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Answer for Reviewer 1 Comments:

Firstly, this paper is clear and well-organized. However, I may require some comments on the following issues.

1. Title: The title is easy to follow and has no mistakes.
   Thank you for the valuable comments.
2. Abstract: This section was well-written and easy to understand. In addition, the objective of the study and the state of the art of the study is clear. Furthermore, the keywords should be represented the study.
   Thank you for the valuable comments. We revised the keywords.
3. Introduction: Based on my review, I recommend that the authors should add various improvements, such as:
   - Hypothesis and objectives.
     Thank you for the valuable comments. We added the objectives in the introduction.
   - Add a statement or sentence about whether there are any similar studies that have been done before.
     Thank you for the valuable comments. We added the sentence about previous studies.
   - Add the important issues.
     Thank you for the valuable comments. We added the important issues in the introduction.

4. Material and Methods: This section should be a more detailed protocol or cite the protocol reference.
   Thank you for the valuable comments. We revised the material and methods and added more detailed protocol.
5. Results: All the part of this section is easy to follow and have no mistakes.
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6. Discussion: The discussion of the study is very comprehensive.
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8. References: The core references are acceptable, but I think need some improvements below:
   - Tacharina MR, Ansori ANM, Plumeriastuti H, Kusnoto , Kurnijasanti R, Hestianah EP. Beneficial effect of grinting grass (Cynodon dactylon) on the streptozotocin induced diabetes mellitus in the mice. Indian Veterinary Journal. 2020;97(4):35-38.
   - Husen SA, Winarni D, Salamun, Ansori ANM, Susilo RJK, Hayaza S. Hepatoprotective Effect of Gamma-mangostin for Amelioration of Impaired Liver Structure and Function in Streptozotocin-induced Diabetic Mice. IOP Conference Series: Earth and Environmental Science. 2019;217(1):012031.
   - Husen SA, Setyawan MF, Syadzha MF, Susilo RJK, Hayaza S, Ansori ANM et al. A novel therapeutic effects of sargassum ilicifolium alginate and okra (Abelmoschus esculentus) pods extracts on open wound healing process in diabetic mice. Research Journal of Pharmacy and Technology. 2020;13(6):2764-2770.
   - Ansori ANM, Susilo RJK, Hayaza S, Winarni D, Husen SA. Renoprotection by Garcinia mangostana L. pericarp extract in streptozotocin-induced diabetic mice. Iraqi Journal of Veterinary Sciences. 2019;33(1):13-19.
   - Hayaza S, Istiqomah S, Kuncoroningrat Susilo RJ, Inayatillah B, Ansori ANM, Winarni D et al. Antidiabetic Activity of Ketapang (Terminalia catappa L.) Leaves Extract in Streptozotocin-Induced Diabetic Mice. Indian Veterinary Journal. 2019;96(12):11-13.

   Thank you for the valuable comments. We added some citations from the mentioned references.

Answer for Reviewer 2 Comments:

I'm pleased to be among the team to review your paper. Although your work might have better used further characterizations such as ELISA and gene expression or varied different types of honey, it is still a good read. I hope you look into that in further studies.

Best regards

Thank you very much for the valuable comments. In future studies, we would like to use further characterizations using more advanced methods and compare the pharmacological activities of different varieties of honey.

Answer for Reviewer 3 Comments:

This manuscript will be considered acceptable after being revised based on the following comments:

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   Thank you for the valuable comments. We have mentioned the ethical clearance and the certificate number in the materials and methods section of the manuscript.
3. References are not according to the rules of the journal.
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4. Acknowledgments.
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Effect of Forest Bee Honey (*Apis dorsata*) Supplementation on Expression of HIF-1α, SOD, and TNF-α in Rats (*Rattus norvegicus*) Liver Exposed to Physical Stress

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Effect of Forest Bee Honey (*Apis dorsata*) Supplementation on Expression of HIF-1α, SOD, and TNF-α in Rats (*Rattus norvegicus*) Liver Exposed to Physical Stress

Abstract

Physical activity in general improves metabolic processes by stimulating muscles to contract actively, improving blood circulation, and supplying oxygen. On the other hand, it will create stressful conditions, which will increase cell damage and inflammatory responses in the liver. This study aimed to determine the effect of forest bee honey supplementation on the expression of HIF-1α, SOD, and TNF-α in rats that underwent the forced swimming test (FST) as a physical stress model. The physical activity conducted is at risk of ~~causing disruption~~disrupting to several organs due to the stress it causes in addition to the health benefits obtained. A total of 24 adult female rats were divided into four groups: Control (C) with FST only; (T1) FST and honey 2 g/day; (T2) FST and honey 4 g/day; (T3) FST and honey 6 g/day. A forced swimming test was conducted for five minutes per day for 14 days. The collected liver organs were histopathologically prepared by immunohistochemical staining for HIF-1α, SOD, and TNF-α proteins. The results showed that hepatic HIF-1α and TNF-α expression decreased in the honey-supplemented group, while hepatic SOD expression increased, although all three showed insignificant differences from each other. The~~ conclusion of this study i~~is study concludes that the supplementation of honey is incapable of increasing the expression of SOD as well as reducing the expression of HIF-1α and TNF-α in the liver of rats model~~l~~led by physical stress.

Keywords

~~Endogenous antioxidants, hypoxia, inflammation, natural medicine, oxidative stress.~~Forest bee honey, hypoxia-inducible factor, physical stress, superoxide dismutase, tumor necrosis factor-α

Introduction

Physical activity in general has a positive effect on metabolic processes, stimulating muscles to actively contract, improving blood circulation, and supplying oxygen [1]. On the other hand, physical activity will also induce stressful conditions, which will subsequently increase cell damage and inflammatory responses in the liver [2, 3]. The production of the cortisol hormone marks the occurrence of stressful conditions in the body [4]. The production of cortisol in the body suppresses the production of brain-derived neurotrophic factor (BDNF), which protects against malondialdehyde (MD) toxicity as a marker of oxidative stress caused by reactive oxygen species (ROS) [5]. Physical activity could trigger an increase in malondialdehyde (MDA) levels and decrease Superoxide Dismutase (SOD) levels in the body [6, 7]. The body naturally has an antioxidant system that aims to prevent damage caused by ROS, including the enzymes Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx), and Catalase [8]. ROS in sufficient levels is beneficial in the cellular immune response, but when produced in excessive amounts beyond the capacity of antioxidant enzymes, it will cause oxidative stress conditions that are ~~destructive to normal cell structures~~detrimental to the cell membrane~~8~~ [9].
Oxidative stress will trigger the activation of Hypoxia Inducible Factor (HIF-1α) through the MAPK pathway~~9~~ [10]. In physiological conditions, activation of HIF-1α plays an important role in wound healing because it triggers angiogenesis. On the other hand, HIF-1α production is often associated with the pathogenesis of various hepatic disorders, such as fibrosis, hepatitis, and even cancer~~10,~~ [11, 12]. Hepatic damage can be induced by an acute inflammatory response due to physical stress characterized by an increase in Tumor Necrosis Factor-α (TNF-α) [13]~~2~~. TNF-α production in the liver is carried out by Kupffer cells, which are involved in inflammation and hepatocyte apoptosis through caspase activation [14]~~3~~. Just like HIF-1α, TNF-α production in the liver is related to the pathogenesis of chronic hepatic inflammation that leads to fibrosis.
Stress due to physical activity is usually not realized by the individual and is often inevitable because it is a consequence of the work undertaken, such as in the case of sports athletes~~13,~~ [14, 15]. Physical activity done with a sufficient portion is beneficial in the metabolic process of the individual while if done with high intensity will cause an increase in cortisol production and oxidative stress [16, 17]. To compensate for the decrease in

endogenous antioxidants due to physical stress, exogenous antioxidants derived from food or beverages are needed.

Antioxidants are substances that can protect biological components from harm caused by chemical processes involving free radicals. They work by breaking down chains or stabilizing molecules [18]. Thus, the best approach to alleviating oxidative stress is to reduce free radicals or optimize the body's defenses by multiplying antioxidants. Furthermore, antioxidants protect the tissue from oxidative damage [19].

Indonesia is rich in biodiversity and has many natural resources that can be beneficial to treat various illnesses [20].

Honey is one of the natural ingredients that can be easily obtained and contains ascorbic acid, carotenoids, phenolic acids, flavonoids, and simple sugars~~15~~ [21]. Antioxidants contained in honey act as free radical scavengers under oxidative stress conditions and were proven to increase the production of GPx, SOD, and CAT as endogenous antioxidants in rats and improve the histological structure of the liver~~16,17~~ [22, 23]. Many animal models of stress and depression have been made using the forced swimming test along with observations on the liver, such as oxidative damage [17], MDA levels [24], and glycogen levels [25] of the liver [17]. This study attempted to determine whether the administration of wild bee honey supplements increased the expression of endogenous antioxidants, especially SOD, and decreased the expression of HIF-1α and TNF-α proteins in the livers of rats modeled by physical stress.~~Research on the effect of honey supplementation in rats modeling physical stress, especially on HIF-1α expression, has not been widely conducted, although HIF-1α is an important early indicator of various types of damage to the liver~~.

Results and Discussion

The mean protein expression of HIF-1α, TNFα, and SOD in the whole population showed the results as shown in Table 1 and Figure 1.

Dose Group (grams/rat)	Mean Rank±SD		
	HIF-1α	SOD	TNF- α
0 (C)	17.06±1.97	13.50±1.45	21.44±0.37
2 (T1)	16.56±0.91	14.63±2.17	17.81±0.89
4 (T2)	16.69±1.85	15.63±1.05	14.69±0.29
6 (t3)	15.69±1.78	22.25±0.26	12.06±0,63
Asymp. Sig.	0.993	0.173	0.205

Table 1. The mean expression of HIF-1α, TNFα, and hepatic SOD in all treatment groups. Based on data analysis, it is said that the results do not have significant differences (p>0,05).

