

Administration of Mangosteen Peel Extract (*Garcinia mangostana*) Prevented the Decrease of Leydig Cells Numbers, Sertoli Cells Numbers, and Testosterone Hormone Levels in Rats (*Rattus norvegicus*) Male Wistar Strains Exposed to Cigarette Smoke

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Abstract: Introduction: Inhalation of cigarette smoke containing nicotine can cause a decrease in the number of Leydig cells and Sertoli cells. Damage to Leydig cells and Sertoli cells causes a deficiency of testosterone which is one of the signs of aging. Mangosteen peel contains xanthone and tannin compounds which have effects as antioxidants, antibacterial, hypoallergenic, antitumor, antihistamine and anti-inflammatory. This study aimed to prove that administration of mangosteen peel extract (*Garcinia mangostana*) prevented a decrease in the number of Leydig cells and Sertoli cells, and the level of testosterone in male Wistar rats (*Rattus norvegicus*) exposed to cigarette smoke. Methods: This was true experimental research using post-test only control group design. The subjects were 36 male Wistar rats aged 12-16 months, weighing 180-200 grams that were randomly divided into 2 groups, namely the control and treatment group. Control group was given 2ml distilled water as placebo and treatment group was given 8mg/200g BW of mangosteen peel extract dissolved in 2 ml distilled water and the rats were then exposed to cigarette smoke 1 hour after administration for 14 days. Blood sample and testes were taken after 14 days to analyze testosterone level and the number of Leydig cells and Sertoli cells. Results: The mean number of Leydig cells in the control group and the treatment group was 4.07 ± 2.19 cells/field of view (FOV) and 7.76 ± 2.28 cells/FOV respectively ($p < 0.001$). The median number of Sertoli cells in the control group and the treatment group was 1.00 cells/FOV and 1.33 cells/FOV respectively ($p = 0.024$). The mean testosterone level in the control group was 2.93 nmol/L, while in the treatment group was 11.25 nmol/L ($p < 0.001$). Conclusion: Administration of mangosteen peel extract (*Garcinia mangostana*) prevented a decrease in the number of Leydig cells, Sertoli cells and testosterone level in male Wistar rats (*Rattus norvegicus*) exposed to cigarette smoke.

Keywords: mangosteen peel extract, Leydig cells, Sertoli cells, testosterone, male Wistar rats

1. Introduction

One of the aging processes can be influenced by the balance between the production of free radicals, especially reactive oxygen species (ROS), then the effectiveness of free radical scavenging systems and body repair systems. Cigarette smoke, which is an aerosol or free gas component which contains a large number of chemical compounds, is a source of free radicals (Buehler, 2012; Anggarani *et al.*, 2020).

Cigarette smoke contains a variety of toxic chemicals, genetic substances and carcinogens, including nicotine and its metabolites, cotinine, radioactive polonium, benzopyrene, dimethylbenzene, naphthalene, methyl naphthalene, polycyclic aromatic hydrocarbons (PAHs) and cadmium. Research shows the impact of inhalation of cigarette smoke containing nicotine can cause a decrease in the number of Leydig cells and Sertoli cells by triggering apoptosis in testicular tissue; it also inhibits the basal function of the hypothalamus pars medialis which causes a decrease in LH secretion whose function is to stimulate Leydig cells and produce testosterone. The nicotine content also interferes

with the steroidogenesis process so that there is a decrease in testosterone hormone secretion (Harlevet *et al.*, 2015; Asare-Anane *et al.*, 2016).

Men with low testosterone level have been observed to have premature atherosclerosis, increased visceral adipose tissue, hyperinsulinemia, and other risk factors for myocardial infarction. Insulin resistance has also been shown to be associated with decreased Leydig cell secretion and testosterone (Bain, 2007).

