SOD2 and HIF-1α expression in rat ovaries (Rattus norvegicus) administered with forest bee honey (Apis dorsata) following physical stress

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SOD2 and HIF-1α expression in rat ovaries (*Rattus norvegicus*) administered with forest bee honey (*Apis dorsata*) following physical stress

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ABSTRACT

Introduction: Excessive physical activity can pose risks to various organs due to induced stress. This study aims to investigate the impact of the supplementation of wild bee honey on the expression of SOD2 and HIF-1α in rats undergoing the forced swimming test (FST) as a model of physical stress.

Methods: As many as 24 adult female rats were categorized into four groups: control (C) with FST only; (T1) FST and honey supplementation of 2 g/day; (T2) FST and honey supplementation of 4 g/day; and (T3) FST and honey supplementation of 6 g/day. The FST was conducted for 5 minutes daily over a period of 14 days. The collected ovaries were histopathologically prepared and subjected to immunohistochemical staining for SOD and HIF-1α proteins.

Results: The findings of this study revealed an increase in SOD2 expression in the group supplemented with honey. Similarly, HIF-1α expression exhibited a similar trend, with a decrease observed in the group receiving 6 g/day of honey. However, these three parameters did not demonstrate significant differences among them.

Conclusion: In conclusion, honey supplementation does not significantly influence the expression of SOD2 and HIF-1α in the ovarian follicles of rat models subjected to physical stress.

Keywords: Endogenous antioxidants, hypoxia, natural medicine, oxidative stress, physical activity.
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INTRODUCTION

Regular physical activity and exercise offer numerous health benefits but can also have implications for female fertility.[1] Engaging in exercise at appropriate levels promotes muscle contraction, improves blood circulation, and enhances oxygen supply.[2] Nonetheless, exercise is known to elicit stress responses in humans and animals, with the degree of stress influenced by exercise type, intensity, and duration.[3]

Intensive swimming, as a form of high-intensity physical activity, has significantly decreased the total number of ovarian follicles compared to moderate physical activity.[1] The hormone cortisol indicates stress in the body[4] and inhibits the synthesis of brain-derived neurotrophic factor (BDNF), which plays a protective role against malondialdehyde (MD) toxicity. MD is a marker of oxidative stress caused by reactive oxygen species (ROS).[5] Excessive ROS production can encompass oxidative stress and contribute to ovarian disorders.[6]

The body relies on the antioxidant defense system to eliminate surplus ROS and maintain ovarian homeostasis.[6] This defense system encompasses both enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx), play crucial roles in maintaining ROS balance.[7] Specifically, the three SOD enzymes (SOD1, SOD2, and SOD3) are activated in the presence of catalytic metals (Cu or Mn) and catalyze the conversion of $O_2^{\bullet-}$ to H_2O_2.[8]

Hypoxic conditions are strongly associated with heightened intracellular oxidative stress, specifically through increased ROS production at the mitochondrial respiratory complex III.[9] In the face of Hypoxia Inducible Factor-1α (HIF-1α) activation has long been recognized as a regulator of exercise-induced oxidative adaptation.[10,11] HIF-1 protein levels rise significantly during low oxygen concentrations.[11] HIF-1 activation facilitates glucose utilization and rapid ATP generation at the cellular level, benefiting proliferating cells and cells facing stressors like nutritional deficiencies and damage from physical, chemical, and mechanical causes.[11]

Indonesia, renowned for its rich biodiversity, boasts abundant natural resources with potential therapeutic applications for various ailments.[12] Forest honey, derived from the nectar collected by *Apis dorsata* bees, represents a variety of polyfloral honey indigenous to Indonesia.[13] Honey, a readily available natural substance, contains carotenoids, ascorbic acid, simple sugars, flavonoids, and phenolic acids.[14] Previous studies have explored the administration of

honey to animal models with various oxidative stress models. Under conditions of oxidative stress, honey's antioxidants act as scavengers of free radicals and have demonstrated an ability to enhance the production of SOD, CAT, and GPx and improve the liver's histological structure in rat models.[15,16] Additionally, administering honey to malnourished rats for five days increased SOD activity, anti-inflammatory cytokines, and regenerated ovarian tissue while decreasing MDA and pro-inflammatory cytokines.[13] This study aims to examine the effects of administering forest bee honey on the expression of SOD2 and HIF-1α proteins in the ovarian follicles of rats subjected to physical stress.

