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Original Article

Evaluation of the Protective Role of Marigold Leaf Extract Ethanol on FSH Levels in Male Laboratory Mice Exposed to Cigarette Smoke

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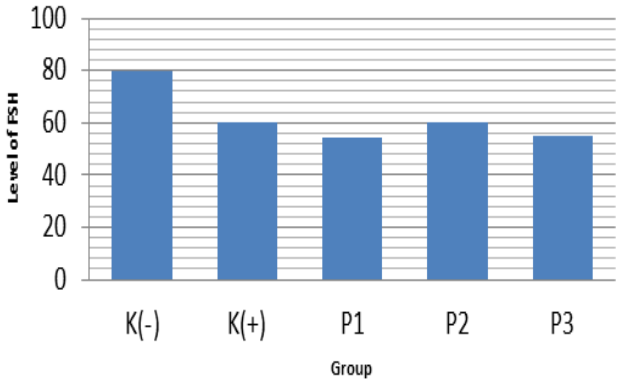
ABSTRACT

Cigarette smoke produces particles such as nicotine, tar, and cadmium which can damage male germ cells and interfere with the hypothalamus, pituitary, and testicles which cause spermatogenesis disorders. The study aimed to analyze the differences in FSH levels in each group of mice (Mus musculus) exposed to cigarette smoke by administering ethanol extract from marigold leaves (*Tegetes erecta*). The laboratory experimental research method used a Randomized post-test-only control group design with exposure to one cigarette smoke 1 cigarette per day and administration of ethanol extract of marigold leaves (*Tegetes erecta*). The grouping of research samples used simple random sampling in 5 groups with experimental mice (*Mus Musculus*) males aged 8-12 weeks and weighing 20-25 grams which were divided into 5 groups, namely K(-) (placebo), K(+) (smoked cigarettes and placebo), P1 (cigarette smoke and marigold leaf extract dose 0.25 g/kg-BW), P2 (smoked cigarettes and marigold leaf extract dose 0.50 g/kg-BW), and P3 (smoked cigarettes and marigold leaf extract dose 1, 0 g/kg-BW). FSH levels were examined using ELISA. The results of the analysis of examining FSH levels showed an average of K(-): 0.80 mIU/ml, K(+): 0.60 mIU/ml, P1: 0.54 mIU/ml (dose 0.25 g/kg-BW), P2:0.60 mIU/ml (dose of 0.50 g/kg-BW), and P3:0.55 mIU/ml (dose of 1.0 g/kg-BW) with p-value ≤0.05. In conclusion, there was a significant effect of the administration of ethanol extract of marigold leaves (*Tegetes erecta*) on the FSH levels of mice exposed to cigarette smoke.

GRAPHICAL ABSTRACT



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Introduction

Cigarettes are processed products from the Nicotiana tobacum and Nicotiana rustica plants where the combustion products can damage male germ cells [1]. Cigarette smoke exposure is among the external factors that can induce oxidative stress in the process of spermatogenesis [2]. Cigarette smoke will produce several harmful gas elements such as ammonia (NH_2), carbon monoxide (CO), carbon dioxide (CO_2), nitrogen oxides (NO), nitrogen dioxide (NO_2), and hydrogen cyanide (HCN) [3]. The chemicals present in the systemic cause Reactive Oxygen Species (ROS) to increase. Increased ROS and not balanced with antioxidants can cause oxidative stress [4, 5]. Oxidative stress in the blood will cause damage to the hypothalamus and affect the pituitary gland which functions in the reproductive system [6]. Oxidative stress will suppress FSH production [7]. The combustion results from the chemical content of cigarette smoke will enter the blood vessels through the lungs and then be forwarded to the brain and stimulate the adrenal medulla to release catecholamines [8]. Excess catecholamines can affect the central nervous system which causes a feedback mechanism between the hypothalamus, and anterior pituitary so that the testes will be disrupted [9]. Research by found 56.23% of male smokers with infertile cases [10]. Also explains that oxidative stress from cigarette smoke can cause infertility in men. Spermatogenesis is strongly influenced by the role of FSH, namely by stimulating Sertoli cells to produce ABP, inhibin synthesis, glycoprotein synthesis, and energy metabolism, and then produces lactic acid which is a source of energy for spermatogenic cells reported that the laboratory reference for normal adult men is (FSH 1.38-9.58 mIU/ml) [11-13]. Marigold (*Tagetes erecta*) or often called the kotok flower as shown in Figure 1 is a plant that grows wild on plantations or around the house [14] indicated that marigold is a type of medicinal plant that contains flavonoids, polyphenols, and two types of carotenoids, namely carotenes, and xanthophylls [15, 16]. Flavonoids present in marigold leaves have been shown to be free radical scanning caused by several factors, one of which is exposure to cigarette smoke [17, 18] showed that flavonoids are antioxidants that can reduce lipid oxidation and suppress the formation of free radicals [17] reported that total polyphenols and flavonoid content in marigolds were 74.8 mg TAE/g and 85.6 mg RE/g. Providing antioxidant supplements can be beneficial in improving sperm function and DNA integrity [19]. Hence, the scientists sought to carry out an experiment on mice exposed to cigarette smoke to evaluate the efficacy of marigold leaf extract [20, 21]. The objective was to determine the impact of marigold leaf ethanol extract, administered at doses of 0.25 g/kg-BW, 0.50 g/kg-BW, and 1.0 g/kg-BW, on FSH levels in cigarette smoke-exposed mice. It is important to note that this particular investigation has not been previously documented in existing studies.