Antioxidants are inhibitors of oxidation processes even at relatively small concentrations, so they have various physiological roles in the body. Antioxidants work by giving electrons (donors), thus inhibiting reactive ROS molecules looking for electron to be used as an extra electron pair and preventing other electrons from becoming unstable, which causes oxidative damage (Veskoukis *et al.*, 2012; Yadav *et al.*, 2016).

Mangosteen (*Garcinia mangostana* L.) is a tropical fruit that has been used for hundreds of years around the world as a

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traditional medicine. Discarded mangosteen peel can be developed as a drug candidate. The content of xanthenes in mangosteen itself is a bioflavonoid with properties as an antioxidant, antibacterial, hypoallergenic, antitumor, antihistamine, and anti-inflammatory. In a study conducted by Umar SH *et al.*, (2015) reported that administration of mangosteen peel extract at a dose of 1 ml for 52 days can increase testosterone production, which is an important hormone involved in the production and maturation of spermatozoa in the seminiferous tubules of the testes (Srihari and Lingganingrum, 2015; Umaret *al.*, 2015; Darmawansyah, 2018; Maligan *et al.*, 2019; Aljunaid *et al.*, 2020).

Through this study, we aim to prove that administration of mangosteen peel extract (*Garciniamangostana*) can prevent a decrease in the number of Leydig cells, Sertolicells, and testosterone level in male Wistar rats (*Rattusnorvegicus*) exposed to cigarette smoke as a source of free radicals.

2. Methods

Study Design and Experimental Animals

This study was a true experimental research with post-test only control group design. The experiment was carried out at the Integrated Biomedical Laboratory Unit, Faculty of Medicine, Udayana University, Bali. Preparation of mangosteen peel extract and phytochemical test were carried out at the Food Analysis Laboratory, Faculty of Agricultural Technology, Udayana University. The sample needed in this experiment was 32 male Wistar rats (n=16), 12-16 weeks old, weighing 180-200 grams. To anticipate drop out, 10% of total sample were added, with the total amount to 36 rats divided into 2 groups: control and treatment group (n=18). This research has been approved by the ethics commission of Faculty of Medicine, Udayana University, Bali, (B/213/UN14.2.9/PT.01.04/2022).

Mangosteen Peel Extract Production

The mangosteen peel was cleaned, washed and dried then milled into simplicial powder at the Faculty of Agricultural Technology Food Analysis, Udayana University, Bali. As much as 1 kg of the powder was placed into the macerator and 96% ethanol \pm water with a ratio of 9:1, which was 3 liters, was added. The maceration process was done for 24 hours. 96% ethanol solvent \pm water with a ratio of 1:1 was added into the macerator, then left for 24 hours while occasionally stirring. All the results of the solvent storage were mixed and then the extract concentration process was carried out using a rotary evaporator, at the Food Analysis Laboratory, Faculty of Agricultural Technology, Udayana University. The phytochemical test for the ethanol extract of mangosteen peel was carried out using the DPPH method at the Faculty of Agricultural Technology, Food Analysis Laboratory, Udayana University.

Experimental

Rats used as experimental animals were adapted for 7 days before treatment and given food in the form of pellets and drink ad libitum. Rats were randomly divided into 2 groups: control group was given a placebo, and the treatment group was given ethanol extract of mangosteen peel and both were exposed to cigarette smoke for 14 days. Placebo was given

to the control group 1 hour before cigarette smoke exposure, while the rats in treatment group were given 8 mg of mangosteen peel extract which was diluted with 2 ml of distilled water through a gastric tube for several times. Exposure to cigarette smoke was done using an aerator. The rats were anesthetized afterwards and the blood was taken to analyze the testosterone level and testicular was taken to examine the number of Sertoli and Leydig cells.

