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The effect of mangosteen peel extract (*Garcinia mangostana Linn*) on the number of spermatogenic cells of mice (*Mus musculus*) exposed to cigarette smoke

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Abstract

Introduction: The aim of this study was to investigate the effects of mangosteen extract on the number of spermatogenic cells in mice exposed to cigarette smoke.

Objective: Thirty mice aged 8 to 12 weeks were randomly divided into 5 groups. The negative control group (K-) received 1% CMC Na/head, the positive control group (K+) was exposed to smoke and received 1% CMC Na/head, (P1) was exposed to smoke and administered mangosteen extract 6,045 mg/head, (P2) was exposed to smoke and administered mangosteen extract 12.09 mg/head, (P3) was exposed to smoke and administered mangosteen extract 24.18 mg/head. Each group was exposed to one cigarette per day, and treatments were administered one hour after smoke exposure. All treatments and smoke exposure lasted for about 45 days. Necropsy was performed after the final day of treatment, and histopathological slides of the testes were processed and stained under a light microscope. The observed parameter was spermatogenic cell count. The data were analyzed using analysis of variance (ANOVA) based on a completely randomized design, and further analyzed using Duncan's multiple range.

Results: Cigarette smoke significantly reduced spermatogenic cells (spermatogonia, primary spermatocytes, and spermatids) ($p<0.05$). Administration of mangosteen peel extract significantly increased spermatogenic cells ($p<0.05$). The highest increase in spermatogenic cells was observed in the group given 12.09 mg/gram BW of mangosteen peel extract (P2). The increase in spermatogenic cells due to the administration of mangosteen peel extract and cigarette smoke did not reach normal conditions (K-).

Conclusion: This study concludes that mangosteen peel extract (*Garcinia mangostana Linn*) could maintain the number of spermatogenic cells in male mice (*Mus musculus*) exposed to cigarette smoke ($p<0.005$).

Keywords: Cigarette Smoke; Mangosteen Extract; Spermatogenic Cell; Mice; Healthcare

1. Introduction

Smoking is an addictive behavior that has become a human lifestyle. Indonesia has the highest number of active smokers with a prevalence of 62.9% in men and 4.8% in women [1]. Exposure to cigarette smoke has adverse health effects, leading to death. Smoking is an unhealthy lifestyle that is still commonly practiced and is the leading cause of death worldwide, claiming 22.000 lives every day. Cigarettes contain more than 4.000 active compounds that can cause various diseases such as cancer, heart disease, respiratory system disorders, and reproductive disorders. Compounds contained in cigarettes include nicotine, tar, carbon monoxide (CO), nitrosamines, nitrogen oxide (NO), and polynuclear aromatic hydrocarbons (PAHs) [2].

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Active smokers who constantly inhale cigarette smoke can increase nicotine levels in the bloodstream. Nicotine will spread to several body systems, such as the reproductive system, which can result in somatic cell mutations and carcinogenic effects directly on male reproductive health. About 60-65% of men have low sperm quality due to smoking [3]. Cigarette smoke exposure can inhibit spermatogenesis, characterized by a decrease in the number of spermatogonia, primary spermatocytes, spermatids, and spermatozoa viability. Additionally, there is a decrease in the diameter of seminiferous tubules in male rats exposed to 4 mg/kg of nicotine [4].

Cigarette smoke exposure can increase the production of reactive oxygen species (ROS). The increase in ROS due to cigarette smoke exposure is counteracted by the antioxidant defense system. When ROS concentration exceeds the antioxidant defense system, oxidative stress occurs. The main target of ROS activity is poly unsaturated fatty acid (PUFA) or double unsaturated fatty acids found in cell membranes. ROS is strengthened by the interaction of hydrogen peroxide and superoxide, leading to lipid peroxidation, resulting in oxidative stress [5]. The consequences of lipid peroxidation negatively affect oxidative phosphorylation in mitochondria, lipid peroxidation in plasma and intracellular membranes damage protease enzymes and disrupt the release of Ca^{2+} into the cytosol, which can lead to necrosis [6].

