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Spermatogenic cell count of mice (*Mus musculus*) after the treatment with polysaccharide-K from *Coriolus versicolor* extract

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The objectives of this study were to determine the effect of different doses of polysaccharide-K from *Coriolus versicolor* extract after 35 days of injection on the number of spermatogenic cells in mice (*Mus musculus*). Twenty eight, 4-8 week old male mice, weight between 20-25 g were used in this study. The animal try were divided into four groups. The first group was the control group (K), treated by giving them aquadest by gavage for 35 days. The other groups (P1, P2, and P3) as the treatment groups, were treated by using three different doses of polysaccharide-K (15, 30, and 60 mg/kg of body weight respectively) injected by gavage for 35 days. Testicular histological slides were prepared to observe the structure of spermatogenic cells. The data of parameters such as the number of spermatogonia cells, spermatocyte cells, and oval spermatid cells were collected. The number of spermatocyte cells and oval spermatid cells was tested using the One Way Anova followed and Duncan. The number of spermatogonia cells was tested using Brown Forsythe. The results showed that spermatogonia and spermatocyte cell numbers were not significantly different in K, P1, P2, and P3 respectively ($P>0.5$). The number of oval spermatid cells was significantly different ($P <0.5$). In P1, the number of spermatid cells decreased 7.9% compared to K. The number of oval spermatid cells P2 and P3 was relatively equal to K. The conclusion of this research showed that the polysaccharide-K treatment from *C. versicolor* extract had no effect on both the number of spermatogonia and spermatocyte cells, but slightly decreased the number of oval spermatids. Polysaccharide-K is still safe to use as a herbal medicine

Keywords: ***Coriolus versicolor*, polysaccharide krestin, spermatogonia, spermatocyte, spermatid**

INTRODUCTION

Coriolus versicolor is a mushroom that is widely used in traditional Asian herbal medicine. There are two main substances that can be extracted from the mushroom, polysaccharide peptide (PSP) and polysaccharide-K (PSK, krestin), which are being studied as cancer treatments (Asterina, 2011). Polysaccharide-K has the appearance of a light powder or a dark-brown substances that dissolves in hot water. Polysaccharide-K is obtained from the body and mycelium of the mushroom. It has a major component of β-glucan, with β-1.4 being the main chain and β-1.3 and β-1.6 being side chains bound to the protein membrane (Cui and Chisti, 2003).

According to Wahyuningsih et al. (2009), polysaccharide-K can improve the conditions of immune-competent cells, restoring and strengthening the function of non-specific immune responses, as well as specific responses in mice that have been injected with *Mycobacterium tuberculosis*. Ho et al. (2006) reported that polysaccharide-K may inhibit leukemia, lymphoma, and hepatoma in vitro as well.

The lethal dose 50 (LD_{50}) from

polysaccharide-K is 231.8 mg/kg of body weight in female, Balb/C strain mice (Wahyuningsih and Darmanto, 2010), whereas the administration of polysaccharide-K from *C. versicolor* extract in the sub-chronic toxicity test for 62 days increased the serum creatinine levels of mice at a dose of 6 mg/kg BB (Wahyuningsih et al. 2009). The polysaccharide-K contains β-glucan as an active compound, which might be toxic at certain doses. According to Murtini et al. (2010), all substances that enter the body have the potential to be toxic depending on the dose consumed and the duration of use.

In male reproductive organs, high doses of polysaccharide-K and the long duration of use can trigger the increase in Reactive Oxygen Species (ROS). According to Hayati (2011), ROS has a potential negative effect on the quality and function of spermatozoa at high levels. The high ROS may increase the number of leucocytes (Effendi, 2003). Leucocyte plays a role in cellular and humoral defenses of organisms against foreign substances. Furthermore, the increased number of leukocyte cells may lead to high levels of ROS, because one of the sources of ROS is derived from the enzymatic reactions in leucocytes cells (Hayati, 2011).

Oxidative stress occurs when there are not enough antioxidants to counter the negative effects of ROS causing pathological effects. Oxidative stress arises as a consequence of an excessive increase in ROS production and the disruption of defense mechanisms by antioxidants (Soehadi, 2009). According to Safarinejad et al. (2009), oxidative stress may lead to a damage to the testicular tissues, especially the seminiferous tubules.

