Biomedicine & Pharmacotherapy 108 (2018) 1384–1392

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Hippocampus and cerebellum damage in sepsis induced by lipopolysaccharide in aged rats - Pregabalin can prevent damage

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ARTICLE INFO

Keywords:
Sepsis
Pregabalin
Hippocampus
Cerebellum
Oxidative stress

ABSTRACT

Backgrounds: The aim of this study was to investigate the oxidative damage and inflammatory effects in the hippocampus and cerebellum in lipopolysaccharide (LPS)-induced sepsis model and possible ameliorating effects of pregabalin (PG).

Methods: Twenty four female Wistar Albino rats (12 month old) were divided into 3 groups as follows: Group I (Control; 0.1 ml/gavage and i.p. saline, single dose), Group II (LPS; 5 mg/kg LPS, i.p, single dose), Group III (LPS + PG; 5 mg/kg LPS, i.p, single dose + 30 mg/kg, gavage, single dose). DNA damage, ischemia-modified albumin (IMA), total oxidant status (TOS), total antioxidant status (TAS) oxidative stress index (OSI), leukocyte (WBC), lymphocyte, neutrophil, hemoglobin (HGB), erythrocyte (RBC), and thrombocyte counts were measured in blood and brain tissues. Histopathological and immunohistochemical evaluation of Caspase- 3, G-CSF, IL-6, SAA, iNOS expressions were conducted using hippocampus and cerebellum tissues.

Results: Comet analysis score, lymphocytes, neutrophils, WBC, IMA, TOS and OSI values were increased in Group II compared with to Group I ($p < 0.05$). IMA levels in blood, TOS and OSI levels in the brain were significantly decreased in Group III compared to Group II ($p < 0.05$). We observed increased hemorrhages, neutrophils, leukocytes infiltrations and neuron degeneration in Group II compared to Group I. Caspase 3, G-CSF, IL-6, SAA, iNOS expressions were increased in group II compared to Group I ($p < 0.001$).

Conclusion: Pregabalin partly ameliorated the damage caused by the exposure to LPS in hippocampus and cerebellum; however, further studies are needed to determine pregabalin's possible protective effects at different doses and with different techniques.

1. Introduction

Despite all the medical developments nowadays, sepsis remains a common and serious infectious disease in the world. In addition, sepsis is a disease that is least known, quite costly to diagnose and with a very high rate of mortality [1]. Sepsis is defined as an uncontrolled hyper-inflammatory immune response developed in response to invading pathogens mediated by the secretion of proinflammatory mediators [2]. Also, sepsis is defined as a systemic inflammatory response syndrome [3]. The distinguishing feature of sepsis is massive cytokine release resulting in oxidative stress, mitochondrial dysfunction, cellular damage and organ dysfunction. Organ dysfunction occurs in heart, liver, lungs, kidneys and brain. Brain injury occurs in the early phase of sepsis, and contributes to the progression severity of sepsis [4,5].

Neuro-inflammation is one of the main factors in the development of brain damage in sepsis. The number of apoptotic neurons increases in neuroinflammation. Therefore, neuroinflammation is a threat to brain cells, which is characterized by increased infiltration of inflammatory cells and excessive production of pro-inflammatory cytokines. Interleukin-1β, TNF-α, IL-6, iNOS, Serum Amyloid A (SAA), and over-activation of microglial cells plays a key role in sepsis [4]. Previous studies have shown that there is an immediate surge of cytokines within a few hours after the administration of sepsis inducing agents in experimental models of sepsis in animals. Increased cytokines such as TNF-α, IL-6, IL-12, iNOS, Serum Amyloid A (SAA) and macrophage migration inhibitor factor (MIF) documented the central nervous system (CNS) inflammation. Thus, the sensitivity of the central nervous system to seizures is increased [6].

In sepsis patients, the level of consciousness, as well as mood and mental state may change; sleep deprivation, restlessness, carelessness,

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https://doi.org/10.1016/j.biopha.2018.09.162
Received 4 June 2018; Received in revised form 12 September 2018; Accepted 28 September 2018
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delirium, and coma are observed [4]. Survival in sepsis patients is decreased by hypoperfusion, coagulopathy, and development of organ insufficiency. Thus, the clinical condition of the patient worsens and therapeutic measures are limited [7]. This frequently results in septic shock, organ dysfunction, multiple organ failure, metabolic acidosis and death. Recent studies began to reveal the pathophysiology of sepsis. However, sepsis is a complex process involving the immune system-related coagulation, endocrine and metabolic pathways, as well as the balance between ROS formation and clearance. For this reason, the pathophysiology of sepsis is not fully understood [5,8].

Pregabalin (PG) is a drug initially developed as an anticonvulsant for epilepsy. Then, it was used for the administration in the treatment of neuropathic pain [9]. Several studies showed that pregabalin reduces consumption of all anesthetic agents and lessens postoperative pain when used preoperatively. However, studies on pregabalin treatment doses are still being conducted [10,11].

Pregabalin is structurally similar to GABA (gamma-aminobutyric acid), but has no direct effect on GABA receptors. The principle mechanism of pregabalin's effects is its ability to bind the α2δ subunit of voltage-gated calcium channels [12]. The α2δ subunit of voltage-gated calcium channels is as common in the central nervous system including in the hippocampus and, modulates excitatory neurotransmitters release such as glutamat, noradrenaline, substance P. Thus, pregabalin causes analgesic, anxiolytic and anticonvulsant activities [9,13].

Therefore, the present study was planned to elucidate brain damage in lipopolysaccharide-induced sepsis model. There is an important reason for using LPS in our study. The same dose of LPS was applied in each animal group. Thus, the groups and sepsis model were standardised in our study. Based on all this information, the purpose of our study was to show the potential protective effects of PG treatment on LPS-induced brain damage.