Seminiferous tubules are the sites of spermatogenesis, which is the process of developing spermatogenic cells starting from the proliferation of germ cells and the maturation of spermatogonia into spermatozoa. Damage to seminiferous tubules due to cigarette smoke exposure can affect spermatogenesis, causing inhibition of cell division for differentiation, thus affecting the number of spermatogenic cells and the quality of spermatozoa [7]. Disorders in the reproductive system of seminiferous tubules can be seen from changes in their microanatomical structure, including a decrease in the number of spermatogenic cells such as spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa, as well as the size of the seminiferous tubules' diameter.

Damage to seminiferous tubules can be minimized using antioxidants. Natural antioxidants from plant extracts are selected as an alternative treatment due to their benefits for human health and consumer concerns about the safety of synthetic antioxidants in food [8]. In vitro studies have shown that mangosteen peel extract has the potential to be an antioxidant with xanthone compound content. Antioxidant compounds in mangosteen peel have 4-5 times higher strength compared to vitamin C and vitamin E, known as potential antioxidants [9]. Scientific research results mention compounds contained in mangosteen peel, namely xanthones including mangostin, mangosterol, α and β-mangostinone, trapezifolixanthone, tovophyllin β, garcinon β, mangostanol, epicatechin flavonoids, and gartanin.

Xanthones in mangosteen peel have about 17.000–20.000 oxygen radical absorbance capacity (ORAC). The high ORAC value of mangosteen illustrates the ability of xanthones to absorb free radicals. Xanthones can neutralize free radicals, including hydroxyl (OH-), superoxide (O2-), and reduce the capacity of Fe^{+2} ions through the fenton reaction, which is a combination of H_2O_2 and Fe^{2+} ions with high oxidation ability, capable of reducing or eliminating the oxidants produced [10]. Xanthone compounds can bind to unpaired electrons from ROS, so ROS will not pull electrons from important cell components to maintain life, including molecules that make up cell membranes, enzymes, and deoxyribonucleic acid (DNA). ROS compounds are highly reactive because they tend to pull electrons in their environment and can convert a molecule into a new free radical [11]. Additionally, xanthones can increase the number of spermatozoa and increase the number of Leydig cells that produce testosterone, which is an important hormone in the spermatogenesis process in the seminiferous tubules of Swiss-Webster mice exposed to cigarette smoke [12]. The activity of xanthones in mangosteen peel is expected to prevent damage to seminiferous tubules due to cigarette smoke exposure. Based on this description, it can be the basis for research to determine the effect of mangosteen peel extract on the number of spermatogenic cells in male mice exposed to cigarette smoke.

2. Material and methods

This research is an experimental study conducted by administering mangosteen peel extract orally to male mice. The research design used was a completely randomized design (CRD). Thirty male mice were randomly divided into 5 groups with 6 repetitions. This research was conducted at the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia. The preparation of mangosteen peel extract was conducted at the Department of Basic Veterinary Medicine, while the male mice were kept in animal cages, and testis histology preparations were made at the Department of Veterinary Pathology.

2.1. Materials

This study used male mice aged around 2-3 months with a weight range of 20 grams, totaling 30 individuals. The materials used in this study included: kretek cigarettes, 70% alcohol, NaCl 0.9%, 10% formalin, and materials used for

mangosteen peel extract: mangosteen peel powder, technical-grade 96% ethanol solvent, 1% carboxy methyl cellulose (CMC Na) suspensor, sterile distilled water.

Equipment used: a cigarette smoke exposure box measuring 40 x 25 x 20 cm with two holes, one hole for inserting cigarettes into the cage and the other hole as ventilation. Mice cages, surgical scissors, blades, scalpels, forceps, probes, organ pots, microscope. The equipment used for the preparation of mangosteen peel extract includes an oven, grinding machine, batch extractor series, thermostat, water bath, vacuum evaporator, analytical balance, moisture analyzer, filter paper, lighter, tripod rack, Bunsen burner.

