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The Effectiveness of Extract Basil Leaves on Motility and Morphology of Spermatozoa Mice Exposed with Cigarette Smoke

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ORIGINAL ARTICLE

The Effectiveness of Extract Basil Leaves on Motility and Morphology of Spermatozoa Mice Exposed with Cigarette Smoke

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ABSTRACT

Introduction: The incidence of infertility is still a problem in the world, including Indonesia (21.3%), and throughout Asia. Fertility in men is strongly influenced by several factors, one of them is smoking. Smoking can increase ROS, reduce antioxidants in semen, cause DNA damage, and morphological abnormalities of spermatozoa. Basil contains flavonoids which are rich in antioxidants, boron, and zinc to stimulate androgen hormones.
Methods: This research used eight weeks old Mus musculus, exposed with smoke of 20 cigarettes/day and was administered of extract basil leaves (*Ocimum sanctum* L.) in dose dependency (100 mg/kg, 200mg/kg, and 300mg/kg) for 4 weeks. We examined the motility and morphology of spermatozoa by microscope. This research consisted of 5 groups. In CS-, CSBL-2 and CSBL-3, it showed that the spermatozoa motility was more progressive than CS+ and CSBL-1.
Results: The morphology of spermatozoa in CS+ and CS- found different results with a significance value of 0.10 ($p < 0.05$) but the results of CSBL- 1, CSBL-2, and CSBL-3 were not significantly different from CS- and CS+ ($p > 0.05$).
Conclusion: There was an effect of giving basil leaves to increase motility and improvement of the morphology of spermatozoa that had been exposed to cigarette smoke especially in dose 300 mg/kgBB, but it was not significant.

Introduction

Infertility is still a problem in the world, especially in Asia. The prevalence of infertility is quite high, as high as 21.3% in Indonesia.[1] Mascarenhas, et al. added that there were 48.5 million productive age couples who could not have children.[2] Fertility in men is strongly influenced by several factors, one of which is due to an unhealthy lifestyle such as smoking.

Cigarettes are one of the addictive substances made from processed tobacco by adding additional ingredients in the form of cloves. Someone who smokes can do harm for individuals and society, known as active smokers and passive smokers. Every single cigarette burned contained 400 kinds of toxic chemicals, but there are three main toxic components contained in cigarette, namely carbon monoxide, nicotine, and tar.[3]

Several studies on the effects of chemicals from cigarettes indicate a disturbance in spermatogenesis. Smoking can increase free radicals (ROS) and reduce antioxidants in semen and can cause DNA damage through cellular DNA fragmentation and cause morphological abnormalities (head, neck, and tail) of spermatozoa. This is proven by an increase in the level of 8-OhdG (marker of DNA fragmentation) by 50% in male active smoker spermatozoa and 23% in passive smokers and lower compared to non-smokers.[3]

Free radicals are molecules that have unpaired electrons, thus they have high reactivity and tend to bind other electrons to produce new free radicals. This is what makes free radicals very dangerous.[4] Some of them are in the form of gases (CO, CO2, NO, NO2, and HCN), and particular nicotine, tar, metal, and phenol.[5] Previous study conducted by Batubara, et al. using animal models exposed to cigarette smoke for 30 days, obtained results that the more cigarettes consumed by the subjects, the normal motility of spermatozoa

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decreased significantly (20.60 ± 3.64).[6] Whereas the percentage of morphology of abnormal spermatozoa also increased significantly (68.00 ± 1.87). Antioxidants are substances to stabilize free radicals by complementing the lack of electrons possessed by free radicals, and inhibiting the occurrence of chain reactions that can cause oxidative stress.[7]

Various types of plants in Indonesia can be used as natural ingredients that can increase fertility. One of them is basil leave (*Ocimum sanctum* L.). According to Kuniawan, empirically basil is used as an aphrodisiac because it contains arginine which can strengthen sperm resistance and prevent sterility.[8] In addition to arginine, basil leaves also contain other secondary metabolites such as boron and zinc which function to release androgen (testosterone), and flavonoids which are antioxidants that play a role in protecting DNA and other important molecules from oxidation and damage, can improve sperm quality, and increase male fertility.9

Based on the previous explanation, this study wanted to further research on the effect of basil leaf extract on the morphology and motility of spermatozoa of male mice (Mus musculus) which were exposed to cigarette smoke.