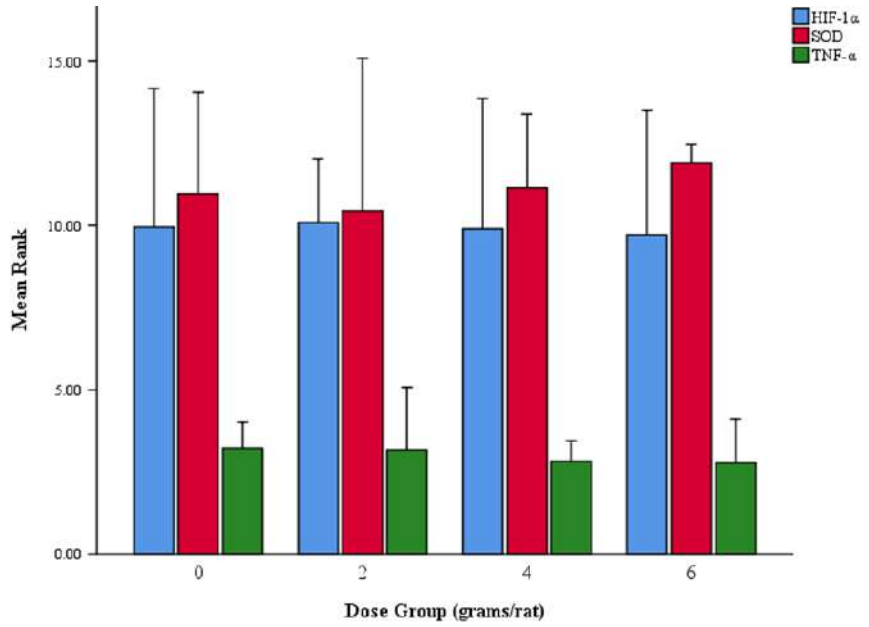

Figure 1. Mean results of hepatic HIF-1α, TNF-α, and SOD protein expression in all treatment groups with honey doses of 0 g (C), 2 g (T1), 4 g (T2), and 6 g (T3). The blue column represents HIF-1α; the red column represents SOD; and the green column represents TNF-α. All study parameters did not appear to differ significantly between treatment groupsgroups.

The forced swimming test as a physical stress model accompanied by honey supplementation was proven to reduce the expression of HIF-1α and TNF-α in the liver but based on statistical analysis, it was considered insignificant ($p>0.05$) as can be observed in Table 1 and Figure 1. The highest expression of HIF-1α and TNF-α was obtained in group C, which only underwent swimming activities without honey supplementation. Based on Table 1 and Figure 1, it can also be observed that between groups of forced swimming test treatment with honey supplementation, hepatic SOD expression gradually increased along with the increase in honey dose, with the least amount in group C, which did not get any honey. Although the values varied, the differences were not significant ($p>0.05$) (Table 1). The expression of HIF-1α, TNFα, and SOD presented in immunohistochemical staining can be further observed in Figures 2, 3, and 4.

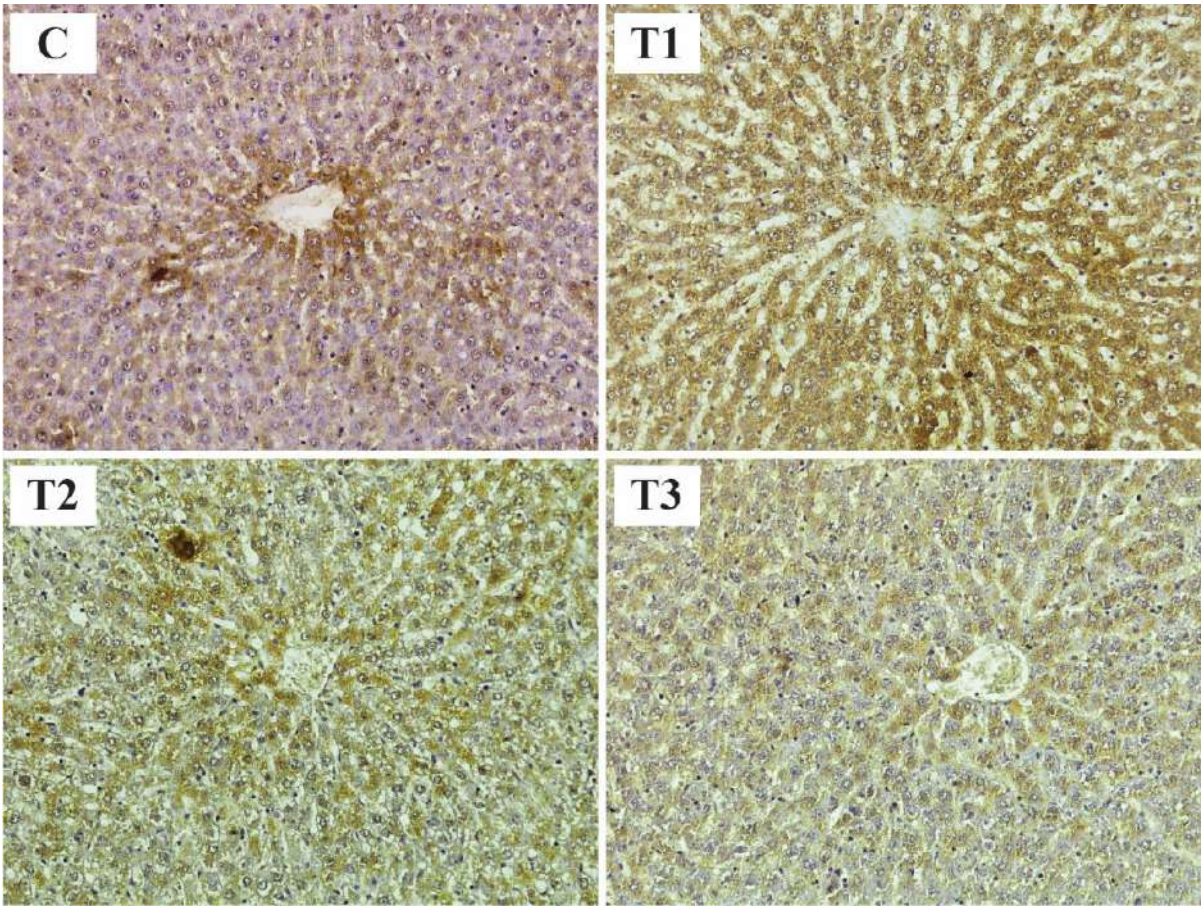

Figure 2. HIF-1α expression in hepatocytes of physical stress model rats by immunohistochemical staining (200x). FST without honey (C); FST with 2 g/day honey (T1); FST with 4 g/day honey (T2); and FST with 6 g/day honey (T3). Brownish Brownish-yellow coloration with weak to moderate intensity could be observed in all treatment groups.

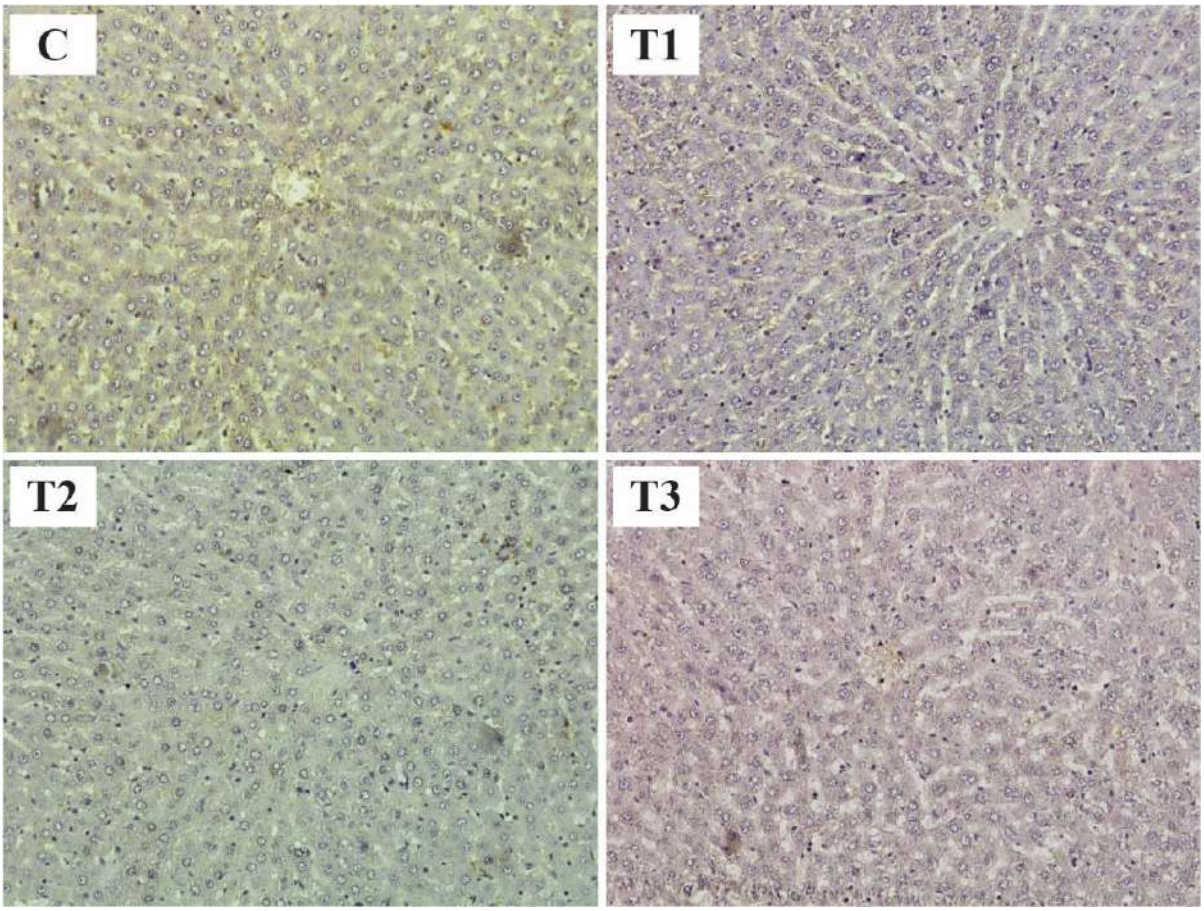

Figure 3. TNF-α expression in the hepatic Kupffer cells of physical stress model rats by immunohistochemical staining (200x). FST without honey (C); FST with 2 g/day honey (T1); FST with 4 g/day honey (T2); and FST with 6 g/day honey (T3). A brownish-yellow color with weak intensity can be observed in group C, while in the honey supplementation group, the intensity is getting smaller to zero.

