

Apilarnil: A Novel Neuroprotective Candidate

Mehmet HAMAMCI¹, Zuleyha DOGANYIGIT², Sibel SILICI³, Aslı OKAN², Emin KAYMAK²,
Seher YILMAZ⁴, Adem TOKPINAR⁴, Levent Ertuğrul INAN¹

Abstract

Purpose: This study was designed to investigate the effect of apilarnil on neuronal damage and related mechanisms in a sepsis model in order to demonstrate whether or not apilarnil has neuroprotective effect.

Methods: In this study, 64 adult male Sprague-Dawley species rats were randomly divided into eight groups. The rats were administered apilarnil and/or lipopolysaccharide (LPS). Superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), xanthine oxidase (XOD) and testican-1 levels were measured in the brain tissue. Proinflammatory cytokines (tumor necrosis factor alpha [TNF- α], interleukin 1 beta [IL-1 β], interleukin 6 [IL-6]) were measured in brain tissue. Histological examinations were performed on hippocampus and cortex tissues in all groups. Apoptotic cell count was estimated using the Tunel method to observe the apilarnil's effect on apoptosis. Purkinje cells were counted in the hippocampus to measure the protective effect of apilarnil on the hippocampus.

Results: Apilarnil reduced the decrease in SOD and CAT levels in the brain developing sepsis. Apilarnil reduced the increase in MDA, XOD, and testican-1 levels in the septic brain. It was observed that the number of degenerated neurons due to sepsis decreased as apilarnil dose increased. Apilarnil reduced the elevated levels of proinflammatory cytokines (IL-6, TNF- α , IL-1 β) induced by sepsis. Apilarnil prevented sepsis-related apoptosis in the brain.

Conclusion: The neuroprotective potential of apilarnil against brain damage in the sepsis model was demonstrated and suggested that it has the potential to contribute to new therapeutic targets against various neurological disorders.

Keywords: Blood-brain barrier; Brain; Sepsis; Apoptosis; Oxidative stress; Neurodegeneration

Acta Neurol Taiwan 2020;29:33-45

1. INTRODUCTION

Neuroinflammation plays a critical role in the

pathology of neurocognitive disorders such as Parkinson's disease, Alzheimer's disease, and cognitive dysfunction induced by sepsis⁽¹⁻³⁾. Lipopolysaccharides (LPS) are one

From the ¹Department of Neurology, Bozok University Medical School, Yozgat, Turkey. ²Department of Histology-Embryology, Bozok University Medical School, Yozgat, Turkey. ³Department of Agricultural Biotechnology, Faculty of Agriculture, Erciyes University, Kayseri, Turkey. ⁴Department of Anatomy, Bozok University Faculty of Medicine, Yozgat, Turkey.

Received February 11, 2020. Revised March 6, 2020.

Accepted March 27, 2020.

Correspondence to: Mehmet HAMAMCI

Department of Neurology, Bozok University Medical School, Yozgat, Turkey.

E-mail: drmehmetmehmet@gmail.com

of the strongest activators of proinflammatory cytokines in lab animals and humans^(4,5). Studies have shown that LPS administration causes memory impairment⁽⁶⁾, chronic inflammation, and advanced neurodegeneration⁽⁷⁾.

LPS is an integral component of the external membrane of gram-negative bacteria and plays an important role in the pathogenesis of septic shock⁽⁸⁾. Sepsis causes dysfunction in multiple organs, especially the brain, which is one of the first organs to be affected^(9,10). Sepsis may cause acute and chronic changes in the central nervous system, particularly the blood-brain barrier, with mechanisms that are largely not understood^(9,11). Sepsis-associated encephalopathy (SAE) is a serious complication that can cause death and severe disabilities⁽¹²⁾. Developments in medical treatment of sepsis has increased survival rates, and subsequently, the number of critical patients with cognitive, functional, and mental impairments⁽¹³⁾. Despite the high mortality rate of SAE, there is no treatment to reduce or minimize brain damage in sepsis patients, and clinical treatment is limited to the underlying infection⁽¹⁴⁾. The most common form of acute brain dysfunction is delirium, which occurs in 80% of sepsis patients⁽¹⁵⁾. In addition, incidence of long-term cognitive impairment in sepsis patients is 46-70% in the first year and 25% in the first six years following discharge^(16,17).

Microglia are immune system cells located in the brain which play an important role in various neurodegenerative diseases. Microglia also have protective and neuroprotective properties against inflammation in the brain⁽¹⁸⁾. Microglia can be activated with the activating effect of proinflammatory factors (tumor necrosis factor alpha [TNF- α], interleukin 1 beta [IL-1 β], interleukin 6 [IL-6])⁽⁷⁾. Excessive microglia activity and high levels of pro-inflammatory factors cause brain damage. Therefore, inhibition of excessive microglial proinflammatory response can treat neurodegenerative diseases such as Parkinson's and Alzheimer's disease^(19,20).

Nowadays, people have a multi-faceted approach to nutritional activity and alter their eating habits in order to prevent and/or treat certain diseases. With the increase in the elderly population, the significance of long-term preservation of brain functions has increased and there is a consensus that protective measures must be discovered as soon as possible. Bee products such as honey, propolis,

royal jelly, beebread, and pollen have been known and utilized by humans for thousands of years⁽²¹⁾. Evidence of the use of bee products by humans as food as well as for medicinal properties has been discovered in Sumerian clay tablets⁽²¹⁾. The anti-inflammatory and antiseptic properties of bee products is widely known^(21,22). Honey has been used in folk medicine to treat infections and colds^(21,22). Apilarnil is the 3-7-day larva period of male bee brood. It has high biological activity due to the sum of nutritional compounds found in both egg and larvae bodies^(21,23).

LPS exposure has an inhibiting effect on steroidogenesis⁽²⁴⁾. Proinflammatory cytokines such as TNF- α and IL-1 have been shown to inhibit gonadal functions especially in steroidogenesis of Leydig cells^(25,26). Research on the histological and biochemical effects of apilarnil are in their initial stages; Bolatovna et al.⁽²³⁾ demonstrated that parenteral apilarnil injection improved qualitative and quantitative production of semen in young pigs. However, no experimental study examining the effect on the brain has been encountered.

This study aims to investigate related mechanisms involved in the neuroprotective effect of apilarnil.

2. METHODS

2.1. Animals

This study obtained ethics approval from the University Animal Experiments Local Ethics Committee (Ethics committee approval No: 18/0063). The rats housed in the Experimental and Clinical Research Center of the University were kept in cages were provided with water and nutrient requirements at normal temperature of 24 \pm 2°C and 12 hours of light/dark environment.

2.2. Experiment Protocol

Lipopolysaccharide was provided by Sigma Aldrich (Escherichia coli LPS, serotype 0127: B8) and Lyophilized Apilarnil was provided by Nutral Therapy Co Ltd (Erciyes University Technopark, Kayseri). The energy content of apilarnil is 472 \pm 2.3 kcal/100g. Apilarnil (100g) contains 4.43g moisture, 4.07g ash, 48.75g protein, 21.62g carbohydrate, and 21.12g lipids. Apilarnil contains 16 different amino acids. Of the essential amino acids, only tryptophan has not been found. Of the amino acids identified in apilarnil, the amino acid of the

highest amount was lysine (7198mg/100g) and the lowest amount was methionine (500 mg/100g). Total phenolic content of apilarnil was 834.05 mg gallic acid equivalents (GAE)/100g and antioxidant activity was 90.91 mg ascorbic acid equivalents (AAE)/g. Antiradical activity inhibition level of apilarnil was found to be 81.61% (27). For the study, 64 Sprague-Dawley species (200-250 gr) male rats were randomly divided into eight equal groups. The doses for LPS and apilarnil were adjusted for the weights of each animal.