Histological Preparation

Mice were necropsied, both testicular organs were taken and washed with NaCl and immersed in 10% buffered formalin solution for 48 hours. Dehydrate gradually in 70% and 80% alcohol, 2x1 hour, then followed by a clearing process with xylene for 2x1 hour and placed in paraffin for 2x1 hour. The tissues were then transferred into a base mold containing liquid paraffin and placed on a cooling surface for 15 minutes until the paraffin solidified. The paraffin block was cut with a microtome with a thickness of 4-5 micrometers, selects the best quality and transferred to a water bath at 45°C to remove wrinkles on the tissue sheet. Place it in the middle of the object glass, let it dry by placing it in a laboratory thermostatic oven with a temperature of 37°C for 24 hours. Rehydration was carried out to remove paraffin using xylol, 95% alcohol, distilled water, and stain with Hematoxylin-Eosin (HE). After the staining process, the preparation slide was placed on a tissue paper on a flat surface. As much as 2-3 drops of mounting medium such as Eukit was used then covered with a cover glass and dried for 24 hours at room temperature.

Leydig and Sertoli Cell Number Examination

Observation of testicular tissue preparations was carried out with Olympus CX21 electric microscope connected to Outilab and computer equipped with Outilab Image Rester software. From each field of view, cells were counted randomly, so that in one preparation, both Leydig and Sertoli cells would be observed. Number of Leydig and Sertoli cells were calculated from 5 visual fields.

Total Testosterone Level Examination

Examination of total testosterone levels was carried out using ELISA kit (BT Lab, Indonesia), with following steps: All ELISA kits were incubated at room temperature for 30-60 minutes before the examination started. Standard and sample solutions (25 μ l each) were added to the wells. Testosterone HRP Conjugate 100 μ l and rat anti-testosterone 50 μ l were added. The reagent mixture was shaken for 30 seconds and incubated for 60 minutes at room temperature. The wells were then washed with 200 μ l wash buffer 3 times. 100 ml of TMB substrate solution was added and incubated for 15 minutes at room temperature. The 50 μ l stop solution was homogenized for 30 minutes, then read with a spectrophotometer at a wavelength of 450nm.

Statistical Analysis

Statistical analysis was performed with SPSS Version 23. Normality test was assessed using Shapiro-Wilk test and homogeneity test was assessed using Levene's test. Comparability test on Leydig cell number was assessed using unpaired t-test because the data was normal, and Mann-Whitney test as used on Sertoli cells and testosterone level because the data was non-normally distributed.

3. Results

In a, it was Normality test on Sertoli and Leydig cell number, and testosterone level was done using Shapiro-wilk test. The data on Leydig cells and testosterone level in control group was normally distributed, while Sertoli cells was non-normally distributed. The data on Leydig cells and Sertoli cells in treatment group was normally distributed, while testosterone level was non-normally distributed.

Levene's homogeneity test on Leydig cells was found homogeneity, while Sertoli cells and testosterone level were heterogeneity.

Comparability test on the number of Leydig cell number was done using unpaired t-test and presented in Table 3.

Table 3: Comparability Test on Leydig Cell Number

Variables	Groups	n	Mean ± SB	P
Leydig Cell Number (cells/FOV)	Control	18	4.07 ± 2.19	<0.001
	Treatment	18	7.76 ± 2.28	

Based on the analysis using the unpaired t-test in table 3, it was found that there was a significant difference in the mean number of Leydig cells between the two groups ($p < 0.05$).

Data comparability analysis was performed to determine differences in the number of Sertoli cells and testosterone level between groups. The analysis was carried out using the Mann-Whitney test with the results presented in Table 4.

Table 4: Median Differences in Sertoli Cells between Groups

Variables	Groups	n	Median (Min-Max)	p
Sertoli Cell (cells/FOV)	Control	18	1.00 (0.33-1.67)	0.024
	Treatment	18	1.33 (0.33-3.33)	
Testosterone Level (ng/mL)	Control	18	2.93 (2.65-3.00)	<0.001
	Treatment	18	11.25 (8.67-12.63)	

Based on the analysis using Mann-Whitney test in table 4, it was found that there was a significant difference in the number of Sertoli cells and testosterone level between groups ($p < 0.05$).