METHODS

Experimental animals

Female Wistar rats (*Rattus norvegicus*) weighing an average of 200 grams at three months were utilized as experimental animals. As many as 24 rats were randomly assigned to four treatment groups. The rats could acclimate for seven days with adequate food and water. During the study, the rats were kept in cages measuring 53 x 30 x 17 cm, furnished with wood chips as bedding, and housed in a room maintained at a humidity level of 50% and a temperature of 34 °C. To induce animal stress and simulate depression, the forced swimming test, as described by Porsolt,[17] was employed. The rats were subjected to daily swimming sessions lasting five minutes for a period of 14 days in a barrel filled with water, with the water level at approximately 2/3 of the barrel's height.

Experimental procedure

The rats were divided into four treatment groups: Group C underwent the forced swimming test (FST) only; Group T1 underwent FST and received 2 g/day of honey supplementation; Group T2 underwent FST and received 4 g/day of honey supplementation; and Group T3 underwent FST and received 6 g/day of honey supplementation. The FST was conducted for 14 days, and honey supplementation was administered orally via gavage. On the 15th day, the rats were sacrificed, and a laparotomy procedure was performed to collect the ovaries.

Tissue processing

The collected organs were processed for histological examination according to the following procedure:[18] the ovaries were fixed in 10% neutral buffered formalin. After that, the tissues were dehydrated using a series of alcohol gradients and cleared with xylene. Subsequently, the tissues were impregnated with molten paraffin at temperatures ranging from 56 to 60 °C. Thin serial sections measuring 3 µm in thickness were cut from the solid tissue blocks, washed, and mounted on glass slides.

Immunohistochemistry staining

After deparaffinization and rehydration, the tissue slides underwent a 10–15-minute incubation with hydrogen peroxide, followed by a 5-minute blocking step. Primary antibodies (SOD, 1:200; HIF-1α, 1:200) were then applied to the slides and incubated for 60 minutes, followed by incubation for 30 minutes with secondary antibodies. The slides were then treated with streptavidin peroxidase for 10 minutes, and DAB chromogen and substrate were added for a duration of 15 minutes. To conclude, counterstaining was performed using Mayer's hemalum solution.

Statistical analysis

The immunohistochemical protein expressions in ovarian follicles were recorded using a Nikon Eclipse Ci microscope at a magnification of 200x, and their interpretation was conducted utilizing the IRS scoring system. Positive expression was identified when a brownish-yellow color change, resulting from antigen-antibody binding in the granulosa cells of ovarian follicles, was observed. Five fields of view were examined, and the results were averaged. The obtained data were subsequently subjected to statistical analysis using the Kruskal-Wallis test in SPSS for Windows.

RESULTS

This study aims to assess the efficacy of *Apis dorsata* forest honey in alleviating ovarian disorders induced by physical stress by evaluating the expression levels of enzymatic antioxidant SOD2 and the key protein HIF-1α under hypoxic conditions.

Immunohistochemical analysis was performed to examine the expression of SOD2 and HIF-1α. The results of the SOD2 analysis revealed increased protein expression in groups T1 (16.06), T2 (17.81), and T3 (20.50) compared to the control group (C) without honey supplementation (11.63). However, these differences did not reach statistical significance ($p>0.05$), as shown in Table 1 and Figure 1.

Similarly, the analysis of HIF-1α expression demonstrated a slight increase in the T1 (17.75), T2 (18.63), and T3 (19.56) groups compared to group C (10.06). Nevertheless, the observed variances were not statistically significant ($p>0.05$), as denoted in Table 1 and Figure 2.