2.2. Method

2.2.1. Preparation of Mangosteen Peel Extract

Fresh mangosteen fruit peel was washed clean, thinly sliced, then dried and aired. The dried peel was ground into powder using a grinder, then the powder was sieved to obtain finer powder. After obtaining fine powder, the extraction process was carried out. This extraction process used 96% ethanol solvent because ethanol solvent has properties that can dissolve all active ingredients contained in natural materials, whether polar, semi-polar, or non-polar active ingredients. Mangosteen peel powder was weighed, then macerated with 96% ethanol for three days at room temperature, then filtered, and the filtrate was collected. Maceration was done three times. The filtrate was concentrated using a rotary vacuum evaporator at 50°C with a speed of 40 rpm until a thick extract was obtained [13]. After becoming an extract, mangosteen peel extract suspension was made using 1% CMC Na as a suspensor.

2.2.2. Experimental Animals

This study used male mice grouped into five groups, consisting of two control groups (K+) and (K-) and three treatment groups (P1), (P2), (P3) that would be given mangosteen peel extract. The mice were adapted in cages and given food and water in the morning and afternoon for seven days. After the adaptation phase for seven days, the next phase was the exposure to cigarette smoke for 45 days. The dose of mangosteen peel extract used as an antioxidant in powder form was 500 mg used three times a day, or 1.500 mg/day. A medicinal material originating from dry powder that will be made into an extract to separate active ingredients suspected of having medicinal properties generally obtained about ± 10% of the dry powder. The dose of medicine used in humans (BW 70 kg) was converted to male mice (BW 20 grams) at 0.0026, so the dose of mangosteen peel extract in mice with BW 20 grams = 1500 mg x 0.0026 = 3.9 mg/day.

K (-): negative control group without exposure to cigarette smoke and given 1% CMC Na. K(+): positive control group with exposure to smoke 1 kretek cigarette for 1 hour without mangosteen peel extract and given 1% CMC Na. P1: treatment group with exposure to smoke 1 kretek cigarette for 1 hour with mangosteen peel extract dose of 6.045 mg/gram BW in 1% CMC Na. P2: treatment group with exposure to smoke 1 kretek cigarette for 1 hour with mangosteen peel extract dose of 12.09 mg/gram BW in 1% CMC Na. P3: treatment group with exposure to smoke 1 kretek cigarette for 1 hour with mangosteen peel extract dose of 24.18 mg/gram BW in 1% CMC Na. Each dose of extract was diluted using 1% CMC Na to a volume of 0.5 ml. Administration of mangosteen peel extract was done using a 1 ml gastric probe once a day for 45 consecutive days. Exposure to cigarette smoke in male mice used smoke from kretek cigarettes. After exposure for 1 hour, the mice were rested, then all mice were gavaged with mangosteen peel extract according to the predetermined doses for P1, P2, and P3 in 1% CMC Na to a volume of 0.5 ml. For K(-) and K(+), 1% CMC Na was given at 0.5 ml/individual/day.

2.2.3. Testis Examination

After 45 days of treatment, all mice were euthanized using ether, then prepared, and the testes were surgically removed and placed in plastic pots containing formalin, then stained using Hematoxylin Eosin staining. Examination was performed under an Olympus microscope supported by Opticlab viewer at magnifications of 100x, then continued at 400x magnification. The number of spermatogenic cells was counted in all fields of view with intact seminiferous tubules. The assessment was made by counting in five different fields of view in one testis histology preparation starting from the left, right, top, bottom, and middle parts. Each field of view was photographed, and spermatogenic cell counts were performed.

2.2.4. Data Analysis

The data obtained will be arranged in tables, and then the number of spermatogenic cells will be analyzed using Analysis of Variance (ANOVA) at a significance level of 5%. If there are significant differences, Duncan's multiple range test will be conducted using Statistical for Social Science (SPSS) for Windows at a significance level of 5%.

3. Results and discussion

The results of the statistical test using ANOVA showed that the data were normally distributed and had homogeneous variance with significance ($p<0.05$). The results of the normality test, homogeneity, statistical data test with ANOVA, and Duncan's multiple range test for observing the number of spermatogenic cells (spermatogonium, primary spermatocyte, and spermatid). In the ANOVA test, the results of the number of spermatogonium, primary spermatocyte, and spermatid showed significant differences in each group with a significance value ($p<0.05$), thus Duncan's multiple range test was continued. Duncan's multiple range test was used for further testing to compare the average number of spermatogonium, primary spermatocyte, and spermatid among treatments in this study.

