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Protective Effect of Apis dorsata Honey against Chronic Monosodium Glutamate-induced Testicular Toxicity in Mus musculus mice,

Apis Dorsata Balının Mus musculus Farelerinde Kronik Monosodyum Glutamat ile İndüklenen Testis Toksisitesine Karşı Koruyucu Etkisi

Short title: Apis dorsata Honey Leydig Monosodium Glutamate

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ABSTRACT
Objectives: This study aims to prove the protective effect of *Apis dorsata* honey against chronic monosodium glutamate-induced testicular toxicity on the Leydig cell necrosis count and Malondialdehyde (MDA) serum level in *Mus musculus* mice. **Materials and Methods:** This study used 25 male mice and were grouped into two large groups: the control group consisting of negative control (C-) and positive control (C+). C+ group was given with 4mg/gBW of MSG followed by distilled water. Treatment group consist of T1, T2, and T3 group with *Apis dorsata* honey dosage 53.82 mg/20g, 107.64 mg/20g, 161.46 mg/20g PO respectively followed by MSG 4 mg/gBW of MSG PO. For the difference analysis between group used the one-way ANOVA test and Duncan test. **Results:** The result of this study showed that there was a significant difference between treatment group and control group ($p<0.05$) in the Leydig cell necrosis count and MDA levels. The highest Leydig cell necrosis count and MDA level was found in C+ with value 13.20 ± 2.05 cell and 37.08 ± 9.17 µmol/L compared to C- while in the treatment group, T3 showed the most lowest Leydig cell necrosis value and MDA level 4.64 ± 0.55 cell and 14.22 ± 2.01 µmol/L compared to C+ group. **Conclusion:** It can be concluded that *Apis dorsata* honey

could reduce the Leydig cell necrosis number and MDA level of mice (*Mus musculus*) exposed to Monosodium Glutamate (MSG).
Keywords : Reproductive health, *Apis dorsata* Honey, MSG, Necrosis, Leydig Cells, MDA
ÖZ

Amaç: Bu çalışma, Apis dorsata balının farelerde Leydig hücre nekrozu sayısı ve Malondialdehit (MDA) serum seviyesi üzerindeki kronik monosodyum glutamat kaynaklı testis toksisitesine karşı koruyucu etkisini kanıtlamayı amaçlamaktadır. **Gereç ve Yöntemler:** Bu çalışmada 5 gruba ayrılmış 25 erkek fare kullanıldı. C- grubunda sadece plasebo olarak distile su ile birlikte verilir. C + grubuna 4 mg / gBW MSG ve ardından distile su verildi. Tedavi grubu sırasıyla 53.82 mg / 20g, 107.64 mg / 20g, 161.46 mg / 20g Apis dorsata bal dozu ve ardından MSG 4 mg / gBW MSG ile T1, T2 ve T3 grubundan oluşur. Gruplar arası fark analizi için tek yönlü ANOVA testi ve Duncan testi kullanıldı. **Bulgular:** Bu çalışmanın sonucu, tedavi grubunda kontrol grubuna göre Leydig hücre nekrozu sayısı ve MDA düzeylerinde anlamlı farklılık olduğunu gösterdi ($p < 0.05$). En yüksek Leydig hücre nekrozu sayısı ve MDA düzeyi, C- ye göre 13.20 ± 2.05 hücre ve 37.08 ± 9.17 µmol / L değerleriyle C + 'da bulunurken, tedavi grubunda en düşük Leydig hücre nekroz değeri ve MDA düzeyi 4.64 ± C + grubuna kıyasla 0.55 hücre ve 14.22 ± 2.01 µmol / L. **Sonuç:** Apis dorsata balının, Monosodyum Glutamata (MSG) maruz kalan farelerin (Mus musculus) Leydig hücre nekroz sayısını ve MDA düzeyini azaltabileceği sonucuna varılabilir.
Anahtar Kelimeler: Üreme sağlığı, Apis dorsata Balı, MSG, Nekroz, Leydig Hücreleri, MDA