Group 1: (control group): only administered 1ml intraperitoneal (i.p.) physiological saline solution (SF) (0.9% NaCl sol.) (n: 8)

Group 2: administered 30 mg/kg/body weight (bw) by 1 ml i.p. LPS (n:8)

Group 3: administered 0.2 g/kg/bw apilarnil by 1 ml oral gavage (daily for 10 days) (n: 8)

Group 4: administered 0.4 g/kg/bw apilarnil by 1 ml oral gavage (daily for 10 days) (n: 8)

Group 5: administered 0.8 g/kg/bw apilarnil by 1 ml oral gavage (daily for 10 days) (n: 8)

Group 6: administered 0.2 g/kg/bw apilarnil by 1 ml oral gavage and 60 min. later LPS (single-dose i.p., 30mg / kg/bw) (n: 8)

Group 7: administered 0.4 g/kg/bw apilarnil by 1 ml oral gavage and 60 min. later LPS (single-dose i.p., 30mg / kg/bw) (n: 8)

Group 8: administered 0.8 g/kg/bw apilarnil by 1 ml oral gavage and 60 min. later LPS (single-dose i.p., 30mg / kg/bw) (n: 8)

2.3. Biochemical analyses

Tissue samples taken from rats were used for biochemical analysis. Superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and xanthine oxidase (XOD) and testican-1 (TCN1) levels were assessed in brain tissue. Rat SOD (Cat. No: 201-11-0169, Sunred Bio), Rat CAT (Cat. No: 201-11-5106, Sunred Bio), Rat MDA (Cat. No: 201-11-0157, Sunred Bio), Rat XOD (Cat. No: 201-11-5140, Sunred Bio), and Rat testican-1 (Cat. No: 201-11-3210, Sunred Bio) levels were measured using protocol instructed in the manufacturer kits. Concentrations were measured with ELISA reader at 450 nm. Results were expressed as ng/ml for SOD, CAT, XOD, and TCN1 and as nmol/ml for MDA.

2.4. Histological Analysis

Brain tissue samples were fixed with 10% formaldehyde solution for histological examinations. After fixation, routine tissue processing steps were applied and the tissues were embedded in paraffin. Sections of 5-6 μ m from paraffin blocks were examined under an Olympus BX53 microscope and stained with hematoxylin-eosin (H&E) in order to observe the general histological structure and imaged with DP25 model digital camera. TNF- α , IL-6 and interleukin 1 beta (IL-1 β) levels were evaluated with immunohistochemical staining in order to assess the anti-inflammatory activity between the groups.

2.5. Immunohistochemical Analysis

Avidin-biotin-peroxidase method was used for labeling in order to determine the differences in TNF- α , IL-6 and IL-1 β expressions in brain tissue. For this purpose, 5-6 μ m sections were kept at 60°C overnight, then rehydrated first by xylene and then by graded alcohol series, washed with phosphate buffer (PBS) three times for five minutes each, and boiled with 5% citrate buffer in a microwave at 600W 3x5 times for antigen recovery. After resting in the buffer solution for 20 minutes at room temperature, sections were rewashed with PBS and treated with 3% hydrogen peroxide (H₂O₂) for 5 minutes to prevent endogenous peroxidase activity. ABC staining kit was used for the next steps. Sections that were rewashed with PBS were treated with blocking serum at room temperature for 20 minutes to ensure that areas outside the antigenic sites were closed. Immediately after, the primary antibody (separate for TNF- α , IL-6, and IL-1 β) was instilled into the sections and left overnight at +4°C and incubated for 20 minutes the following day. PBS was used instead of primary antibody for negative control. After the washing process, the sections were incubated with biotinylated secondary antibody for 30 minutes and washing was repeated. The sections were then treated with Avidin-Biotin (AB) enzyme reagent for 30 minutes and washed and treated with diaminobenzidine (DAB) based peroxidase substrate for five minutes to make immunoreactivity visible, then washed with deionized H₂O for five minutes. The sections stained with Gill's hematoxylin were washed several times with deionized H₂O. As the final step, water was removed with increased series of alcohol and the sections were passed through xylene and closed with entellan.

The images were observed under an Olympus BX53 light microscope and the images were obtained with the DP25 digital camera and evaluated using image j software in terms of differences in expression.

2.6. TUNEL Method

Apoptotic cells were identified in sections obtained from the subjects using the Roche brand In Situ Cell Detection Apoptosis Fluorescein Kit. The staining process was performed according to the kit's protocol. Brain tissue sections taken at 5µm thickness were deparaffinized and then rehydrated and washed with PBS for 5 minutes twice. They were then incubated in 0.01 M 5% sodium citrate buffer at 350 W in a microwave oven for five minutes for antigen recovery, then allowed to cool for 10 minutes at room temperature. The tissues that were washed with PBS for five minutes twice were then placed in a humidity chamber at 37°C with TUNEL reaction mixture from the kit and incubated in the oven for 60 minutes. The tissues washed with PBS for 5 minutes twice were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Tissues covered with glycerol solution were viewed under an Olympus BX-51 fluorescence microscope at a wavelength of 450-500 nm. For apoptotic index, apoptotic cells of a total of 50 different regions were counted at 20X objective.

2.7. Pyramidal cell count in the hippocampus

In order to estimate the number of pyramidal cells in the hippocampus CA region, the optical disector method^(28,29) was used in the calculations, sections were selected according to random systematic sampling.

Stereology workstation (Kayseri, Turkey) was used for stereological analyses, consisting of a CCD digital camera, an image capture card, a personal computer, a computer-controlled motorized specimen stage (Prior Scientific, Cambridge, UK), a microcator (Heidenhein Traunreut, Germany), and a light microscope (Leica, Nubloch, Germany). The software program Macrobrightfield was used to measure and record stereological data and capture digital images of the sections. Microscopy images [100x Leica HCX Plan Apo objective] were generated by the software and displayed on a computer monitor to count the number of pyramidal cells in the hippocampal CA regions. The total number of pyramidal cells was calculated

according to the pyramidal cell count and sampling probability.

The total number of pyramidal neurons (N) in the CA region of the hippocampus was calculated using the following formula:

$$N = \Sigma Q \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{1}{tsf}$$

(N: total number of neurons, ΣQ: total disector neuron number, ssf: section sampling fraction, asf: area sampling fraction, tsf: thickness sampling fraction)

In order to obtain unbiased results of hippocampal cell counts, the analysis was performed by blinded researchers.