2. Materials and methods

2.1. Experimental design

All experiments were conducted in accordance with the guidelines for animal research from the National Institutes of Health. Also, the procedures performed on rats were reviewed and approved by the Animal Experiments Local Ethics Committee of Mehmet Akif Ersoy University (Ethic No: 309, August 2017/02).

All the experiments were performed using 12-month-old female Wistar-Albino rats, weighing 300–350 g. (Mehmet Akif Ersoy University, Burdur, Turkey). Animals were kept under the standard laboratory conditions (temperature, 21–23 °C, humidity, 55–60% and 12 h light/dark cycle). All rats were fed with standard commercial chow diet (Korkuteli yem) and given tap water. Other environmental factors were kept the same in all groups. Before the experiment began, the animals had been monitored for seven days and adjusted to their orientation. Female Wistar-Albino rats were randomly divided into three groups (eight rats in each group): Group I: (Control); rats were administered a single dose of 0.1 ml saline by gavage and intraperitoneal injection.

Group II: (LPS group); the rats in the LPS group were administered a single dose of 5 mg/kg LPS, single dose, i.p (048K4126, Sigma Aldrich, USA). LPS was dissolved in saline. Six hours after LPS administration, the rats were sacrificed. The selected dose of LPS was based on previous studies. It was shown previously that rats given 5 mg/kg LPS experienced endotoxemia [14].

Group III: (LPS + PG group); 5 mg/kg LPS (i.p, a single dose) and 30 mg/kg pregabalin (gavage, pregabalin-Lyrica, Pfizer, Turkey). Pregabalin was administered one hour before LPS. Pregabalin was dissolved in the normal saline. The selected dose of pregabalin was based on previous neuroprotective studies [14,15]. It was shown in previous studies that, rats treated with 30 mg / kg of pregabalin demonsrated lower apoptotic cell count and better neurological function [15].

Animals were sacrificed by exsanguination, following the standardised ethical procedures, six hours after LPS administration. Blood samples were collected from each animal for biochemical and hematological analyses and commet assay. Serum samples were obtained by centrifugation at 4000 rpm for 10 min and stored at −80 °C until analysed. Brain was rapidly dissected. Brain tissue was precisely divided into two hemispheres. Right hemisphere and cerebellum were kept in 10% formalin solution for histological examination. r Another hemisphere was homogenized and stored at −80 °C until analysed biochemically.

2.2. Hematological analyses

Blood samples were collected from the sacrificed animals at the end of the experiment. Serum and plasma were obtained from blood samples. Blood samples (2 ml) were placed in the anticoagulant tube. Red Blood Cell (RBC), Hemoglobin (Hb), White Blood Cell (WBC), Neutrophil, Lymphocyte and Thrombocyte counts were measured from heparinized blood samples. These cells were counted with automated hematology analyzer (Code No: CR163712, Sysmex, Japan).

2.3. Biochemical analyses

The brain tissue extracted from the sacrificed animals was first placed in phosphate buffer (pH 7, 4). Briefly, the tissues were disrupted with a homogenizer (IKA Ultra-Turrax T25 Basic; Labortechnic, Staufen, Germany) and a sonicator (UW-2070 Bandelin Electronic, Germany). Later, the tissue samples were centrifuged at 10,000*g* for 10 min at + 4 °C. The Colorimetric and automated methods developed by Erel for evaluation of Total anti-oxidative status (TAS) and Total oxidative status (TOS) in tissue samples were used [16,17]. The change in absorbance of samples was measured at 660 nm using a spectrophotometer (Shimadzu UV1601 spectrophotometer, Japan) and the results were expressed as mmol Trolox Eq/mg protein. Oxidative stress index (OSI) was defined by the formula OSI (arbitrary unit) = [(TOS, mmol/L)/(TAS, mmol Trolox equivalent/ L)/100].

2.4. Measurement of ischemia-modified albumin (IMA)

Ischemia modified albumin test measures the ability of cobalt binding of amino (N-terminal) end of albumin. Briefly, 200 μl of patient serum and 50 ml of 0.1% $CoCl_2$ x $6H_2O$ (Sigma Aldrich, Missouri, USA) were compared in a glass tube. After, the mixture was incubated for 10 min. Then, 50 ml of 1.5 mg/ml dithiothreitol (DTT) was added and noncovalent cobalt was measured colorimetrically by dithiothreitol. The colorimetric method was developed by Bar-Or et al. [18]. Absorbance was recorded at 470 nm using a Shimadzu UV1601 spectrophotometer (Tokyo, Japan). Color formation in specimens with DTT was compared with color formation in the blank tubes and the results were expressed as absorbance units.

2.5. DNA damage determination by comet assay

DNA damage was investigated using the comet assay (single-cell gel electrophoresis). Blood samples, previously kept at −80, were dissolved in water bath (37 °C) for 2 min. 20 μl of whole blood and 150 μl of low-melting agarose (LMA) were mixed at 37 °C. 140 μl of solution was placed on the slides precoated with 1% normal melting agarose (NMA) and the slides were stored at 4 °C for 5 min. The slides were lysed (lysis solution: pH10; 4 °C) for at least one hour. After an hour, the slides were washed three times with dH_2O. The slides were placed in electrophoresis unit filled with freshly prepared cold alkaline electrophoresis buffer (1 mmol/L EDTA and 300 mM NaOH, pH > 13) at 4 °C for 30-minute incubation to allow the DNA to unwind. The electrophoresis was performed at 25 V, 300 mA for 25 min. Afterwards, the

Table 1
Statistical analysis of hematological parameters.