Methods

Eight weeks old male Mus musculus were used. Water and food were given ad libitum and no replacement of animals was used in case of death. Cigarette smoking experiment was performed in a closed chamber once a day and administration of basil leaf extract for 28 days. The mice were randomly divided into 5 groups; CS- (without exposure to cigarette smoke and treatment of basil leaf extract), CS+ (without treatment of basil leaf extract but exposed to smoke of 5 cigarettes/day), CSBL-1 (exposed to smoke of 5 cigarettes/day + 100mg/kg basil leaf extract), CSBL-2 (exposed to smoke of 5 cigarettes/day + 200mg/kg basil leaf extract), and CSBL-3 (exposed to smoke of 5 cigarettes/day + 300mg/kg basil leaf extract).

Materials: male mice, basil leaves, 70% ethanol, cigarettes, standard food for mice, materials for preparing sperm analysis (NaCl solution, Eosin Y 1%), Aquades

NaCMC. Tools: Experimental animal cages, digital scales, Rotavapor tools, blenders, glass beakers, Maceration tubes, intragastric sonde, measuring pipettes, Handschon, cotton, filter paper, surgical instruments, glass jars with caps, glass objects, glass cover, light microscope, syringe, lighters, petri dish.

The experimental animals were anesthetized using a dose of 0.5 ml timolol and dissected using a dissecting kit to take the organs of the testis and cauda epididymis. The Cauda epididymis was separated by cutting the proximal part of the corpus epididymis and the distal vas deferens.

Cauda epididymis that had been separated was placed in a petri dish containing 1 ml of 0.9% NaCl. Cauda epididymis was cut until smooth and stirred, thus it was suspended with 0.9% NaCl to form a spermatozoa suspension. Furthermore, the suspension was observed using a light microscope.

Observation of spermatozoa motility was by using suspension of spermatozoa dropped on the object's glass by using a dropper pipette and the lid of the glass object using a closing glass. Observing the preparation under a light microscope with a magnification of 400 times the spermatozoa movements after that categorize the results.

Observation of spermatozoa motility was by using suspension of spermatozoa dropped on top of the object's glass to make smear preparations and dried in the air. The smear preparations were fixed with Bunsen fire to dry and must not evaporate, then colored with 1% Y eosin solution, left to dry. The preparations were washed with distilled water and dried. The preparation was observed under a light microscope with a magnification of 400 times to determine the morphology of 100 spermatozoa of mice. Calculate the percentage of normal and abnormal spermatozoa.

The data was recorded in Microsoft Excel 2016 and exported to SPSS Version 25 for statistical analysis. Quantitative variables were reported as amounts (%). The values were given as mean ± standard deviation (SD). The result were analyzed by Kruskal-Wallis test followed by Mann-Whitney test. The significance level used was 95% with a significance value of 5%. If the value of p showed $p < 0.05$, then it was considered statistically significant.

Results

The motility category of spermatozoa was: Motility A: spermatozoa movement was straight and fast (progressive), Motility B: spermatozoa movement was turned, difficult to progress straight/slow, Motility C: spermatozoa moved in place, Motility D: spermatozoa were silent or did not appear to move. Normal motility was the amount of Motility A and Motility B.

Based on the results of the study (Table 1), there were differences in spermatozoa motility between CS-, CS+, CSBL-1, CSBL-2, and CSBL-3. In CS-, CSBL-2, and CSBL-3, it showed that the spermatozoa motility was more progressive than CS+ and CSBL-1 which showed spermatozoa motility were silent or did not appear to move and several spermatozoa moved in place.

Table 1. Description of Spermatozoa Motility

Group	Motility of Spermatozoa
Cigarettes Smoke (-)	Spermatozoa movement was straight and fast (progressive)
Cigarettes Smoke (+)	Spermatozoa were silent or did not appear to move and several spermatozoa moved in place
Cigarettes Smoke + Basil Leaf 1	Spermatozoa moved in place and several spermatozoa movement was turned and slow.
Cigarettes Smoke + Basil Leaf 2	Spermatozoa movement was turned, difficult to progress straight or slow
Cigarettes Smoke + Basil Leaf 3	Spermatozoa movement was turned, and several spermatozoa movement was straight and fast (progressive)

From the results of data analysis, it showed that there was a decrease and increase in the average percentage of normal spermatozoa morphology after being exposed to cigarette smoke and given basil leaf extract. In Table 2, using the Kruskal-Wallis Test, it was found that the average percentage of spermatozoa morphology showed a significant difference value of 0.032 ($p < 0.05$).