The seminiferous tubules act as functional units for the process of spermatogenesis.The main components of seminiferous tubules are germ cells and somatic cells (Sertoli cells) (Hayati, 2011). Spermatogenesis is directly regulated by the gonadotropin hormone known as Luteinizing Hormone (LH). This hormone stimulates Leydig cells to produce testosterone which is very important in the regulation of spermatogenesis. Testosterone and Follicle Stimulating Hormone (FSH) in the seminiferous tubules, stimulate the spermatogenesis (Hayati, 2011).

Damage to Sertoli cells results in a disruption of spermatogenesis and spermiogenesis, while the destruction of Leydig cells causes a disruption of the synthesis of testosterone, thus decreasing plasma testosterone levels, which may interfere with spermatogenesis (Siti, 2009). According to Indyastuti (1990), Impaired spermatogenesis can be characterized by a decreased spermatogenic cell count and its effect on seminiferous tubular size. The indicator of the disrupted process of spermatogenesis is a change in the number of spermatogenic cells in the seminiferous tubules at a particular stage. The number of spermatocytes is an indicator for meiosis whereas spermatids are indicators for spermiogenesis as a whole. The decreasing number of spermatogenic cells is one of the causes of declining fertility in males.

MATERIALS AND METHODS

This research was conducted from June to November 2015 at the Laboratory of Reproductive Biology, and Institute of Tropical Diseases, Department of Biology, Faculty of Science and Technology, Airlangga University. The research used 48 male mice strain Balb/C, 4-8 week old, and weight 20-25g. The materials in the present study included the *C. versicolor* mushroom, aquadest, phenol, H_2SO_4, formaldehyde buffer, 70% ethanol, 80% ethanol, 96% ethanol, absolute ethanol, xylol, paraffin, Meyer's albumin, Hematoxylin, Eosin, and entellan.

Isolation of the polysaccharide-K

The extracts were made by cleaning the mushroom and then drying it to reduce the water content, the dried mushrooms were then ground into a powder. The extraction and isolation of polysaccharide-K was carried out according to the methods reported by Cui and Chisti (2003) and Wahyuningsih et al. (2009). The measurement of PSK with phenol-sulfuric acid was done according to Wahyuningsih et al. (2009).

Experimental design

The animals were acclimatized for 14 days before the treatment, and were allowed to eat as much as they wanted (ad libitum) during the treatment. It was divided into four groups. The first group (K) was the control group, only giving the aquadest. The other groups were P1, P2, and P3. Its were treated by using three different doses of polysaccharide-K (15, 30, and 60 mg/kg of body weight respectively) injected by gavage for 35 days.

Spermatogenic cell count

The testicles were collected after the treatment. Testicular histological slides were prepared to observe the structure of spermatogenic cells. Data of parameters such as the number of spermatogonia cells, spermatocyte

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cells, and oval spermatids cells were collected. The calculation of spermatogenic cell count is according to Hayati (2011). The number of spermatocyte cells and oval spermatids cells were tested using One Way Anova and followed by Duncan test. The number of spermatogonia cells were tested using Brown Forsythe.

RESULTS

The data of spermatogenic cell count of the control group (K) and the treated groups (P1, P2, and P3) after the administration of polysaccharide-K for 35 days are shown in Table 1.

Table 1. **Spermatogenic cell count of the control group (K) and the treatment groups (P1, P2, and P3) after the administration of polysaccharide-K for 35 days.**

Treatment	The dose of polysaccharide-K (mg/kg)	Cell count (cell/tubules)		
		Spermatogonium	Spermatocyte	Oval Spermatid
K	0	81.17± 9.70[a]	118.67±12.35[a]	104.94±6.07[bc]
P1	15	83.64±14.67[a]	125.50±18.97[a]	96.67±6.65[a]
P2	30	88.97± 5.30[a]	128.46±11.16[a]	101.64±5.70[ab]
P3	60	91.74± 4.64[a]	129.90± 6.29[a]	112.20±5.80[c]