Introduction

The development of human lifestyles in the era of globalization has led to significant changes in the needs and means of fulfilling nutrition. The fast lifestyle causes people to choose fast food as a fast and cheap alternative. Fast food is an option because of savory taste due to additive added to enhance taste, the most common additive is monosodium glutamate (MSG).[1] MSG consumption has increased every year in Indonesia from 1.53g/capita/day in 1998 to 9.62 g/capita/day in 2011.[2] This excessive consumption behaviour could damage the reproductive system due to the production of excess free radicals subsequently infertility.[3]

MSG can cause infertility due to the activation of several glutamatergic receptors such as Metabotropic Glutamic Receptor (mGluR), Ionotropic Glutamic Receptor (iGluR), and N-Methyl D-Aspartate Receptor (NMDAR). Activation of these receptors will initiate PLC signaling due to activation of G protein and increase intracellular calcium from cells.[4] Increased calcium levels will increase the production of Reactive Oxygen Species (ROS) in the synapses of hypothalamic neurons and cause ablation. On the other hand, the ablation will disrupt the hypothalamic signaling axis - anterior pituitary - testes and interfere with the production of reproductive hormones such as Follicle Stimulating Hormone (FSH) and Interstitial Cell Stimulating Hormone (ICSH).[3]

Leydig cell damage is also caused by excessive production of ROS in the tubules and causes cells to be in a state of oxidative stress, which is characterized by increasing levels of Malondialdehyde (MDA) as a waste product of lipid peroxidation reactions and decreasing glutathione. The damage caused by ROS can be prevented with exogenous antioxidants because it has ability to donor the hydrogen ions and neutralize ROS. [5] *Apis dorsata* forest honey is multiflora honey that produced from multi flower and nectar. It has a more diverse bioactive antioxidant content than *Apis mellifera* honey, which only harvested from one flower.[6] Based on the explanation above, This study aims to prove the protective effect of *Apis dorsata* honey

against chronic monosodium glutamate-induced testicular toxicity with the parameter of the Leydig cell necrosis count and Malondialdehyde (MDA) serum level in *Mus musculus* mice.

Materials and Methods

Ethical approval

This research received ethical clearance number: 1.KE.075.08.2020 released by Animal Care and Use Committee, Faculty of Veterinary Medicine Universitas Airlangga.

This research is an experimental laboratory study using a completely randomized design (CRD) of 25 male mice *(Mus musculus)* divided into five treatment groups using preventive doses and five replications. Mice *(Mus musculus)* were obtained from the Center for Veterinary Farma (PUSVETMA). The mice were then acclimatized for 7 days to minimize stress. The mice were then given a standard feed of Hi-Pro-Vite Medicated 593 Feed.

Mice were grouped into two large groups: the control group consisting of negative control (C-) and positive control (C+) and the treatment group consisting of treatment 1 (T1), treatment 2 (T2), and treatment 3 (T3). The C- was only given a placebo (aqua dest), the C+ was induced with 4 mg/gBW MSG and given aqua dest post 1 hour.The treatment group including T1, T2, and T3 was given with Apis dorsata forest honey with dosages 53,82mg/20gBW, 107,64mg/20gBW, and 161,45mg/20gBW PO respectively, and post 1 hour, they were induced with MSG 4 mg/gBW PO. The dosage is based on research conducted by [7] for Apis dorsata forest honey and [8] for MSG doses. All treatments were carried out for 52 days.

At the end of the treatment, the mice were euthanized using atlantooccipital cervical dislocation, then the testes were prepared and put in 10% formalin solution for histopathological examination with HE staining and intra-cardiac blood collection for MDA levels measurement.

Histopathological examination was performed using a Nikon Eclipse microscope with 400x magnification to see the number of necrotized Leydig cells. Leydig cell necrosis was counted in five visual fields and then averaged. The MDA examination was carried out by using serum samples and using the ELISA colorimetric method. MDA levels have units of μmol/L.

Data Analysis

For the difference analysis between group used the one-way ANOVA test and Duncan test and the data obtained were analyzed statistically by SPSS 20.00 version. To understand which groups are significant each other, the superscript (a,b,c,d) show the different value and different superscript show significant differences between group.