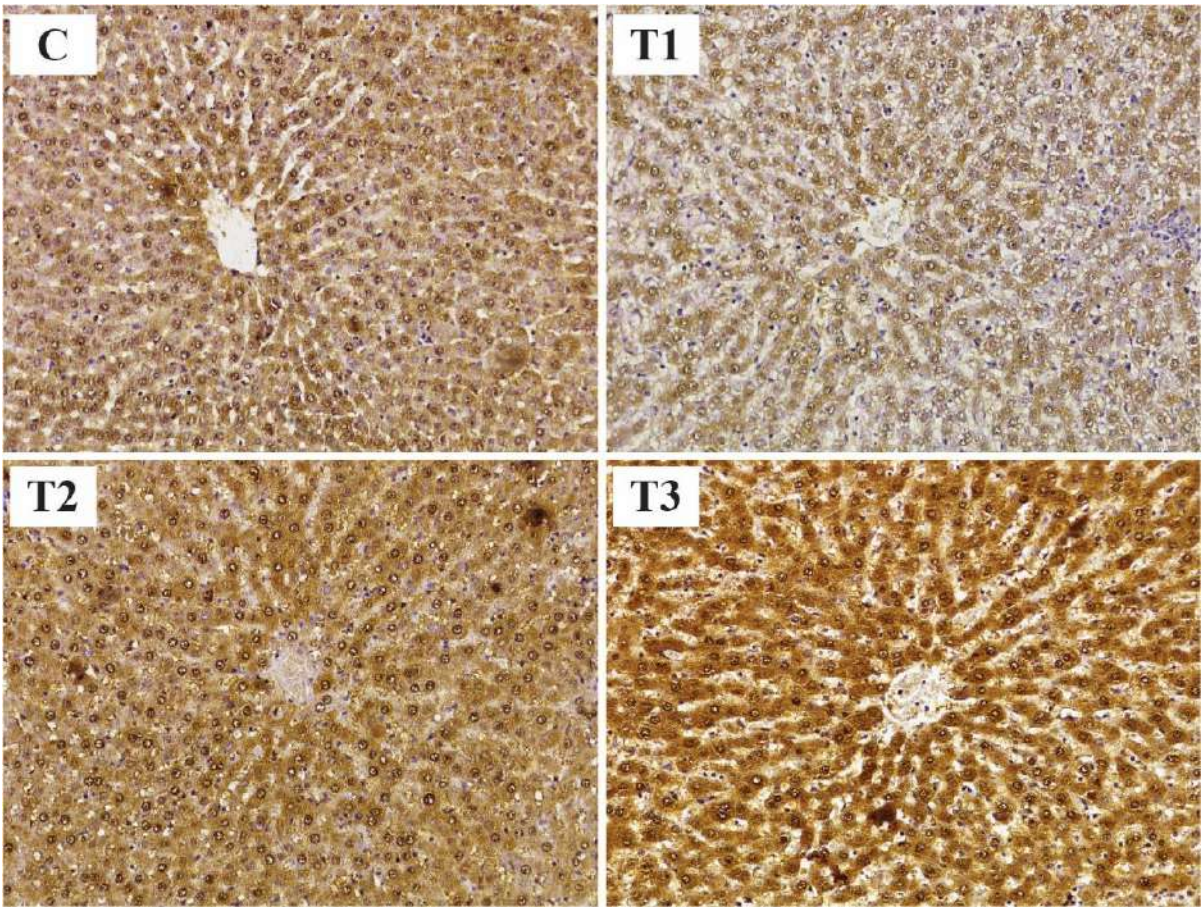

Figure 4. SOD expression in hepatocytes of rats modeled by physical stress by immunohistochemical staining (200x). FST without honey (C); FST with 2 g/day honey (T1); FST with 4 g/day honey (T2); and FST with 6 g/day honey (T3). A ~~brownish~~ brownish-yellow color with moderate to strong intensity can be observed in all groups, with the strongest intensity seen in group T3.

Based on the immunohistochemical overview of the liver shown in Figure 2, it can be observed that there is an expression of HIF-1α in the hepatic parenchyma of rats ~~modeled~~modelled by physical stress, even though there is no significant difference in expression between groups. ROS that arises due to stress will stimulate MAP/ERK Kinase (MEK) to phosphorylate p300 so that there is an increase in the transcription of the HIF-1α molecule in the liver even under normoxia [11]~~0~~. While in Figure 3 it appears that TNF-α expression is very weak to almost zero in the liver. TNF-α production ~~on~~ in the liver is carried out by Kupffer cells, which play a role in inflammation and hepatocyte apoptosis through caspase activation [13]~~2~~. Apoptosis begins with a decrease in BCl-2 activity on the mitochondrial membrane~~,~~ so that membrane permeability changes. These changes cause the release of cytochrome C into the cytosol, which will activate Apaf-1. This activation is followed by a caspase cascade on pro-caspase 9 to process caspase 3, which will damage DNA~~18~~ [26]. From Figure 4, it can be seen that the expression of SOD in the group with 6 g/day honey supplementation appears to be stronger in color change when compared to other groups, although in statistical analysis the difference was ~~apparently~~ considered insignificant.

The FST procedure used in this study was shown to be associated with HIF-1α production in the liver. This result is in line with other studies that revealed that the physical stress of swimming can increase the production of ROS, which will induce the activation of HIF-1α in the liver~~19~~ [27]. In addition, the FST procedure also proved to be positively correlated with hepatic TNF-α production, ~~in accordance with~~following other studies that prove that physical stress can increase TNF-α expression in the liver [28]~~0~~. The antioxidant content of honey is known to suppress cell damage due to physical stress and ROS~~21,22~~ [29, 30]. In addition, honey has also been shown to increase the production of the endogenous hepatic antioxidants SOD as well as GPx, GSH, and catalase~~16,17~~ [22, 23].

However, the efficacy of honey was not significantly proven in this study. This may be due to the concentration of ROS being too high, so the dose level used in this study was insufficient to compensate for the cell damage that occurred even though there was an increase in SOD expression. Other studies have shown that excessive consumption of honey can ~~actually cause distortion of~~distort the hepatic sinusoid circuit and necrosis of hepatocytes~~2~~ [31]. Similar results also revealed that honey consumption can cause hematological changes as well as damage to hepatic and renal cells~~24~~ [32]. This damage is thought to be due to the accumulation of heavy metals such as Pb and Cd in honey from the environment where the honey is taken. The accumulation of these metals then initiates higher ROS~~24~~ [32]. Therefore, the length of time honey is given and the amount of dose given should be of utmost concern so that the honey consumed provides benefits to the body and not the other way around. The dose level of honey and the duration of the study in this study are not sufficient to prove that honey supplementation can prevent general hepatic damage in rats ~~modeled~~modelled under physical stress. However, the observed parameters in this study do not fully represent the hepatic quality of an individual; other supporting data such as AST, ALT, and GGT profiles are needed for higher validity.

Materials and Methods

Ethical approval

This study had been approved by the Ethics Commission of the Faculty of Veterinary Medicine~~, at~~ Universitas Airlangga (1.KEH.041.04.2022).

Experimental animals

The experimental animals used were three-month-old female Wistar rats (*Rattus norvegicus*) with an average body weight of 200 grams. The total population of 24 rats was divided into four treatment groups using a complete randomization system~~,~~ so that each group contained six rats. Rats were acclimatized with adequate food and water for seven days. During the study, the rats were placed in cages measuring 53 x 30 x 17 cm with a base of wood chips and placed in a room with a temperature of 34 °C and 50% humidity. The forced swimming test is a method used to model animal stress and depression, according to Porsolt~~24~~ [34]. Rats were exercised in a 50 cm diameter and 60 cm high barrel filled with water to approximately 2/3 of the height of the barrel for five minutes every day for 14 days.

Experimental procedure

There were four treatment groups: C with the forced swimming test (FST) alone; T1 with the FST and 2 g/day honey; T2 with the FST and 4 g/day honey; and T3 with the FST and 6 g/day honey. Honey was given by oral gavage. FST was performed for 14 days, and the rats were sacrificed on day 15. Rats were sacrificed with a combination of ketamine and xylazine injections. A laparotomy procedure was performed to collect the liver.

~~The liver was then made into immunohistochemical preparations with HIF-1α, TNFα and SOD antibodies.~~*Tissue processing*

The organs were prepared for histological examination as follows [33]: each animal's liver was fixed in 10% neutral buffered formalin. The fixed tissues were cleared with xylol after being dehydrated in a graded series of alcohols. The tissues were then infiltrated with molten paraffin at 56–60 °C. From a solid block of tissue, serial sections of 3 µm thickness were cut, washed, and placed in object glass.

Immunohistochemistry staining

The tissues were deparaffinized and rehydrated. Then the tissue slides were incubated with hydrogen peroxide for 10–15 minutes, and blocking was performed for 5 minutes. Antibody incubations were conducted using primary antibodies (HIF-1α (1:200), TNFα (1:50), and SOD (1:200)) for 60 minutes and secondary antibodies for 30 minutes. Subsequently, the slides were incubated with streptavidin peroxidase for 10 minutes. Furthermore, the addition of DAB chromogen and substrate was carried out for 15 minutes. Counterstaining was carried out using Mayer's hemalum solution.

Statistical analysis

Hepatic immunohistochemical protein expressions were documented using a Nikon Eclipse Ci microscope at 200x magnification and interpreted using the IRS scoring system. Expression was considered positive if there was a brownish-yellow color change due to antigen-antibody binding in hepatocytes. Observations were made in five~~5~~ fields of view, and the results were averaged. The data obtained were then statistically analyzed by ANOVA using SPSS for Windows.

Conclusions

The conclusion that can be drawn from this study is that supplementation of *Apis dorsata* honey at the dose level given is unable to reduce the expression of HIF-1α and TNFα and increase the expression of SOD in physical stress model rats. In future studies, it is suggested to use a more customized dose of honey~~,~~ and to determine the overall hepatic profile, other research parameters such as AST, ALT, and GGT are needed.

Acknowledg~~e~~ements~~:~~

~~This study was funded by Universitas Airlangga with Grant Number 251/UN3/2022.~~ The authors also express gratitude to Agung Budianto Achmad, DVM, M.Sc., who supported the data analysis in this study.

Conflict of interest

The authors declared that they have no conflict of interest.

Consent for publications

All authors have read and approved the final manuscript for publication.

Availability of data and material

The authors declare that they embedded all data in the manuscript.

Authors' Contributions

HP and WW designed the research concept and conducted the experiments. MVPS conducted the draft writing. AP performed data analysis and manuscript editing. AP and MVPS conducted the manuscript revision.

Funding

This study was funded by Universitas Airlangga with Grant Number 251/UN3/2022.

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Original Article

Effect of Forest Bee Honey (*Apis dorsata*) Supplementation on Expression of HIF-1α, SOD, and TNF-α in Rats (*Rattus norvegicus*) Liver Exposed to Physical Stress

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ARTICLE INFO

Article history

Receive: 2023-04-05
Received in revised: 2023-05-10
Accepted: 2023-05-14
Manuscript ID: JMCS-2304-2018
Checked for Plagiarism: **Yes**
Language Editor:
Dr. Fatima Ramezani
Editor who approved publication:
Dr. Zeinab Arzehgar

DOI:10.26655/JMCHEMSCI.2021.x.x

KEYWORDS

Forest bee honey
Hypoxia-inducible factor
Physical stress
Superoxide dismutase
Tumour necrosis factor-α

ABSTRACT

Physical activity in general improves metabolic processes by stimulating muscles to contract actively, improving blood circulation, and supplying oxygen. On the other hand, it will create stressful conditions, which will increase cell damage and inflammatory responses in the liver. This study aimed to determine the effect of forest bee honey supplementation on the expression of HIF-1α, SOD, and TNF-α in rats that underwent the forced swimming test (FST) as a physical stress model. The physical activity conducted is at risk of disrupting to several organs due to the stress it causes in addition to the obtained health benefits. A total of 24 adult female rats were divided into four groups: Control (C) with FST only, (T1) FST and honey 2 g/day, (T2) FST and honey 4 g/day, (T3) FST and honey 6 g/day. A forced swimming test was conducted for five minutes per day for 14 days. The collected liver organs were histopathologically prepared by immunohistochemical staining for HIF-1α, SOD, and TNF-α proteins. The results showed that hepatic HIF-1α and TNF-α expression decreased in the honey-supplemented group, while hepatic SOD expression increased, although all three showed insignificant differences from each other. This study concludes that honey supplementation is incapable of increasing the expression of SOD as well as reducing the expression of HIF-1α and TNF-α in the liver of rats modelled by physical stress.

