RESEARCH ARTICLE

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The Administration of Cordyceps Militaris Inhibited the Decrease of Leydig Cell Number and Testosterone Level in Male Wistar Rats (*Rattus Norvegicus*) Exposed with Cigarette Smoke

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ABSTRACT

Large amounts of free radicals generate oxidative stress resulting in cell damage. Cigarettes are a source of exogenous free radicals containing various toxic substances. Cigarettes exposure decreased Leydig cells and testosterone. Cordyceps militaris has strong antioxidant effect that can counteract the effects of free radicals. This research was aimed to evaluate the administration of Cordyceps militaris in inhibiting the decrease in Leydig cell count and testosterone levels in male wistar rats (Rattus norvegicus) exposed to cigarette smoke. This study was a true experimental study with a post-test only control group design, using 30 male wistar rats which were randomly divided into three groups: control (P0) given aquadest, treated with Cordyceps militaris extract 50 mg/kgBW rats (P1), and 200 mg/kgBW rats (P2). All groups were exposed to cigarette smoke for 30 days. Examination of testosterone and the amount of testicular Leydig cells were examined on the 31st day. The results showed that the mean amount of Leydig cells at P0 was 16.10±3.14/HPF, P1 was 18.87±1.76/HPF, and P2 was 30.34±0.72/HPF (p<0.001). The mean values of P0 testosterone were 4.02±0.11 nmol/ml, P1 was 4.31±0.11 nmol/ml, and P2 was 9.43±0.17 nmol/ml (p<0.001). In conclusion, administration of Cordyceps militaris inhibited the decrease in Leydig cell count and testosterone levels in male Wistar rats (Rattus norvegicus) exposed to cigarette smoke.

Keywords: cigarette smoke, *Cordyceps militaris*, Leydig cells, testosterone.

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I. Introduction

The number of Indonesian smokers is increased to 34.7% in 2010 from 27.2% in 1995. The percentage of men who smoke rose from 53.9% in 1995 to 67.0% in 2010 [1]. In terms of smoking countries, Indonesia comes in fourth with 239 billion cigarettes consumed yearly [2].

There are gases, evaporated liquids, and particles in cigarette smoke. The chemical reactions of hydrogenation, pyrolysis, oxidation, decarboxylation, and dehydration result in the release of about 4,000 chemicals. Nicotine and its metabolites, cotinine, radioactive polonium, benzopyrene, dimethylbenzanthracene, methylnaphthalene, polycyclic aromatic hydrocarbons (PAHs), and cadmium are only a few of the hazardous compounds, mutagenesis agents, and carcinogens found in cigarette smoke. Nicotine is tobacco's principal psychoactive ingredient. The substance that causes addiction to tobacco is nicotine [3]. Benzo(a)Pyrene (BaP) contained in cigarettes decreases StAR expression which ultimately decreases testosterone production [4]. Nicotine induces oxidative stress which can be seen from a significant decrease in the antioxidant enzymes SOD, CAT, GSH and also an increase in levels of Thiobarbituric acid reactive susbtances (TBARS). Nicotine increases ROS production which eventually interferes with the process of streroidogenesis at the stage of cholesterol transfer into the mitochondria by suppressing the expression of StAR protein [5]. Smoking also correlates with an increase in free radicals and leads to oxidative stress, accompanied by an increase in oxidative stress markers [3].

Smoking is one of the external factors causing aging, leading to several diseases and death. Smoking causes stress oxidative as a result of the free radical increase in the body. Prevention of the aging trigger will help to prevent the aging process and organ destruction, thus increasing the quality of life, as the principal of antiaging medicine [6]. Prevention can be achieved through free radical suppression. Free radicals are normally produced through cell metabolism processes. However, in large quantities, free radicals cause oxidative stress, damaging cells [7]. Supplementation of antioxidant compounds can reduce the number of free radicals and the emergence of oxidative stress in the body. Cordyceps militaris polysaccharide (CMP) is an antioxidant compound that can prevent ROS activity. Cordyceps is an entomopathogenic fungus on insects. Most cordyceps species

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are found on the heads of Lepidoptera moth larvae [8]. For 300 years ago, cordyceps, a member of the Ascomycetes family of mushrooms, has had great popularity in traditional Chinese medicine. In China, cordyceps is also referred to as Dong Chong Xia Cao, which translates as "worm in winter, grass in summer" [9]. Adenosine, proteoglycans, terpenoids, amphinols, steroids, ergosterols, and lectins are just a few of the bioactive substances found in cordyceps. It also contains exopolysaccharides, cordycepin, phenolic compounds, polysaccharides, cordycepic acids, and cordycepin. Additionally, B1, B2, B12, E, and K vitamins and minerals (Fe, Ca, Mg, Ni, Sr, Na, Ti, Pi, Se, Mn, Zn, Al, Si, K, Cr, Ga, V, and Zr) are present in cordyceps [10]. Study showed that CMP could significantly increase SOD activity, CAT activity, GSH-Px activity, and Total Antioxidant Capacity (TAOC) activity in the heart, liver, and kidney. In addition, CMP significantly lowers MDA, indicating that CMP is an effective scavenger of various types of oxygen free radicals and their derivatives [11]. CMP supplementation in medium (100 mg/kg/day) and high doses (200 mg/kg/day) significantly inhibited MDA formation in the kidney, liver, and heart. High doses of CMP effectively capture various types of oxygen-free radicals and protects against oxidative stress [12].