Mann-Whitney Test was conducted between groups CS-, CS+, CSBL-1, CSBL-2, CSBL-3. In CS- and CS+, there were significant differences values ($p < 0.05$). Meanwhile, in CSBL-1, CSBL-2, CSBL-3, there were no significant difference value ($p > 0.05$) with CS- or CS+, but based on Table 2, it was found that the average percentage of normal spermatozoa morphology of CSBL-1, CSBL-2, and CSBL-3 increased. In CSBL-3, the average percentage was 75.50 ± 9.19 which was almost the same as the average CS- percentage. Thus, there was an effect of giving basil leaves extract to increase the average percentage of normal spermatozoa morphology, especially CSBL-3, but it was not significant.

Table 2. Mean and standard deviation value of normal morphology of spermatozoa

Group	Mean (%) ± SD
Cigarettes Smoke (-)	81.25 ± 3.10
Cigarettes Smoke (+)	55.17 ± 10.13
Cigarettes Smoke + Basil Leaf 1	58.00 ± 12.73
Cigarettes Smoke + Basil Leaf 2	62.75 ± 19.14
Cigarettes Smoke + Basil Leaf 3	75.50 ± 9.19

From the results of data analysis, it showed that there was an increase and decrease in the average percentage of abnormal spermatozoa morphology after being exposed to cigarette smoke and given basil leaf extract. In Table 3, using the Kruskal-Wallis Test, it was found that the average percentage of abnormal spermatozoa morphology showed no significant difference value of 0.068 ($p > 0.05$).

Based on Table 3, it showed that the average percentage of abnormal spermatozoa morphology CSBL-1, CSBL-2, and CSBL-3 decreased. In C3, the average percentage was 24.50 ± 9.19 which was almost the same as the average CS- percentage. Thus, there was an effect of giving basil leaves extract to decrease the average percentage of abnormal spermatozoa morphology, especially CSBL-3, but it was not significant.

Table 3. Mean and standard deviation valueof abnormal morphology of spermatozoa

Group	Mean (%) ± SD
Cigarettes Smoke (-)	18.75 ± 3.10
Cigarettes Smoke (+)	44.83 ± 10.13
Cigarettes Smoke + Basil Leaf 1	42.00 ± 12.73
Cigarettes Smoke + Basil Leaf 2	34.75 ± 22.20
Cigarettes Smoke + Basil Leaf 3	24.50 ± 9.19

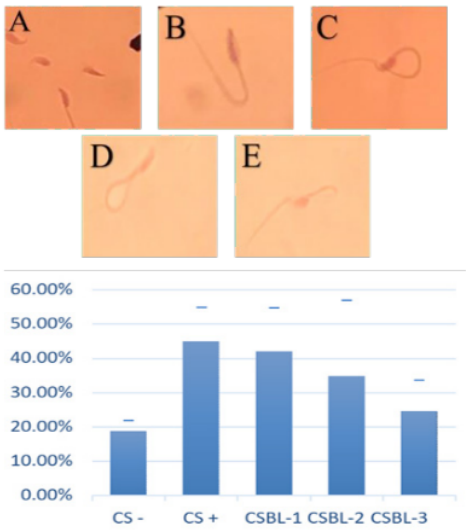

Figure 1. The results of representation of abnormal spermatozoa morphology in the microscope; A. Only head, B & D. curved like the letter C, C & E. 1 Head with 2 Tails. Bar: increase and decrease mean percentage of abnormal spermatozoa morphology. CS-: Cigarettes Smoke -, CS+: Cigarettes Smoke +, CSBL-1: Cigarettes Smoke + Basil Leaf 1, CSBL-2: Cigarettes Smoke + Basil Leaf 2, CSBL-3: Cigarettes Smoke + Basil Leaf 3.

Discussion

Correlation From the results of the study, it showed that the ethanol extract of basil leaves was effective against increased sperm motility of mice. Ethanol extract of basil leaves was effective against the increase of motility of mice because it contained flavonoids which could neutralize the high levels of free radicals generated by cigarette smoke. These results are in accordance with previous studies conducted by Desi, et al. where the flavonoid content of basil leaves could prevent a decrease in the percentage of spermatozoa motility of MSG-induced male Wistar rats (*Rattus norvegicus*).[10]

The results of the study also showed that the ethanol extract of basil leaves was effective against the increase in the average percentage of normal spermatozoa of mice but statistically significant results were not obtained. This result is slightly different from previous studies conducted by Murod which showed that the administration of basil leaves gave a significant increase in the morphology of spermatozoa.[11]