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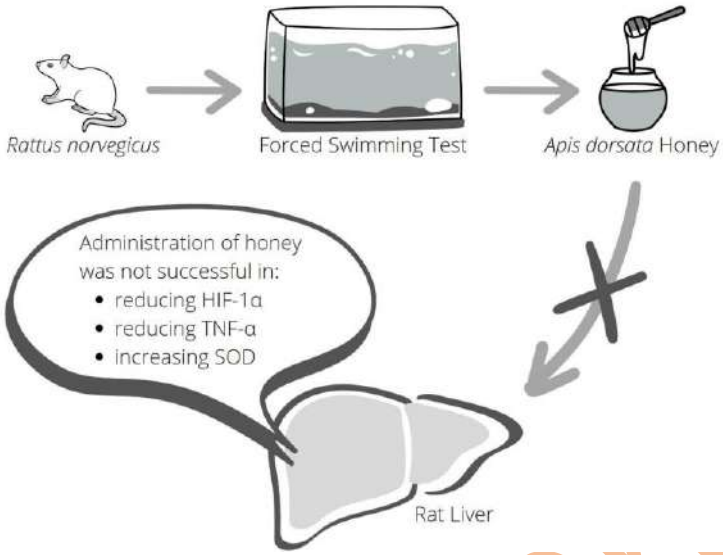
GRAPHICAL ABSTRACT



Introduction

Physical activity in general has a positive effect on metabolic processes, stimulating muscles to actively contract, improving blood circulation, and supplying oxygen [1]. On the other hand, physical activity will also induce stressful conditions, which will subsequently increase cell damage and inflammatory responses in the liver [2, 3]. The production of the cortisol hormone marks the occurrence of stressful conditions in the body [4]. The production of cortisol in the body suppresses the production of brain-derived neurotrophic factor (BDNF), which protects against malondialdehyde (MD) toxicity as a marker of oxidative stress caused by reactive oxygen species (ROS) [5]. Physical activity could trigger an increase in malondialdehyde (MDA) levels and decrease Superoxide Dismutase (SOD) levels in the body [6, 7]. The body naturally has an antioxidant system that aims to prevent damage caused by ROS, including the enzymes Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx), and Catalase [8]. ROS in sufficient levels is beneficial in the cellular immune response, but when produced in excessive amounts beyond the capacity of antioxidant enzymes, it will cause oxidative stress conditions that are detrimental to the cell membrane [9].

Oxidative stress will trigger the activation of Hypoxia Inducible Factor (HIF-1α) through the MAPK pathway [10]. In physiological conditions, activation of HIF-1α plays an important role in wound healing because it triggers angiogenesis. On the other hand, HIF-1α production is often associated with the pathogenesis of various hepatic disorders, such as fibrosis, hepatitis, and even cancer [11, 12]. Hepatic damage can be induced by an acute inflammatory response due to physical stress characterized by an increase in Tumor Necrosis Factor-α (TNF-α) [13]. TNF-α production in the liver is carried out by Kupffer cells, which are involved in inflammation and hepatocyte apoptosis through caspase activation [14]. Just like HIF-1α, TNF-α production in the liver is related to the pathogenesis of chronic hepatic inflammation that leads to fibrosis.

Stress due to physical activity is usually not realized by the individual and is often inevitable because it is a consequence of the work undertaken, such as in the case of sports athletes [14, 15].

Physical activity done with a sufficient portion is beneficial in the metabolic process of the individual while if done with high intensity will cause an increase in cortisol production and oxidative stress [16, 17].

To compensate for the decrease in endogenous antioxidants due to physical stress, exogenous antioxidants derived from food or beverages are needed.

Antioxidants are substances that can protect biological components from harm caused by chemical processes involving free radicals. They work by breaking down chains or stabilizing molecules [18]. Thus, the best approach to alleviating oxidative stress is to reduce free radicals or optimize the body's defences by multiplying antioxidants. Furthermore, antioxidants protect the tissue from oxidative damage [19].

Indonesia is rich in biodiversity and has many natural resources that can be beneficial to treat various illnesses [20].

Honey is one of the natural ingredients that can be easily obtained and contains ascorbic acid, carotenoids, phenolic acids, flavonoids, and simple sugars [21]. Antioxidants contained in honey act as free radical scavengers under oxidative stress conditions and were proven to increase the production of GPx, SOD, and CAT as endogenous antioxidants in rats and improve the histological structure of the liver [22, 23]. Many animal models of stress and depression have been made using the forced swimming test along with observations on the liver, such as oxidative damage [17], MDA levels [24], and glycogen levels [25] of the liver [17]. This study attempted to determine whether the administration of wild bee honey supplements increased the expression of endogenous antioxidants, especially SOD, and decreased the expression of HIF-1α and TNF-α proteins in the livers of rats modelled by physical stress.

Results and Discussion

The mean protein expression of HIF-1α, TNFα, and SOD in the whole population showed the results, as indicated in Table 1 and Figure 1.

Table 1: The mean expression of HIF-1α, TNFα, and hepatic SOD in all treatment groups. Based on data analysis, it is mentioned that the results do not have significant differences (p>0,05)

Dose Group (grams/rat)	Mean Rank±SD		
	HIF-1α	SOD	TNF- α
0 (C)	17.06±1.97	13.50±1.45	21.44±0.37
2 (T1)	16.56±0.91	14.63±2.17	17.81±0.89
4 (T2)	16.69±1.85	15.63±1.05	14.69±0.29
6 (t3)	15.69±1.78	22.25±0.26	12.06±0,63
Asymp. Sig.	0.993	0.173	0.205

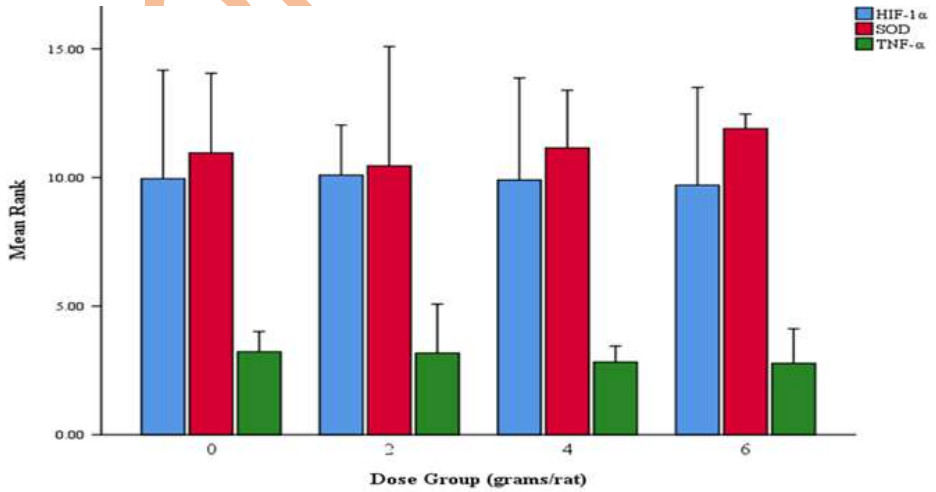


Figure 1: Mean results of hepatic HIF-1α, TNF-α, and SOD protein expression in all treatment groups with honey doses of 0 g (C), 2 g (T1), 4 g (T2), and 6 g (T3). The blue column represents HIF-1α; the red column represents SOD; and the green column represents TNF-α. All study parameters did not appear to differ significantly between treatment groups

The forced swimming test as a physical stress model accompanied by honey supplementation was proven to reduce the expression of HIF-1α and TNF-α in the liver but based on statistical analysis, it was considered insignificant ($p>0.05$) as can be observed in Table 1 and Figure 1. The highest expression of HIF-1α and TNF-α was obtained in group C, which only underwent swimming activities without honey supplementation. Based on Table 1 and Figure 1, it can be further observed that between groups of forced swimming test treatment with honey supplementation, hepatic SOD expression gradually increased along with the increase in honey dose, with the least amount in group C, which did not get any honey. Although the values varied, the differences were not significant ($p>0.05$) (Table 1). The expression of HIF-1α, TNFα, and SOD presented in immunohistochemical staining can be further observed in Figures 2, 3, and 4.

Based on the immunohistochemical overview of the liver depicted in Figure 2, it can be observed that there is an expression of HIF-1α in the hepatic parenchyma of rats modelled by physical stress, even though there is no significant difference in expression between groups. ROS that arises due to stress will stimulate MAP/ERK Kinase (MEK) to phosphorylate p300 so that there is an increase in the transcription of the HIF-1α molecule in the liver even under normoxia [11]. While in Figure 3, it is appeared that TNF-α expression is very weak to almost zero in the liver. TNF-α production in the liver is carried out by Kupffer cells, which play a role in inflammation and hepatocyte apoptosis through caspase activation [13]. Apoptosis begins with a decrease in BCl-2 activity on the mitochondrial membrane so that membrane permeability changes. These changes cause the release of cytochrome C into the cytosol, which will activate Apaf-1. This activation is followed by a caspase cascade on pro-caspase 9 to process caspase 3, which will damage DNA [26]. From Figure 4, it can be observed that the expression of SOD in the group with 6 g/day honey supplementation appears to be stronger in colour change when compared to other groups, although in statistical analysis the difference was considered insignificant.