Groups	RBC	HGB	WBC	Lymphocyte	Neutrophil	Thrombocyte
Group I	7,97 ± 0,21	15,21 ± 0,45	3,73 ± 0,44	0,95 ± 0,25	1,12 ± 0,15	569,85 ± 79,01
Group II	7,43 ± 0,20	14,08 ± 0,36	6,90 ± 0,60[a]	4,37 ± 0,39[a]	2,17 ± 0,24[a]	210,00 ± 40,42[a]
Group III	7,18 ± 0,31	13,66 ± 0,37[a]	3,43 ± 0,41[b]	1,45 ± 0,39[b]	1,12 ± 0,14[b]	341,66 ± 48,17

Data were expressed as means ± SE. The relationships between groups and results are assessed by Bonnferroni one-way ANOVA. ap < 0.001 when compared to the Group I. bp < 0.001 when compared to the Group II. Thrombocyte results were considered significantly different at p < 0.05 by Kruskal–Wallis test.

slides were kept in a neutralizing solution (0.4 M Tris, pH 7.4) for 5 min. The slides were coded, and the images of 100 randomly selected cells stained with ethidium bromide were analysed separately under a fluorescent microscope Olympus BX-50 (Olympus, Japan).

According to the degree of damage, cells were classified into five categories (as 0–4) from the undamaged (no DNA migrated) to severely damaged (DNA migrated). All the process steps were performed in the dark [19,20].

2.6. Histopathological method

Brain and cerebellum samples were collected during the necropsy immediately after the euthanasia and fixed in 10% buffered formalin. After the routine processing by an automatic tissue processor equipment (Leica ASP300S, Wetzlar, Germany), tissues were embedded in paraffin and sectioned at 5-μm thickness by a Leica RM2155 rotary microtome (Leica Microsystems, Wetzlar, Germany). Tissue sections were stained with hematoxylin-eosin and examined microscopically. Histopathological changes were graded in a blinded manner and lesions scored to evaluate the pathological findings. Scores were made related to hyperemia, edema, emphysema, inflammatory reaction, and necrosis.

2.7. Immunohistochemical metod

Selected brain and cerebellum samples were immunostained with caspase-3 antiserum active caspase-3 [Anti-Caspase-3 anti-body (ab4051)], granulocyte colony-stimulating factor [Anti-G-CSF antibody (ab9691)], interleukin-6 [Anti-IL6 antibody, (ab9324)], iNOS [Anti-iNOS antibody (ab15323)], and Serum Amyloid A [Anti-Serum Amyloid A antibody [mc1] (ab655)] by streptavidin-biotin technique to evaluate inflammatory and apoptotic activity. Primary antibodies were used in 1/100 dilution. All primary serums and secondary antibodies were purchased from Abcam (Cambridge, UK). The sections were incubated with the primary antibodies for a period of 60 min, and immunohistochemistry was carried out using biotinylated secondary antibody and streptavidin-alkaline phosphatase conjugate. EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436) was used as a secondary antibody. The antigens were demonstrated by using diaminobenzidine (DAB) as the chromogen. For negative controls the primary antiserum step was omitted. Pathological specimens were evaluated by an expert pathologist from another university who was blinded to the study groups. The percentage of positively immunostained cells for each marker was counted in 10 different fields for every section at X40 objective magnification in all groups. Results obtained from the image analyzer were subjected to statistical analyses. Morphometric analyses were performed using the Database Manual Cell Sens Life Science Imaging Software System (Olympus Co., Tokyo, Japan).

2.8. Statistical analysis

Calculations were made using SPSS 15.0 program pack (SPSS Inc., Chicago, IL, USA). Kolmogorov Smirnov and Shapiro-Wilk tests were used for normal distribution of data. Levene test was used for the homogeneity of variance. Data characterized by a normal distribution were expressed as mean ± standard deviation. $P < 0.05$ was considered statistically significant. The groups were compared using nonparametric KruskalWallis test and Mann-Whitney-*U* test. Biochemical parameters were shown to fit with the normal distribution, and ANOVA and post hoc LSD tests were used to compare the groups

3. Results

3.1. Hematological analyses

LPS group, WBC, neutrophil and lymphocyte counts were significantly increased compared to the control group ($p = 0.001$, $p = 0.001$, $p = 0.001$; respectively). PG treatment significantly decreased these parameters in LPS + PG group compared to LPS group ($p = 0.001$, $p = 0.001$, $p = 0.001$; respectively). Thrombocyte counts were significantly reduced in LPS group compared to the control group ($p = 0.001$). Thrombocyte counts were increased in LPS + PG group compared to LPS group, but different were not significant. RBC counts were not significantly different between groups ($p = 0.094$). HGB levels were not significantly different between the control group and LPS group ($p = 0.196$). However, HGB level was significantly decreased in LPS + PG group compared to the control group ($p = 0.047$) (Table 1).

3.2. Biochemical analyses

Oxidative stress markers, TOS and OSI levels were significnatly increased in LPS group compared to the control group 6 h after LPS injection ($p = 0.022$, $p = 0.019$, respectively). Pregabalin treatment was significantly decreased these parameters in LPS + PG group compared to the LPS group ($p = 0.026$, $p = 0.042$, respectively). TAS levels were not significantly different between groups (Table 2).

Table 2
Oxidative stress markers of brain tissue.

Groups	TAS (mmolTroloxequivalents/L)		TOS (mmolH_2O_2 Equiv./L)		OSI	
	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value
Group I	1,33 ± 0,07	NS	13,76 ± 1,96	NS	0,98 ± 0,18	NS
Group II	1,27 ± 0,13	NS	16,66 ± 1,99[a]	a:0.022	1,21 ± 0,12[a]	a:0.019
Group III	1,29 ± 0,08	NS	13,82 ± 1,91[b]	b:0.026	1,01 ± 0,17[b]	b:0.042

Data were expressed as means ± SD. The relationships between groups were evaluated by Bonnferroni one-way ANOVA. a: $p < 0.05$ compared with Group I, b: $p < 0.05$ compared with Group II. NS: Not siginificant. TAS: Total anti-oxidative status; TOS: Total oxidative status, OSI: Oxidative stres index.