Figure 1: Marigold plant (*Tegetes erecta*)

Materials and Methods

Research design

This research is a laboratory experiment with a Randomized post-test-only control group design with exposure to one cigarette smoke 1 cigarette per day and administration of ethanol extract of marigold leaves (*Tegetes erecta*). Grouping of research samples using simple random sampling. The experimental animals used were mice (*Mus musculus*) with the criteria of male mice aged 8-12 weeks and weighing 20-25 grams as many as 8 samples per group. The group consisted of 5 groups, namely 2 control groups and 3 treatment groups. The control group (-) was given a placebo, and the control group (+) was exposed to kretek cigarette smoke and a placebo.

Meanwhile, treatment groups 1, 2, and 3 were exposed to kretek cigarette smoke and were given marigold leaf ethanol extract in gradual doses, namely P1 0.25 g/kg, P2 0.50 g/kg, and P3 1.0 g/kg. This study consisted of independent variables, namely Marigold Leaf Ethanol Extract with a P1 dose of 0.25 g/kg BW, P2 0.50 g/kg BW, and P3 1.0 g/kg BW, the dependent variable was FSH, the control variable was 1 cigarette per day in the modified cage. The experimental animals were adapted for 7 days after which the positive control group was only exposed to cigarette smoke and the treatment group was exposed to cigarette smoke and given marigold leaf ethanol extract P1 0.25 g/kg-BW, P2 0.50 g/kg-BW, and P3 1.0 g/ kg for 35 days. The production of marigold leaf ethanol extract was conducted at the Pharmacology Laboratory of the Faculty of Veterinary Medicine, Airlangga University. The exposure to cigarette smoke was performed in a modified cage with a connecting hole between the pump and the cage. The administration of marigold leaf ethanol extract was carried out using a mouse probe, with doses of 0.25 g/kg BW P1, 0.50 g/kg BW P2, and 1.0 g/kg B3 P3, given every morning for 35 days, based on the mice's stomach volume of 0.5 ml

Examination of FSH levels was carried out in the morning at 10.00 a.m. Prior to the collection of FSH serum, the mice were fasted for 4 hours prior to anesthesia. Mice's blood was taken from the heart with a disposable syringe of 1-2 ml and put into a test tube then put into a centrifuge to separate serum and blood cells. The serum taken was then examined in the laboratory to determine FSH levels using the ELISA method.

Data processing and analysis

The obtained data is arranged in a table and tested for normality with the Shapiro-Wilk test. If the distribution is normal, the variance homogeneity test is continued. The results of the homogeneity test of the variant if the variant is homogeneous are followed by a One-way ANOVA test at a significant level of p-value ≤ 0.05 and continued with the LSD test [22].

