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ORIGINAL ARTICLE

The Impact of Prophylactic Lacosamide on LPS-Induced Neuroinflammation in Aged Rats

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Abstract— Sepsis-induced central nervous system damage is called sepsis-associated encephalopathy (SAE). In addition to neuroinflammation, oxidative stress and apoptosis act in the development of SAE. In the current study, we evaluated the protective effects of lacosamide (LCM) on neuroinflammation induced by lipopolysaccharide (LPS). Twenty-four Wistar albino rats were divided into 3 groups as controls, LPS group (5 mg/kg i.p.), and LPS plus LCM group (5 mg/kg i.p and 40 mg/kg i.p, respectively). In the rat brain, LPS-induced tissue damage was revealed histopathologically as hyperemia and microhemorrhages. LCM pretreatment ameliorated these histopathological changes. LPS decreased brain TAS levels and significantly increased MDA, CRP, HSP, IL-1β, and TNF-α expressions in the cortex, hippocampus, and cerebellum. Western analysis revealed increased brain tissue levels of TNF-α, NF-Kβ, and caspase-3 following LPS. Prophylactic LCM treatment reversed these parameters including oxidative stress, inflammation, and apoptosis in the cortex, hippocampus, and cerebellum.

KEY WORDS: LPS; lacosamide; sepsis; oxidative stress; neuroinflammation; brain.

INTRODUCTION

Sepsis, used by Hippocrates for the first time in the medical literature, is currently defined as a life-threatening organ dysfunction caused by dysregulated host response to infection [1, 2]. Chronic medical problems, immune suppression, and advanced age are the main risk factors that may cause sepsis [3], and both incidence and mortality rates of sepsis are increased by advancing age [4].

One of the common complications of sepsis which is not well understood is "sepsis-associated encephalopathy" (SAE) [5]. As there is not a direct central nervous system infection, instead of septic encephalopathy, SAE is the preferred term [6]. Disruption of blood–brain barrier (BBB), apoptosis, neuroinflammation (NI), and oxidative stress (OS) are thought to be involved in the pathogenesis of SAE [7–9]. Lipopolysaccharides (LPS) are known to induce OS leading to the formation of reactive oxygen species (ROS) and lipid peroxidation products [10, 11]. Therefore, OS is one of the main mechanisms involved in the development of SAE.

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During sepsis, NI can occur as a reflection of exaggerated systemic inflammatory response. Many immune inflammatory cells notably microglial cells and macrophages may increase NI that is mediated by cytokines and chemokines [12, 13]. As shown in experimental NI models induced by LPS, tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1β levels were found to be increased in both blood and the hippocampus [14], related to the activation of nuclear factor kappa B (NF-kβ) signaling [14, 15]. NF-kβ which is bound with inhibitor of NF-kβ (IkB) is activated by the phosphorylation of IkB. It is known that toll-like receptor-4, TNF-α receptor, and IL-1 receptor stimulate NF-kβ, leading to inflammatory cytokine secretion [16].

The BBB, which is a critical component for the normal cerebral function, disintegrates in the course of SAE. During NI, LPS and increased cytokines such as TNF-α enhance BBB dysfunction [5]. Induction of apoptosis which is triggered by the increased expression of caspases (Cas) secondary to cytokines can also contribute to the breakdown of the BBB [6, 17].

Lacosamide (LCM) that was recently approved in the USA and Europe is a novel drug for the treatment of epilepsy [18]. LCM reduces pathological hyperexcitability by selectively enhancing slow sodium channel inactivation without altering the physiological activity of neurons [19, 20]. In experimental models, LCM has been shown to protect hippocampal pyramidal neurons against ischemic injury, probably due to catalase and glutathione peroxidase expression [21] and to inhibit inflammation and lipid peroxidation by its antioxidant and anti-inflammatory activity [22]. Considering that OS, inflammation, and apoptosis are involved in LPS-induced tissue damage, we hypothesized that prophylactic LCM could be protective against LPS-induced brain injury in aged rats. We also aimed to evaluate the role of NF-kβ signaling pathway following prophylactic LCM in a NI animal model.