Result and Discussion

The average number of necrotic Leydig cells was observed on histopathological preparations using the Nikon Eclipse E-100 and calculated using a raster image application with a magnification of 400x in five fields of view. MDA levels were measured using a colorimetric method using a spectrophotometer with an absorbance of 450nm. The MDA level was then compared with a standard curve. Generally, the results showed that there was a significant differences ($p<0,05$) between control group and treatment group in the Leydig cell necrosis count and MDA serum level.

In the Leydig cell necrosis count, there were significant differences between the control group and the treatment group as shown in Table 1. In the control group, the highest necrosis cells count was found in C+ with 13.20 ± 2.05 cells, this value is significantly different with T1, T2, T3, and C- (as shown with different superscript), meanwhile, the lowest necrosis cell count was found in C- with 2.56 ± 0.51 cells and significantly different with C+, T1, T2, and T3. In the treatment group consisting of T1, T2, and T3, the T3 group with the highest dose of Apis dorsata forest honey has the lowest necrosis cell count with 4.64 ± 0.55 cells and is significantly

different compared to another treatment group (T1, and T2) and control group (C- and C+). These results indicated that along with an increasing dose of Apis dorsata honey given in monosodium glutamate-induced testicular toxicity, there was a decrease in Leydig cell necrosis count even though T3 is still significantly different with the lowest value in C-.

The MDA serum level results, as shown in Table 2. There were significant differences between groups. In the control group, C+ is significant with C- and all treatment groups (T1, T2, and T3) but C- is only significant with C+, and T1 but not significantly different with T2 and T3. The C+ had the highest value (37.08 ± 9.17) compared to all groups and the lowest MDA value was found in C- (11,87±3,81). In the treatment group consisting of T1, T2, and T3, the T3 group with the highest dose of Apis dorsata forest honey has the lowest MDA serum level 14.22 ± 2.01 although it was not significantly different with T2 17.65 ± 5.72 and compared to C- in the control group. The results also showed that all the treatment group values including T1, T2, and T3 are significantly different from C+ in the control group. These results indicated that the MDA value of each treatment group is decreasing along with the dose of Apis dorsata honey in the treatment group (T1, T2, and T3) and statistically significant compared to C+ even though the lowest value of MDA is in C- group.

Chronic consumption of monosodium glutamate will increase L-glutamate levels in blood vessels which will activate the Metabotropic Glutamic Receptor (mGluR) then will increase the binding activity of D-Aspartate with N-Methyl D-Aspartate Receptor (NMDAR).[8] Normally, in the steroidogenesis process, NMDAR is activated via the MAPK and cAMP signaling pathways to activate the STAR (Steroidogenic Acute Regulatory Protein) complex which actively converts cholesterol into testosterone through biosynthesis of testosterone.[9]

Chronic high L-glutamate levels in the blood will increase the influx of Ca^{2+} in the hypothalamic nerve synapses and will cause nerve cell death due to excessive excitation known as excitotoxicity.[4] This condition will cause ablation of the hypothalamic neuron cells and affect the hypothalamus-pituitary-testis axis and affect the production of ICSH directly.[3] This is evidenced by a study conducted by [10] that there was a significant decrease in ICSH levels along with the increase in the dose of MSG induction.

The disruption of the endocrine axis will cause a hypostimulation state in Leydig cells.[3] On the other hand, excessive NMDAR stimulation facilitates excessive intracellular Ca^{2+} secretion and stimulates activation of ROS-forming enzymes such as Xanthine oxidase, Lipoxygenase, and NADPH Oxidase. Excessive production of ROS will result in a state where endogenous antioxidants such as glutathione (GSH) and Superoxide Dismutase (SOD) are unable to keep up with the production of ROS known as oxidative stress.[11] The excessive activation will disrupt the MAPK signaling pathway so that it will interfere with the STAR-mediated steroidogenesis process.[12]

ROS will bind to Polyunsaturated Fatty Acid (PUFA) and initiate a lipid peroxidation event where a chain reaction occurs which results in a radical lipid. Oxidized lipid cell membranes will produce Malondialdehyde (MDA) and 4-Hydroxinonenal (4-NHE) which are toxic to tissues, especially reproductive tissue.[11] Increased levels of MDA were positively correlated with cell necrosis and tissue damage.[13] This statement was proven by administering MSG 4 mg/gBW in the C+ which caused an increase in the number of necrotic Leydig cells (13.20 ± 2.05) and an increase in MDA levels (37.08 ± 9.17 μmol/L) compared to the C- and the treatment groups (T1, T2, and T3).