Table 1 Average Spermatogenic Cell Counts in Mice After Exposure to Cigarette Smoke and Mangosteen Peel Extract

Group	Spermatogonium	Primary Spermatocyte	Spermatid
K-	$56.08^{e} \pm 3.97$	$76.83^{e} \pm 1.57$	$82.84^{e} \pm 2.63$
K+	$28.2^{a} \pm 1.75$	$30.32^{a} \pm 3.97$	$44.28^{a} \pm 4.72$
P1	$40.48^{c} \pm 2.51$	$53.20^{c} \pm 3.46$	$66.84^{c} \pm 5.97$
P2	$49.88^{d} \pm 3.94$	$62.68^{d} \pm 3.34$	$76.80^{d} \pm 1.81$
P3	$33.92^{b} \pm 0.76$	$47.68^{b} \pm 4.27$	$55.56^{b} \pm 4.02$

Note: Superscripts (^{a,b,c}) that differ in the same column indicate significant differences ($p<0.05$). K (-): group without exposure to cigarette smoke and given 1% CMC Na. K(+): group exposed to cigarette smoke without mangosteen peel extract. P1: group exposed to cigarette smoke with mangosteen peel extract at a dose of 6.045 mg/gram BW. P2: group exposed to cigarette smoke with administration of mangosteen peel extract at a dose of 12.09 mg/gram BW. P3: group exposed to cigarette smoke with administration of mangosteen peel extract at a dose of 24.18 mg/gram BW.