MATERIALS AND METHODS

Experimental Design

All experiments were performed in accordance with the guidelines for animal research from the National Institutes of Health, and approved by the Committee on Animal Research of Mehmet Akif Ersoy University, Burdur (No. 02.08.2017-308). Twenty-four female adult Wistar albino rats (300–350 g) were placed in a temperature (21–22 °C) and humidity (60 ± 5%) controlled room with a 12:12-h light/dark cycle. All the rats were fed with standard commercial chow diet (Korkuteli yem, Antalya, Turkey). The rats were distributed into three groups (eight in each group): (I) control ($n = 8$), rats were given a single dose of 1 ml intraperitoneal (i.p.) normal saline to the left and right inguinal areas; (II) LPS ($n = 8$), a single dose of 5 mg/kg i.p. LPS (Sigma-Aldrich, Sweden) and 1 ml i.p. normal saline were injected to the left and right inguinal areas, respectively [23]; (III) LPS plus LCM ($n = 8$), rats were given a single dose of 40 mg/kg i.p. LCM (Benvida 100 mg tb/Adeka Farmacy, Turkey) dissolved in normal saline for 3 days to the right inguinal area. After 30 min of the last treatment, a single-dose 5 mg/kg i.p LPS (Sigma-Aldrich, Sweden) was injected to the left inguinal area [22, 24].

Six hours after LPS or normal saline administrations, all rats were anesthetized by i.p. injections of 90 mg/kg ketamine (Alfamin, Alfasan IBV) and 10 mg/kg xylazine (Alfazin, Alfasan IBV) for euthanasia. Half of the tissues including cortex, hippocampus, and cerebellum from each animal were placed into liquid nitrogen and stored at − 20 °C until biochemical analysis. The remaining tissues were collected and fixed in 10% buffered formalin for histopathological and immunohistochemical analyses.

Biochemical Analyses

Determination of Oxidative Stress Markers in Brain Tissues

Brain tissue samples were thawed and mixed using a vortex mixer (Thermo, USA) for oxidant-antioxidant analysis. Tissue total antioxidant status (TAS) and total oxidant status (TOS) were measured spectrophotometrically using modified Erel method [25, 26]. Oxidative stress index (OSI) was calculated using the formula OSI = TOS/TAS [27].

TAS was measured using a novel automated colorimetric measurement method developed by Erel. In this method, antioxidants in the sample reduce dark blue-green–colored 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical to colorless reduced ABTS form. The change of absorbance at 660 nm is related to the total antioxidant level of the sample. This method determines the antioxidative effect of the sample against the potent free radical reactions initiated by the produced hydroxyl radical. The results are expressed as millimolar Trolox equivalent per liter (mmol Trolox Eq/l) [25]. The TOS was measured using a novel automated colorimetric method described by Erel. In this method, oxidants present in the sample oxidize the ferrous ioneo-dianisidine

complex to the ferric ion. The oxidation reactions are enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion forms a colored complex with xylenol orange in an acidic medium. The color intensity measured spectrophotometrically is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide, and the results are expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 Eq/l) [26].

Protein Preparation and Immunoblotting

Brain tissues were dissected, homogenized, and lysed in ice-cold RIPA buffer (1× PBS, 1% nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS, pH 8.0) containing protease inhibitor. Following centrifugation at 14,000 rpm for 10 min at 4 °C, insoluble materials were removed. Protein concentrations were determined by the BCA protein assay kit (Thermo Scientific, Pittsburgh, PA, USA). Typically, 40 μg of total protein was used for immunoblotting. Samples were denatured in 4× SDS-PAGE loading buffer (200 mM Tris-HCl, pH 6.8, 8% SDS, 0.4% bromophenol blue, 40% glycerol, 4% 14.7 β-mercaptoethanol, 10% 0.5 M EDTA) at 95 °C for 5 min and were separated on hand-cast polyacrylamide gels. Following electrophoresis, proteins were transferred to the PVDF membrane (Millipore). After routine immunoblotting steps (blocking, incubating with primary and secondary antibodies, respectively), specific proteins were visualized using Pierce ECL Western blotting substrate kit (Thermo Scientific, Pittsburgh, PA, USA) by ChemiDoc™ Imaging System (BioRAD). Polyclonal anti-Cas-3 (ab4051) was from Abcam; anti-TNF-α (3707), monoclonal anti-NF-kβ (D14E12), and anti-p-NF-kβ (Ser536) (93H1) were from Cell Signaling Technology; anti-β-Actin (A5316) was from Sigma-Aldrich; and HRP-conjugated anti-mouse or anti-rabbit IgG was purchased from Pierce.