Various studies have shown that CMP can produce strong antioxidant activity and is potentially used in treating diseases induced by oxidative stress. This study is *in vivo* study to evaluate the activity of *Cordyceps militaris* in improve Leydig cell and testosterone of male Wistar rats (Rattus norvegicus) exposed to cigarette smoke as a source of free radicals.

II. METHOD

This research was a true experimental study with a post-test-only control group design. The animal experiment was carried out in the Integrated Biomedical Laboratory Unit, Faculty of Medicine, Udayana University, Bali. Testosterone was examined at the Integrated Biomedical Laboratory Unit, Faculty of Medicine, Udayana University Biochemistry Division and Biomolecular Division. Leydig cell examination was carried out at the Bali Pathology Diagnostic Center Laboratory. This research received a certificate of animal ethics approval from the Animal Ethics Committee of the Faculty of Veterinary Medicine, Udayana University (Number B/107/UN14.2.9/PT.01.04/2022).

A. Animal Experiment

This experiment used 30 male Wistar rats (*Rattus norvegicus*). Experimental animals were acclimatized for seven days to laboratory conditions before the experiment was carried out and given standard food and drink. All experimental animals were randomly divided into three groups, 10 experimental animals for each group. The first group was the control (P0) was given 1ml/200grBW/day of aquadest as a placebo orally, 1 hour before cigarette smoke exposure. The second (P1) and third (P2) groups were treated with Cordyceps militaris extract (CME) at a dose of 50 mg/kg BW and 200 mg/kg BW in 1 ml of distilled water, given orally 1 hour before cigarette smoke exposure. Treatment in the three groups was given for 30 days. Animal blood samples were taken on day 31 through the medial canthus of the orbital sinus to be used for testing testosterone. Testicular tissue samples were taken to be used in making histopathological preparations

B. Leydig Cell Quantification

The amount of Leydig cells was examine on both left and right testicular tissue preparations using through histopathology images stained with Hematoxylin & Eosin (HE). Olympus CX41 microscope (Japan) was used at 400x magnification in five random fields of view. The amount of Leydig cells is present as the average amount of Leydig cells observed in each field of view, expressed in units of the amount of Leydig cells/high power field (HPF).

C. Testosterone Level Examination

Testosterone was examined by indirect ELISA using the Bioassay Technology Laboratory Kit with Cat No. E0259Ra, following the manufacturer's instructions. Results are expressed in units of nmol/mL.

D. Data Analysis

The data obtained were analyzed statistically using One-Way Anova and Bonferroni with a 95% confidence level. The significance value (p)<0.05 indicates a difference between groups.

III. RESULT

The amount of Leydig cells in this study was observed using histopathological preparations of testicular tissue. The Leydig cells in the three groups are shown in Table I and Fig. 1. The amount of Leydig cells with 200 mg/kg BW treatment (30.34±0.72) was higher than the control group (16.10±3.14) and 50 mg/kg BW treatment (18.87±1.76). Statistical analysis showed significant differences in the three groups (p<0.01), which showed that CME administration significantly inhibited decreased Leydig cells in male rats exposed to cigarette smoke.

TABLE I: LEYDIG CELL AND TESTOSTERONE LEVEL OF THREE EXPERIMENTAL GROUPS

Group	Leydig Cell (/HPF)	Testosterone level (nmol/mL)
Control group (P0)	16.10±3.14	4.02±0.11
CME 50mg/kg BW (P1)	18.87±1.76	4.31±0.11
CME 200mg/ kg BW (P2)	30.34±0.72	9.43±0.17

Results are expressed in mean ± standard deviation (n=10). Values with different letters indicate statistical differences (p<0.05).

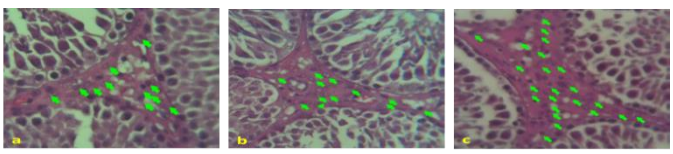

Fig. 1. Leydig cell histology (green arrows) P0 (a), P1 (b), and P2 (c) stained with Haematoxylin-Eosin 400x magnification. Leydig cells are marked with green arrows.

Testosterone hormones are shown in Table I. The results showed significantly higher testosterone hormone in CME 200mg/kg BW treatment (9.43±0.17) as compared to 50mg/kg BW treatment (4.31±0.11) and the control group

(4.02±0.11) ($p<0.01$). The results showed that administration of CME significantly inhibited the decrease in Testosterone in male rats exposed to cigarette smoke.