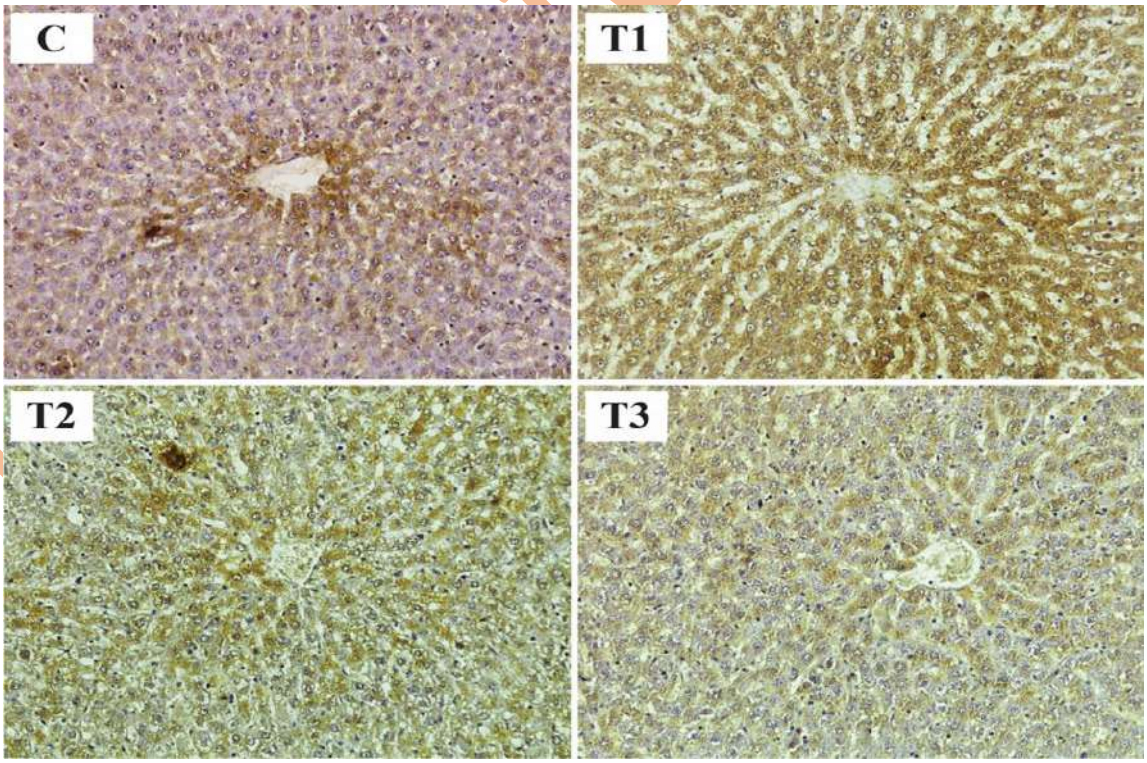


Figure 2: HIF-1α expression in hepatocytes of physical stress model rats by immunohistochemical staining (200x). FST without honey (C); FST with 2 g/day honey (T1); FST with 4 g/day honey (T2); and FST with 6 g/day honey (T3). Brownish-yellow coloration with weak to moderate intensity could be observed in all treatment groups

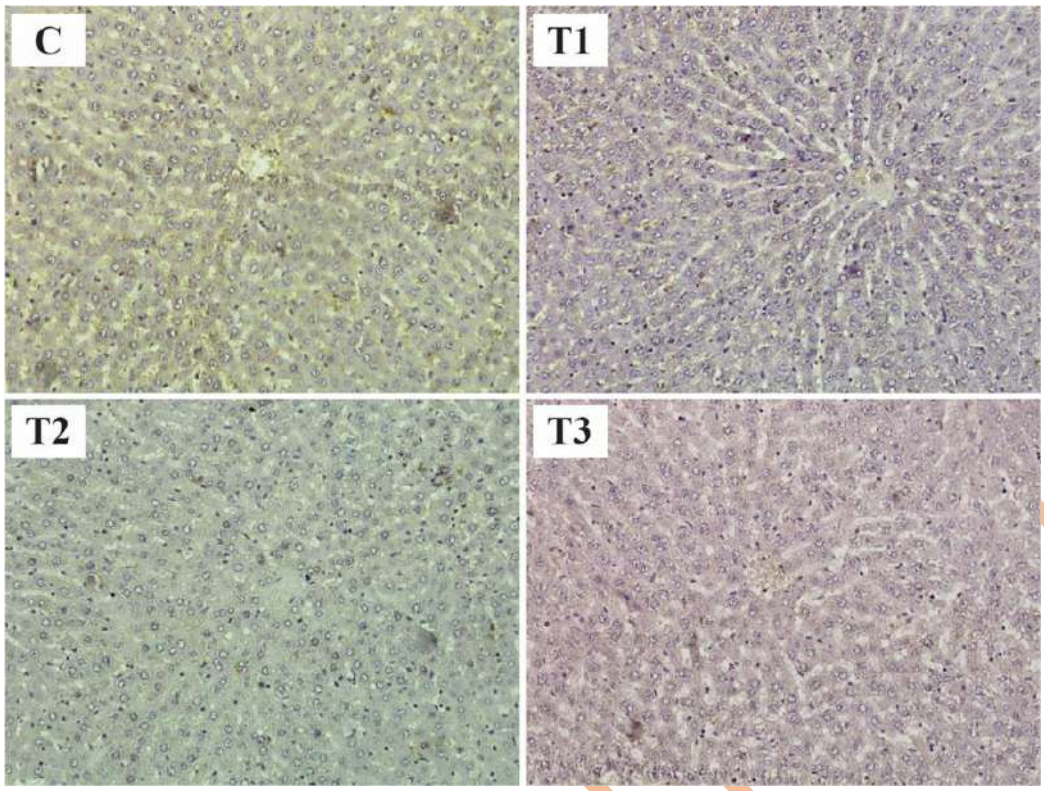


Figure 3: TNF-α expression in hepatic Kupffer cells of physical stress model rats by immunohistochemical staining (200x). FST without honey (C); FST with 2 g/day honey (T1); FST with 4 g/day honey (T2); and FST with 6 g/day honey (T3). A brownish-yellow colour with weak intensity can be observed in group C, while in the honey supplementation group, the intensity is getting smaller to zero

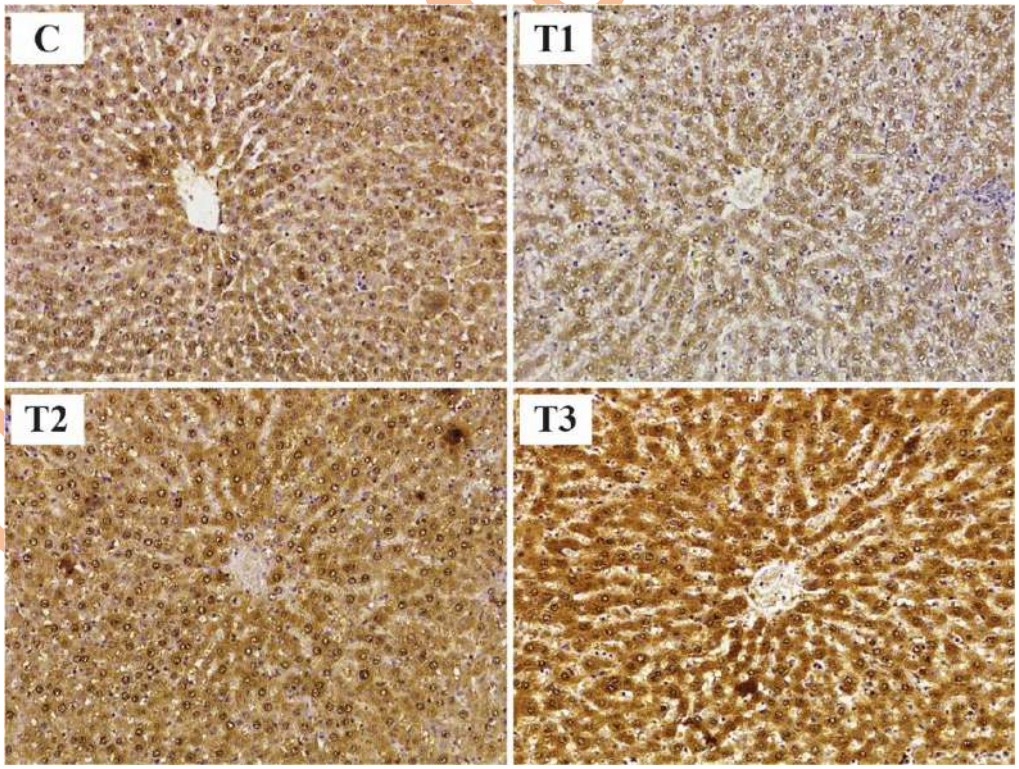


Figure 4: SOD expression in hepatocytes of rats modeled by physical stress by immunohistochemical staining (200x). FST without honey (C); FST with 2 g/day honey (T1); FST with 4 g/day honey (T2); and FST with 6 g/day honey (T3). A brownish-yellow color with moderate to strong intensity can be observed in all groups, with the strongest intensity seen in group T3

The FST procedure used in this study was shown to be associated with HIF-1α production in the liver. This result is in line with other studies that revealed that physical stress of swimming can increase the ROS production, which will induce the activation of HIF-1α in the liver [27]. In addition, the FST procedure proved to be positively correlated with hepatic TNF-α production, following other studies that prove that physical stress can increase TNF-α expression in the liver [28]. The antioxidant content of honey is known to suppress cell damage due to physical stress and ROS [29, 30]. Furthermore, honey has also been shown to increase the production of endogenous hepatic antioxidants SOD as well as GPx, GSH, and catalase [22, 23].

However, honey efficacy was not significantly proven in this study. This may be due to the concentration of ROS being too high, so the dose level used in this study was insufficient to compensate for the cell damage that occurred even though there was an increase in SOD expression. Other studies have shown that excessive consumption of honey can distort the hepatic sinusoid circuit and necrosis of hepatocytes [31]. Similar results also revealed that honey consumption can cause haematological changes as well as damage to hepatic and renal cells [32]. This damage is thought to be due to the accumulation of heavy metals such as Pb and Cd in honey from the environment where the honey is taken. The accumulation of these metals then initiates higher ROS [32]. Therefore, the length of time honey is given and the amount of dose given should be of utmost concern so that honey consumed provides benefits to the body and not the other way around. The dose level of honey and the duration of the study in this study are not sufficient to prove that honey supplementation can prevent general hepatic damage in rats modelled under physical stress. However, the observed parameters in this study do not fully represent the hepatic quality of an individual; other supporting data such as AST, ALT, and GGT profiles are needed for higher validity.

Materials and Methods

Ethical approval

This study had been approved by the Ethics Commission of the Faculty of Veterinary Medicine, Universitas Airlangga (1.KEH.041.04.2022).

Experimental animals

The experimental animals used were three-month-old female Wistar rats (*Rattus norvegicus*) with an average body weight of 200 grams. The total population of 24 rats was divided into four treatment groups using a complete randomization system so that each group contained six rats. Rats were acclimatized with adequate food and water for seven days. During the study, the rats were placed in cages measuring 53 × 30 × 17 cm with a base of wood chips and placed in a room with a temperature of 34 °C and 50% humidity. The forced swimming test is a method used to model animal stress and depression, according to Porsolt [34]. Rats were exercised in a 50 cm diameter and 60 cm high barrel filled with water to approximately 2/3 of the height of the barrel for five minutes every day for 14 days.