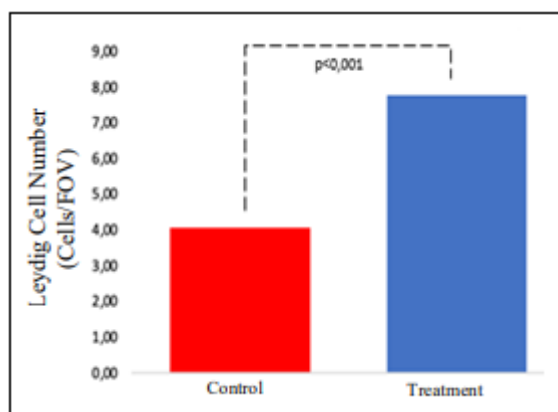


Figure 1: Comparison of Mean Leydig Cell Numbers between Groups

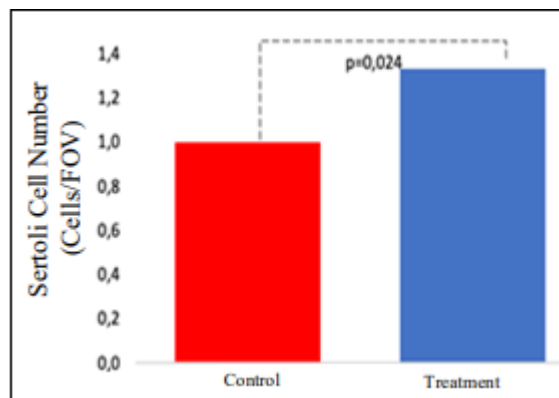


Figure 2: Comparison of Mean Sertoli Cell Numbers between Groups

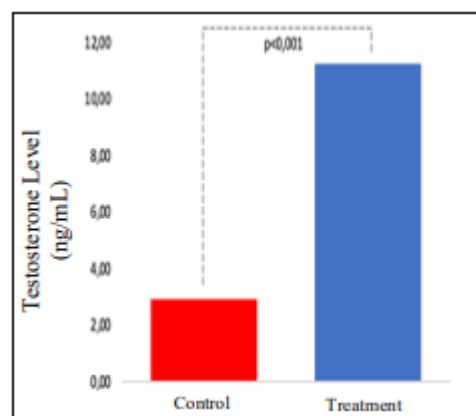


Figure 3: Comparison of Sertoli Cell Number between Groups

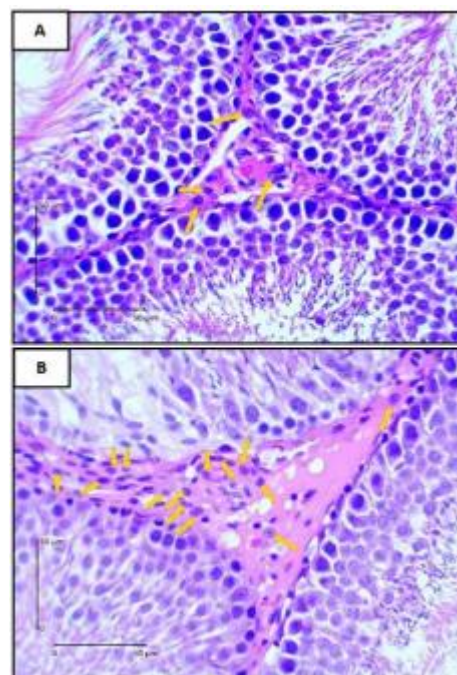


Figure 4: Comparison of Sertoli Cell Numbers between Groups

Description:

(A) Control Group. (B) Treatment Group. The yellow arrow indicates Leydig cells. Leydig cells have a round nucleus and pale cytoplasm with fat vacuoles. These cells have a smooth endoplasmic reticulum that is dominant in their cytoplasm (H&E, 400x).