The same letters indicate no significant differences and different letters indicate a significant differences (α=0.05)

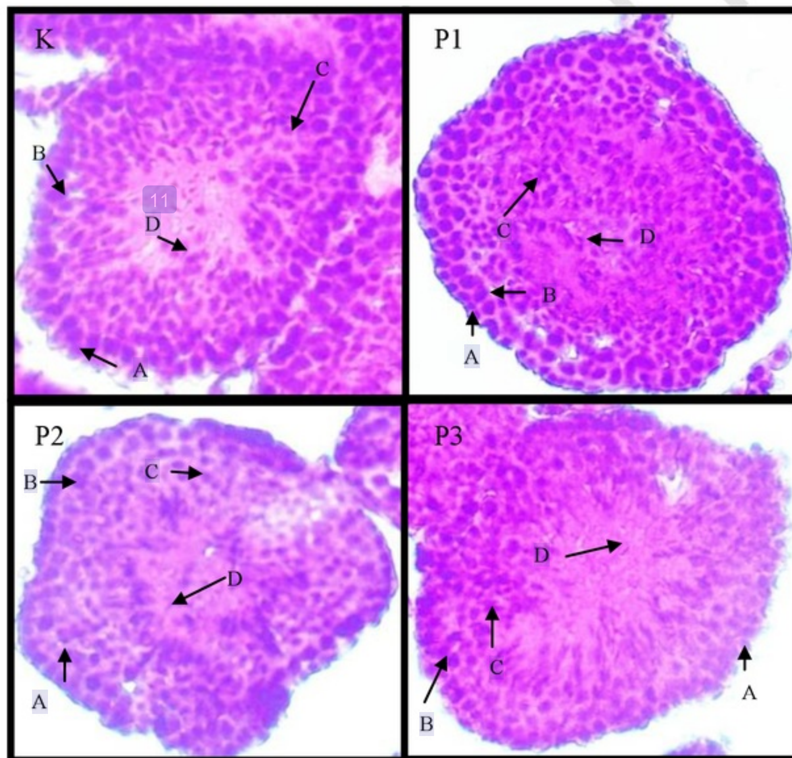

Figure 1. Testicular histological slides of K, P1, P2, and P3 group.K= PSK 0 mg/Kg of BW, P1=PSK 15 mg/Kg of BW, P2= PSK 30 mg/Kg BB, P3= PSK 60 mg/Kg BB, A=Spermatogonium, B=Primary spermatocyte C=Secondary spermatocyte, D=Oval Spermatid, 40x magnification.

DISCUSSION

Polysaccharide-K can improve the conditions of immunocompetent cells, and restore and strengthen the function of non-specific immune responses, and specific responses in mice due to infection *Mycobacterium tuberculosis* (Wahyuningsih et al. 2009). Polysaccharide-K contain a major component of β-glucan, with β-1,4 being the main chain and the β-1.3 and β-1.6 side chains being bound to the protein membrane (Cui and Christi, 2003). Polysaccharide-K of *C. versicolor* might be toxic at certain doses. According to Murtini et al. (2010), all substances that enter the body have the potential to be toxic depending on the dose consumed and the duration of use.

Reactive oxygen species or free radicals sources are derived from enzymatic factors (internal) and non-enzymatic (external) factors. Internal factors include enzymatic oxidation in mitochondria, phagocyte cells, reaction with metals (Fe), peroxisomes, inflammation, and ischemia. External factors include X-ray radiation, ozone, cigarettes, air pollution, pesticide drugs and chemicals or solvents used in industry (Hayati, 2011).

These free radicals may cause a disruption to spermatogenesis and the spermatozoa membranes, thus decreasing sperm motility and the ability to penetrate the ovum. This cell membrane disorder is caused because the cell membrane is one of the main targets of cell damage or injury caused by various external stimuli including free radicals (Sutarina and Edward, 2004). The damage to the cell membrane may occur because there is a covalent bond between the free radical and the membrane component, resulting in a structural change of the receptor function, and thiol group oxidation of the membrane component by free radical which cause the membrane transport process to be disturbed, or even a peroxidation reaction of the membrane lipid containing poly unsaturated fatty acids (PUFAs) (Slatter, 1984). Free radicals can also cause damage to spermatozoa DNA especially the integrity of the DNA, in the nucleus, which can further lead to cell death (Termelen, 2008).