2.8. Statistical Analysis

SPSS 22.0 package program was used for statistical analysis. Results were expressed as mean and standard deviation. One-way ANOVA test was used for comparison between groups with normal distribution. Post-Hoc Tukey test was used for paired comparisons with normal distribution. Kruskal Wallis test was used for comparison of values without normal distribution. Mann-Whitney-U non-parametric test was used for paired comparisons of values with normal distribution. P <0.05 was considered statistically significant.

3. RESULTS

3.1. Biochemical Results

There was no significant difference between the control group and the groups that were only administered apilarnil (Groups 3, 4, and 5) according to SOD, CAT, MDA, XOD, and TCN-1 levels. When the biochemical results of the brain were examined, there was a significant decrease in SOD and CAT levels in the LPS group compared to the control group. SOD and CAT levels in the LPS+0.8 g/kg apilarnil group were significantly higher compared to the LPS group. MDA, XOD, and testican-1 levels increased significantly in the LPS group compared to the control group. MDA, XOD, and testican-1 levels in LPS+0.8 g/kg apilarnil group were significantly lower compared to the LPS group (Table 1).

Table 1: Biochemical results of the groups

Groups	Control	LPS	0.2 g/kg Apilarnil	0.4 g/kg Apilarnil	0.8 g/kg Apilarnil	LPS+0.2 g/kg Apilarnil	LPS+0.4 g/kg Apilarnil	LPS+0.8 g/kg Apilarnil	<i>P</i>
SOD	23.95 ±1.21 ^a	17.58 ±0.97 ^b	21.19 ±2.65 ^{a,b}	22.66 ±2.90 ^a	19.82 ±2.64 ^{a,b}	20.45 ±2.50 ^{a,b}	21.32 ±3.03 ^{a,b}	22.92 ±2.85 ^a	0.014
CAT	78.58 ±5.48 ^a	65.45 ±2.16 ^b	73.19 ±3.52 ^{a,b}	74.48 ±1.18 ^{a,b}	76.52 ±5.31 ^a	73.25 ±6.08 ^{a,b}	70.58 ±5.53 ^{a,b}	75.18 ±7.77 ^a	0.016
XOD	14.68 ±1.14 ^c	19.24 ±1.27 ^a	16.41 ±0.89 ^{b,c}	17.79 ±1.19 ^{a,b}	16.45 ±1.15 ^{b,c}	18.12 ±1.73 ^{a,b}	16.94 ±1.29 ^{a,b,c}	15.88 ±1.34 ^{b,c}	0.001
MDA	16.15 ±2.19 ^a	26.49 ±2.66 ^b	15.98 ±2.52 ^a	15.26 ±5.43 ^a	18.42 ±6.49 ^{a,b}	20.94 ±4.93 ^{a,b}	20.49 ±9.01 ^{a,b}	17.07 ±2.48 ^a	0.038
Testican-1	2.01 ±0.63 ^a	3.56 ±0.31 ^b	2.91 ±0.45 ^{a,b}	2.91 ±0.58 ^{a,b}	2.65 ±0.46 ^b	2.62 ±0.87 ^{a,b}	2.66 ±0.60 ^{a,b}	2.82 ±0.67 ^a	0.010

SOD: Superoxide dismutase, CAT: catalase, XOD: xanthine oxidase, MDA: malondialdehyde, Data is expressed as ± standard deviation.

P < 0.05 was considered significant.

There was no significant difference between the groups containing the same letter (a, b, and c).

3.2. Histological Results

There was no significant difference between the control group and the groups that were only administered apilarnil (Groups 3, 4, and 5) according to pathological changes in the cortex and hippocampus in histologic examination. Enlarged blood vessels and some neurons degeneration were observed in the brain cortex in the LPS group. The number of degenerated neurons observed in the brain cortex of the LPS group decreased as apilarnil dose increased (Figure 1A). Dilated blood vessels and degeneration of some neurons were observed in the hippocampus of the LPS group. In the groups that were administered LPS and apilarnil together, observed pathology decreased as apilarnil dose increased (Figure 1B).

3.3. Immunohistochemical Results

The control group and the groups that were only administered apilarnil (Groups 3, 4, and 5) had similar TNF- α , IL-6, and IL-1 β levels. TNF- α expression in the cortex was significantly increased in the LPS and LPS+0.2 g/kg apilarnil groups compared to the control group. Increased TNF- α expression was significantly lower in the LPS+0.4 g/kg and LPS+0.8 g/kg apilarnil groups. TNF- α expression in the hippocampus was significantly increased in LPS, LPS+0.2 g/kg and LPS+0.4 g/kg apilarnil groups. Increased TNF- α expression was found to be significantly reduced in the LPS+0.8 g/kg apilarnil group. IL-6 expression in the cortex was significantly higher in the LPS, LPS+0.2 g/kg, LPS+0.4 g/kg and LPS+0.8 g/kg apilarnil groups compared to the control group. IL-6 expression in the hippocampus was suppressed in groups in which LPS and apilarnil were administered together. IL-6 expression in the cortex was significantly increased in LPS, LPS+0.2 g/kg and LPS+0.4 g/kg apilarnil groups compared to the control group. IL-6 expression in the hippocampus was suppressed in groups in which LPS and apilarnil were administered together. IL-1 β expression in the cortex was significantly higher in the LPS, LPS+ 0.2 g/kg, and LPS+0.4 g/kg apilarnil groups compared to the control group. IL-1 β expression in the cortex was suppressed in groups in which LPS and apilarnil were administered together. IL-1 β expression in the hippocampus was significantly increased in LPS, LPS +0.2 g/kg and LPS+0.4 g/kg apilarnil groups compared to

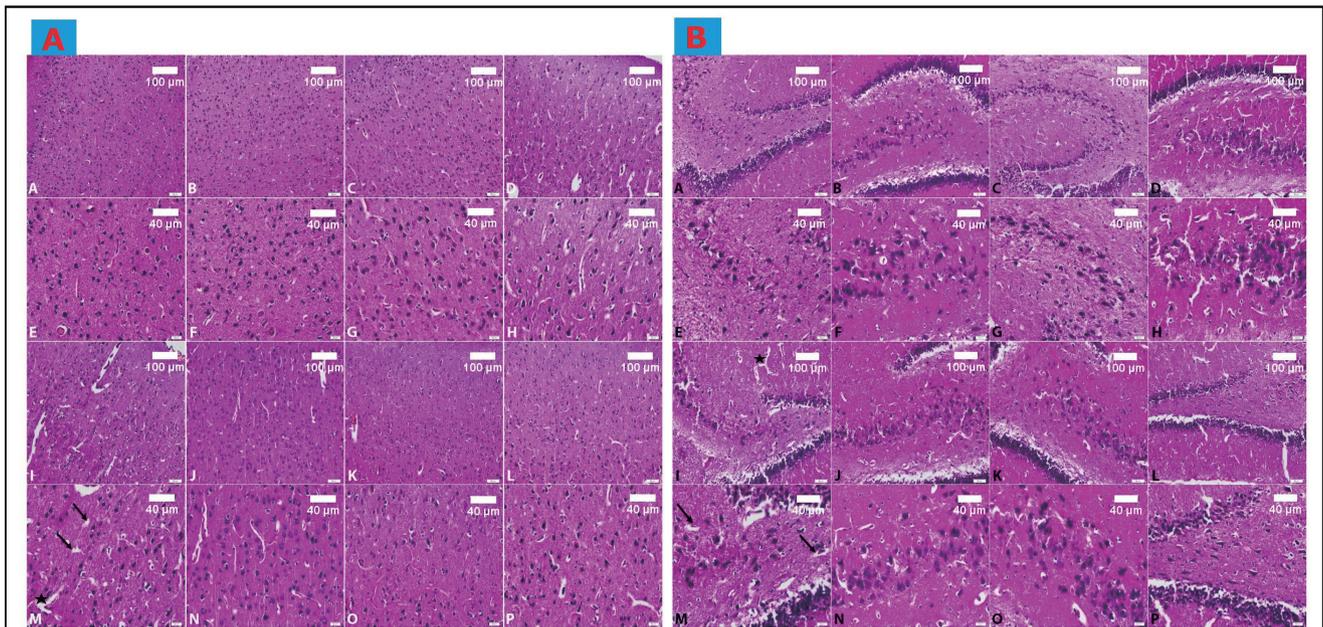


Figure 1.

