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Antioxidant activity in red mulberries on sperm development exposed by cigarette smoke

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ABSTRACT

Introduction: The adverse effects of exposure to cigarette smoke have been discovered for a long time. However, most people assume that exposure to cigarette smoke merely causes respiratory problems. The increase in free radicals from cigarette smoke will also affect sperm development, decreasing the number of sperm produced. The intake of antioxidants derived from red mulberry is expected to reduce cigarette smoke exposure on sperm count. The study aimed to analyze the antioxidant activity of red mulberries on sperm development exposed by cigarette smoke in experimental animals.
Methods: This study employed experimental animals, Wistar Rats (*Rattus norvegicus*), divided into five groups with different treatments in each group. The treatment in the form of exposure to cigarette smoke and red mulberry was undertaken for 30 days. The parameters used were histological preparations from the testes by calculating the number of Leydig cells, spermatocytes, and spermatids in each tubule.
Results: The results showed that the negative control group had the highest mean, while the positive control group had the lowest mean. The treatment group showed that the more red mulberry juice was given, the more sperm count was increased. The study group had a significant difference ($p < 0.05$) and a strong relationship between groups ($r > 0.80$).
Conclusion: In conclusion, consuming red mulberry juice can increase sperm quantity exposed by cigarette smoke.

Keywords: Cigarette, leydig cell, red mulberries, spermatocytes, spermatid.
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INTRODUCTION

The usage of tobacco cigarettes has caused various health problems. The nicotine content in tobacco cigarettes also causes addictive effects. The usage of tobacco cigarettes in the United States at the age of 18 has reached 21.3% of the total population.[1] Cigarettes have been designed to send high doses of nicotine from cigarette smoke through the lungs and enter the brain about 10-20 seconds after being inhaled. That will accelerate and amplify the effects of nicotine, causing dependence.[2] Various attempts have been made to reduce the number of smokers through cigarette tax increases, regulations related to smoke-free areas, and anti-tobacco campaigns. These efforts have had a significant impact in the last ten years. Thus it was estimated that the number of world smokers has decreased by 2.8%. The decreasing number prevalence was not in line with the decreasing number of smokers due to the increasing population, thus it was estimated that the number of smokers worldwide remains around 1.1 billion people.[3]

The damage caused by smoking can emerge in various ways, from developing chronic diseases to cancer. This impact is not only on smokers but also on people exposed to second-hand smoke.[4] The deterioration of health caused by cigarette smoke also depends on the duration of smoking and exposure to cigarette smoke that enters the airways. This damage is caused by increased free radicals in cigarette smoke, resulting in cell damage.[5] Cigarette smoke frequently causes various diseases, including respiratory system disorders, cardiovascular system disorders, and cancer. Moreover, the reproductive system can also be affected by it, triggering infertility in men.[6] Hazardous substances contained in cigarettes will deliver a negative impact on the number of sperm produced, leading to infertility.[7] Decreased sperm quantity in male smokers includes decreased numbers of Leydig cells, spermatocytes, and spermatids. These are due to the increase in free radicals that enter the body through cigarette smoke exposure, resulting in an increase in unsaturated fatty acids in cell membranes and a decrease in the number of antioxidants in cells.[8] Enzymatic antioxidants such as superoxide dismutase (SOD) and glutathione peroxidases (GSH-Px) cannot protect sperm from lipid peroxidation and DNA damage in sperm cells.[9] So, sperm reduction concentration among smokers is estimated at 22%. This condition is also determined by the length and number of cigarettes inhaled.[10]

The increase of antioxidants in the body can reduce free radicals obtained from cigarette smoke. Various antioxidants given through intake can help neutralize excessive amounts of free radicals, thereby reducing the impact of sperm cell damage and improving sperm cell count. For example, flavonoids are a type of antioxidant made from natural ingredients that can reduce free radicals.[11,12] These

Table 1. The mean value of Leydig, spermatocyte, and spermatid cells in each group

Group	Note	Mean ± SD		
		Leydig Cell	Spermatocyte	Spermatid
I	Negative control group, only food intake	17,2±1,48	42,6±1,95	57,6±1,95
II	Positive control group, given food intake and exposure to cigarette smoke	8,4±1,14	30,6±1,14	35,0±1,58
III	Treatment group with exposure to cigarette smoke and *Morus rubra* peroral fruit 3.25 g / day	11,6±1,52	36,6±1,14	41,0±1,00
IV	Treatment group with exposure to cigarette smoke and *Morus rubra* peroral fruit 6.5 g / day	12,8±1,30	38,4±1,14	48,0±1,58
V	Treatment group with exposure to cigarette smoke and *Morus rubra* peroral 13 g / day	15,0±1,00	41,2±1,64	53,0±1,58