IV. Discussion

This study evaluates the effect of Cordyceps militaris on the amount of Leydig cells and levels of the hormone testosterone in experimental animals exposed to cigarette smoke. In this study, the average amount of Leydig cells in the control group of Wistar rats exposed to cigarette smoke for 30 days was 16.10±3.14. The average amount of Leydig cells in Wistar rats aged 8-12 weeks with a body weight of 180-200 mg is around 56.50±13.03 cells [13]. Normal serum testosterone in male Wistar rats are around 11.66±4.825 nmol/mL [14], while testosterone serum levels in control rats in this study were 4.02±0.11 nmol/mL. These results showed that exposure to cigarette smoke for 30 days reduced the amount of Leydig cells and serum testosterone of male Wistar rats.

Testosterone production is initiated through the transfer of cholesterol from the outer mitochondrial membrane into the inside of Leydig cells by the steroidogenic acute regulatory protein (StAR). Benzo(a)Pyrene (BaP) contained in cigarettes decreases StAR expression which ultimately decreases testosterone production [4]. Nicotine induces oxidative stress which can be seen from a significant decrease in the antioxidant enzymes SOD, CAT, GSH and also an increase in levels of Thiobarbituric acid reactive susbtances (TBARS). Nicotine increases ROS production which eventually interferes with the process of streroidogenesis at the stage of cholesterol transfer into the mitochondria by suppressing the expression of StAR protein [5]. Nicotine also increases peroxidative damage, inhibiting FSH and LH release, causing decreased Leydig cell stimulation. Previous studies have shown a dose-dependent relationship of nicotine to rat testicular damage through acetylcholine receptors in the cell membrane or directly inhibiting Leydig cell steroidogenesis.

Benzo(a)pyrene as a free radical causes DNA damage in Leydig cells and increases testosterone metabolism in the liver. High levels of Cd and Ni metals in cigarette smoke can also cause severe bleeding, edema, Leydig cell necrosis and reduce testosterone concentrations in plasma and testes, reduce germ cell junctions in the seminiferous tubules and eliminate integral membrane proteins at the Sertoli blood–testis cell interface barrier (BTB), as well as decreasing sperm count and motility [15].

Toxins in cigarette smoke will cause oxidative stress, which is associated with an imbalance of ROS and the body's antioxidant system. These oxidative stress conditions activate a cascade of mitogen-activated protein kinases (MAPKs), particularly the p38 MAPK pathway, which plays an important role in nuclear factor kappa B (NF-B) activation and proinflammatory expression. NF-B is one of the main factors that increase the inflammatory response and causes the expression of proinflammatory cytokines such as TNF- and IL-6 [16]. The combination of damaged Leydig cells and decreased serum testosterone cause testosterone deficiency which, if left untreated, will result in clinical andropause syndrome, which reduces the overall quality of life for men [17].

The results showed a significant difference in the three groups' mean amount of Leydig cells and testosterone. The treatment with 50mg/ kg BW of CME had a significantly higher mean amount of Leydig cells and testosterone than the control group. Meanwhile, treatment of 200mg/ kg BW of CME produced a higher mean amount of Leydig cells and testosterone than the control group and a lower dose. These results indicate that preventing a decrease in the average amount of Leydig cells and testosterone is dose-dependent. A dose of Cordyceps militaris extract 200 mg/kg BW rats gives better results than a dose of 50 mg/kg BW rats.

A study of cordyceps militaris (200, 400, and 800 mg/kg body weight/day) for four weeks significantly reduced BPA-induced reproductive harm by raising testicular superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), and glutathione (GSH); and by lowering serum malondialdehyde. This was done in the presence of bisphenol A/BPA, which has been shown to increase reproductive harm (MDA). The amount of mature sperm considerably increased in the seminiferous tubules of mouse testicular tissue that had been co-treated with BPA and Cordyceps militaris (200, 400, and 800 mg/kg, respectively) [18].

Cordyceps militaris polysaccharide (CMP) has been studied to prevent Cyclophosphamide-induced ROS activity. The results showed that CMP significantly increases SOD activity, CAT activity, GSH-Px activity, and Total Antioxidant Capacity (TAOC) activity in the heart, liver, and kidney. In addition, CMP significantly lowers MDA, indicating that it is effective as a scavenger of various types of oxygen-free radicals and their derivatives [11].

Another study showed that CMP administration in medium (100 mg/kg/day) and high doses (200 mg/kg/day) significantly inhibited MDA formation in the kidney, liver and heart, indicating that high doses of CMP were effective in capturing various types of oxygen free radicals and protects against oxidative stress [12].

V. Conclusion

There were significant differences in the amount of Leydig cells and testosterone in the three groups. The amount of Leydig cells and testosterone in the treatment group was higher than in the control group, and higher doses (200 mg/kg BW) had a better effect. Cordyceps militaris has been proven to inhibit the negative effects of free radicals in reducing the amount of Leydig cells and testosterone. This can be the basis for administering the extract to men to prevent a decrease in testosterone caused by exposure to excess free radicals. However, the optimal dose in humans needs to be studied further, along with the side effects of long-term use.

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