A. Hematoxylin eosin cross-sectional images belonging to the rat brain cortex. Dilated blood vessels (asterisks) and degenerate neurons (arrows) with cavities are observed in the brain cortex of the LPS group.

B. Hematoxylin eosin cross-sectional images belonging to the rat hippocampus. Dilated blood vessels (asterisks) and degenerate neurons (arrows) with cavities are observed in the hippocampus of the LPS group.

(Control (A and E), 0.2 g/kg apilarnil (B and F), 0.4 g/kg apilarnil (C and G), 0.8 g/kg apilarnil (D and H), LPS (I and M), LPS + 0.2 g/kg apilarnil (J and N), LPS + 0.4 g/kg apilarnil (K and O) and LPS + 0.8 g/kg apilarnil (L and P). A-D and I-L 20X magnification, bar = 100μm. E-H and M-P 40X magnification, bar = 40μm.)

the control group. IL-1 β expression in the hippocampus was suppressed in the LPS+0.8 g/kg apilarnil group (Figure 2).

3.4. TUNEL Results

There was no significant difference between the control group and the groups that were only administered apilarnil (Groups 3, 4, and 5) according to number of apoptotic cells. According to the results of apoptosis, there was a significant increase in the number of apoptotic cells in the brain cortex in the LPS group compared to the control group. Number of apoptotic cells were significantly less in the LPS, LPS+0.2 g/kg, LPS+0.4 g/kg, and LPS+0.8 g/kg apilarnil groups compared to the LPS group (Table 2, Figure 3).

3.5. Hippocampal Pyramidal cell count

There was no significant difference between the

control group and the groups that were only administered apilarnil (Groups 3, 4, and 5) according to number of pyramidal cells. The mean number of pyramidal cells was 554.185 ± 28.178 in the LPS group and 607.912 ± 16.164 in the control group, and this difference was significant ($p < 0.001$). The mean number of pyramidal cells in the LPS+0.8 g/kg apilarnil group was 593.990 ± 27.346 and was significantly higher compared to the LPS group ($p < 0.008$) (Table 3, Figure 4).

4. DISCUSSION

Sepsis-associated encephalopathy (SAE) is diffuse cerebral dysfunction thought to be caused by systemic inflammatory response. Although the mechanism of cerebral dysfunction is unclear, the central nervous system has been proven to be one of the first organs to be affected by sepsis^(9,12). LPS is a strong stimulant of inflammatory

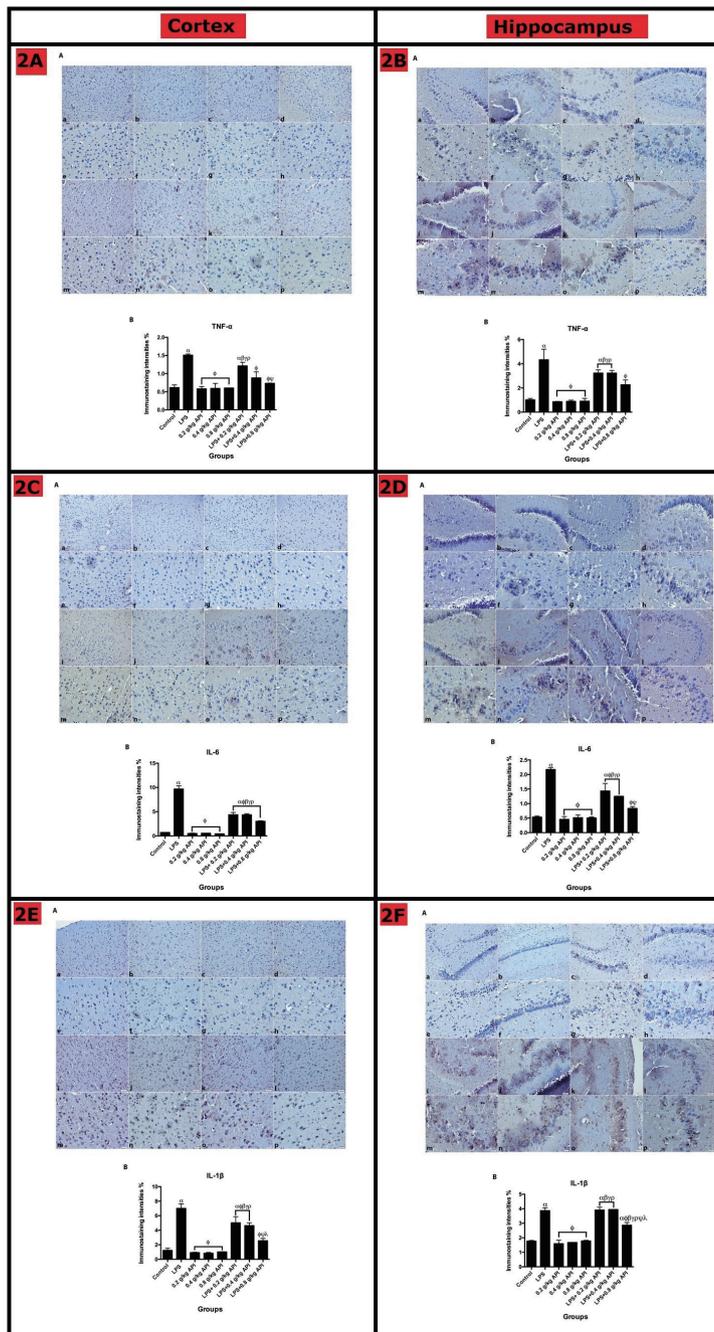


Figure 2.

2A. TNF- α immune staining in rat brain cortices: Increased TNF- α expression in the LPS group was significantly reduced in the groups that were administered LPS + 0.4 g/kg/bw and LPS + 0.8 g/kg/bw apilarnil.

2B. TNF- α immune staining in rat brain hippocampus: Increased TNF- α expression in the LPS group was significantly reduced in the group that was administered LPS + 0.8 g/kg/bw apilarnil.

2C. IL-6 immune staining in rat brain cortices: Increased IL-6 expression in the LPS group was suppressed in the groups that were administered LPS and apilarnil together.

2D. IL-6 immune staining in rat brain hippocampi: Increased IL-6 expression in the LPS group was suppressed in the groups that were administered LPS and apilarnil together.

