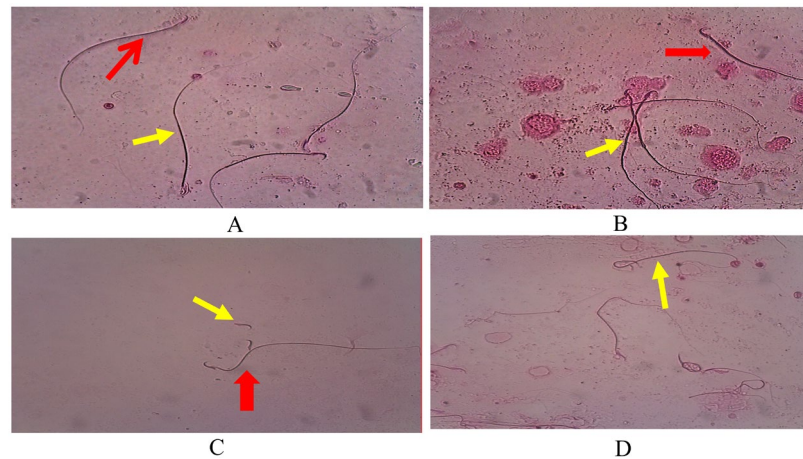


Figure 1. Sperm morphology in normal control and negative control group at 400x magnification.

A. Normal and abnormal sperm morphology in the normal control group

B. Normal and Abnormal sperm morphology in the normal control group

C. Normal and abnormal sperm morphology in the negative control group

D. Abnormal sperm morphology in the negative control group

\*red arrow: normal

\*yellow arrow: abnormal

tail. Figure 1B shows a picture of an abnormal sperm morphology, namely the tail of the sperm is not straight and curved, one of the sperm also appears to have a broken tail. In Figure 1C, it can be seen that the morphological normal rat sperm is the same as the normal control. Figures 1C and 1D show abnormal sperm morphology in the form of abnormalities in the head or tail. It appears that the head and tail are separated from other parts, the tail is folded and also broken.

In Figure 2A it can be seen that there are sperm with normal morphology, but some sperm have abnormal morphology. Sperm with abnormal morphology, among others, there are images of sperm with separate heads and tails (isolated head and isolated tail) and sperm with folded tails. In Figure 2B it can be seen that there are sperm with normal morphology, but some sperm have abnormal morphology. Sperm with abnormal morphology, including the appearance of sperm with a folded tail, a broken tail and sperm with a short tail. Figure 2C also shows sperm with normal and abnormal morphology. Sperm with abnormal morphology, among others, there is a picture of sperm with

an imperfect hook on the head, sperm with a broken tail and separated tail from the head (isolated tail).

Table 1 shows that the group with the highest percentage of normal sperm is in the normal control group and the lowest is in the negative control group. Groups with treatment 2 and 3 had almost the same percentage of normal sperm and both were higher than the negative control group.

The results of the analysis of normality and homogeneity using the Saphiro Wilk test and Levene test show that all data produce a significance of 0.05, that's mean the data obtained have a normal and homogeneous distribution. Therefore, the data can be analyzed using the ANOVA parametric test. The significance of ANOVA showed  $p=0.0001$ , then continued with the post-hoc test.

In table 2. it can be seen that the treatment group 2 and treatment group 3 showed no difference, they both appeared to be in the same subset, while for the other groups from normal control, negative control and treatment 1 were in different subsets.