In the treatment group, there was a decrease in the number of necrotic Leydig cells sequentially along with the increase in the preventive dose of Apis dorsata forest honey. In the

T3 group, the minimum number of necrotic Leydig cells was 4.64 ± 0.55 cells and significantly different compared to C+ 13.20 ± 2.05 cells ($p<0,05$). In the MDA levels analysis using the colorimetric method, the T3 group showed the lowest MDA level of 14.22 μmol/L and significantly different compared to the C+ group 37.08 ± 9.17 μmol/L ($p<0,05$) and not significantly different ($p> 0.05$) with C- 11.87 ± 3.81 μmol/L. These results are closely related to the potential of Apis dorsata forest honey as an antioxidant and testicular protector potential.

The content of *Apis dorsata* forest honey consists of flavonoids, phenolic components, enzymatic antioxidants such as (glucose oxidase, catalase), carotenoids, amino acids, and vitamin C (ascorbic acid).[6] Phenolic analysis of Apis dorsata forest honey by [14] showed the highest yield of 352.73 gallic acid equivalent compared to *Apis mellifera* honey at 186.70 gallic acid equivalent and *Apis cerana* at 206.33 gallic acid equivalent. *Apis dorsata* forest honey also has antioxidant potential measured using the DPPH method of 5453.57 ppm IC50.[15] This high antioxidant potential can overcome the formation of ROS caused by MSG.

The phenolic compounds present in *Apis dorsata* forest honey play an important role in the inactivation of ROS produced by excessive NMDAR activation. Anthraquinone compounds reduce ROS such as singlet oxygen, hydroxyl radical, and superoxide and make these radicals inactive and unable to bind to PUFAs thus preventing auto-oxidation.[16] The content of vitamin C in *Apis dorsata* forest honey also acts as a chain-breaking antioxidant that protects PUFAs. The content of flavonoids also plays a role in chelating transition metals such as Fe (II), Fe (III), and Cu (II) which play a role in the formation of ROS. [17] In this study, giving forest honey as a preventive dose was proven to reduce the number of necrotic Leydig cells and reduce MDA levels.

On the other hand, forest honey also has a role in preventing hypothalamic ablation caused by excitotoxicity and reducing oxidative stress that occurs in the brain due to excessive excitatory postsynaptic stimulation of neurons. Repair in the hypothalamus-pituitary-testicular axis directly normalizes ICSH production from the anterior pituitary and normalizes the function of steroidogenesis.[3] Through this mechanism, giving *Apis dorsata* forest honey a preventive dose can prevent oxidative stress caused by chronic MSG consumption by reducing the number of necrotic Leydig cells and decreasing MDA levels.

Conclusion

This study concludes that giving *Apis dorsata* forest honey as a preventive dose can reduce the Leydig cells necrotic counts and MDA levels in mice (Mus musculus) that are chronically exposed to MSG..

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Conflicts of Interest:

The authors declared no conflict of interest.

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Table 1. The average number of necrotic Leydig cells in each group

Group	**Leydig cell necrosis number (Mean ± S.D)**
C-	$2,56^{e} \pm 0,51$
C+	$13,20^{a} \pm 2,05$
T1	$9,84^{b} \pm 0,74$
T2	$8,12^{c} \pm 1,08$

T3	$4,64^{d} \pm 0,55$

Different superscript show significant differences ($p < 0.05$). C-: Control (distilled water). C+: MSG PO 4mg / gBW + distilled water. T1: Apis dorsata PO (53.82mg / 20g) + MSG PO 4mg / gBW. T2: Apis dorsata Honey PO (107.64 mg / 20g) + MSG PO 4 mg / gBW. T3: Apis dorsata PO Honey (161.46g / 20g) + MSG PO 4mg / gBW. All treatments were carried out for 52 days.

