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Effect of Temulawak *(Curcuma xanthorrhiza)* Extract on Sperm Counts, Morphology, and Motility of Nicotine-Induced Mice

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ABSTRACT

Background & Objective: Infertility is a health problem that affects 72.4 million couples worldwide. Infertility can occur as a result of an unhealthy lifestyle, such as a smoking habit. Cigarettes contain nicotine, a toxic compound that can trigger oxidative stress and eventually decrease fertility. Temulawak is a medicinal plant that is rich in antioxidants and anti-inflammatory properties. It has been used as a traditional remedy and is believed to improve fertility. We aim to study the effect of temulawak extract on the morphology, motility, and sperm counts of nicotine-induced mice.

Materials & Methods: A total of 20 mice were divided into four groups (n = 5): control normal (K0); nicotine-induced (K1); and nicotine-induced treated with two different concentrations of temulawak extract, 4 mg/20 g body weight (P1) and 8 mg/20 g body weight (P2). The mice were given nicotine and temulawak for 28 days and then killed by neck dislocation. Both testes and vas deferens were collected for sperm analysis.

Results: Mice treated with nicotine (K1) showed lower sperm counts *($p=0.012$)* and motility *($p<0.001$)* compared to mice in the control normal (K0) but had no difference in morphology. Treatment with temulawak (4 mg/20 g body weight) on nicotine-induced mice (P1) significantly increased sperm motility *($p< 0.001$)*. A double dose of temulawak extract (P2) resulted in significantly higher motility and sperm counts than in group K1.

Conclusion: Temulawak treatment (8 mg/20 g body weight) on nicotine-induced mice can significantly improve motility and sperm counts.

Keywords: Nicotine, Temulawak, Spermatozoa, Infertility

Introduction

Infertility is a problem that affects approximately 72.4 million couples worldwide, with 40% of infertility problems occurring in men (1, 2). Infertility can impact a man's income, psychology, and social relationships, all of which may cause long-term trauma and depression (3, 4). Specifically, infertility in men is often associated negatively with masculinity, which causes a lack of confidence and increased anxiety (5).

Smoking is a known risk factor for many diseases. Indonesia has the highest prevalence of smokers globally, with 33% of fifteen-year-old children being active smokers (6). A cigarette contains tar, carbon monoxide, *tobacco specific-nitrosamine,* B-a-P *(Benzo-a-pyrene)*, nicotine, and pesticide residue. Nicotine can trigger the production of radical molecules that can damage DNA and the lipid membrane of spermatozoa. The radical superoxide (ROS) and hydrogen peroxide (H_2O_2) can break the plasma membrane of spermatozoa and inhibit ATP production, which is vital for spermatozoa movement (7). As a result, men who smoke tend to have low sperm counts, abnormal spermatozoa motility and morphology, and low testosterone levels, leading to infertility. Furthermore, nicotine can induce the production of pro-inflammatory cytokine-like IL – 1 β, TNF α, and MCP-1 (8–10). It also inhibits Gonadotropin-Releasing Hormone (GnRH) secretion. As a result, the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) do not produce enough testosterone in spermatogenic cells to maintain the spermatogenic process in seminiferous tubules (9, 11, 12).

Temulawak *(Curcuma xanthorrhiza* Roxb.*)* is a traditional medicinal plant found easily in Indonesia.

Temulawak is well known for its benefits as an anti-inflammatory, antioxidant, diuretic, anti-cancer, anti-hepatotoxic, anti-dysmenorrhea, anti-spasmodic, anti-leucorrhoea, anti-bacterial, and anti-fungal drug (13). The antioxidant effect of temulawak in the binding of free radical molecules has been reported as better than that of vitamin E and beta carotene (9, 14). Moreover, the curcumin compound in temulawak can influence the androgenesis process by increasing testosterone and LH levels. It also increases the FSH level, making Sertoli cells produce higher germinal cells and androgen binding proteins (9). Besides their antioxidant effects, the curcumin and xanthorrhizol in temulawak also work as an anti-inflammatory, which decreases the IL – 1β, IL-6, and TNF α (9, 15). However, the use of temulawak to protect the quality of sperm against the effects of nicotine has not been studied so far. Therefore, this study aims to investigate the effect of temulawak extract on sperm quality of nicotine-induced mice.

Materials and Methods

Chemicals

Ethanol 97% (MERCK), nicotine (RTS Vapes, USA)

Extract Preparation

Temulawak extract was made by macerating 200 g of temulawak (*Curcuma xanthorrhiza* Roxb.) powder with two liters of 97% ethanol. The mixture was filtrated and evaporated for four days to get a thick liquid sample of temulawak extract. The extract was given orally to the mice.