Both endogenous and exogenous antioxidants are important for body function, because they are able to reduce the negative effects of oxidants in the body. Endogenous antioxidants include superoxide dismutase (SOD) enzymes, catalase, and glutathione peroxidase (GSH-Px), whereas exogenous antioxidants include vitamin E, vitamin C, β-carotene, flavonoids, uric acid, bilirubin and albumin. The effective utilization of exogenous antioxidant compounds is necessary to prevent oxidative stress. Exogenous antioxidants are preventive defense systems, where the antioxidants work by cutting off the chain oxidation reaction of the free radicals or by capturing them (Winarsi, 2007). Exogenous sources of antioxidants are deemed important to increase the endogenous antioxidant sources (Abu, 2015).

ß-Glucan has various biological activities such as antitumor, antioxidant, anticholesterol, anti-aging, and immune system enhancers (Miura et al. 2003). The benefits include the fact that β-glucan as antioxidants neutralize the presence of ROS that can interfere with the spermatogenesis process (Table 1). The results showed similar spermatogonium cell counts (Table 1) (K: 81.17, P1: 83.64, P2: 88.97, and P3: 91.74) cell/tubules. This was because spermatogonium has strong resistance to foreign substances compared to other spermatogenic cells. This was in accordance with the statements of Rumanta *et al.* (2001) and Hayati *et al.* (2004), in a study of the effects of 2-methoxyethanol on the histologic structure of testicular mice (*Mus musculus*); the results showed the spermatogenic cells that had a greater resistance to the influence of 2-methoxyacetic acid (MAA), which are spermatogonium A and pra-leptoten spermatocytes. Spermatogonium A and praleptotene spermatocytes are more resistant to MAA because these two cells are located in the basal compartment, outside of the adluminal compartment, so they are protected by the presence of barriers formed by Sertoli cells

The spermatocyte cell count (Table 1) was not significantly differen between the groups (K: 118.67, P1: 125.50, P2: 128.46, and P3: 129.90) cell/tubules.This result showed the effect of polysaccharide-K from *C. versicolor* extract as an antioxidant because spermatocyte cells have very high sensitivity to external influences. Johnson and Everitt (1990) stated that spermatocytes are very sensitive to external influences and tend to decay after the first meiotic prophase especially in the pachytene stage, that is, during the crossover between homologous chromosomes.

The result of oval spermatid cell count (Table 1) showed no significant differences between the groups (K: 104.94, P1: 96.67, P2: 101.64, and P3: 112.20) cell/tubules. The increase in the number of oval spermatid cells was directly proportional to

the increase in the number of spermatocyte cells. Anas and Asterina (2011) reported that spermatogenesis is an elaborate and orderly cycle; the increase from the previous stage will affect the subsequent stage. In this study, the number of spermatocyte cells increased, although this was not significant; as the spermatocyte cell number increased so did the spermatid cell number. However, there was a decrease in the number of oval spermatid cells in mice given polysaccharide-K extract at a dose of 15 mg/kg BW, possibly because the low doses of polysaccharide-K may affect spermatid formation, which tends to decrease spermatid count by inhibiting spermatid formation. In accordance with the statement of Murtini et al. (2010), all substances that enter the body have the potential to be toxic depending on the dose consumed and the duration of use.

CONCLUSION

It can be concluded from the results that treatment polysaccharide-K from *C. versicolor* extract had no effect on the number of spermatogonia cells and spermatocyte cells, but decreased the number of oval spermatid cells at a dose of 15 mg/kg of body weight.

CONFLICT OF INTEREST

The authors declare that all researchers familiar with this work contributed in all this work items without conflicts.

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AUTHOR CONTRIBUTIONS

SPAW and SK designed and performed theexperiments and also wrote the manuscript. We both also prepared and isolation the extract, performed animal treatments, collect and data analysis. All authors read and approved the final version..

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