2E. IL-1 β immune staining in rat brain cortices: Increased IL-1 β expression in the LPS group was suppressed in the groups that were administered LPS and apilarnil together.

2F. IL-1 β immune staining in rat brain hippocampi: Increased IL-1 β expression in the LPS group was suppressed in the groups that were administered LPS and apilarnil together.

[A. Images of immunostaining: control (a and e), 0.2 g/kg apilarnil (b and f), 0.4 g/kg apilarnil (c and g), 0.8 g/kg apilarnil (d and h), LPS (i and m), LPS+0.2 g/kg apilarnil (j and n), LPS+0.4 g/kg apilarnil (k and o) and LPS+0.8 g/kg apilarnil (l and p) groups. a-d and i-l 20X magnification, e-h and m-p 40X magnification. B. Histograms represent the intensity values in percent of immunostaining obtained using Image J software. (α P < 0.05 different from control group; ϕ P < 0.05 difference from LPS group; β P < 0.05 difference from 0.2 g/kg apilarnil group; γ P < 0.05 difference from 0.4 g/kg apilarnil group; δ P < 0.05 difference from 0.8 g/kg apilarnil group; ψ P < 0.05 difference from LPS+0.2 g/kg apilarnil group; λ P < 0.05 difference from LPS+0.4 g/kg apilarnil group)]

cytokines and is generally used as a model to examine sepsis, neuroinflammation, and cognitive impairment. LPS is also known to cause brain damage as a result of neuronal death induced by oxidative stress and inflammation^(30,31).

In this study, LPS-induced endotoxic sepsis model was used to investigate the effect of apilarnil on responses to inflammation and oxidative stress. Apilarnil was found to prevent decrease in SOD and CAT levels while also

preventing increase in XOD and MDA levels. The body possesses a complex antioxidant defense mechanism based on endogenous enzymatic and non-enzymatic antioxidants. These molecules act collectively against free radicals to resist the harmful effects against vital biomolecules and, ultimately, body tissues. Antioxidants can be categorized as primary, secondary, tertiary, and even quaternary lines of defense according to their responses against the

Table 2. TUNEL results of the groups

Groups	Control	LPS	0.2 g/kg Apilarnil	0.4 g/kg Apilarnil	0.8 g/kg Apilarnil	LPS+ 0.2 g/kg Apilarnil	LPS+ 0.4 g/kg Apilarnil	LPS+ 0.8 g/kg Apilarnil	<i>p</i>
TUNEL	0.08±0.34 ^a	0.5±0.61 ^b	0.02±0.14 ^a	0.06±0.23 ^a	0.10±0.30 ^a	0.18±0.56 ^a	0.04±0.28 ^a	0.06±0.23 ^a	0.001

Data is expressed as ± standard deviation. $P < 0.05$ was considered statistically significant. There was no significant difference between groups containing the same letter (a and b).

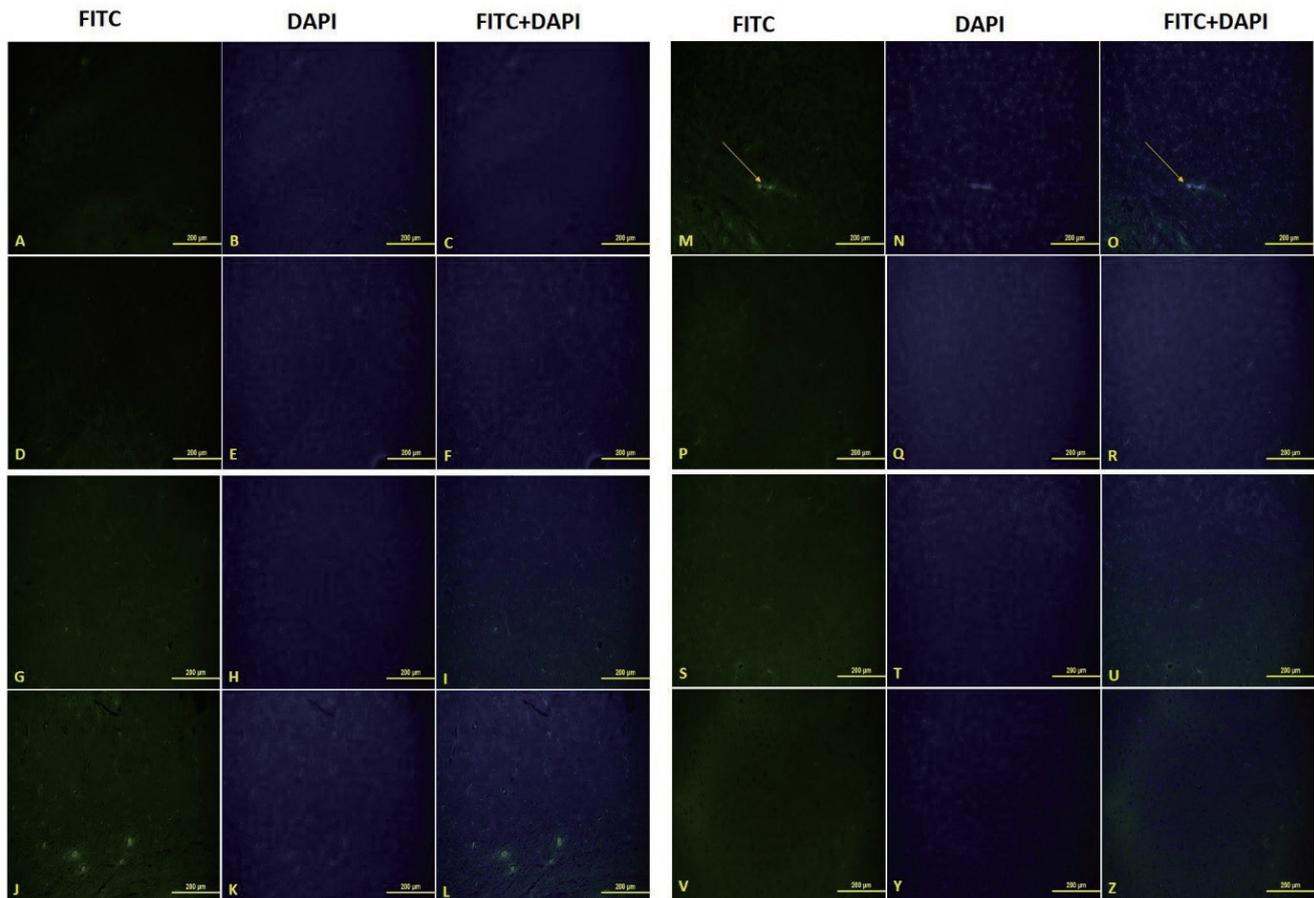


Figure 3. Control group (A, B, C), 0.2 g/kg apilarnil group (D, E, F), 0.4 g/kg apilarnil group (G, H, I), 0.8 g/kg apilarnil group (J, K, L), LPS group (M, N, O), LPS+0.2 g/kg apilarnil group (P, Q, R), LPS+0.4 g/kg apilarnil group (S, T, U), and LPS+0.8 g/kg (V, Y, Z); TUNEL positive cells in brain tissue (Cortex) are indicated with arrows. X200 magnification.