The immunoblotting results were densitometrically analyzed and presented in the graph in the form of fold increase compared with that of the control group. β-Actin was used as a loading control.

Histopathological Analysis

During the necropsy, cortex, hippocampus, and cerebellum samples were collected and fixed in 10% neutral formalin. Samples were then routinely processed by automatic tissue processor equipment (Leica ASP300S, Wetzlar, Germany) and embedded in paraffin wax. Tissue sections were cut 5 μm in thickness by a rotary microtome (Leica RM2155, Leica Microsystems, Wetzlar, Germany). Then, they were stained by hematoxylin and eosin (H&E), covered with a coverslip with a mounting medium and examined under a light microscope. Histopathological changes were graded in a blinded manner, and lesions were evaluated by a pathologist from another university. Lesions such as hyperemia, edema, and hemorrhages were evaluated.

Immunohistochemical Analyses

Cortex, hippocampus, and cerebellum samples were immunostained with MDA (Anti-Malondialdehyde antibody (ab6463)), C-reactive protein (Anti-C Reactive Protein antibody - Aminoterminal end (ab65842)), heat shock protein (Anti-Hsp70 antibody [5A5] (ab2787)), IL-1β (Anti-IL1 beta antibody (ab2105)), and TNF-α (Anti-TNF alpha antibody (ab6671)) by streptavidin biotin technique. All primary serums and secondary antibodies were purchased from Abcam (Cambridge, UK), and all primary antibodies used 1/100 dilution. Primary antibodies were incubated for a period of 60 min, and immunohistochemistry was carried out using biotinylated secondary antibody and streptavidin-alkaline phosphatase conjugate. Ready-to-use kits (Expose Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436)) were used as secondary antibody and 3,3-diaminobenzidine (DAB) used as chromogen for 5 min. For negative controls, the primary antiserum step was omitted. All examinations were performed on blinded samples. To evaluate the percentage of immune-positive cells for each marker, 100 cells were counted in 10 different fields for every section at a magnification under × 40 objective for all groups. Image analyzer results were statistically evaluated. Morphometric analyses were performed using the Database Manual Cell Sens Life Science Imaging Software System (Olympus Co., Tokyo, Japan).

Statistical Analysis

Variables were presented as mean ± standard deviations. One-way analysis of variance ANOVA and *post hoc* LSD tests were used to compare biochemical, histopathological, and immunohistochemical scores between the groups. $p < 0.05$ was set as statistical significance.

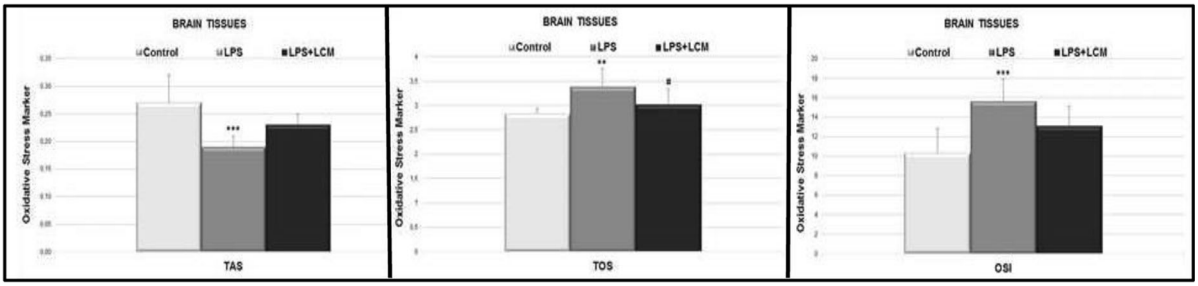

Fig. 1. Oxidative stress markers of brain tissues. Values are presented as means ± SD. The relationships between groups and results of biochemical markers are assessed by one-way ANOVA test (*post hoc* LSD test). $^{**}p < 0.01$, $^{***}p < 0.001$, LPS compared with control; $^{\#}p < 0.05$, LPS + LCM, compared with LPS.