Table 3
DNA damage score and IMA levels of blood tissue.

Groups	Comet score (Arbitrary Unit)		IMA (ABSU)	
	Mean ± SD	P value	Mean ± SD	P value
Group I	65,62 ± 35,01	NS	0,647 ± 0,55	NS
Group II	173,87 ± 50,95[a]	a:0.001	0,697 ± 0,14[a]	a:0.026
Group III	74,50 ± 62,89[b]	b:0.001	0,648 ± 0,15[b]	b:0.028

Data were expressed as means ± SD. The comparison between groups were evaluated by Bonnferroni one-way ANOVA. a: $p < 0.05$ compared with group I, b: $p < 0.05$ compared with group II. NS: Not siginificant. IMA: Ischemia modified albümin.

3.3. Ischemia-modified albumin (IMA) analyses

Ischemia-modified albumin (IMA) was significantly increased in LPS group compared to the control group (p = 0.026). IMA level was significantly reduced in the LPS + PG group by pregabalin treatment compared to the LPS group (p = 0.028). The results of this parameter are presented in Table 3.

3.4. DNA damage score

The comet analysis scores of blood were significantly increased in LPS group compared to the control group (p = 0.001) and these scores were significantly decreased in LPS + PG group compared to LPS group (p = 0.0001). At the same time, comet scores of LPS + PG group were found similar to the control group (Table 2). The comet assay image of all groups is presented in Fig. 1.

3.5. Histopathological analyses

At the histopathological analyses of the brains, normal histology was observed in the control group. There were no hemorrhages, neutrophils, leukocytes infiltrations and neuron degeneration in this group. Numerous small hemorrhages, neutrophil leukocytes infiltrations and increased number of degenerated neurons in brains and cerebellums were observed in LPS group. The most commonly affected area of the brains was hippocampus. In cerebellums, Purkinje cells were most commonly degenerated. In addition, desquamation in endothelial cells was observed in LPS group. Histopathologically marked amelioration was observed in the pregabalin-treated group (Figs. 2 and 3).

3.6. Immunohistochemical analyses

In LPS group, immunohistochemically there was a marked increase in Caspase-3, G-CSF, IL-6, SAA and iNOS immunoreaction in the cells of CNS compared to the control group ($p < 0.001$). But with pregabalin treatment, the expressions of Caspase-3, G-CSF, IL-6, SAA and iNOS immunoreaction were decreased ($p < 0.001$) Figs. 4–9). Immunopositive cell numbers in pregabalin-treated group were similar to the control group ($p > 0.05$). The results of immunopositive cell counts are presented in Table 4.

4. Discussion

In the present study it was shown that, LPS caused oxidative stress, inflammation and apoptosis in hippocampus, cerebellum and blood. Especially, comet score and IMA reflected the blood toxicity and novel biomarkers of SAA, iNOS and IL-6, caspase-3 reflected inflammation on hippocampus, serebellum and plexus chroideus. Another new feature of this study was that PG partly ameliorated LPS-induced brain damage.

Leukocytes migrating to the damaged tissue during inflammation often cause vasodilatation and endothelial cell damage with cytokines. Then, the permeability of the capillaries increases and there is an increase in the target cells are increase in hydrogen peroxide and reactive oxygen metabolites. Consequently, hypoxia in tissues, capillary leakage and edema develop [21]. Kumar et al. demonstrated that neutrophil counts in sepsis patients were decreased [22]. We found that WBC, neutrophil and lymphocyte counts were significantly increased six hours after LPS exposure. The increase in leukocytes may be regarded as an indicator of inflammation in rats caused by LPS exposure Pretreatment with PG could effectively protect rats against LPS-induced acute inflammation. The study findings suggest that pregabalin may have anti-inflammatory effect; immunohistochemical results supported these findings.

The platelets are recognised as key players in immune system and their critical role in inflammation, means that platelets are significantly involved in the pathogenesis of sepsis. [23,24]. In a study of intensive care patients, sepsis was identified as a major risk factor for thrombocytopenia [25]. The effect of inflammation on platelet function is continuous and progressive. The development of sepsis thrombocytopenia is time-related. Thrombocytopenia usually develops within the first days following sepsis [24]. Previous studies have shown efforts to find a way to explain the mechanism of sepsis-thrombocytopenia [26,27]. We found that the count of platelets decreased six hours after LPS exposure and PG treatment increased thrombocyte count. We think that PG treatment may have a significant effect on LPS-induced brain damage. However, the relationship between PG and sepsis should be investigated in detail. Our study will make an important contribution to the literature that subject.

Sepsis induces a wide range of effects on the red blood cells. Changes in erythrocyte metabolism can be prevented by treatment, and cell membrane deformations should be investigated. Researchers evaluated erythrocyte volume [28], distribution width [29,30], hemoglobin content [31], antioxidant status [32], and membrane proteins [33]. These investigators found that these parameters were decreased in sepsis. However, we found that RBC and HGB counts were not different after LPS exposure. We think in future studies that the relationship in between PG, sepsis and erythrocyte parameters can investigated detailed.