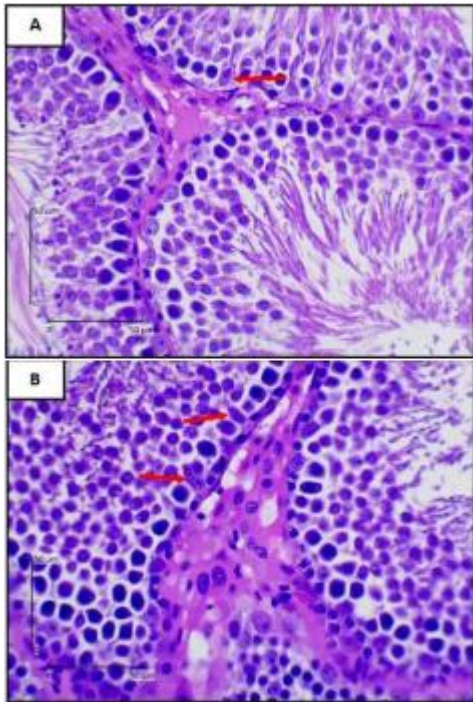


Figure 5: Median Differences in Testosterone Level between Groups

Description:

(A) Control Group. (B) Treatment Group. The red arrow indicates Sertoli cells. Sertoli cells are somatic cells in the seminiferous tubules with an irregular columnar shape and a pale ovoid nucleus (H&E, 400x).

4. Discussion

In this study, the mean number of Leydig and Sertoli cells in the control group was 4.07 cells/FOV and 0.9 cells/FOV. This finding is in line with previous studies evaluating the effects of antioxidants on a number of spermatogenesis parameters including the number of tubular tissue cells. The study reported that the control group that was exposed to cigarette smoke for 21 days had an average number of Sertoli and Leydig cells of 6.13 ± 0.74 and 5.63 ± 0.56 cells/FOV, respectively, which was much lower than the treatment group that was given vitamin C, E, glutathione, and zinc for 21 days (Yuniarifa *et al.*, 2021). Other studies also reported similar findings, namely the mean number of Leydig cells and Sertoli cells were 3.10 ± 0.74 and 6.75 ± 0.84 respectively in the 20-day cigarette smoke exposure group, which was much lower than the treatment group administered with antioxidant vitamin C for 20 days (Juniatiningrum *et al.*, 2018).

In this study, the control group had much lower numbers of Leydig cells and Sertoli cells, as well as testosterone levels compared to the treatment group. This finding could be due to the effects of cigarette smoke exposure on testicular tissue. Previous *in vivo* studies reported that the lamina basalis of the seminiferous tubules in rats exposed to tobacco smoke every day was significantly worse and disorganized than the group without exposure to tobacco smoke, leading to inhibition of spermatogenesis (Abdul-Ghani *et al.*, 2014). Other studies also reinforce this finding where exposure to chronic cigarette smoke can induce

apoptosis in rat testes, and reduce the number of germ cells, Leydig cells and Sertoli cells (La Maestra *et al.*, 2015).

Nicotine was reported to significantly reduce testosterone levels in rats. This could be the main mechanism for finding low testosterone levels in the control group and higher levels in the treatment group. Other studies have revealed that nicotine can induce apoptosis of Leydig cells and inhibit androgen biosynthesis in mouse Leydig cells (Nesseim *et al.*, 2011; Dai *et al.*, 2015).

Effect of Mangosteen Peel Extract on Leydig Cell Count in Male Wistar Rats Exposed to Cigarette Smoke

This study found significant differences in the mean number of Leydig cells between groups. The group that was given 8 mg/200 g BW of mangosteen peel extract for 14 days had a higher number of Leydig cells (7.76 ± 2.28). The same thing was shown by Prahandita's research in 2019 which was conducted on 25 rats aged 8 to 12 weeks which showed that when rats were exposed to cigarette smoke, mangosteen peel extract significantly increased the number of Leydig cells ($p < 0.05$). The highest concentration of Leydig cells was found at a dose of 12.09 mg of mangosteen peel extract per 1 rat (Prahandita, 2019).