RESULTS

Biochemical Results

LCM Reversed LPS-Induced Changes in Oxidative Parameters

As shown in Fig. 1, LPS decreased the brain tissue TAS levels significantly ($p < 0.001$). With LCM treatment, a non-significant increase was found ($p > 0.05$). On the other hand, TOS was increased by LPS and decreased by LCM treatment significantly ($p < 0.01$ and $p < 0.05$, respectively). OSI levels were significantly increased by LPS ($p < 0.001$). LCM treatment non-significantly decreased OSI levels ($p > 0.05$).

LCM Decreased Inflammatory and Apoptotic Markers in Western Blot Analyses

As shown in Fig. 2, phospho-NF-Kβ ($p < 0.05$), TNF-α ($p < 0.05$), and cleaved Cas-3 protein levels were significantly increased in the LPS group compared with the control group ($p < 0.05$ for all comparisons). LCM treatment significantly decreased p-NF-Kβ, TNF-α, and cleaved Cas-3 expressions compared with LPS-exposed animals ($p < 0.05$ for all comparisons).

Histopathological Results

At microscopical examination, no pathological findings were observed in the cortex, hippocampus, and cerebellum of the control animals. Following LPS exposure (group 2), severe central nervous system hyperemia and microhemorrhages were commonly observed. Some neuronal cells exhibited degenerative changes. However, LCM treatment ameliorated these histopathological findings (Fig. 3).

Immunohistochemical Results

Immunohistochemical analysis revealed that LPS significantly increased MDA, CRP, HSP, IL-1β, and TNF-α expressions in the cortex, hippocampus, and cerebellum compared with the controls ($p < 0.001$ for all comparisons) (Figs. 4, 5, 6, 7, and 8). LCM treatment significantly decreased immunoexpressions of the evaluated markers in all regions compared with LPS ($p < 0.01$ for cortical and hippocampal IL-1β, $p < 0.001$ for other comparisons).

DISCUSSION

As we have shown in the present experimental study, OS and NI can be important in the pathogenesis of brain injury induced by LPS. Brain cells are more vulnerable tissues to the OS that occurs as a result of imbalance between oxidant and antioxidant systems due to high oxygen consumption and lipid content [28]. OS triggered by LPS leads to free radical formation and subsequent protein oxidation, lipid peroxidation, and DNA damage leading to tissue injury [10, 11]. In our study, the OS parameters TAS, TOS, and OSI were altered to reflect increased OS in brain tissues of LPS-exposed animals, supporting the previous data that showed LPS-induced brain injury [29, 30]. Moreover, we have shown that LCM treatment partly ameliorated these effects. This protective effect was pronounced specifically for the TOS values.

LCM has been shown to have antioxidant effects by increasing the catalase and glutathione peroxidase expressions in addition to TAS [21, 31]. Al-Massri et al. [31] reported increased TAS levels with long-term LCM treatment. Non-significant elevations in TAS levels with LCM treatment in our design were probably due to short treatment time. Further studies should evaluate whether longer