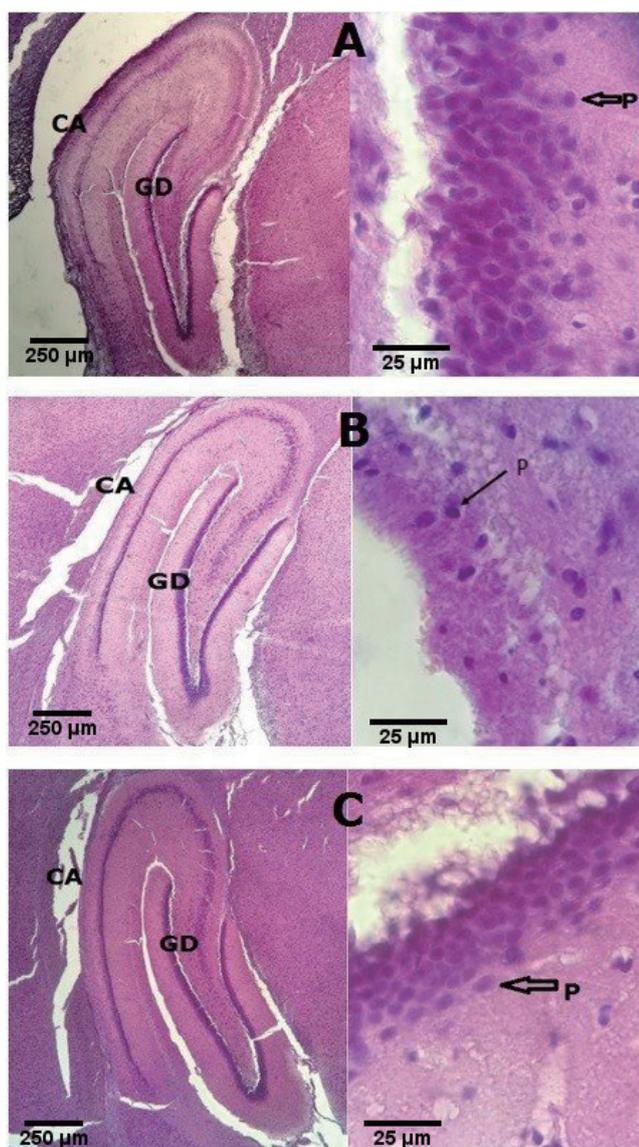
invasion of free radicals. SOD and CAT are antioxidants that are fundamentally in the primary line of defense⁽³²⁾. On the other hand, XOD activity results in the formation of superoxide anion and hydroperoxide radicals. Xanthine oxidase may cause oxidative damage such as brain edema, ischemia, and changes in vessel permeability, and increased serum XOD levels have been reported in brain tumor patients⁽³³⁾. In addition, one of the most commonly

used oxidative stress markers is MDA, a secondary lipid peroxidation product that occurs after exposure to reactive oxygen species and free radicals^(34, 35). MDA, which is widely used in the determination of oxidative damage, is a general indicator of the formation of lipid peroxidation under oxidative stress. Evaluation of the results of this study together with the literature indicates that apilarnil prevents oxidative damage in the brain by

Table 3. Number of pyramidal cells in hippocampus CA region by groups

Study groups	Total number of pyramidal cells (Mean± SD)	<i>p</i>
Control	607.912±16.164 ^b	0.001
LPS	554.185±28.178 ^a	
0,2 gr/kg Apilarnil	603.591±14.164 ^b	
0,4 gr/kg Apilarnil	604.467±15.304 ^b	
0,8 gr/kg Apilarnil	598.771±29.113 ^b	
0,2 gr/kg Apilarnil + LPS	564.086±29.113 ^a	
0,4 gr/kg Apilarnil + LPS	577.907±26.408	
0,8 gr/kg Apilarnil + LPS	593.990±27.346 ^b	

Data is expressed as ± standard deviation. $P < 0.05$ was considered statistically significant. There was no significant difference between groups containing the same letter (a and b).

**Figure 4.**

A. Appearance of hippocampus and pyramidal cells of the control group;
 B. Hippocampus and pyramidal cells in the LPS group;
 C. Hippocampus and pyramidal cells in the LPS+0.8 g/kg apilarnil group.
 (CA: Cornu ammonis, GD: Gyrus dentatus, P: Pyramidal cell)

producing positive effects on the responsible mechanisms of oxidative damage.

In this study, TNF- α , IL-6 and IL-1 levels were significantly increased in the LPS group and decreased in groups administered apilarnil. Accumulated evidence has shown that the release of pro-inflammatory cytokines and inflammation of the central nervous system play an important role in cognitive impairment⁽³⁶⁾. In particular, inflammatory mediators such as TNF- α , IL-1, IL-6, and the complement system play an important role in the pathophysiology of brain dysfunction in SAE. TNF- α induces neutrophil infiltration of brain tissue, changes in blood-brain barrier, neuronal cell apoptosis, and brain edema. IL-1 and IL-6 stimulate prostaglandin E-2 production by brain endothelial cells responsible for the activation of the adrenal axis of the hypothalamus, thereby causing fever and behavioral changes⁽³⁷⁾. However, animal studies have confirmed that postoperative cognitive impairment may be associated with neuroinflammation⁽³⁸⁾. Inhibition of proinflammatory factors has been observed to significantly reverse cognitive impairment caused by the hippocampus⁽³⁹⁾. Neuroinflammation has been associated with melancholy, multiple sclerosis, and Alzheimer's disease in elderly adults⁽⁴⁰⁾. These findings indicate that apilarnil exhibits an effective anti-inflammatory effect and inhibits mechanisms that cause neuronal damage. In addition, testican-1, a molecule with some newly identified attributes, is a proteoglycan expressed in the thalamus. Testican-1 has been correlated with severe sepsis and also its release shown to be induced by brain damage^(41,42). In this study, apilarnil administration was observed to significantly decrease the increase in testican-1 levels due to LPS. For these reasons, it can be argued that apilarnil may play a critical role in reducing neuronal damage caused by infiltration, thereby preventing neurological dysfunction.

SAE development likely involves all of a number of mechanisms, including reduced cerebral blood flow and oxygen content, impaired blood-brain barrier, brain edema, brain inflammation, neuronal degeneration, and activation of glial cells^(10,43-45). Although the pathophysiology of sepsis and SAE remains unclear and various mechanisms have been proposed, it has been well established that apoptosis is a fundamental mechanism throughout the immunopathogenesis of sepsis^(12,46,47). Indeed, apoptotic

deaths of neurons have been shown to increase in various brain regions in response to sepsis^(48,49). In this study, we observed that apilarnil administration decreased the extent of dilated blood vessels and neuronal degeneration in the histological examination of sepsis model groups. We also found that there was a significant increase in the number of apoptotic cells only in the LPS group, whereas the number of apoptotic cells was lower in the LPS + apilarnil groups. Furthermore, apilarnil administration was observed to prevent the decrease in the number of Purkinje cells due to sepsis. These findings are indicative that apilarnil may play a critical role in preventing sepsis-induced neuronal damage.