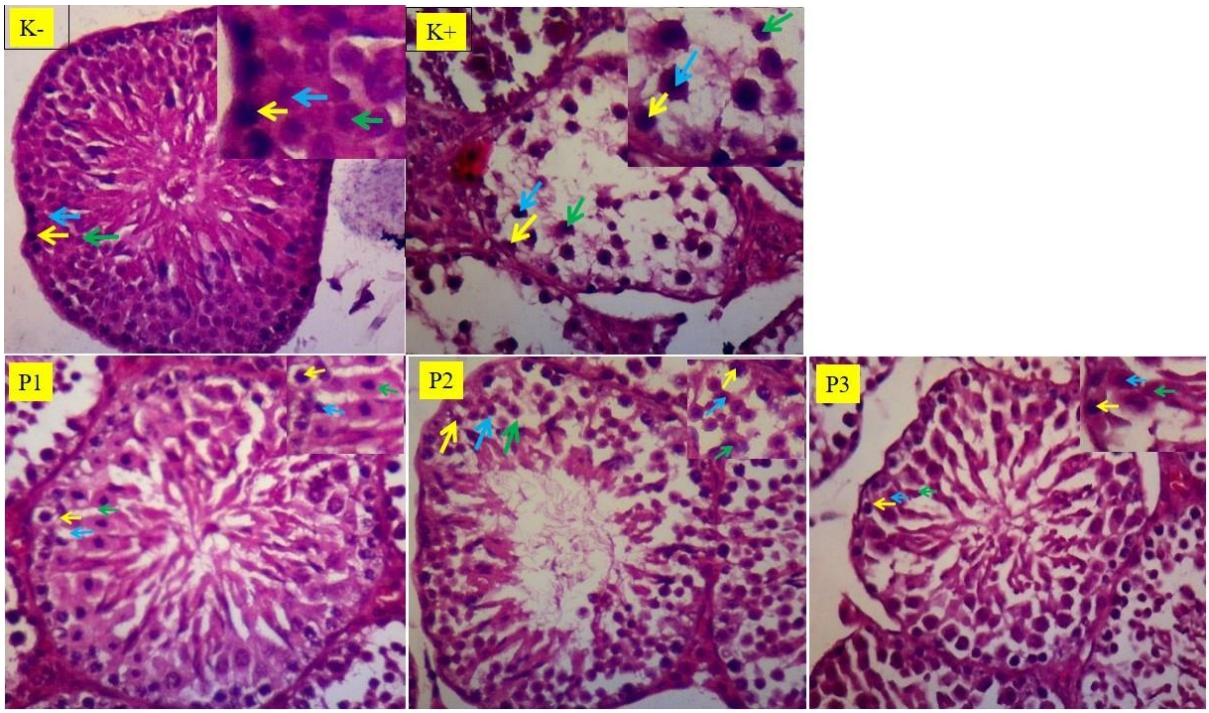

Figure 1 Histopathological of the seminiferous tubules of mice (Mus musculus). Yellow arrows indicate spermatogonium cells, blue indicates primary spermatocyte cells, and green indicates spermatid cells. K (-): group without exposure to cigarette smoke and given 1% CMC Na. K(+): group exposed to cigarette smoke without mangosteen peel extract. P1: group exposed to cigarette smoke with mangosteen peel extract at a dose of 6.045 mg/gram BW. P2: group exposed to cigarette smoke with administration of mangosteen peel extract at a dose of 12.09 mg/gram BW. P3: group exposed to cigarette smoke with administration of mangosteen peel extract at a dose of 24.18 mg/gram BW.

Cigarette smoke can significantly reduce spermatogenic cells (spermatogonium, primary spermatocyte, and spermatid) ($p<0.05$). Administration of mangosteen fruit peel extract can significantly increase spermatogenic cells ($p<0.05$). The

highest increase in spermatogenic cells was achieved in the group given mangosteen peel extract at 12.09 mg/gram BW (P2). The increase in spermatogenic cells due to the administration of mangosteen fruit peel extract and cigarette smoke has not reached normal conditions (K-).

This study aimed to prove the effect of mangosteen peel extract on the number of spermatogenic cells in male mice exposed to cigarette smoke. The results of the mean spermatogenic cell count analysis quantitatively prove that administration of mangosteen peel extract at different doses is able to maintain and increase the number of spermatogenic cells in male mice exposed to cigarette smoke.

Cigarette smoke has the potential to increase free radicals such as reactive oxygen species (ROS) in the body such as superoxide (O_2), hydroxyl radicals (OH^-), and peroxyl radicals (H_2O_2) [14]. Excessive free radicals will cause oxidative stress. Prolonged oxidative stress conditions will cause cell necrosis. Free radicals damage cells by first damaging the cell membrane by covalently binding free radicals with enzymes or receptors on the cell membrane, thereby altering the activity of membrane components. Cell damage causes changes in the structure of the membrane and disables the binding of the membrane to receptors or enzymes that can disrupt normal cell function [15]. The testes are the site of spermatogenesis, which is highly susceptible to oxidation and free radicals due to having a small amount of Superoxide Dismutase (SOD). These free radicals can disrupt spermatogenesis and the spermatogenic cell membrane. The spermatogenic cell membrane contains a large amount of polyunsaturated fatty acids with double bonds [16].

ROS will bind to the polyunsaturated fatty acids in the cell membrane, leading to lipid peroxidation of the membrane, resulting in the production of malondialdehyde (MDA), which is one of the signs of oxidative stress in the body [17]. Another consequence of increased free radical production is the decrease in antioxidant enzymes such as superoxide dismutase (SOD) and glutathione (GSH), resulting in the uncontrolled production of superoxide anions and hydroxyl radicals. Various oxidative damages experienced by mitochondria in various cells in the testicular organ can trigger mitochondria to release cytochrome-c and apoptosis induction factor (AIF) from mitochondria into the cell cytoplasm, thus triggering apoptosis and necrosis in the spermatogenic cells of male mice, leading to a decrease in spermatogenic cells [18].