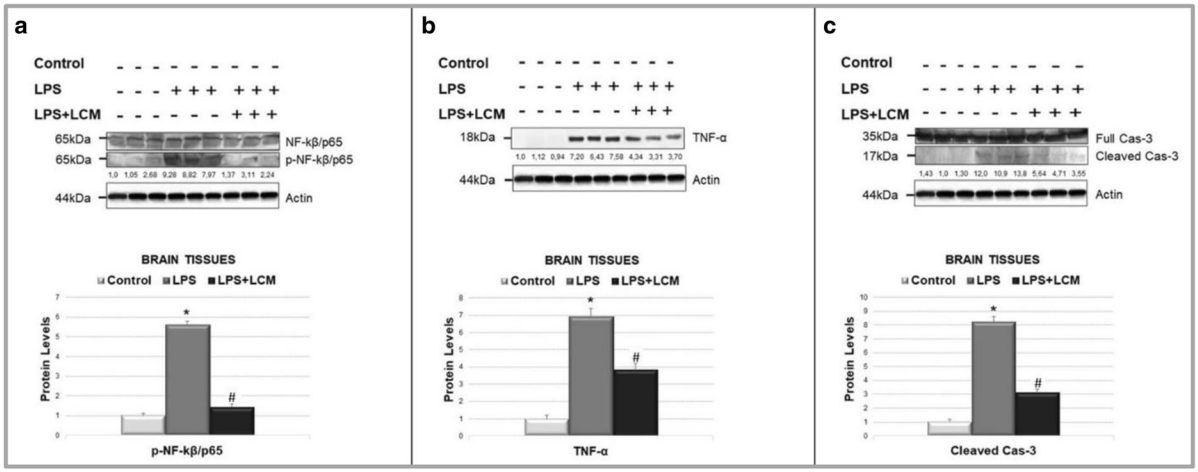

Fig. 2. Western blot analysis of p-NF-kβ/p65 (A), TNF-α (B), and cleaved Cas-3 (C) levels on brain tissues. $^{*}p < 0.05$, LPS compared with control; $^{\#}p < 0.05$, LPS + LCM compared with LPS. Error bars: ± SEM.

durations of LCM administrations lead to significant changes in TAS and OSI.

LPS was previously shown to increase MDA, an end product of lipid peroxidation in brain tissues [32, 33]. We also confirmed this effect in our model. Furthermore, LCM ameliorated increased MDA expressions in the cortex, hippocampus, and cerebellum of LPS-exposed animals in our design. In a study using early and delayed melatonin

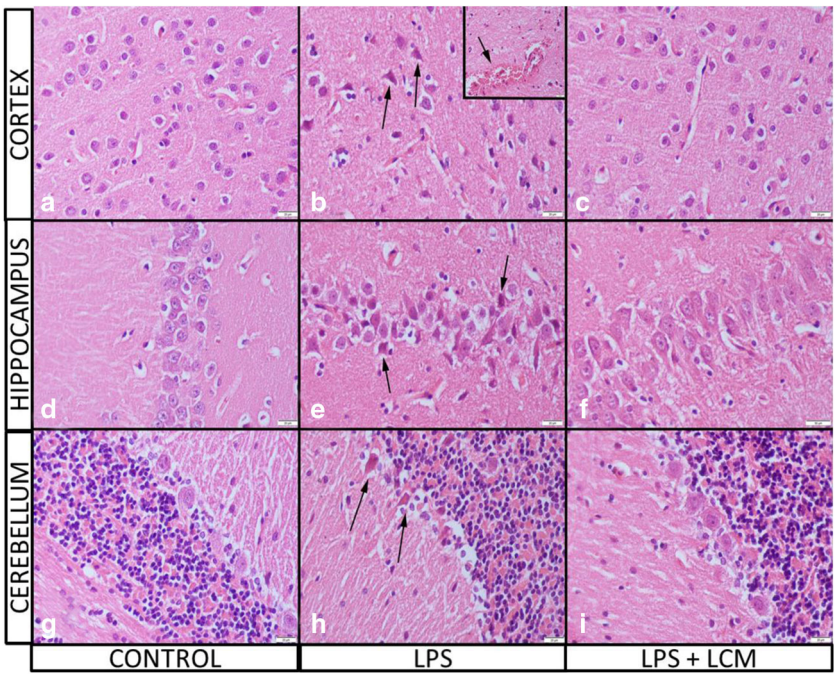

Fig. 3. Histopathological appearance of cortex (A, B, C), hippocampus (D, E, F), and cerebellum (G, H, I) tissues. (A) Normal cortex tissue architecture in control group. (B) Degenerated neurons (arrows) and hemorrhages (arrow) (inset) in cortex belonging to LPS group. (C) No pathological findings in cortex in LPS + LCM group. (D) Normal hippocampus tissue architecture in control group. (E) Degenerated cells (arrows) in hippocampus in LPS group. (F) No pathological findings in hippocampus in LPS + LCM group. (G) Normal cerebellum tissue architecture in control group. (H) Degenerated Purkinje cells (arrows) in cerebellum in LPS group. (I) No pathological findings in cerebellum in LPS + LCM group. H&E, bar = 20 μm.