Ethical clearance

Approval for this study has been obtained from the Animal Ethics Commission of the Faculty of Veterinary Medicine at Airlangga University, with the ethical reference no. 2.KEH.092.08.2022.

Results and Discussion

According to Table 1, the normality test results for FSH levels in mice (Mus musculus) indicated that K (-), K (+), P1, P2, and P3 followed a normal distribution (p-value ≥0.05).

According to Table 2, the homogeneity test results indicate a p-value ≥0.05, leading to the conclusion that the data demonstrates homogeneity.

According to Table 3, the results of the One-way ANOVA test yielded a value of 0.001 (p-value ≤0.05). Hence, it can be inferred that the impact of various doses of marigold leaf extract (*Tegetes erecta*) on FSH levels in mice exposed to cigarette smoke demonstrated significantly distinct outcomes.

According to Figure 2, it shows that the average FSH level in *Mus musculus* without treatment K(-) has the highest FSH level and P1 has the lowest FSH level.

K(-): A group of mice without exposure to cigarette smoke and without being given ethanol extract from marigold leaves.

K(+): A group of mice that were only exposed to cigarette smoke without being given marigold leaf ethanol extract.

P1: The first treatment group was subjected to cigarette smoke exposure and administered marigold leaf ethanol extract at a dose of 0.25 g/kg BW.

Effect of administration of ethanol extract of marigold leaves (Tegetes erecta) on FSH levels of mice (Mus musculus) exposed to cigarette smoke

The One-way ANOVA test on FSH levels yielded significant results with a p-value ≤0.05. The measurement of FSH levels in the K(-) group was 0.80, while in the K(+) group, it was 0.60. These findings indicate a decrease in FSH levels in the K(+) group, which experienced cigarette smoke exposure. This decline in FSH levels aligns with Guyton's study (2000), which demonstrated that the presence of free radicals in cigarette smoke

Table 1: Normality test (Shapiro Wilk) for FSH levels

Group	*P*-value	Distribution
K(-)	0.135	Normal
K(+)	0.362	Normal
P1	0.667	Normal
P2	0.895	Normal
P3	0.077	Normal

Table 2: Homogeneity test for FSH levels

Variable	Assumption	*P*-value	Conclusion
Kadar FSH	Homogenous	0.062	Homogenous

Table 3: One-way ANOVA test for FSH levels

Group	Mean±Std	*P*-value
K-	0.80±0.18 mIU/ml	0.001*
K+	0.60±0.11 mIU/ml	
P1	0.54±0.08 mIU/ml	
P2	0.60±0.11 mIU/ml	
P3	0.55±0.10 mIU/ml	

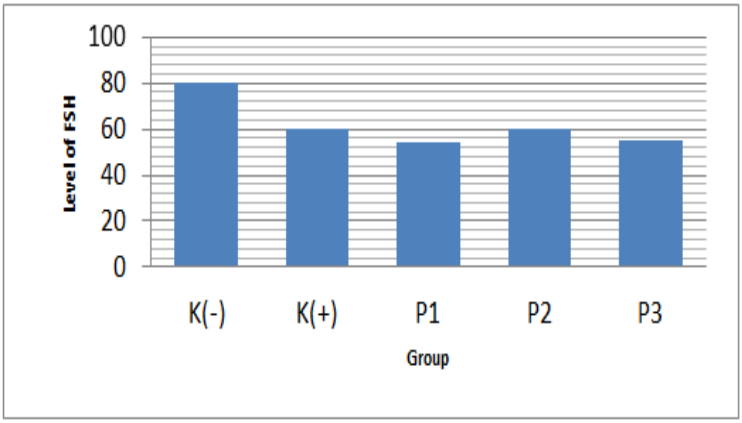

Figure 2: Average FSH levels

Table 4: LSD test for FSH levels

Group	K(-)	K(+)	P1	P2	P3
K(-)	-	0.003*	0.314	0.945	0.375
K(+)	0.003*	-	0.000*	0.002*	0.000*
P1	0.314	0.000*	-	0.345	0.902
P2	0.945	0.002*	0.348	-	0.413
P3	0.375	0.000*	0.902	0.413	-

can lead to the peroxidation of unsaturated fatty acids and subsequently cause oxidative stress [23].