Recent studies have shown that oxidative stress plays an important role in the development of many diseases such as cancer,

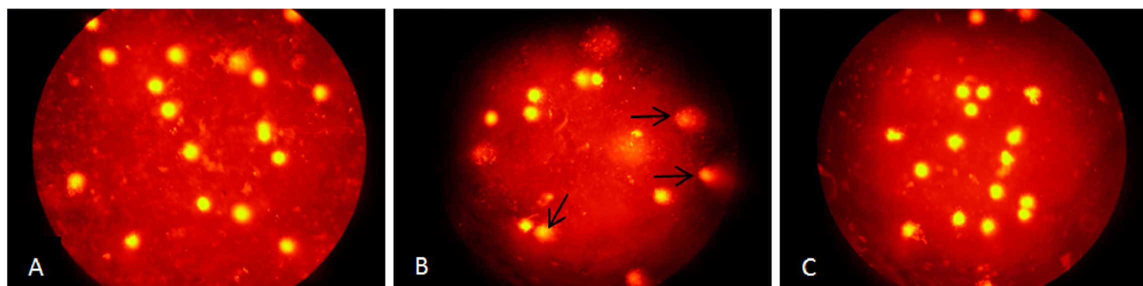

Fig. 1. Comet assay findings in the among the group. (A) DNA migration (3 and 4 °) was not occur in the control group, (B) 3 and 4 ° DNA migration was occur in LPS group, (C) In pregabalin treatment DNA migration was similar to control group.

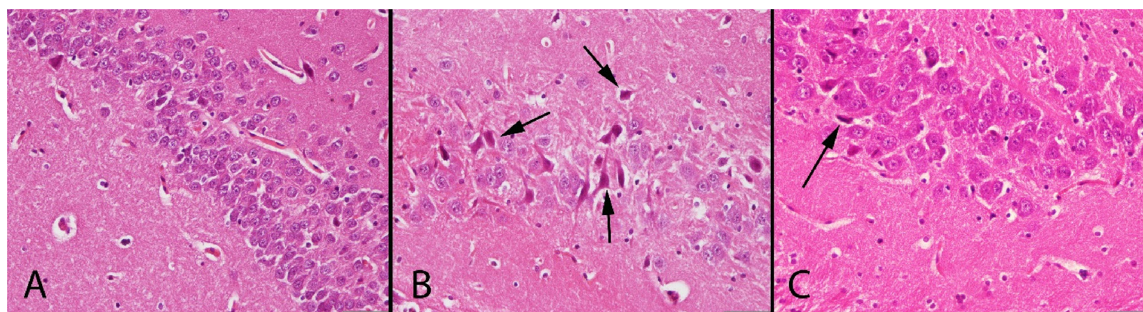

Fig. 2. Histopathological findings in the hippacampus among the groups. (A) Normal hippocampal architecture in control group, (B) Numerous degenerated neurons (arrows) in LPS group, (C) decreased number of degenerated neurons (arrow) in PG treated group, HE, Bar = 50 µm.

cardiovascular, neurodegenerative diseases and psychiatric disorders [34]. Liu et al. demonstrated that LPS causes increase in MDA level in rat lung and decrease in CAT and SOD activities in rat lungs [7]. Kumar et al. investigated the assessment of oxidative stress and antioxidant status in sepsis. They suggested that oxidative damage played a role in LPS- induced brain injury [22]. Karamese et al. evaluated that antioxidant and anti-inflammatory effects of apigenin in a rat model of sepsis. They investigators foun that lipid peroxidase was increased by LPS application [35]. In other study, NADPH oxidase, oxidative stress, and dopaminergic neurodegeneration develop after systemic LPS administration (5 mg/kg, ip, single dose) [36]. Similary, we found that TOS and OSI levels in the brain increased six hours after LPS exposure. In our study, PG treatment was increased TAS levels. But, different were not significant. We think that the dose of pregabalin may have been insufficient. In the future, we suggest that different doses should be tried for the antioxidant properties of pregabalin.

Researchers evaluated the IMA levels; in multiple sclerosis, chronic obstructive pulmonary disease, in association between IMA, inflammation and hypergliycemia in type 2 diabetes mellitus and in cord blood of pregnant smokers. They suggested that IMA is a serum marker of the oxidative stress [37–39]. In a clinical study of patients with severe sepsis, IMA levels were siginificantly increased. This was considered a risk factor that accelerated death in patients [40]. We found that the oxidative stress injury markers, IMA, were increased following LPS exposure and were reduced by PG therapy. Also, TAS, TOS, OSI, and comet scores supported these findings. Our finding indicated that the effects of sepsis were more obvious in hippocampus and cerebellum, but PG treatment was antioxidant effects in brain tissue.

According to the inflammatory mechanism described in several studies, LPS-stimulated cytokines and other mediators that bind to the target cell surface receptors. These cytokines play a role in nitric oxide (NO) synthesis from endothelium, macrophages and smooth muscle cells. NO reacts with superoxide radicals that lead to the formation of cytotoxic oxygen radicals. Subsequently, the oxidative state in the target cell can lead to DNA damage [41,42]. DNA damage in peripheral blood lymphocytes was investigated by the comet assay. The comet assay is a simple, sensitive, and rapid way for detecting DNA breaks. It is commonly used in studies to investigate the protective effects of pharmacological agents and antioxidants on DNA damage [43]. In this study, DNA damage was investigated after LPS exposure. Our study findings are similar to the findings of studies on DNA damage in LPS-induced sepsis [44]. In the current study, pregabalin treatment in the sepsis-induced group was found to significantly decrease DNA damage. According to the study results, we think that pregabalin may prevent DNA damage in sepsis. Therefore, this study will shed light on future research and enrich the literature.