Experimental procedure

There were four treatment groups: C with the forced swimming test (FST) alone; T1 with the FST and 2 g/day honey; T2 with the FST and 4 g/day honey; and T3 with the FST and 6 g/day honey. Honey was given by oral gavage. FST was performed for 14 days, and the rats were sacrificed on day 15. Rats were sacrificed with a combination of ketamine and xylazine injections. A laparotomy procedure was performed to collect the liver.

Tissue processing

The organs were prepared for histological examination as follows [33]: each animal's liver was fixed in 10% neutral buffered formalin. The fixed tissues were cleared with xylol after being dehydrated in a graded series of alcohols. The tissues were then infiltrated with molten paraffin at 56–6 ºC. From a solid block of tissue, serial

sections of 3 µm thickness were cut, washed, and placed in object glass.

Immunohistochemistry staining

The tissues were deparaffinized and rehydrated. Then the tissue slides were incubated with hydrogen peroxide for 10-15 minutes, and blocking was performed for 5 minutes. Antibody incubations were conducted using primary antibodies (HIF-1α (1:200), TNFα (1:50), and SOD (1:200)) for 60 minutes and secondary antibodies for 30 minutes. Subsequently, the slides were incubated with streptavidin peroxidase for 10 minutes. Furthermore, the addition of DAB chromogen and substrate was carried out for 15 minutes. Likewise,counterstaining was carried out using Mayer's hemalum solution.

Statistical analysis

Hepatic immunohistochemical protein expressions were documented using a Nikon Eclipse Ci microscope at 200x magnification and interpreted using the IRS scoring system. Expression was considered positive if there was a brownish-yellow colour change due to antigen-antibody binding in hepatocytes. Observations were made in five fields of view, and the results were averaged. The data obtained were then statistically analysed by ANOVA using SPSS for Windows.

Conclusion

The conclusion that can be drawn from this study is that supplementation of *Apis dorsata* honey at the dose level given is unable to reduce the expression of HIF-1α and TNFα and increase the SOD expression in physical stress model rats. In future studies, it is suggested to use a more customized dose of honey and to determine the overall hepatic profile, other research parameters such as AST, ALT, and GGT are needed.

Acknowledgments

The authors express their gratitude to Agung Budianto Achmad, DVM, M.Sc., who supported the data analysis in this study.

Disclosure Statement

No potential conflict of interest was reported by the authors.

Funding

This study was funded by Universitas Airlangga with Grant No. 251/UN3/2022.

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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HOW TO CITE THIS ARTICLE

Hani Plumeriastuti, Widjiati Widjiati, Mey Vanda Pusparina Sajida, Annise Proboningrat. Effect of Forest Bee Honey (*Apis dorsata*) Supplementation on Expression of HIF-1α, SOD, and TNF-α in Rats (*Rattus norvegicus*) Liver Exposed to Physical Stress. *J. Med. Chem. Sci.*, 2023, x(x) xx-xx

DOI: 10.26655/JMCHEMSCI.2023.xx

SPC

Journal of Medicinal and Chemical Sciences

Journal homepage: http://www.jmchemsci.com/

Original Article

Effect of Forest Bee Honey (*Apis dorsata*) Supplementation on Expression of HIF-1α, SOD, and TNF-α in Rats (*Rattus norvegicus*) Liver Exposed to Physical Stress

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ARTICLE INFO

Article history

Receive: 2023-04-05
Received in revised: 2023-05-10
Accepted: 2023-05-14
Manuscript ID: JMCS-2304-2018
Checked for Plagiarism: **Yes**
Language Editor:
Dr. Fatima Ramezani
Editor who approved publication:
Dr. Zeinab Arzehgar

DOI:10.26655/JMCHEMSCI.2021.x.x

KEYWORDS

Forest bee honey
Hypoxia-inducible factor
Physical stress
Superoxide dismutase
Tumour necrosis factor-α

ABSTRACT

Physical activity in general improves metabolic processes by stimulating muscles to contract actively, improving blood circulation, and supplying oxygen. On the other hand, it will create stressful conditions, which will increase cell damage and inflammatory responses in the liver. This study aimed to determine the effect of forest bee honey supplementation on the expression of HIF-1α, SOD, and TNF-α in rats that underwent the forced swimming test (FST) as a physical stress model. The physical activity conducted is at risk of disrupting to several organs due to the stress it causes in addition to the obtained health benefits. A total of 24 adult female rats were divided into four groups: Control (C) with FST only, (T1) FST and honey 2 g/day, (T2) FST and honey 4 g/day, (T3) FST and honey 6 g/day. A forced swimming test was conducted for five minutes per day for 14 days. The collected liver organs were histopathologically prepared by immunohistochemical staining for HIF-1α, SOD, and TNF-α proteins. The results showed that hepatic HIF-1α and TNF-α expression decreased in the honey-supplemented group, while hepatic SOD expression increased, although all three showed insignificant differences from each other. This study concludes that honey supplementation is incapable of increasing the expression of SOD as well as reducing the expression of HIF-1α and TNF-α in the liver of rats modelled by physical stress.

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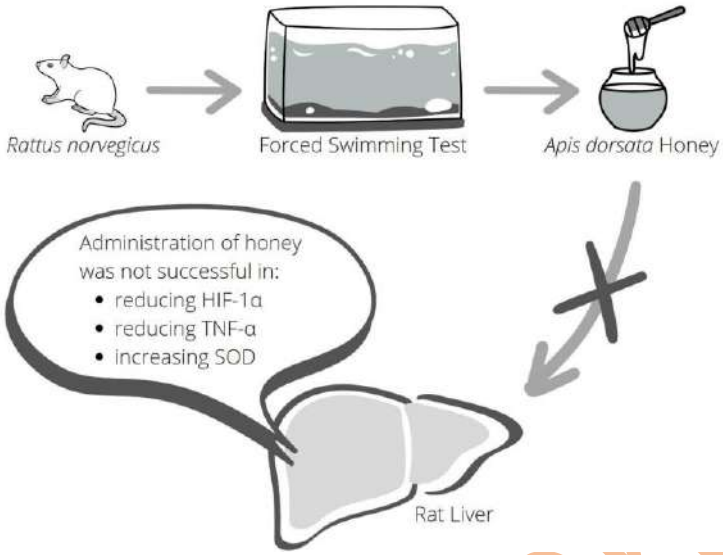
GRAPHICAL ABSTRACT



Introduction

Physical activity in general has a positive effect on metabolic processes, stimulating muscles to actively contract, improving blood circulation, and supplying oxygen [1]. On the other hand, physical activity will also induce stressful conditions, which will subsequently increase cell damage and inflammatory responses in the liver [2, 3]. The production of the cortisol hormone marks the occurrence of stressful conditions in the body [4]. The production of cortisol in the body suppresses the production of brain-derived neurotrophic factor (BDNF), which protects against malondialdehyde (MD) toxicity as a marker of oxidative stress caused by reactive oxygen species (ROS) [5]. Physical activity could trigger an increase in malondialdehyde (MDA) levels and decrease Superoxide Dismutase (SOD) levels in the body [6, 7]. The body naturally has an antioxidant system that aims to prevent damage caused by ROS, including the enzymes Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx), and Catalase [8]. ROS in sufficient levels is beneficial in the cellular immune response, but when produced in excessive amounts beyond the capacity of antioxidant enzymes, it will cause oxidative stress conditions that are detrimental to the cell membrane [9].

Oxidative stress will trigger the activation of Hypoxia Inducible Factor (HIF-1α) through the MAPK pathway [10]. In physiological conditions, activation of HIF-1α plays an important role in wound healing because it triggers angiogenesis. On the other hand, HIF-1α production is often associated with the pathogenesis of various hepatic disorders, such as fibrosis, hepatitis, and even cancer [11, 12]. Hepatic damage can be induced by an acute inflammatory response due to physical stress characterized by an increase in Tumor Necrosis Factor-α (TNF-α) [13]. TNF-α production in the liver is carried out by Kupffer cells, which are involved in inflammation and hepatocyte apoptosis through caspase activation [14]. Just like HIF-1α, TNF-α production in the liver is related to the pathogenesis of chronic hepatic inflammation that leads to fibrosis.

Stress due to physical activity is usually not realized by the individual and is often inevitable because it is a consequence of the work undertaken, such as in the case of sports athletes [14, 15].

Physical activity done with a sufficient portion is beneficial in the metabolic process of the individual while if done with high intensity will cause an increase in cortisol production and oxidative stress [16, 17].

To compensate for the decrease in endogenous antioxidants due to physical stress, exogenous antioxidants derived from food or beverages are needed.

Antioxidants are substances that can protect biological components from harm caused by chemical processes involving free radicals. They work by breaking down chains or stabilizing molecules [18]. Thus, the best approach to alleviating oxidative stress is to reduce free radicals or optimize the body's defences by multiplying antioxidants. Furthermore, antioxidants protect the tissue from oxidative damage [19].

Indonesia is rich in biodiversity and has many natural resources that can be beneficial to treat various illnesses [20].

Honey is one of the natural ingredients that can be easily obtained and contains ascorbic acid, carotenoids, phenolic acids, flavonoids, and simple sugars [21]. Antioxidants contained in honey act as free radical scavengers under oxidative stress conditions and were proven to increase the production of GPx, SOD, and CAT as endogenous antioxidants in rats and improve the histological structure of the liver [22, 23]. Many animal models of stress and depression have been made using the forced swimming test along with observations on the liver, such as oxidative damage [17], MDA levels [24], and glycogen levels [25] of the liver [17]. This study attempted to determine whether the administration of wild bee honey supplements increased the expression of endogenous antioxidants, especially SOD, and decreased the expression of HIF-1α and TNF-α proteins in the livers of rats modelled by physical stress.

Results and Discussion

The mean protein expression of HIF-1α, TNFα, and SOD in the whole population showed the results, as indicated in Table 1 and Figure 1.