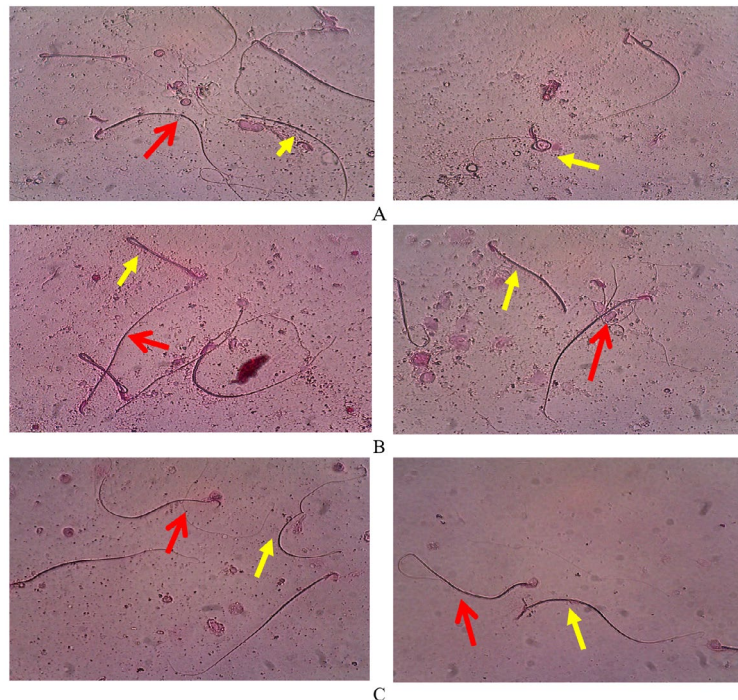


Figure 2. Sperm Morphology of Groups III, IV and V or Treatment Groups 1,2 and 3  
 A. Normal and abnormal sperm morphology in treatment group I (extract with the dose of 125 mg/kg BW)  
 B. Normal and abnormal sperm morphology in treatment group II (extract with the dose of 250 mg/kg BW)  
 C. Normal and abnormal sperm morphology in treatment group III (extract with the dose of 500 mg/kg BW)  
 \*red arrow: normal  
 \*yellow arrow: abnormal

Table 1. Average percentage of sperm morphology of Wistar strain male rats exposed to cigarette smoke and ethanolic extract of white oyster

Groups	Sperm Morfologi	
	Normal (%)	Abnormal (%)
Normal control	79 ± 0.79	21 ± 0.76
Negative control	39 ± 0.55	61 ± 0.55
Treatment 1	56 ± 0.15	44 ± 0.15
Treatment 2	65 ± 0.54	35 ± 0.54
Treatment 3	66 ± 0.21	34 ± 0.21

Note:

Normal control Group: is a group of rats that get normal water and feed and are not exposed to cigarette smoke.  
 Negative Control Group: is a group of rats that get water and normal feed, CMC and get exposure to cigarette smoke 30 minutes/day.

Treatment group 1: was a group of rats that received ethanolic extract of white oyster mushroom 125 mg/kg BW and exposed to cigarette smoke 30 minutes/day.

Treatment group 2: was a group of rats that received ethanolic extract of white oyster mushroom 250 mg/kg BW and exposed to cigarette smoke 30 minutes/day.

Treatment group 3: is a group of rats that received 500 mg/kg BW white oyster mushroom ethanol extract and were exposed to cigarette smoke 30 minutes/day.

Table 2. Tukey's test on sperm morphology of Wistar strain male rats exposed to cigarette smoke and white oyster mushroom ethanol extract.

Initial Groups	Comparison Groups	Sign.
Normal control	Negative control	.000*
	Treatment 1	.000*
	Treatment 2	.000*
	Treatment 3	.001*
Negative control	Normal control	.000*
	Treatment 1	.000*
	Treatment 2	.000*
	Treatment 3	.000*
Treatment 1	Normal control	.000*
	Negative control	.000*
	Treatment 2	.039*
	Treatment 3	.018*
Treatment 2	Normal control	.000*
	Negative control	.000*
	Treatment 1	.039*
	Treatment 3	.997
Treatment 3	Normal control	.001*
	Negative control	.000*
	Treatment 1	.018*
	Treatment 2	.997

Note:

\*Significance if p value < 0.05.

Normal control Group: is a group of rats that get normal water and feed and are not exposed to cigarette smoke.

Negative Control Group: is a group of rats that get water and normal feed, CMC and get exposure to cigarette smoke 30 minutes/day.

Treatment group 1: was a group of rats that received ethanol extract of white oyster mushroom 125 mg/kg BW and exposed to cigarette smoke 30 minutes/day.

Treatment group 2: was a group of rats that received ethanol extract of White Oyster Mushroom 250 mg/kg BW and exposed to cigarette smoke 30 minutes/day.