Research by Hayati *et al.*, showed that at a low dose of 0.4 mg/kg BW of mangosteen peel ethanol extract was able to have antioxidants effects and provide benefits to the testes due to an increase in spermatogenic cells and spermatozoa cells. In addition, the quality of sperm which is assessed from its motility, morphology and viability also increases (Hayati *et al.*, 2014; Umar *et al.*, 2015). In addition, research by Permatasari showed that administration of mangosteen peel ethanol extract at a dose of 200 mg/kgBW was able to significantly increase the average number of spermatozoa in rats induced by cigarette smoke during 30 days (Permatasari, 2014).

Effect of Mangosteen Peel Extract on Sertoli Cell Count in Male Wistar Rats Exposed to Cigarette Smoke

In this study, there was a significant difference in the number of Sertoli cells between the treatment and control groups. These findings are in line with previous studies evaluating the effect of mangosteen peel extract on the tissue structure of the testicular seminiferous tubules in diabetic rats induced with streptozotocin (STZ). The study reported that administration of 83.3 mg/kg extract improved the structure of the testicular seminiferous tubule network as reflected in an increase in lumen diameter ($703.25 \mu\text{m}$), an increase in the number of spermatogonia, and spermatogenesis in a population of male Wistar rats aged 2 - 3 months, weighing 120-150 grams (Primiani and Lestari, 2019).

Similar findings were also reported in studies evaluating the effect of white tea extract or white tea (*Camellia sinensis L.*) on Sertoli cells, which showed decreased levels of protein and mRNA GLUT1 and increased intracellular LDH, accompanied by increased production of lactate and alanine which are antiapoptotic in tubular tissue (Martins *et al.*, 2014).

The effect of mangosteen peel extract on increasing Sertoli cells can be mediated by the content of the main phenolic compounds in mangosteen peel, including mangosteen and xanthenes. Other phenolic content in mangosteen fruit can also play a role, similar to the flavonoid and anthocyanin groups. One of the compounds in the xanthenes group, namely catechin compounds, has been reported to have a number of effects on testicular tubular tissue, including Sertoli cells (Rizaldy *et al.*, 2022). Other studies also reported that catechin compounds can work to increase the development of Sertoli cells through receptors on the plasmatic membrane and testosterone receptors which show non-classical pathways in Sertoli cells from rat testes (Cavalari *et al.*, 2012).

Effect of Mangosteen Peel Extract on Testosterone Levels in Male Wistar Rats Exposed to Cigarette Smoke

This study found significant differences in median testosterone levels between the two groups. The group given mangosteen peel extract had higher testosterone levels [11.15 (8.67-12.62) ng/mL] than the control group. This is also shown in the research by Nelli which found a significant effect of mangosteen peel extract on testosterone levels. It is known that testosterone plays an important role in the production and maturation of sperm (Nelli, 2013). Mangosteen peel antioxidants called xanthenes can increase testosterone production and play a role in the development and maturation of spermatozoa in the seminiferous tubules of the testes. This mechanism prevents free radical damage to cell membranes, ensures that the bonds between hormones involved in spermatogenesis and hormone receptors remain intact and the process of spermatogenesis runs more efficiently (Muhartono *et al.*, 2017). Testosterone is formed by Leydig cells, so if there is damage to Leydig cells it can cause a decrease in testosterone levels, while Sertoli cells play a role in providing nutrition to sperm, thereby maintaining sperm quality (Walker, 2011).

5. Conclusion

The administration of mangosteen peel (*Garcinia mangostana*) extract prevented a decrease in the number of Leydig cell and Sertoli cell, and the testosterone level in male Wistar rats (*Rattus norvegicus*) exposed to cigarette smoke.

Conflict of Interest

All researchers declare that there is no conflict of interest related to this article

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Author's Contribution

All authors contribute equally in compiling this research article

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