Cigarette smoke has a negative effect besides affecting the health of the respiratory system as well as disrupting the reproductive system in the form of a disruption of the process of spermatogenesis in the seminiferous tubules and influencing testosterone hormone levels. In the study of Batubara, et al. it showed that the increasing number of exposure to cigarette smoke was given in line with the decreasing percentage in abnormal motility and morphology of spermatozoa.[6]

The contents of each clove cigarette are 1-3 mg nicotine which are a toxoid alkaloid which can enter the brain barrier quickly in approximately 10 seconds and circulate to all parts of the body in 15-20 minutes at the time of the last suction. Nicotine in cigarette smoke can stimulate the adrenal medulla

to release catecholamines which can affect the central nervous system, thus the mechanism of feedback between the hypothalamus, anterior pituitary, and testicles is disrupted, as a result of the synthesis of testosterone hormone and the process of spermatogenesis. The effect of nicotine on sperm cells depends on the body's resistance to mice because each mouse has different sensitivity.[12]

Cigarette smoke can increase the production of ROS so much that oxidative stress occurs. Oxidative stress is an important factor in male fertility. ROS is a metabolite derived from oxygen which can modify cell function and endanger cell survival. Spermatozoa are susceptible to ROS because the plasma and cytoplasmic membranes contain large amounts of unsaturated fatty acids, thus lipid peroxides can be formed.[13]

Cigarette smoke has free radical compounds that can increase the amount of lipid peroxidation and cause damage or decrease in membrane spermatozoa integrity.[14] The lipid peroxide reaction will cause an increase in membrane fluidity, membrane integrity disorder, and inactivation of membrane bonds with enzymes and receptors.[15] This will cause inhibition of nutritional needs and ATP in cells. If the supply of ATB is exhausted or decreases, the spermatozoa flagellum does not contract and does not move.[16]

The motility of spermatozoa can also decrease due to abnormalities of the spermatozoa. The progressive progress of the spermatozoa depends on the balance of the shape of the tail. Spermatozoa with abnormal morphology will inhibit the movement and balance the tail.[17] Protective effects on cells can be done by giving antioxidants before exposure.[18]

In the CS+ group, the average percentage of abnormal morphological numbers of spermatozoa increased and decreased after being given basil leaf extract. This was due to exposure to cigarette smoke caused increased oxidative stress which affected the process of spermatogenesis, the quality of semen, and changes in testosterone levels which caused the formation of abnormal spermatozoa morphology.[17] In addition, in the study of Faranita, it is said that high free radicals can damage the mitochondrial membrane and DNA integrity in the spermatozoa nucleus.[19] This can induce cell apoptosis which causes changes in the morphology of spermatozoa during spermatogenesis.

Basil is a herbal plant that has high antioxidant. Basil contains flavonoids which are antioxidants that play a role in protecting DNA and other important molecules from oxidation and damage, and can improve sperm quality, thus it can increase male fertility.[9] Flavonoids are phenolic compounds that act as free radical scavengers. Flavonoids will push hydrogen ions, thus they can neutralize the toxic effects of these free radicals and increase the normal motility and morphology of spermatozoa.[18, 20]

Aside from flavonoids, basil also contains arginine. Arginine is a non-essential and polar amino acid that is indispensable in protein synthesis and has an important role in the body's immune system and cellular immunity. According to Srivastava, et al. arginine also plays an active role in the process of spermatozoa formation.[21] This is supported by previous study by Mahendra and Setyo Kurniawan that said arginine in basil leaves increases the motility and concentration of spermatozoa, thus it can strengthen sperm resistance and prevent sterility.[22, 23]

The results of this study indicated that CSBL-3 had more progressive spermatozoa motility and a lower percentage of abnormal spermatozoa (24.50 ± 9.19) than CSBL-1 and CSBL-2. This was due to in CSBL-3 (300 mg/kg), the antioxidant of basil leaf extract was quite optimal in protecting against cell membrane damage, increasing motility, and decreasing the abnormal morphology of spermatozoa in mice.

Conclusion

Based on the results of the analysis and discussion of this study, it can be concluded that the effectiveness of extract basil leaves on motility and morphology of spermatozoa mice exposed with cigarette smoke showed no significant effect, but on an average percentage there was an increase in motility and a decrease in the abnormal morphological amount of spermatozoa in mice.

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Conflict of Interest

The authors declared no conflict of interest in the conduct and reporting of this study.

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