In conclusion, by utilizing the LPS-induced endotoxin sepsis model, we propose to the scientific community that apilarnil reduces secretion of proinflammatory proteins in the brain, reduces oxidative stress, and preserves the number of neurons by reducing apoptosis. This study not only shows the neuroprotective effect of apilarnil against sepsis-induced brain damage but also its potential to contribute to new therapeutic targets to combat various neurological disorders.

Compliance with ethical standards: The University Animal Experiments Local Ethics Committee approved the study protocol (Ethics committee approval No: 18/0063).

Conflicts of interests: None.

Sources of financial support: We were supported by Yozgat Bozok University, Department of Scientific Research Projects, Turkey (Project no: 6602b-TF/19-246).

REFERENCES

1. Concannon RM, Okine BN, Finn DP, Dowd E. Differential upregulation of the cannabinoid CB2 receptor in neurotoxic and inflammation-driven rat models of Parkinson's disease. *Experimental neurology*. 2015;269:133-41. doi: 10.1016/j.expneurol.2015.04.007.
2. Heneka MT, Carson MJ, El Khoury J, Landreth GE, Brosseron F, Feinstein DL, et al. Neuroinflammation in Alzheimer's disease. *The Lancet Neurology*. 2015; 14: 388-405. doi: 10.1016/S1474-4422(15)70016-5.
3. Medeiros R, LaFerla FM. Astrocytes: conductors of the Alzheimer disease neuroinflammatory symphony.

- Experimental neurology. 2013;239:133-8. doi: 10.1016/j.expneurol.2012.10.007.
4. Zhang X, Wang J, Qian W, Zhao J, Sun L, Qian Y, et al. Dexmedetomidine inhibits inducible nitric oxide synthase in lipopolysaccharide-stimulated microglia by suppression of extracellular signal-regulated kinase. *Neurological research*. 2015;37:238-45. doi: 10.1179/1743132814Y.0000000426.
 5. Zhang X, Yan F, Feng J, Qian H, Cheng Z, Yang Q, et al. Dexmedetomidine inhibits inflammatory reaction in the hippocampus of septic rats by suppressing NF- κ B pathway. *PloS one*. 2018;13:e0196897. doi: 10.1371/journal.pone.0196897.
 6. Hsing C-H, Hung S-K, Chen Y-C, Wei T-S, Sun D-P, Wang J-J, et al. Histone deacetylase inhibitor trichostatin ameliorated endotoxin-induced neuroinflammation and cognitive dysfunction. *Mediators of inflammation*. 2015; 163140. doi: 10.1155/2015/163140.
 7. Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS, et al. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia*. 2007;55: 453-62. doi: 10.1002/glia.20467.
 8. Korish AA, Arafa MM. Propolis derivatives inhibit the systemic inflammatory response and protect hepatic and neuronal cells in acute septic shock. *The Brazilian Journal of Infectious Diseases*. 2011;15:332-8. doi: 10.1016/S1413-8670(11)70201-X.
 9. Gofton TE, Young GB. Sepsis-associated encephalopathy. *Nature Reviews Neurology*. 2012; 8, 557-566. doi: 10.1038/nrneurol.2012.183.
 10. Sonnevile R, Verdonk F, Rauturier C, Klein IF, Wolff M, Annane D, et al. Understanding brain dysfunction in sepsis. *Annals of intensive care*. 2013;3:15. doi: 10.1186/2110-5820-3-15.
 11. Nwafor DC, Brichacek AL, Mohammad AS, Griffith J, Lucke-Wold BP, Benkovic SA, et al. Targeting the Blood-Brain Barrier to Prevent Sepsis-Associated Cognitive Impairment. *Journal of central nervous system disease*. 2019;11:1-14. doi: 10.1177/1179573519840652.
 12. Zhu T, Zhao Y, Hu H, Zheng Q, Luo X, Ling Y, et al. TRPM2 channel regulates cytokines production in astrocytes and aggravates brain disorder during lipopolysaccharide-induced endotoxin sepsis. *International immunopharmacology*. 2019;75:105836. doi: 10.1016/j.intimp.2019.105836.
 13. Cheung AM, Tansey CM, Tomlinson G, Diaz-Granados N, Matté A, Barr A, et al. Two-year outcomes, health care use, and costs of survivors of acute respiratory distress syndrome. *American journal of respiratory and critical care medicine*. 2006;174:538-44. doi:10.1164/rccm.200505-693OC.
 14. Robba C, Crippa IA, Taccone FS. Septic Encephalopathy. *Current neurology and neuroscience reports*. 2018;18:82. doi: 10.1007/s11910-018-0895-6.
 15. Ely EW, Shintani A, Truman B, Speroff T, Gordon SM, Harrell Jr FE, et al. Delirium as a predictor of mortality in mechanically ventilated patients in the intensive care unit. *Jama*. 2004;291:1753-62. doi: 10.1001/jama.291.14.1753.
 16. Hopkins RO, Weaver LK, Chan KJ, ORME JF. Quality of life, emotional, and cognitive function following acute respiratory distress syndrome. *Journal of the International Neuropsychological Society*. 2004;10:1005-17. doi:10.1017/S135561770410711X.
 17. Rothenhäusler H-B, Ehrentraut S, Stoll C, Schelling G, Kapfhammer H-P. The relationship between cognitive performance and employment and health status in long-term survivors of the acute respiratory distress syndrome: results of an exploratory study. *General hospital psychiatry*. 2001;23:90-6. doi:10.1016/S0163-8343(01)00123-2.
 18. Ransohoff RM, Brown MA. Innate immunity in the central nervous system. *The Journal of clinical investigation*. 2012;122:1164-71. doi: 10.1172/JCI 58644.
 19. Hirsch EC, Vyas S, Hunot S. Neuroinflammation in Parkinson's disease. *Parkinsonism & related disorders*. 2012;18:S210-S2. doi: 10.1016/S1353-8020(11)70065-7.
 20. Maccioni RB, Rojo LE, Fernandez JA, Kuljis RO. The role of neuroimmunomodulation in Alzheimer's disease. *Annals of the New York Academy of Sciences*. 2009;1153:240-6. doi: 10.1111/j.1749-6632.2008.03972.x.
 21. Sawczuk R, Karpinska J, Miltik W. What do we know and what we would like to know about drone homogenate. *Journal of ethnopharmacology*. 2018; 5, 111581. doi: 10.1016/j.jep.2018.10.042.