Numerous small hemorrhages, neutrophil leukocytes infiltrations and increased number of degenerated neurons in brains and cerebellums were observed in LPS group. In cerebellums, Purkinje cells were most commonly degenerated. Also, desquamation in endothelial cells was observed in LPS group. The most commonly affected area of the brains was hippocampus. Improvement in these lesions was observed after PG treatment. Therefore, PG might be tested at different doses for the prevention of inflammation and apoptosis. Similary, Yokoo et al. investigated neurodegenerative evidence in mice brains with cecal ligation and puncture-induced sepsis, and they showed that sepsis causes serious neuronal degeneration, and the hippocampus appeared to be the most affected area [45]. Toklu et al. showed that sepsis causes injury in cerebral cortex and cerebellum tissue. Also, an increase in blood brain barrier permeability and brain edema were observed [46].

The importance of caspases in the activation and application of apoptosis in the organism: Caspase-3 is activated in a protease cascade that rapidly interferes with important structural proteins and signals of improper activation of homeostatic and repair enzymes [47]. Apoptosis is initiated by stabilization of p53, increased Bax/Bcl-2 ratio and release of cytochrome c, which activates caspase, especially Caspase-3 [37]. We found increased Caspase-3 expression in LPS group and Caspase-3 activity was improvement following PG therapy.

G-CSF significantly affects the survival of mature neutrophils by inhibiting apoptosis. The G-CSF receptor is located in many regions of the brain. G-CSF crosses the blood-brain barrier. Therefore, it is thought

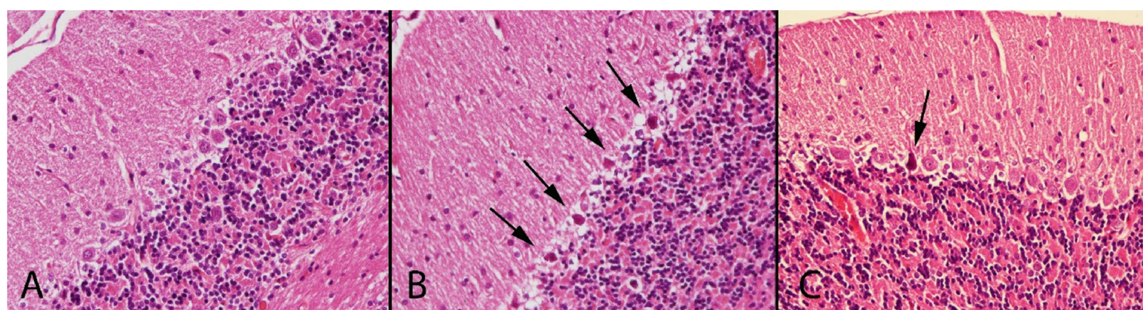

Fig. 3. Histopathological findings in the cerebellum among the groups. (A) Normal cerebellar architecture in control group, (B) Numerous degenerated Purkinje cells (arrows) in LPS group, (C) Decreased degenerated neurons (Purkinje cells) in PG treated group, HE, Bar = 50 µm.

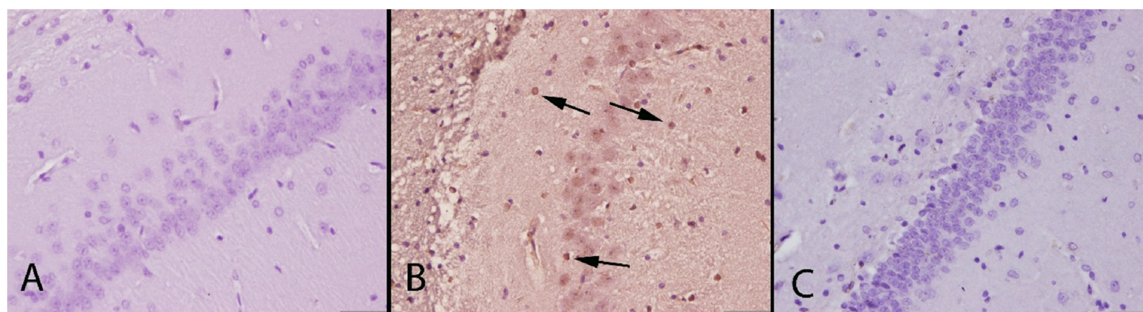

Fig. 4. Caspas-3 immunoreaction of the hippocampus among the groups. (A) No caspase-3 expression in control group, (B) Numerous Caspase-3 positive astrocytes (arrows) in LPS group (C) Negative immunoreaction in astrocytes in PG treated group, Streptavidin biotin peroxidase method, Bar = 50 μm.

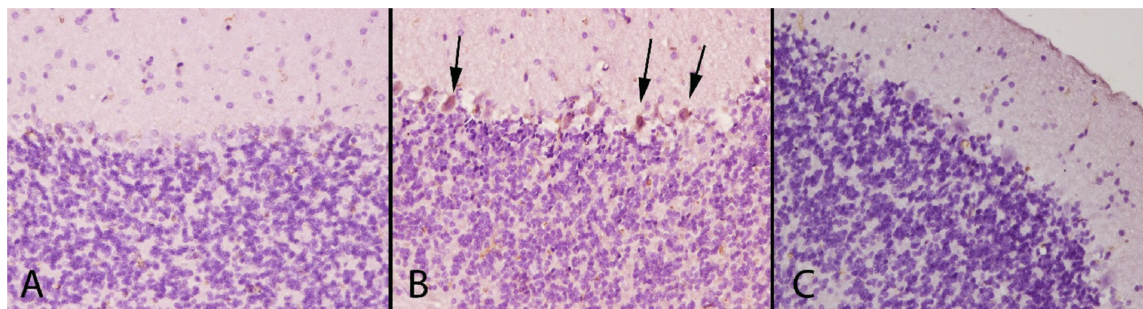

Fig. 5. Caspas-3 immunoreaction of the cerebellums among the groups. (A) No caspase-3 expression in control group, (B) Numerous Caspase-3 positive Purkinje cells (arrows), (C) Decreased immunoreaction in Purkinje cells in PG treated group, Streptavidin biotin peroxidase method, Bar = 50 μm.