Table 1: The mean expression of HIF-1α, TNFα, and hepatic SOD in all treatment groups. Based on data analysis, it is mentioned that the results do not have significant differences (p>0,05)

Dose Group (grams/rat)	Mean Rank±SD		
	HIF-1α	SOD	TNF- α
0 (C)	17.06±1.97	13.50±1.45	21.44±0.37
2 (T1)	16.56±0.91	14.63±2.17	17.81±0.89
4 (T2)	16.69±1.85	15.63±1.05	14.69±0.29
6 (t3)	15.69±1.78	22.25±0.26	12.06±0,63
Asymp. Sig.	0.993	0.173	0.205

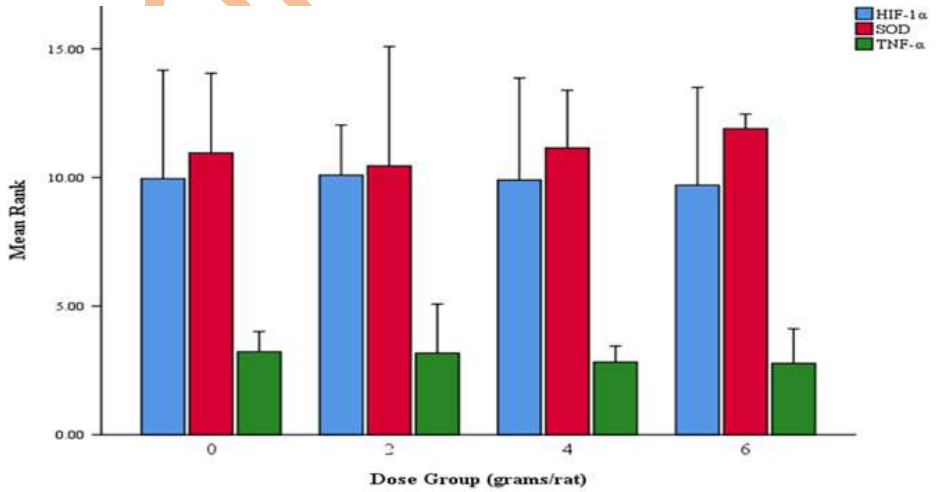


Figure 1: Mean results of hepatic HIF-1α, TNF-α, and SOD protein expression in all treatment groups with honey doses of 0 g (C), 2 g (T1), 4 g (T2), and 6 g (T3). The blue column represents HIF-1α; the red column represents SOD; and the green column represents TNF-α. All study parameters did not appear to differ significantly between treatment groups

The forced swimming test as a physical stress model accompanied by honey supplementation was proven to reduce the expression of HIF-1α and TNF-α in the liver but based on statistical analysis, it was considered insignificant ($p>0.05$) as can be observed in Table 1 and Figure 1. The highest expression of HIF-1α and TNF-α was obtained in group C, which only underwent swimming activities without honey supplementation. Based on Table 1 and Figure 1, it can be further observed that between groups of forced swimming test treatment with honey supplementation, hepatic SOD expression gradually increased along with the increase in honey dose, with the least amount in group C, which did not get any honey. Although the values varied, the differences were not significant ($p>0.05$) (Table 1). The expression of HIF-1α, TNFα, and SOD presented in immunohistochemical staining can be further observed in Figures 2, 3, and 4.

Based on the immunohistochemical overview of the liver depicted in Figure 2, it can be observed that there is an expression of HIF-1α in the hepatic parenchyma of rats modelled by physical stress, even though there is no significant difference in expression between groups. ROS that arises due to stress will stimulate MAP/ERK Kinase (MEK) to phosphorylate p300 so that there is an increase in the transcription of the HIF-1α molecule in the liver even under normoxia [11]. While in Figure 3, it is appeared that TNF-α expression is very weak to almost zero in the liver. TNF-α production in the liver is carried out by Kupffer cells, which play a role in inflammation and hepatocyte apoptosis through caspase activation [13]. Apoptosis begins with a decrease in BCl-2 activity on the mitochondrial membrane so that membrane permeability changes. These changes cause the release of cytochrome C into the cytosol, which will activate Apaf-1. This activation is followed by a caspase cascade on pro-caspase 9 to process caspase 3, which will damage DNA [26]. From Figure 4, it can be observed that the expression of SOD in the group with 6 g/day honey supplementation appears to be stronger in colour change when compared to other groups, although in statistical analysis the difference was considered insignificant.

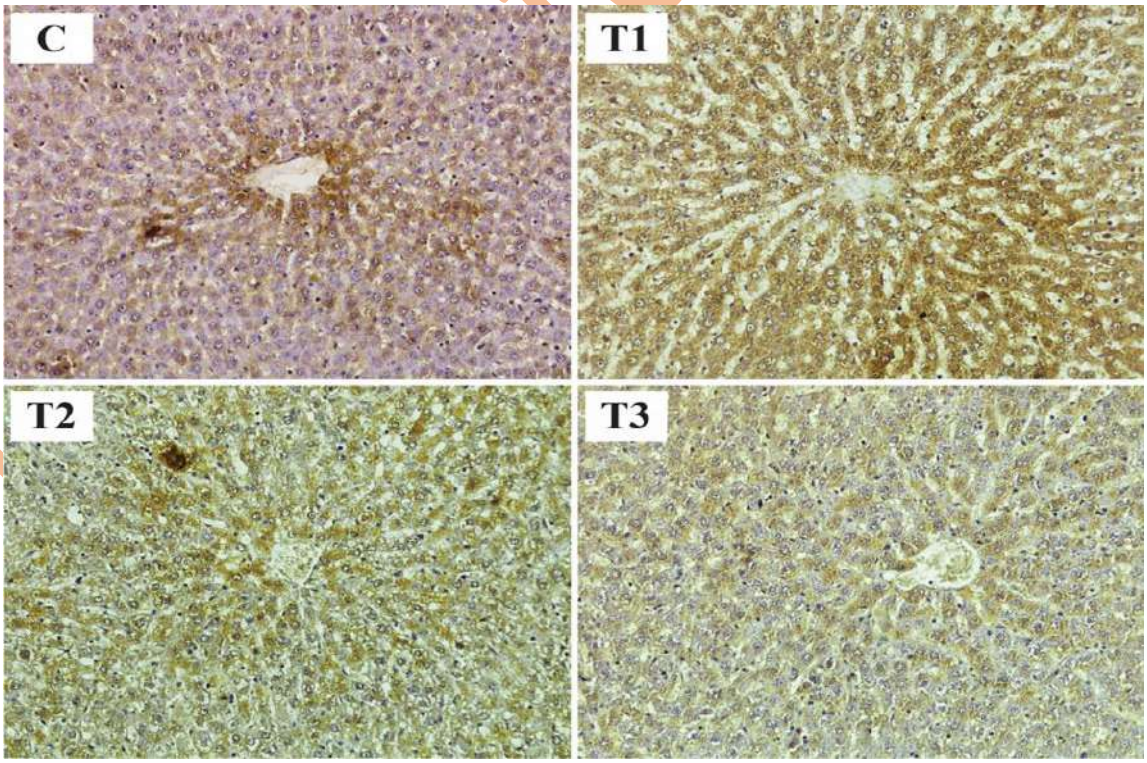


Figure 2: HIF-1α expression in hepatocytes of physical stress model rats by immunohistochemical staining (200x). FST without honey (C); FST with 2 g/day honey (T1); FST with 4 g/day honey (T2); and FST with 6 g/day honey (T3). Brownish-yellow coloration with weak to moderate intensity could be observed in all treatment groups

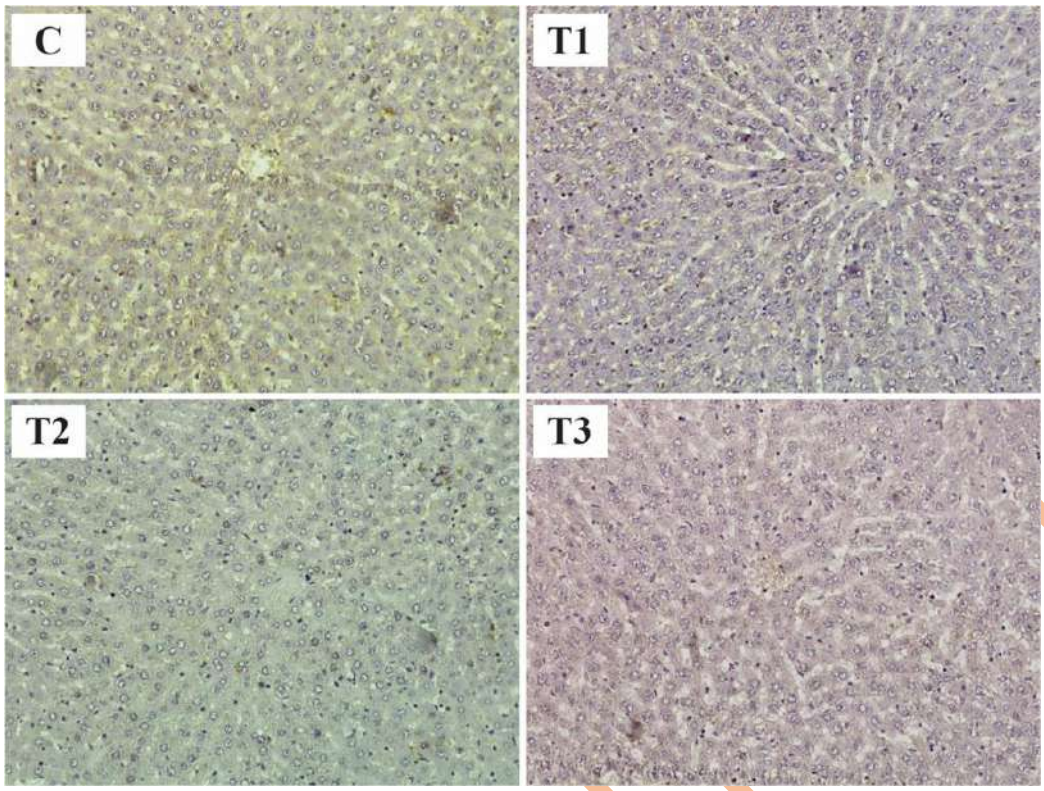


Figure 3: TNF-α expression in hepatic Kupffer cells of physical stress model rats by immunohistochemical staining (200x). FST without honey (C); FST with 2 g/day honey (T1); FST with 4 g/day honey (T2); and FST with 6 g/day honey (T3). A brownish-yellow colour with weak intensity can be observed in group C, while in the honey supplementation group, the intensity is getting smaller to zero