Table 2. ANOVA test results in every group

Group	p-value
Leydig Cell	0,000
Spermatocytes	0,000
Spermatids	0,000

Table 3. Pearson test results among groups

Groups	Leydig Cell (r)	Spermatocytes (r)	Spermatids (r)
Leydig Cell	-	-	-
Spermatocytes	0,853	-	-
Spermatids	0,927	0,917	-

antioxidants are found in many red mulberries. Therefore, this study aims to determine the increase of sperm count in histological preparations against the provision of antioxidants in the form of red mulberry juice due to exposure to cigarette smoke.

METHODS

The experimental animal in this study was male Wistar rats (*Rattus novergicus*). The inclusion criteria in this study were experimental animals weighing 200 - 250 gram with no macros abnormalities were found and had never been used in the study. Meanwhile, the dropout criteria in this study were animals that were sick or died during the study. The maintenance and treatment of experimental animals during the research refers to the 3R principle (Replacement, Reduction, and Refinement). The size of the experimental animal cage was 20x20x16 cm and cleaned twice a week. The experimental animal room was set with a temperature of 25-26 ^{0}C, humidity 40-60%, and the lighting cycle was carried out alternately 12 hours of the light cycle and 12 hours of the dark cycle with the light intensity of 75-125 fc. Food is given twice a day as much as 20 grams with a balanced composition of carbohydrates, proteins, and fats. Drinking water is changed every morning.

The research method used experimental research with a posttest control group design. The experimental animals used in this study were 30 male Wistar rats (*Rattus novergicus*) for 30 days which were obtained and maintained from the Faculty of Medicine, Universitas Airlangga. The experimental animal group was divided into 5, namely a negative control group, a positive control group, and three treatment groups. The research was conducted at the Faculty of Medicine, Universitas Airlangga, and was approved by the Health Research Ethics Committee (No. 135 / KE / VI / 2020). The parameters used in this study were the number of Leydig cells, spermatocytes, and spermatids in testicular preparations.

Red mulberry fruit (*Morus rubra*) obtained will be checked first to get the excellent condition of the fruit. Making juice using a blender is followed by a filtering process to obtain red mulberry juice. Then, the levels of flavonoids would be measured in each gram and obtained ±38mg. The levels of flavonoids that have been obtained will be converted from the daily needs of adults (± 190mg/Kg) to the daily intake dose of Wistar rats of ± 240mg /head using the Laurence and Bacharach table. The dosage of red mulberry juice to be given to the treatment group was divided into three types, such as 3.25 g, 6.5 g, and 13 g.

Preparations and histological observations of the airways were made, starting with the anesthetic and euthanasia processes in experimental animals. Then, the testicular organ was taken to make preparations. Preparations were made in a BNF (Buffer Normal Formalin) solution using the HE (Hematoxylin Eosin) staining method. Identification of increased sperm count based on the number of Leydig cells, spermatocytes, and spermatids in the seminiferous tubules. Observations were made of 5 seminiferous tubules in each experimental animal. The increase in the number of sperm cells was expressed based on comparing the number of Leydig cells, spermatocytes, and spermatids with the control group.

This research was conducted for 30 days by dividing it into five groups. The first group was a negative control group, where the experimental animal did not receive any treatment, only daily intake. The second group was a positive control group, where the experimental animals receive daily intake and exposure to tobacco smoke as much as two sticks per day. While the three treatment groups were the group that received exposure to

tobacco smoke as much as two sticks per day, daily intake, and mulberry fruit juice with different amounts, such as 3.25 g, 6.5 g, and 13 g.

The research results will be obtained ratio data in the form of the mean number of Leydig cells, spermatocytes, and spermatids in each group. Data analysis was performed using Analysis of Variance (ANOVA) analysis with SPSS version 22 to see any differences between groups.

RESULTS

The study results were conducted by comparing Leydig cells, spermatocytes, and spermatids cell numbers between groups.

Table 1 shows the average number in each group. In group I, the negative control showed the highest mean compared to other treatment groups. In contrast, in group II, the positive control showed the lowest mean compared to other treatment groups. Groups III, IV, and V were the groups that were given different doses of red mulberry juice. The results showed that the higher the red mulberry juice was given, the higher the mean of sperm cells.