Based on the above exposure, it is consistent with the results of this study showing the smallest number of spermatogenic cells obtained in the group of mice exposed to cigarette smoke without the administration of mangosteen peel extract therapy. This data proves that cigarette smoke causes toxicity to spermatogenic cells by reducing the number of spermatogonia, primary spermatocytes, and spermatids in the mice's testes. The formation of free radicals in body tissues is a normal activity, but their presence can damage cell function, including spermatozoa function. To counteract this, cells produce antioxidants as their scavenging enzymes. In normal conditions, the balance between the number of free radicals and antioxidants is maintained. The lack of antioxidants in the body with increased free radicals can result in infertility disorders [19]. The ability of mangosteen pericarp fractions as antioxidants can neutralize free radicals. Moongkarndi et al. [20] reported that at certain doses, mangosteen peel can act as an antioxidant.

Naturally, there are endogenous antioxidants such as SOD that can protect cells from free radicals [21]. There are several enzymes in cells that originate from non-enzymatic systems that function to deactivate free radicals. SOD, catalase, and GSH are enzymes that function to protect cells from free radical injuries. The catalase enzyme in peroxides converts H_2O_2 into $O_2 + 2H_2O$. In addition, exogenous antioxidants include vitamin E, A, and C and β-carotene, which can also inhibit free radical activity [22]. The results of this study indicate that mangosteen peel extract can maintain and increase the number of spermatogenic cells exposed to cigarette smoke, as shown by the average number of spermatogenic cells in treatment groups P1, P2, and P3 being larger compared to group K+. Cigarette smoke, which induces oxidative stress, can be inhibited by compounds contained in mangosteen peel extract.

Mangosteen peel contains xanthones that can stimulate SOD enzymes, which are primary antioxidants in the body and also play a role in inhibiting lipid peroxidation [23]. SOD functions to reduce the formation of new free radicals by breaking the chain reaction by providing one electron and converting it into a more stable compound, thereby inhibiting free radicals caused by cigarette smoke. SOD functions to protect cell destruction in the body. In a study conducted by Arsana (24), it was shown that mangosteen peel extract significantly increased SOD levels.

The mechanism of action of xanthones through 3 pathways, namely, as hydrogen donors, inhibiting lipid peroxidation, and scavenging free radicals. It is known from Kurniawati's research [25] that the antioxidant potential in mangosteen peel is relatively high as a free radical scavenger with an IC50 value (11-21 ppm). The antioxidant properties in mangosteen peel extract exceed those of vitamin E and vitamin C, so xanthones are highly needed in the body to balance peroxides and inhibit cell damage. This study is supported by research by Puspitasari, which showed that mangosteen

peel extract can increase SOD levels, thus increasing the number of spermatogenic cells in male mice exposed to cigarette smoke for 32 days [26].

Based on the results of this study, it can be concluded that the P2 group with a dose of 12.09 mg/gram BW is the optimum dose of mangosteen peel extract antioxidant in maintaining and increasing the number of spermatogenic cells due to exposure to cigarette smoke compared to groups P3 and P1. Administration of mangosteen peel extract in the P3 group with a dose of 24.18 mg/day resulted in a decrease in the number of spermatogonia, primary spermatocytes, and spermatids compared to groups P1 and P2. This occurs because antioxidants in mangosteen peel extract are suspected of producing prooxidants [27]. According to research by Hayati et al. [28] xanthone compounds in high concentrations function as oxidants by inhibiting the function of aromatase using γ-mangostin. Aromatase is an enzyme that catalyzes testosterone into estrogen. Therefore, high doses of mangosteen peel extract will increase testosterone. Excessive testosterone can disrupt testis function, leading to a decrease in the number of spermatogenic cells.

4. Conclusion

Based on the results of this study, it can be concluded that mangosteen peel extract (*Garcinia mangostana.* Linn) can maintain the number of spermatogenic cells in male mice (*Mus musculus*) exposed to cigarette smoke. Further research is needed on the effect of mangosteen peel extract (*Garcinia mangostana*. Linn) on testosterone hormone levels.

Compliance with ethical standards

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Disclosure of Conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

The study was approved by the faculty of Dentistry Animal Ethics Committee of Universitas Airlangga. All variables were considered in accordance with the Ethics Committee related to the animal handling to ensure no discomfort or pain was caused to the animals during sampling(certificate registration number: 021/HRECC.FODM/I/2019.

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