Table 2. MDA levels in serum

Group	MDA Level (µmol/L) (Mean ± S.D)
C-	$11,87^{c} \pm 3,81$
C+	$37,08^{a} \pm 9,17$
T1	$23,87^{b} \pm 11,88$
T2	$17,65^{bc} \pm 5,72$
T3	$14,22^{bc} \pm 2,01$

Different superscript show significant differences ($p < 0.05$). C-: Control (distilled water). C+: MSG PO 4mg / gBW + distilled water. T1: Apis dorsata PO (53.82mg / 20g) + MSG PO 4mg / gBW. T2: Apis dorsata Honey PO (107.64 mg / 20g) + MSG PO 4 mg / gBW. T3: Apis dorsata PO Honey (161.46g / 20g) + MSG PO 4mg / gBW. All treatments were carried out for 52 days.

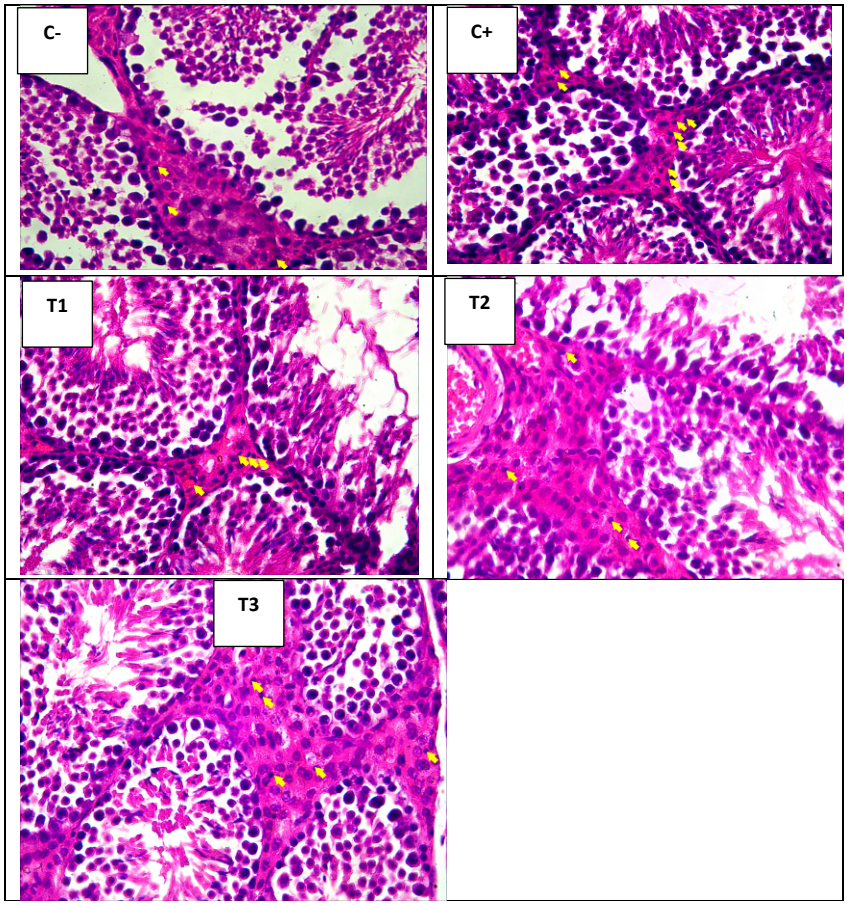

Figure 1 The testicular histopathology (HE) of mice (Mus musculus) given Apis dorsata forest honey as a preventive dose with a magnification of 400x, yellow arrows showed necrotic Leydig cells marked with pyknotic. C-: Control (distilled water). C+: MSG PO 4mg / gBW + distilled water. T1: Apis dorsata PO (53.82mg / 20g) + MSG PO 4mg / gBW. T2: Apis dorsata Honey PO (107.64 mg / 20g) + MSG PO 4 mg / gBW. T3: Apis dorsata PO Honey (161.46g / 20g) + MSG PO 4mg / gBW. All treatments were carried out for 52 days.