ANOVA test results in table 2 had been carried out to find the differences between groups. Previously, the normality and homogeneity tests had been carried out in each group, and the p-value was >0.05. Hence it could be concluded that the data obtained were normally distributed and homogeneous. The ANOVA test results in the three groups showed a $p<0.05$, and it can be concluded that there was a significant difference.

Table 3 shows the correlation test results using the Pearson test to determine the relationship between the three groups. This test aims to show the relationship between the decrease in Leydig cells, which will impact the entire process of sperm formation. The Pearson test results showed a value of $r>0.80$, so it can be concluded that the three groups have a strong relation.

DISCUSSION

The increase in free radicals due to exposure to cigarette smoke can cause a decrease in sperm count. An excessive increase in free radicals may cause the inability of antioxidants in the body to neutralize, known as oxidative stress, which can lead to cell damage and death.[13] Free radicals in cigarette smoke have the most role in influencing the condition of sperm count and are classified as Reactive Oxygen Species (ROS), namely superoxide.[14] Superoxide radicals can be neutralized by the antioxidant superoxide dismutase (SOD) into hydrogen peroxidase (H_2O_2), which will be converted again into water (H_2O) and oxygen (O_2) through the antioxidant glutathione peroxidase (GSH-Px) and catalase (CAT).[15,16] However, excessive free radical production will imbalance the number of free radicals and antioxidants. Giving antioxidants from outside the body can reduce the impact of damage caused by free radicals. Increasing the provision of antioxidants will further increase the reduction in free radicals caused by cigarette smoke.[17]

One of the health risk factors caused by exposure to cigarette smoke is reproductive health related to the spermatogenesis process.[18] Disruption in the spermatogenesis process is influenced by Leydig cells, which secrete androgen hormones and are influenced by Luteinizing Hormone (LH).[8] Various studies show a decrease in the hormone testosterone in smokers compared to non-smokers. Nicotine is the leading cause of the secretion of the hormone testosterone, so that it will directly interfere with the spermatogenesis process through apoptosis in Leydig cells.[19] Moreover, it will affect the formation of spermatogonia in the basal compartment, which is the forerunner of the sperm. The spermatogonia will turn into spermatocytes and will undergo meiotic division which occurs to develop into spermatids. Meiosis is the process of cell division by reducing the number of chromosomes so that each sperm contains only 1 chromosome (haploid).[20]

Flavonoids have a significant role in suppressing ROS synthesis by obstructing the formation of pro-oxidant enzymes or eliminating various elements that can trigger the formation of free radicals.[21] Flavonoids can neutralize ROS by reacting with reactive radical compounds. There will be high reactivity in the hydroxyl groups of flavonoids from this process, causing radical ions to become inactive.[22] In addition, flavonoids can prevent hydroxyl radicals from binding to nitric oxide (NO), so peroxynitrite formation does not occur, and cell damage can be avoided. Flavonoids induce SOD and GPx, which are enzymatic antioxidants and function to neutralize free radicals. Flavonoids inhibit enzymes involved in ROS formation, namely microsomal, monooxygenase, glutathione-S-transferase, mitochondrial succioxidase, and NADH oxidase.[21] Glutathione-S-transferase (GST) inhibited by flavonoid activity will induce glutathione (GSH) activation so that testicular germ cells can defend against free radicals. This allows spermatogonia cells to survive oxidative stress and suppress the amount of spermatogonia cell apoptosis induced by oxidative stress. The excessive increase in free radicals will result in imperfect sperm formation, and the body will carry out the process of apoptosis.[23]

Provision of antioxidants in the form of red mulberry juice containing flavonoids showed an increase in the number of Leydig cells, spermatocytes, and spermatids directly. It shows that the antioxidants in red mulberry juice can neutralize free radicals in cigarette smoke.[24,25] Therefore, the use of red mulberry fruit as an additional intake of antioxidants from outside the body can be considered to prevent a decrease in sperm cell count due to exposure to cigarette smoke. This study has limitations, namely in determining the age of the fruit and the soil conditions of origin of red mulberry fruit, which will affect the flavonoid content.

CONCLUSION

Cigarette smoke exposure affects the quantity of sperm by decreasing the number of Leydig cells, spermatocytes, and spermatids. The entry of free radicals contained in cigarette smoke will cause cell damage in the testes. The provision of red mulberry juice as an antioxidant can inhibit and is directly proportional to the decrease in sperm count due to exposure to cigarette smoke.

DISCLOSURE

Authors Contribution

All authors contributed equally to the study.

Disclosures

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