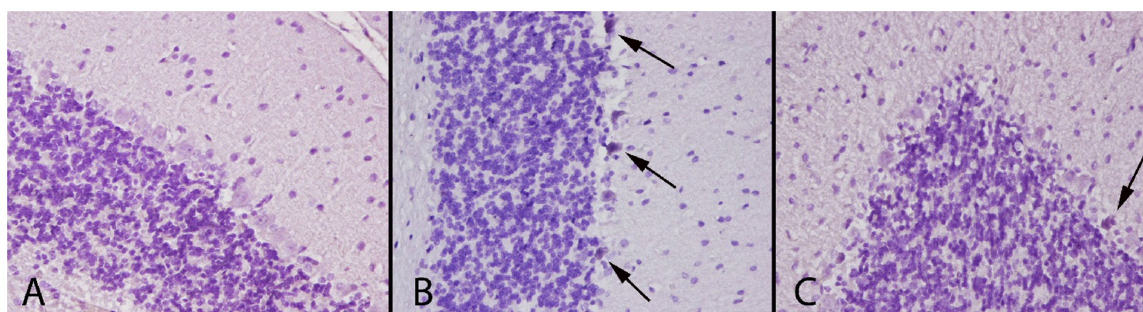

Fig. 6. G-CSF immunoreaction of the cerebellums among the groups. (A) No caspase-3 expression in control group, (B) Increased expressions in Purkinje cells (arrows) in LPS group, (C) Decreased immunoreaction in Purkinje cells (arrow) PG treated group, Streptavidin biotin peroxidase method, Bar = 50 μm.

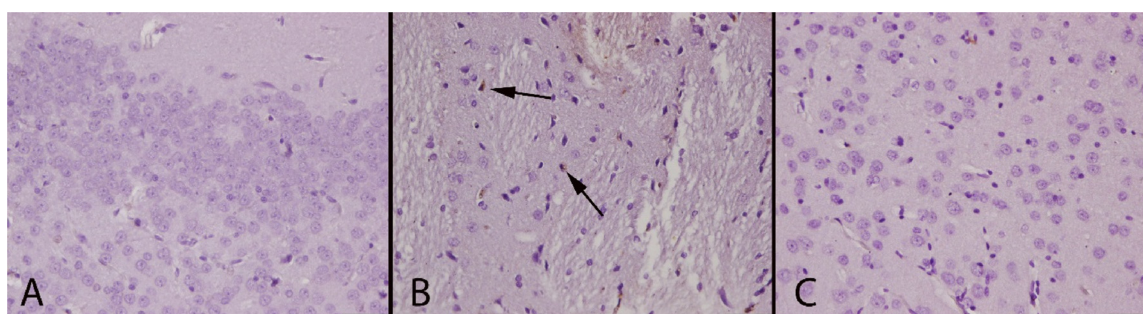

Fig. 7. IL-6 immunoreaction of the hippocampus among the groups. (A) Negative expression in control group, (B) Small number of immunopositive astrocytes (arrows) in LPS group (C) No immunoreaction in PG treated group, Streptavidin biotin peroxidase method, Bar = 50 μm.

to be effective in neurogenesis. Also, G-CSF may be used in for many other neurodegenerative and psychiatric disorders in which neuronal cell death or neurogenesis are implicated [48]. We found that LPS caused an increased in expression, which was decreased by PG therapy.

IL6 is a cytokine with proinflammatory and anti-inflammatory effects. IL-6 is greatly upregulated in the serum of patients with bacterial infection or sepsis. In many experimental sepsis studies, the effect of IL-6 on the outcome is controversial [49]. But, most scientific studies support the current data that proinflammatory cytokines contribute to the inflammation and damage method [35,50,51]. We found that IL-6 was increased in hippocampus tissue after LPS exposure in LPS goups. Also, PG therapy was decreased IL-6 expression.

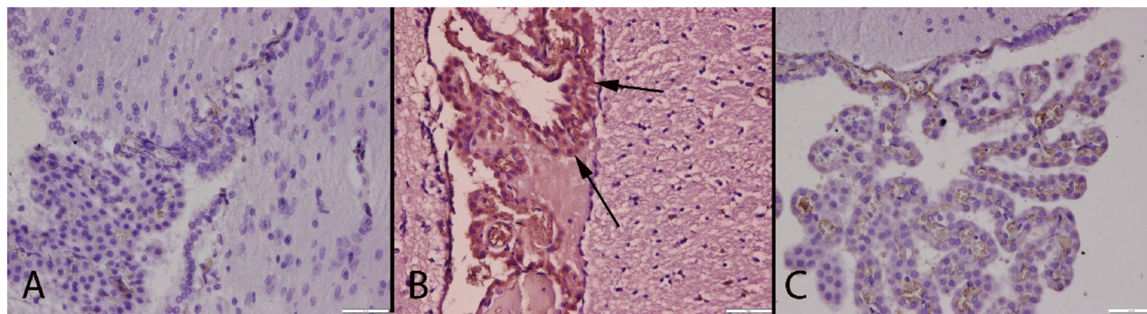

Fig. 8. SAA immunoreaction of the choroid plexus among the groups. (A) No expression in control group, (B) Numerous immunopositive ependymal cells (arrows) in LPS group, (C) Decreased immunoreaction in PG treated group, Streptavidin biotin peroxidase method, Bar = 50 µm.