DISCUSSION

The forced swimming test, coupled with honey supplementation, demonstrated an increase in the expression of SOD2, with the highest response observed in the T3 group receiving a dose of 6 g/day of honey.

Table 1. Mean of SOD and HIF-1α expression in all treatment groups with no significant difference ($p>0.05$)

Dose Group (grams/rat)	Mean Rank	
	SOD	HIF-1α
0 (C)	11.63	10.06
2 (T1)	16.06	17.75
4 (T2)	17.81	18.63
6 (T3)	20.50	19.56
Asymp. Sig.	0.278	0.157

Notes: C refers to the control group receiving only the forced swimming test (FST); T1 represents the treatment group receiving FST and a daily dose of 2 g honey; T2 corresponds to the treatment group receiving FST and a daily dose of 4 g honey; T3 denotes the treatment group receiving FST and a daily dose of 6 g honey.

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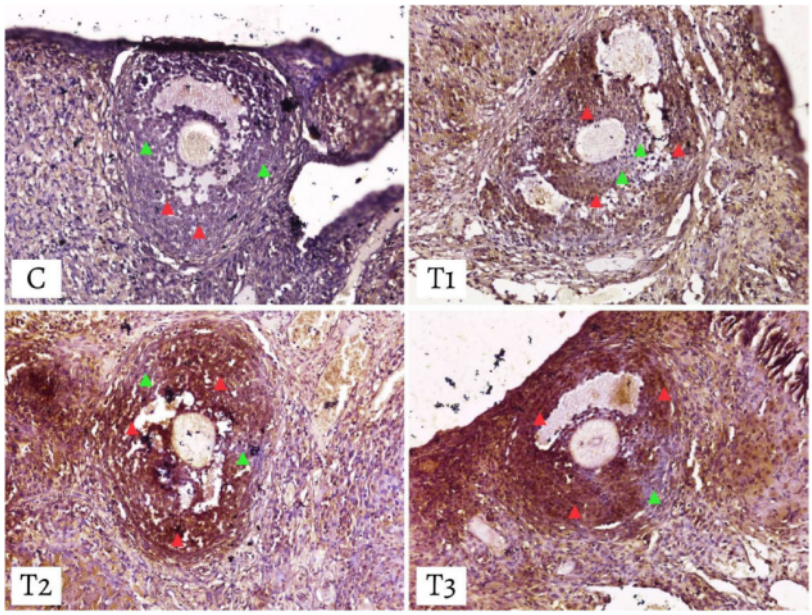

Figure 1. Immunohistochemical staining of SOD2 expression in hepatocytes of rats subjected to physical stress. The four groups depicted are: FST without honey (C), FST with a daily dose of 2 g honey (T1), FST with a daily dose of 4 g honey (T2), and FST with a daily dose of 6 g honey (T3). The green arrowheads indicate granulosa cells that did not exhibit SOD2 expression, while the red arrowheads indicate granulosa cells expressing SOD2. Notably, all groups exhibit brownish-yellow coloration indicative of protein expression, ranging from moderate to strong intensity. The T3 group demonstrates the strongest intensity among the groups. The image is magnified at 200x.

Similarly, the expression of HIF-1α in ovarian follicles exhibited an upward trend, reaching its peak in the T3 group (honey supplementation, 6 g/day). However, statistical analysis indicated that these findings were not significant ($p>0.05$), as depicted in Table 1. Figures 1 and 2 visually represent the immunohistochemical staining, illustrating the expression of SOD2 and HIF-1α.

Honey is known for its antioxidant properties, which are beneficial in mitigating cell damage caused by physical stress and reactive oxygen species (ROS). Additionally, previous studies have shown that honey can enhance the production of endogenous antioxidants, including GPx, catalase, and SOD.[15,16] However, in this study, the significant efficacy of honey supplementation was not established (Figure 2). This result could possibly be attributed to the high concentration of ROS, rendering the dosage used in this study inadequate to counteract the cellular damage. Consequently, careful consideration should be given to the duration and dosage of honey administration to ensure its optimal benefits to the body.