Nicotine contained in cigarette smoke can stimulate the adrenal medulla to release catecholamines thereby increasing the hypothalamic pulse and stimulating the release of GnRH from the hypothalamus to the anterior pituitary [24]. Catecholamines can increase the frequency of GnRH stimulation resulting in decreased gonadotropins (LH and FSH) [25]. In P1, the dosage of 0.25 g/kg-BW exhibited an average of 0.54, while in P2, the dosage of 0.50 g/kg-BW resulted in an average of 0.60 [18].

Demonstrated that an increase in P2 with a dosage of 0.50 g/kg-BW was associated with marigold (*Tegetes erecta*) leaf extracts containing flavonoids, which have the ability to reduce lipid oxidation and the formation of free radicals that can disrupt the hormonal system. Nevertheless, this discovery contradicts the research carried out by [26], which found a correlation between higher FSH levels and a lower sperm count per milliliter, suggesting a decline in spermatogenic function. The study also highlighted that recent advancements in clinical genetics have identified FSH gene polymorphisms that can result in mutations in the FSH receptor and androgen receptor, thereby influencing the interpretation of hormone test outcomes. It was observed that certain patients exhibited heightened FSH receptor sensitivity despite relatively low FSH concentrations. The FSH assessment still needs to be considered in assessing male fertility [26]. Laboratory clinical parameters such as testicular volume, histopathological results, and FSH levels have a diagnostic accuracy of only 74% [27, 28] explained that an increase in serum FSH levels accompanied by low testosterone (<300 ng/dl) indicates damage to the testicles. Congress of the Asia Pacific Initiative on Reproduction (2016) also states that an increase in serum FSH levels and a decrease in testosterone are associated with abnormal spermatogenesis [29]. The value of FSH levels if > 10.36 mIU/ml has a sensitivity of 82.1% with a specificity of 79.5% for azoospermia [12]. Similar studies also found that increasing FSH levels could adversely affect the reproduction of male mice [30]. The American Society for Reproductive Medicine and Society for Male Reproduction and Urology found that an FSH level that is twice as high indicates spermatogenic failure [31]. At P3 (dose 1.0 g/Kg-BW), an average of 0.55 was found. At P3, there was a decrease again; this needs to be suspected of the presence of other ingredients besides flavonoids in the ethanol extract of marigold leaves [16]. Explained that there are several country markets that sell flowers from the marigold plant to be used as mosquito repellent and vegetable insecticides [28]. Also explained that low gonadotropin levels below 1.2 mUI/ml are common in men with NOA (non-obstructive azoospermia). It occurs due to the suppression of spermatogenic by excessive administration of exogenous androgens [32]. In addition, decreased FSH levels can occur due to other factors such as the presence of congenital GnRH, for example, Kallman's syndrome, or the presence of identified lesions in the hypothalamus. Human reproduction is regulated by the combined action of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) on the gonads. Although FSH is predominantly used in female reproduction, especially in women undergoing assisted reproductive techniques to stimulate multi-follicular growth, its efficacy in men with idiopathic infertility is not clearly demonstrated [33]. An increase in FSH levels after the administration of Marigold leaf extract may indicate an interaction between the components of the extract and the reproductive hormone system. Marigold leaf extract is believed to contain active compounds that can influence the regulation of reproductive hormones [34]. The elevation of FSH levels after Marigold leaf extract administration can have a stimulating effect on the ovaries [35]. FSH is an important hormone in regulating the growth of ovarian follicles and the secretion of estrogen hormones [36]. By increasing FSH levels, Marigold leaf extract may stimulate the follicles development in the ovaries and enhance the production of related reproductive hormones. However, the therapeutic benefits in men experiencing changes in sperm production despite normal serum FSH levels, remains unclear.

Conclusion

The administration of marigold leaf ethanol extract (*Tegetes erecta*) has an impact on the FSH levels of mice (*Mus musculus*) that have been exposed to cigarette smoke. However, it is not certain that the ethanol extract of marigold leaves (*Tegetes erecta*) has good benefits for spermatogenesis. It is necessary to examine other parameters such as testosterone, inhibin, testicular histopathology, hypothalamus, and pituitary.

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Authors' Contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

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