Study Design and Animal Grouping

The study adopted the post-test-only control group design. The ethic for this research was declared on June 19, 2020, by the Ethical Committee (KEPK) Faculty of Medicine Diponegoro University Semarang, with an approval number of 60/EC/H/FK-UNDIP/VI/2020.

A total of 20 healthy male BALB/C mice aged six to eight weeks with a weight range of 20 – 30 g and no physical abnormalities were obtained from Semarang State University. The animals were kept at room temperature under controlled environmental conditions with alternating 12 hours of light and dark. The mice were acclimatized for a week before the beginning of the study. Then, the mice were randomly divided into four groups (n=5): the negative control group (K0), the positive control group (K1), and the experimental groups (P1 and P2). The positive control group was given nicotine (2 mg/kg BW) and the experimental groups were given nicotine (2 mg/kg BW) plus temulawak extract (4 mg/20 g body weight [P1] and 8 mg/20 g body weight [P2]) for 28 consecutive days. The nicotine was given by inhalation, and the temulawak extract was given orally. The mice were terminated on the 29th day, and then both the testis and vas deferens were collected to get a semen sample.

Specimen Collection and Microscopic Examination

To check the sperm quality of each group, the sperm inside the vas deferens was taken out and put on a petri dish containing 0.5 mL of NaCl solution. The suspension was gently shaken to make it homogeneous. This suspension was used to analyze sperm parameters, including morphology, motility, and sperm counts.

The sperm morphology sample preparations were analyzed by eosin staining. The stained smear was examined under a microscope with 400x magnification. Sperm morphology is said to be normal if it has a single head, neck, and tail. The motility of the sperm was determined by placing a drop of sperm in a glass chamber. It was then analyzed under a microscope with 100x magnification for three fields. It is considered normal if the sperm has progressive motility. The sperm counts were estimated by diluting the suspension and transferring it to the *Neubauer improved* chamber and allowing it to settle for ten minutes. The sperm on the four corners of the central square were calculated under 400x magnification.

Statistical Analysis

The data were analyzed using SPSS 21 program. The data normality and homogeneity were determined with Shapiro-Wilk and Levene tests. Since the data were normally distributed *($p > 0.05$)*, the hypothesis was tested using one-way ANOVA followed by a post hoc test, and both will conclude as significant if $p<0.05$.

Results

As shown in Table 1, there was a decrease in the percentage of normal morphology, progressive motility, and sperm counts of the nicotine-treated group compared to those of the control normal (K1 vs K0). Giving temulawak extract to the nicotine-induced mice (groups P1 and P2) increased normal morphology, progressive motility, and sperm counts compared to those of the control nicotine group (K1). The difference in the percentage of normal morphology spermatozoa was not significant. In contrast, the differences in the progressive motility and sperm counts were significant *($p < 0.01$ and $p = 0.007$).*

Table 1. The sperm characteristics of male mice in experimental groups.

Group	Normal morphology (%)	Progressive motility (%)	Sperm counts (x 10^3 unit)
Control normal (K0)	93.6 ± 2.9	47.98 ± 6.48	630± 69.93

Group	Normal morphology (%)	Progressive motility (%)	Sperm counts (x 10^3 unit)
Control nicotine (K1)	86.4 ± 5.2	6.64 ± 7.05	415± 85.88
Nicotine-temulawak (4 mg/20 g BW) (P1)	88.6 ± 4.9	36.62 ± 10.25	495± 83.67
Nicotine-temulawak (8 mg/20 g BW) (P2)	89.6 ± 7.19	41.96 ± 13.45	615 ± 128.21
p	0.218	<0.001*	0.007*

Values are expressed as means ±SD. * : Significant if $p<0.05$.

Post hoc tests were performed to compare the experimental groups. From Table 2, it is clear that administering nicotine lowered the progressive motility of the sperm compared to that of the control normal *($p<0.001$).* Giving temulawak extract resulted in higher progressive motility compared to that of the nicotine-administered group *($p=0.001$* and *$p<0.001$).* In addition, providing 8 mg/20 g BW temulawak extract (P2) improved the progressive motility, but it did not reveal any significant effect when compared with the P1 group.

Table 3 shows that nicotine results in lower sperm counts than those of the control group *($p=0.012$).* On the other hand, giving temulawak extract improved the sperm counts compared to those of the control normal group (K0). Moreover, providing 8 mg/20 g BW of temulawak extract resulted in significantly higher sperm counts compared to those of the nicotine control group *($p=0.019$),* but there was no significant result when compared to the P1 group.