Treatment group 3: is a group of rats that received 500 mg/kg BW White Oyster Mushroom ethanol extract and were exposed to cigarette smoke 30 minutes/day.

Table 2. The results of the Tukey test of sperm morphology of Wistar strain male rats exposed to cigarette smoke and white oyster mushroom ethanol extract.

Groups	N	Subset for alpha = 0.05			
		1	2	3	4
Normal control	6	.2067			
Treatment 3	6		.3417		
Treatment 2	6		.3517		
Treatment 1	6			.4400	
Negative control	6				.6050
Sig.		1.000	.997	1.000	1.000

## DISCUSSION

Research on the effect of smoking on male reproduction is quite a lot, but the conclusions are still mixed. Cigarettes are thought to cause sperm damage by an unclear mechanism. Cigarette components are thought to be transferred across the testicular blood barrier, causing an increase in seminal ROS and causing DNA damage. Free radicals can also damage unsaturated fatty acids which are important ingredients in the formation of prostaglandins. Prostaglandins are important in the process of spermatogenesis.<sup>6-8</sup> The effects of smoking are thought to cause damage to sperm morphology, increase in seminal leukocytes, decrease sperm motility, and decrease seminal ascorbic acid levels. The effect of secondhand smoke on fertility has never been studied.<sup>3,9,11</sup>

Cigarette smoke in this study is a very large source of oxidants that will cause oxidative stress. This can be seen from the results of the research and statistical tests conducted, that the negative control was significantly different from the normal control. In the study, it was seen that the group that was exposed to cigarette smoke showed a significant increase in the number of abnormal sperm compared to the normal control group who was not exposed to cigarette smoke. The herbal exogenous antioxidant used in this study to counter the negative effects of exposure to cigarette smoke is the ethanolic extract of white oyster mushroom.

White oyster mushroom on phytochemical test results, showed that the ethanol extract contained alkaloids, flavonoids, tannins and steroids and phenolic components. One of the phenolic components is tannins, and these secondary metabolites are often found conjugated with other metabolites such as flavonoids and sterols. Phenolic components are non-essential food components that are often found in plants and have various biological effects including antioxidant effects.<sup>4,5</sup> The antioxidant activity of phenolics is mainly due to its ability as a reducing agent for hydrogen donors and singlet oxygen quenchers, and has a potential metal chelation effect.<sup>6</sup> The phenolic

components of mushrooms have been shown to have a strong antioxidant effect and are not mutagenic. Previous research has proven that mushroom extracts have a radical scavenging effect from primary and secondary radicals in certain concentrations.<sup>7-9</sup>

Oyster mushrooms (one of which is white oyster mushrooms) overall when compared to winter and shitake mushrooms, the oyster mushroom have higher antioxidant activity, reducing power, scavenging abilities and higher total phenol content.<sup>8,10</sup>

The results of the study showed that at a starting dose of 125 mg, the ethanol extract of white oyster mushroom had shown a fairly good antioxidant effect, compared to the negative control there was a significant difference. Doses of 250 mg and 500 mg also showed significant results but between the two doses did not show a significant difference.

Ethanol extract of white oyster mushroom was proven to have good antioxidant ability to prevent cell damage due to cigarette smoke. Another study showed that the ethanolic extract of white oyster mushrooms can also inhibit cell damage in the lung alveoli and prevent the increase in malondialdehyde levels in rats exposed to cigarette smoke.<sup>11</sup> This study showed that the ethanolic extract of white oyster mushrooms also had a good ability to prevent damage to sperm cells in rats exposed to cigarette smoke. This is very useful because antioxidants are one of the compounds that can be developed in the future to treat various diseases, not only as a preventive but also curative and rehabilitative.

## CONCLUSION

Ethanolic extract of white oyster mushroom can prevent the increase in the number of abnormal sperm morphology in male rats exposed to cigarette smoke with the optimal doses at 250 mg/kg BW .

## CONFLICT OF INTEREST

The author doesn't have a conflict of interest with the publication. The author does not have



a financial agreement with certain institutions or companies related to this research.

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