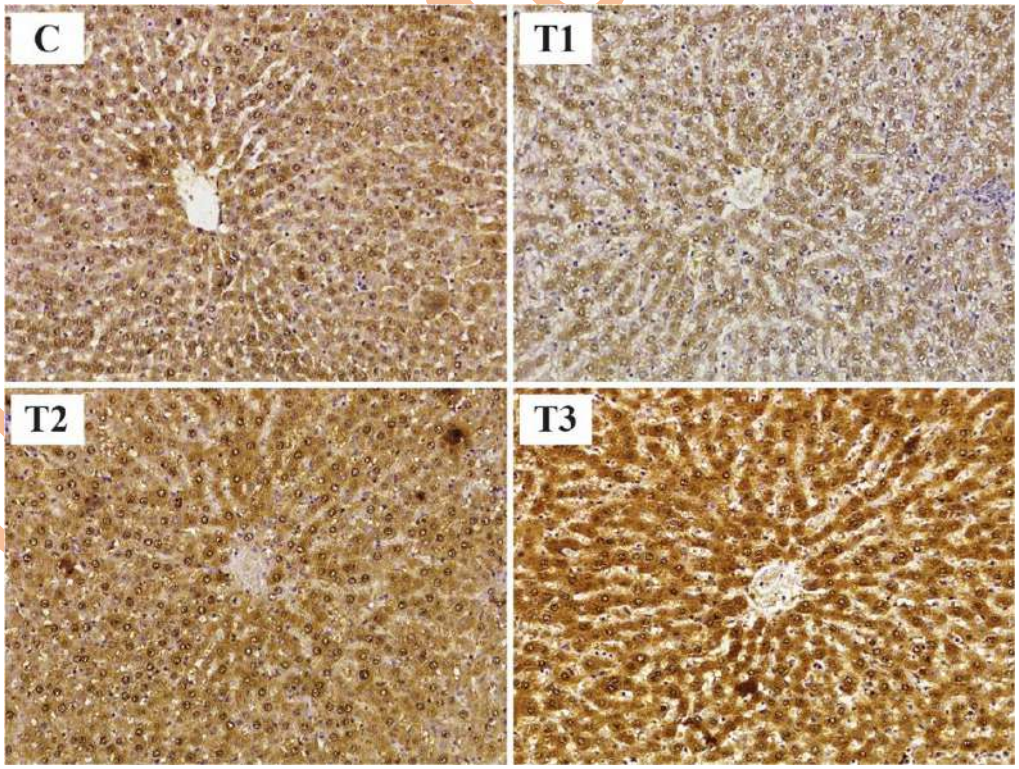


Figure 4: SOD expression in hepatocytes of rats modeled by physical stress by immunohistochemical staining (200x). FST without honey (C); FST with 2 g/day honey (T1); FST with 4 g/day honey (T2); and FST with 6 g/day honey (T3). A brownish-yellow color with moderate to strong intensity can be observed in all groups, with the strongest intensity seen in group T3

The FST procedure used in this study was shown to be associated with HIF-1α production in the liver. This result is in line with other studies that revealed that physical stress of swimming can increase the ROS production, which will induce the activation of HIF-1α in the liver [27]. In addition, the FST procedure proved to be positively correlated with hepatic TNF-α production, following other studies that prove that physical stress can increase TNF-α expression in the liver [28]. The antioxidant content of honey is known to suppress cell damage due to physical stress and ROS [29, 30]. Furthermore, honey has also been shown to increase the production of endogenous hepatic antioxidants SOD as well as GPx, GSH, and catalase [22, 23].

However, honey efficacy was not significantly proven in this study. This may be due to the concentration of ROS being too high, so the dose level used in this study was insufficient to compensate for the cell damage that occurred even though there was an increase in SOD expression. Other studies have shown that excessive consumption of honey can distort the hepatic sinusoid circuit and necrosis of hepatocytes [31]. Similar results also revealed that honey consumption can cause haematological changes as well as damage to hepatic and renal cells [32]. This damage is thought to be due to the accumulation of heavy metals such as Pb and Cd in honey from the environment where the honey is taken. The accumulation of these metals then initiates higher ROS [32]. Therefore, the length of time honey is given and the amount of dose given should be of utmost concern so that honey consumed provides benefits to the body and not the other way around. The dose level of honey and the duration of the study in this study are not sufficient to prove that honey supplementation can prevent general hepatic damage in rats modelled under physical stress. However, the observed parameters in this study do not fully represent the hepatic quality of an individual; other supporting data such as AST, ALT, and GGT profiles are needed for higher validity.

Materials and Methods

Ethical approval

This study had been approved by the Ethics Commission of the Faculty of Veterinary Medicine, Universitas Airlangga (1.KEH.041.04.2022).

Experimental animals

The experimental animals used were three-month-old female Wistar rats (*Rattus norvegicus*) with an average body weight of 200 grams. The total population of 24 rats was divided into four treatment groups using a complete randomization system so that each group contained six rats. Rats were acclimatized with adequate food and water for seven days. During the study, the rats were placed in cages measuring 53 × 30 × 17 cm with a base of wood chips and placed in a room with a temperature of 34 °C and 50% humidity. The forced swimming test is a method used to model animal stress and depression, according to Porsolt [34]. Rats were exercised in a 50 cm diameter and 60 cm high barrel filled with water to approximately 2/3 of the height of the barrel for five minutes every day for 14 days.

Experimental procedure

There were four treatment groups: C with the forced swimming test (FST) alone; T1 with the FST and 2 g/day honey; T2 with the FST and 4 g/day honey; and T3 with the FST and 6 g/day honey. Honey was given by oral gavage. FST was performed for 14 days, and the rats were sacrificed on day 15. Rats were sacrificed with a combination of ketamine and xylazine injections. A laparotomy procedure was performed to collect the liver.

Tissue processing

The organs were prepared for histological examination as follows [33]: each animal's liver was fixed in 10% neutral buffered formalin. The fixed tissues were cleared with xylol after being dehydrated in a graded series of alcohols. The tissues were then infiltrated with molten paraffin at 56–6 ºC. From a solid block of tissue, serial

sections of 3 µm thickness were cut, washed, and placed in object glass.

Immunohistochemistry staining

The tissues were deparaffinized and rehydrated. Then the tissue slides were incubated with hydrogen peroxide for 10-15 minutes, and blocking was performed for 5 minutes. Antibody incubations were conducted using primary antibodies (HIF-1α (1:200), TNFα (1:50), and SOD (1:200)) for 60 minutes and secondary antibodies for 30 minutes. Subsequently, the slides were incubated with streptavidin peroxidase for 10 minutes. Furthermore, the addition of DAB chromogen and substrate was carried out for 15 minutes. Likewise,counterstaining was carried out using Mayer's hemalum solution.

Statistical analysis

Hepatic immunohistochemical protein expressions were documented using a Nikon Eclipse Ci microscope at 200x magnification and interpreted using the IRS scoring system. Expression was considered positive if there was a brownish-yellow colour change due to antigen-antibody binding in hepatocytes. Observations were made in five fields of view, and the results were averaged. The data obtained were then statistically analysed by ANOVA using SPSS for Windows.

Conclusion

The conclusion that can be drawn from this study is that supplementation of *Apis dorsata* honey at the dose level given is unable to reduce the expression of HIF-1α and TNFα and increase the SOD expression in physical stress model rats. In future studies, it is suggested to use a more customized dose of honey and to determine the overall hepatic profile, other research parameters such as AST, ALT, and GGT are needed.

Acknowledgments

The authors express their gratitude to Agung Budianto Achmad, DVM, M.Sc., who supported the data analysis in this study.

Disclosure Statement

No potential conflict of interest was reported by the authors.

Funding

This study was funded by Universitas Airlangga with Grant No. 251/UN3/2022.

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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Manuscript Type	Original Article
Running Title	Effect of Forest Bee Honey (Apis dorsata) Supplementation to Physical Stress
Main Subjects	**Drug** / Drug design and discovery - Offered Subjects: Histopathology
Abstract	Physical activity in general improves metabolic processes by stimulating muscles to contract actively, improving blood circulation, and supplying oxygen. On the other hand, it will create stressful conditions, which will increase cell damage and inflammatory responses in the liver. This study aimed to determine the effect of forest bee honey supplementation on the expression of HIF-1α, SOD, and TNF-α in rats that underwent the forced swimming test (FST) as a physical stress model. The physical activity conducted is at risk of disrupting to several organs due to the stress it causes in addition to the health benefits obtained. A total of 24 adult female rats were divided into four groups: Control (C) with FST only; (T1) FST and honey 2 g/day; (T2) FST and honey 4 g/day; (T3) FST and honey 6 g/day. A forced swimming test was conducted for five minutes per day for 14 days. The collected liver organs were histopathologically prepared by immunohistochemical staining for HIF-1α, SOD, and TNF-α proteins. The results showed that hepatic HIF-1α and TNF-α expression decreased in the honey-supplemented group, while hepatic SOD expression increased, although all three showed insignificant differences from each other. This study concludes that the supplementation of honey is incapable of increasing the expression of SOD as well as reducing the expression of HIF-1α and TNF-α in the liver of rats modelled by physical stress.
Keywords	Forest bee honey, hypoxia-inducible factor, physical stress, superoxide dismutase, tumor necrosis factor-α
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> Hayaza S, Istiqomah S, Kuncoroningrat Susilo RJ, Inayatillah B, Ansori ANM, Winarni D et al. Antidiabetic Activity of Ketapang (Terminalia catappa L.) Leaves Extract in Streptozotocin-Induced Diabetic Mice. Indian Veterinary Journal. 2019;96(12):11-13.

Reviewer 2:

Reviewer Comment For Author:

I'm pleased to be among the team to review your paper. Although your work might have better used further characterizations such as ELISA and gene expression or varied different types of honey, it is still a good read. I hope you look into that in further studies.

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Thu, May 18, 4:23 PM

Final Acceptance Letter and Publishing

Manuscript ID: **JMCS-2304-2018 (R1)**

Manuscript Title: **Effect of Forest Bee Honey (Apis dorsata) Supplementation on Expression of HIF-1α, SOD, and TNF-α in Rats (Rattus norvegicus) Liver Exposed to Physical Stress**

Authors: **Hani Plumeriastuti, Widjiati Widjiati, Mey Vanda Pusparina Sajida, Annise Proboningrat**

Dear **Dr. Hani Plumeriastuti**

This is to confirm that after technical and in-house evaluation, the above mentioned manuscript has been finalized and accepted for publication in the journal. Scopus link of the journal: **https://www.scopus.com/sourceid/21101046187**, Scimago link: **https://www.scimagojr.com/journalsearch.php?q=21101046187&tip=sid&clean=0**, **CiteScore 2022=1.5, Q3, H-index=9, SJR 2022=0.22**. You can see all the articles uploaded to Scopus in this **link** directly.

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6. Publication

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Manuscript Title: **Effect of Forest Bee Honey (Apis dorsata) Supplementation on Expression of HIF-1α, SOD, and TNF-α in Rats (Rattus norvegicus) Liver Exposed to Physical Stress**

Authors: Hani Plumeriastuti, Widjiati Widjiati, Mey Vanda Pusparina Sajida, Annise Proboningrat

Dear Dr. Hani Plumeriastuti

Your paper published in journal.

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Tue, May 23, 1:37 AM

Manuscript ID: JMCS-2304-2018 (R1)

Manuscript Title: **Effect of Forest Bee Honey (Apis dorsata) Supplementation on Expression of HIF-1α, SOD, and TNF-α in Rats (Rattus norvegicus) Liver Exposed to Physical Stress**

Authors: Hani Plumeriastuti, Widjiati Widjiati, Mey Vanda Pusparina Sajida, Annise Proboningrat

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