Table 2. Comparison of progressive motility between each group

	K0 (control normal)	K1 (control nicotine)	P1 Nicotine-temulawak (4 mg/20 g BW)	P2 Nicotine-temulawak (8 mg/20 g BW)
K0 (control normal)	-	<0.001*	0.288	0.763
K1 (control nicotine)	<0.001*	-	0.001*	<0.001*
P1 (Nicotine-temulawak (4 mg/20g BW))	0.288	0.001*	-	0.821
P2 (Nicotine-temulawak (8 mg/20 g BW))	0.763	<0.001*	0.821	-

*: significant, $p<0.05$

Table 3. Comparison of sperm counts between each group

	K0 (control normal)	K1 (control nicotine)	P1 Nicotine-temulawak (4 mg/20 g BW)	P2 Nicotine-temulawak (8 mg/20 g BW)
K0 (control normal)	-	0.012*	0.150	0.994
K1 (control nicotine)	0.012*	-	0.553	0.019*
P1 (Nicotine-temulawak	0.150	0.553	-	0.226

	K0 (control normal)	K1 (control nicotine)	P1 Nicotine-temulawak (4 mg/20 g BW)	P2 Nicotine-temulawak (8 mg/20 g BW)
(4 mg/20 g BW))				
P2 (Nicotine-temulawak (8 mg/20 g BW))	0.994	0.019*	0.226	-

*: significant, $p<0.05$

Discussion

This experimental study showed that giving temulawak extract, both 4 mg/20 g body weight and 8 mg/20 g body weight, to nicotine–treated mice markedly improves sperm quality. The temulawak could improve sperm quality due to its curcumin and xanthorrhizol compounds, which are known for their antioxidant and anti-inflammatory properties.

Several documents have reported the benefits of curcumin and xanthorrhizol compounds. Curcumin works by lowering the toxicity effect of nicotine in spermatogenic cells by producing more ROS scavenging molecules to bind the free radicals **(16–18)**. Both curcumin and xanthorrhizol inhibit IL – 1β, IL-6, TNF alpha, and MCP 1. Moreover, they also suppress the increase of COX-2 that usually results from the presence of nicotine **(9, 15)**. Additionally, curcumin increases the total antioxidant capacity in seminal plasma, which is closely related to male fertility, to provide a suitable environment for sperm swimming **(17)**. The curcumin also improves the androgenesis in the testis and increases the testosterone and LH levels to improve the spermatogenesis process **(9)**.

In our study, we also observed that giving nicotine by inhalation significantly lowered sperm quality in mice. Results from this study are in line with the previous research. Nicotine significantly reduced the testosterone level, sperm counts, viability and motility of sperm, testis weight, and seminiferous tubules diameters in the nicotine (2.5 mg/kg body weight) treated group compared to those of the control (normal saline) **(19)**. Another study showed that giving nicotine in graded doses (0.5 mg/kg body weight to 4 mg/kg body weight) could lower the percentage of normal morphology of sperm and sperm motility **(7)**. However, the decrease in sperm morphology was not significant, which was perhaps due to an incomplete spermatogenesis cycle. The research was conducted in 28 days, while complete spermatogenesis in mice takes 35 days **(20)**. Moreover, previous research mainly used intraperitoneal injection for the nicotine administration to control the exact dose for each animal, while our research used inhalation. Intraperitoneal injection has a slower absorption time that results in a higher concentration of nicotine inside the body **(21)**.

Our study is in line with previous research. Intraperitoneal (IP) injection of 0.5 ml/kg body weight nicotine for 28 days significantly reduced sperm counts and the spermatozoa's motility and normal morphology. Curcumin supplementation (10 mg/kg body weight, 30 mg/kg body weight, and 60 mg/kg body weight) to the nicotine-treated mice increased the sperm counts, motility, and normal morphology significantly **(16)**. Another study showed that giving 4 mg/kg BW of nicotine via IP injection for eight weeks could significantly reduce testis weight, epididymis, seminal vesicles, ventral prostate, sexual hormones, antioxidant, and spermatozoa concentration. A significant increase was also observed in the concentration of cytokine, pro-inflammatory mediators, superoxide anions, lipid peroxide, dead sperm, and abnormal morphology of spermatozoa. However, giving curcumin prevents these degenerative changes caused by nicotine **(9)**.

Conclusion

Based on the results of this study, giving temulawak extract improves the sperm parameters in nicotine-treated groups. The most effective improvement was observed in the dose of 8 mg/ 20 g BW.

Acknowledgments

None.

Conflict of Interest

The authors declare no conflicts of interest.

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