22. Stanhope J, Carver S, Weinstein P. Health outcomes of beekeeping: a systematic review. *Journal of Apicultural Research*. 2017;56:100-11. doi:10.1080/00218839.2017.1291208.
23. Bolatovna K, Rustenov A, Eleuqalieva N, Omirzak T, Akhanov U. Improving Reproductive Qualities of Pigs Using the Drone Brood Homogenate. *Biol Med (Aligarh)*. 2015;7:2.
24. Reddy MM, Mahipal SV, Subhashini J, Reddy MC, Roy KR, Reddy GV, et al. Bacterial lipopolysaccharide-induced oxidative stress in the impairment of steroidogenesis and spermatogenesis in rats. *Reproductive Toxicology*. 2006;22:493-500. doi: 10.1016/j.reprotox.2006.03.003.
25. Herrmann M, Schölmerich J, Straub R. Influence of cytokines and growth factors on distinct steroidogenic enzymes in vitro: a short tabular data collection. *Annals of the New York Academy of Sciences*. 2002;966:166-86. doi:10.1111/j.1749-6632.2002.tb04213.x.
26. Hong CY, Park JH, Ahn RS, Im SY, Choi H-S, Soh J, et al. Molecular mechanism of suppression of testicular steroidogenesis by proinflammatory cytokine tumor necrosis factor alpha. *Molecular and Cellular Biology*. 2004;24:2593-604. doi:10.1128/MCB.24.7.2593-2604.2004.
27. S. Silici Chemical content and bioactive properties of drone larvae (*Apis mellifera*) Mellifera. 2019;19(2):14-22.
28. Hausdorf J, Lemmens MA, Kaplan S, Marangoz C, Milz S, Odaci E, et al. Extracorporeal shockwave application to the distal femur of rabbits diminishes the number of neurons immunoreactive for substance P in dorsal root ganglia L5. *Brain research*. 2008;1207:96-101. doi: 10.1016/j.brainres.2008.02.013.
29. Sarsilmaz M, Kaplan S, Songur A, Colakoglu S, Aslan H, Tunc AT, et al. Effects of postnatal formaldehyde exposure on pyramidal cell number, volume of cell layer in hippocampus and hemisphere in the rat: a stereological study. *Brain research*. 2007;1145:157-67. doi: 10.1016/j.brainres.2007.01.139.
30. Huang Z, Cheng C, Jiang L, Yu Z, Cao F, Zhong J, et al. Intraventricular apolipoprotein ApoJ infusion acts protectively in Traumatic Brain Injury. *Journal of neurochemistry*. 2016;136:1017-25. doi: 10.1111/jnc.13491.
31. Yürüker V, Nazıroğlu M, Şenol N. Reduction in traumatic brain injury-induced oxidative stress, apoptosis, and calcium entry in rat hippocampus by melatonin: Possible involvement of TRPM2 channels. *Metabolic brain disease*. 2015;30:223-31. doi: 10.1007/s11011-014-9623-3.
32. Ighodaro O, Akinloye O. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine*. 2018;54:287-93. <https://doi.org/10.1016/j.ajme.2017.09.001>
33. Lavelli V, Peri C, Rizzolo A. Antioxidant activity of tomato products as studied by model reactions using xanthine oxidase, myeloperoxidase, and copper-induced lipid peroxidation. *Journal of Agricultural and Food Chemistry*. 2000;48:1442-8. doi:10.1021/jf990782j.
34. Bernecker C, Ragginer C, Fauler G, Horejsi R, Möller R, Zelzer S, et al. Oxidative stress is associated with migraine and migraine-related metabolic risk in females. *European journal of neurology*. 2011;18:1233-9. doi:10.1111/j.1468-1331.2011.03414.x.
35. Lorente L. New prognostic biomarkers in patients with traumatic brain injury. *Archives of trauma research*. 2015;4, e30165. doi: 10.5812/atr.30165.
36. Arai K, Matsuki N, Ikegaya Y, Nishiyama N. Deterioration of spatial learning performances in lipopolysaccharide-treated mice. *The Japanese journal of pharmacology*. 2001;87:195-201. doi:10.1254/jjp.87.195.
37. Van Dam A, De Vries H, Kuiper J, Zijlstra F, De Boer A, Tilders F, et al. Interleukin-1 receptors on rat brain endothelial cells: a role in neuroimmune interaction? *The FASEB Journal*. 1996;10:351-6. doi: 10.1096/fasebj.10.2.8641570.
38. Sun J, Zhang S, Zhang X, Zhang X, Dong H, Qian Y. IL-17A is implicated in lipopolysaccharide-induced neuroinflammation and cognitive impairment in aged rats via microglial activation. *Journal of neuroinflammation*. 2015;12:165. doi: 10.1186/s12974-015-0394-5.
39. Belarbi K, Jopson T, Tweedie D, Arellano C, Luo W, Greig NH, et al. TNF- α protein synthesis inhibitor

- restores neuronal function and reverses cognitive deficits induced by chronic neuroinflammation. *Journal of neuroinflammation*. 2012;9:23. doi: 10.1186/1742-2094-9-23.
40. Hovens IB, Schoemaker RG, van der Zee EA, Absalom AR, Heineman E, van Leeuwen BL. Postoperative cognitive dysfunction: involvement of neuroinflammation and neuronal functioning. *Brain, behavior, and immunity*. 2014;38:202-10. doi:10.1016/j.bbi.2014.02.002.
 41. Iseki K, Hagino S, Zhang Y, Mori T, Sato N, Yokoya S, et al. Altered expression pattern of testican-1 mRNA after brain injury. *Biomedical Research*. 2011;32:373-8. doi: 10.2220/biomedres.32.373.
 42. Lee Y, Lee W, Chang HH, Kim SW, Kim J, Bae JS. Testican-1, as a novel diagnosis of sepsis. *Journal of cellular biochemistry*. 2018;119:4216-23. doi: 10.1002/jcb.26661.
 43. Kuperberg SJ, Wadgaonkar R. Sepsis-associated encephalopathy: the blood–brain barrier and the sphingolipid rheostat. *Frontiers in immunology*. 2017;8:597. doi: 10.3389/fimmu.2017.00597.
 44. Silvio Taccone F, Scolletta S, Franchi F, Donadello K, Oddo M. Brain perfusion in sepsis. *Current vascular pharmacology*. 2013;11:170-86. doi:10.2174/1570161111311020007.
 45. Michels M, Danieslki LG, Vieira A, Florentino D, Dall'Igna D, Galant L, et al. CD40-CD40 ligand pathway is a major component of acute neuroinflammation and contributes to long-term cognitive dysfunction after sepsis. *Molecular medicine*. 2015;21:219-26. doi: 10.2119/molmed.2015.00070.
 46. Cheyuo C, Jacob A, Wu R, Zhou M, Qi L, Dong W, et al. Recombinant human MFG-E8 attenuates cerebral ischemic injury: its role in anti-inflammation and anti-apoptosis. *Neuropharmacology*. 2012;62:890-900. doi:10.1016/j.neuropharm.2011.09.018.
 47. Yao Y, Deng H, Li P, Zhang J, Zhang J, Wang D, et al. α -lactose improves the survival of septic mice by blockade of TIM-3 signaling to prevent NKT cell apoptosis and attenuate cytokine storm. *Shock: Injury, Inflammation, and Sepsis: Laboratory and Clinical Approaches*. 2017;47:337-45. doi: 10.1097/SHK.0000000000000717.
 48. Deng M, Hofacer R, Jiang C, Joseph B, Hughes E, Jia B, et al. Brain regional vulnerability to anaesthesia-induced neuroapoptosis shifts with age at exposure and extends into adulthood for some regions. *British journal of anaesthesia*. 2014;113:443-51. doi: 10.1093/bja/aet469.
 49. Sharshar T, Annane D, de la Gradmaison GL, Brouland JP, Hopkinson NS, Gray F. The neuropathology of septic shock. *Brain pathology*. 2004;14:21-33. doi:10.1111/j.1750-3639.2004.tb00494.x.