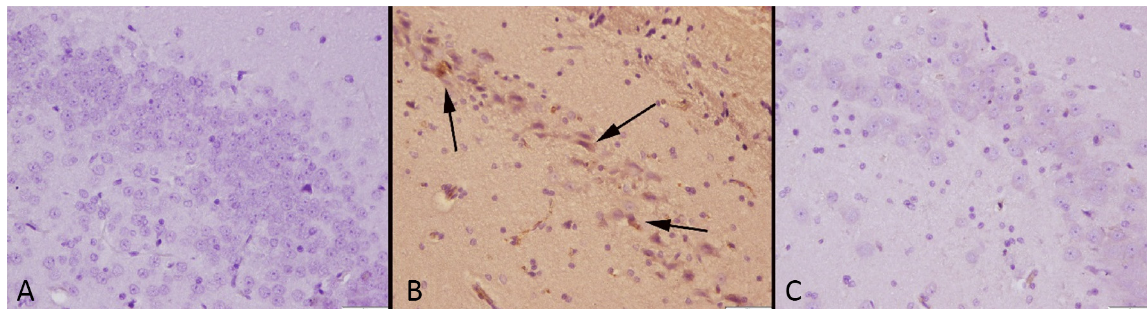

Fig. 9. iNOS immunoreaction of the hippocampus among the groups. (A) No reaction in control group, (B) Numerous immunopositive neurons (arrows) in the LPS group, (C) Negative immunoreaction in PG treated group, Streptavidin biotin peroxidase method, Bar = 50 µm.

Table 4
Statistical analysis of immunohistochemically positive cell numbers.

Markers	Organs	Group I	Group II	Group III	Pairwise comparisons
Caspase-3	Hippocampus	0.00 ± 0.00	8.12 ± 2.58[a]	0.00 ± 0.00[b]	a: $p < 0.001$ b: $p < 0.001$
	Cerebellum	0.00 ± 0.00	6.12 ± 1.88[a]	1.75 ± 1.06[b]	a: $p < 0.001$ b: $p < 0.001$
G-CSF	Cerebellum	0.00 ± 0.00	10.00 ± 0.59[a]	0.62 ± 0.26[b]	a: $p < 0.001$ b: $p < 0.01$
IL-6	Hippocampus	0.00 ± 0.00	5.25 ± 2.31[a]	1.12 ± 0.36[b]	a: $p < 0.001$ b: $p < 0.05$
SAA	Plexus chroideus	0.00 ± 0.00	13.25 ± 4.74[a]	0.50 ± 0.18[b]	a: $p < 0.001$ b: $p < 0.01$
iNOS	Hippocampus	0.00 ± 0.00	15.62 ± 3.77[a]	0.00 ± 0.00[b]	a: $p < 0.001$ b: $p < 0.001$ align="0cm 0cm 0.0001pt"

Data expressed as means ± SD. The relationships between groups and results of immunohistochemical scores are assessed by Bonnferroni and one-way ANOVA. a: $p < 0.05$ compared with group I, b: $p < 0.05$ compared with group II. G-CSF: granulocyte colony-stimulating factor, IL-6: Interleukin-6, SAA: Serum Amyloid A, iNOS: Inducible Nitric Oxide Synthase.

Serum amyloid A (SAA), one of the three major HDL (high-density lipoprotein) associated acute-phase proteins. SAA is predominantly produced by hepatocytes as a major response to cytokines such as IL-1, IL-6 and TNF-α, which play a central role in the pathogenesis of sepsis [52]. Extrahepatic SAA was histologically demonstrated in other tissues [53]. The choroid plexus, a source of secretion of vitamins, peptides and hormones for neurons, provides brain homeostasis [54]. Increased SAA in the plexus choroidal cord may impair neuronal homeostasis. In this study, we found that SAA expression was increased in plexus chroideus in LPS group. Also, PG treatment reduced SAA activity. We think that relationship between plexus chroideus and SAA in sepsis should investigation.

Previous studies have shown that the severity of sepsis correlates with the systemic release of proinflammatory cytokines with an accompany increase in inducible nitric oxide synthase (iNOS). Excessive production of iNOS and nitric oxide (NO) leads to impaired hemodynamic parameters in the late phase of severe sepsis [55]. Kadoi and Goto found that iNOS activity and nitrotyrosine in the brain in sepsis (by cecal ligation and puncture) increase in parallel with hyperdynamic changes [56]. Farias Correa et al. investigated vasopressin, iNOS and NO levels in plasma and hypotalamus during polymicrobial sepsis. These investigators found that iNOS and NO levels increased in sepsis group compared to the control group [57]. Also, Schwartz et al. showed that NO levels were increased in rats exposed to LPS [58]. Moreover, Vincent et al. investigated the role of three main NOSs, iNOS, cNOS and eNOS. Investigators have shown that NO produced by iNOS or cNOS can have both beneficial and deleterious effects on many organ systems in sepsis [59]. Therefore, we investigated iNOS activity in hippocampus. Similary, this study showed that iNOS expression was increased in the hippocampus after LPS exposure. Also, iNOS expression was reduced by PG treatment in hippocampus.

5. Conclusion

In summary, Caspase-3, G-CSF, IL-6, SAA, iNOS expressions were reduced by PG treatment administered one hour before LPS exposure. Additionaly, it led to a decreased oxidative stress in brain tissue. PG may be a therapeutic option for prevention of sepsis-induced organ injury, especially brain damage when we consider all the parameters. Although these results suggest that PG have the potential to prevent the hazardous effects of sepsis or protect the organs from these effects, molecular mechanisms and cellular targets remain to be determined. However, our study has shed some light on the antioxidative and anti-inflammatory effects of pregabalin.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors has declared that there is no conflict of interest that regarding the publication of this article.

Acknowledgement

The authors would sincerely like to thank Head of the Department of Physiology, Faculty of Medicine, Assoc. Prof. Dr. Mustafa SAYGIN for technical support.

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