The immunohistochemistry findings depicted demonstrate an elevation in HIF-1α expression in the ovaries of rats subjected to the physical stress model. However, no significant differences were observed among the groups. From a physiological perspective, the existence of Hypoxia Inducible Factor-1α (HIF-1α) in the ovary is connected with the avascular state found in the dominant follicle.[19] As the antrum of the follicle expands, the distance between the oocyte and theca interna cells, responsible for oxygen distribution, increases, resulting in a hypoxic environment.[19] The activation of HIF-1α in cumulus cells intensifies during the luteinizing hormone (LH) surge and peaks at ovulation.[19-22] This activation initiates the production of the Vascular Endothelial Growth Factor (VEGF) to facilitate angiogenesis, supporting energy transport in the oocytes even under hypoxic conditions. Although honey supplementation at doses of 2 g/day, 4 g/day, and 8 g/day demonstrated a reduction in cellular damage and subsequent increase in HIF-1α activation compared to the non-supplemented group, the differences were not statistically significant. These findings suggest that the dosage and duration of honey administration in this study were insufficient to establish the preventive effects of honey supplementation on general ovarian damage in the physical stress model rats.

CONCLUSION

In conclusion, this study determined that the administration of *Apis dorsata* forest bee honey at the specified dosage did not result in a significant augmentation of SOD2 and HIF-1α expression in the ovaries of rat models exposed to physical stress. Future studies should consider using a more tailored honey dosage and incorporate additional research parameters, such as follicle count and the quantity and quality of ovulated oocytes, to obtain a comprehensive profile of ovarian activity.

ETHICAL CLEARANCE

This study has been approved by the Ethics Commission of the Faculty of Veterinary Medicine, Universitas Airlangga (No. 1.KEH.041.04.2022).

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CONFLICTS OF INTEREST

The author reports that there are no conflicts of interest in this work.

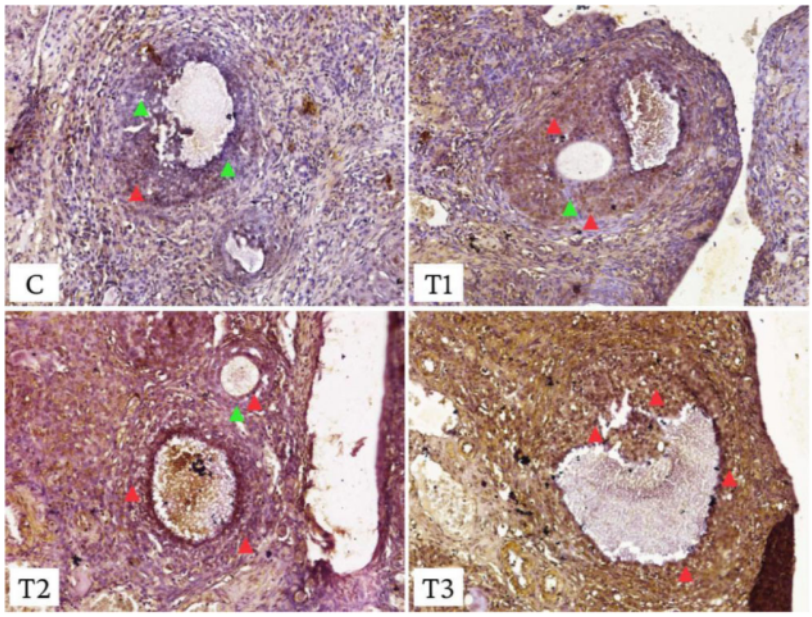

Figure 2. Immunohistochemical staining of HIF-1α expression in hepatocytes of rats subjected to physical stress. The image is magnified at 200x. The four groups shown are: FST without honey (C), FST with a daily dose of 2 g honey (T1), FST with a daily dose of 4 g honey (T2), and FST with a daily dose of 6 g honey (T3). The brownish-yellow colorations in the image represent protein expression, ranging from moderate to strong intensity, found in all groups. Notably, the strongest intensity is observed in the T3 group.

AUTHOR'S CONTRIBUTION

All authors contributed equally to this review article

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