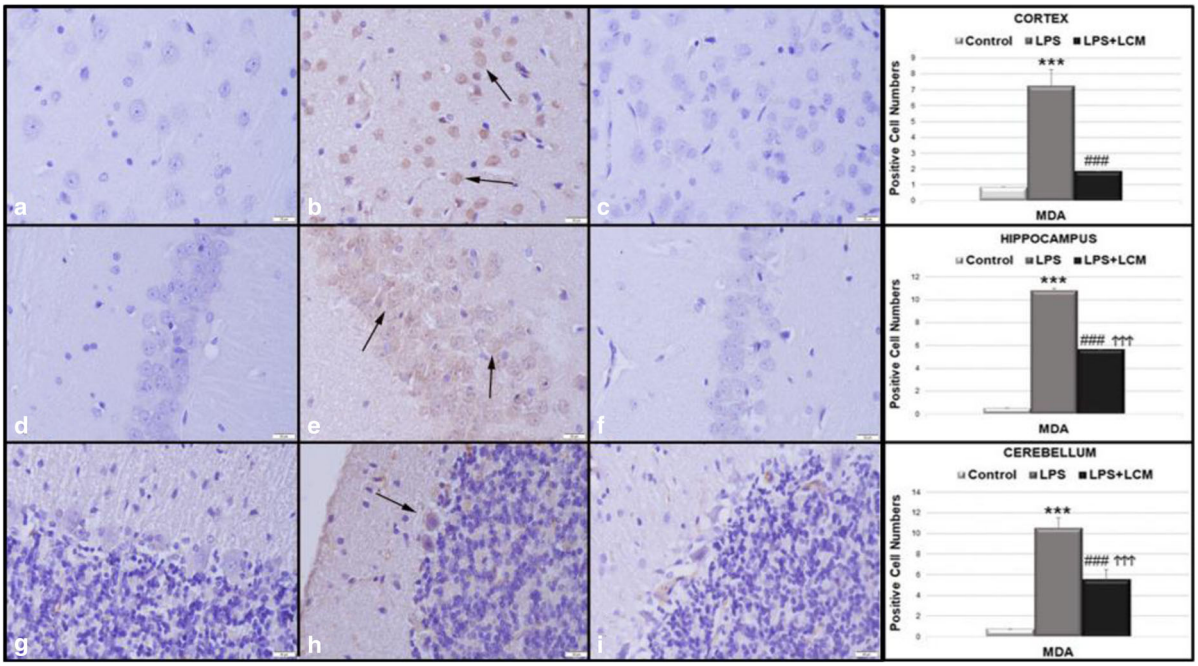

Fig. 4. MDA immunoreaction and statistical analysis of immunohistochemical positive cell numbers of cortex (A, B, C), hippocampus (D, E, F), and cerebellum (G, H, I) tissues. (A) Negative immunoreaction in control group in cortex. (B) Increased expression in neuronal cells (arrows) in LPS group in cortex. (C) Negative immunoreaction in LPS + LCM group in cortex. (D) No expression in control group in hippocampus. (E) Increased expression in cells (arrows) in LPS group in hippocampus. (F) Decreased immunoreaction in LPS + LCM group in hippocampus. (G) No expression in control group in cerebellum. (H) Increased expression in neuronal cells (arrows) in LPS group in cerebellum. (I) Decreased immunoreaction in LPS + LCM group, in cerebellum. Streptavidin biotin peroxidase method, bar = 20 μm. Values are presented as means ± SD. The relationships between groups and results of immunohistochemical parameters are assessed by one-way ANOVA (*post hoc* LSD test). ***$p < 0.001$, compared with control; $^{###}p < 0.001$ LPS + LCM, compared with LPS; $p < 0.001$, LPS + LCM, compared with control.

treatments against SAE [34], early melatonin treatment reversed elevated MDA levels in the hippocampus of mice showing the protective effects of antioxidants in short-term therapy. In our study, LCM administrated at a dose of 40 mg/kg reversed MDA expressions in all brain tissues. In another experimental study evaluating the protective effects of LCM on sepsis-induced polyneuropathy, induced by cecal ligation and puncture (CLP), two different doses of LCM (20 mg/kg and 40 mg/kg) caused a significant decrease in plasma MDA levels with no significant difference between the doses [22]. These results support the earlier studies about the imbalance between oxidants and antioxidants in sepsis development and reveal the antioxidant effect of LCM.

Free radicals generated by OS impair many structures including Na^+/K^+/ATPase. This impairment causes the release of glutamate that induces excitotoxicity [35]. Direct antioxidant effect of LCM by increased expression of antioxidant molecules can be one of the protective mechanisms. Further studies investigating an indirect effect of LCM on tissue protection by decreasing excitotoxicity *via* inhibition of OS are warranted.

It has been well known that enhanced free radicals as a consequence of OS trigger the production of inflammatory cytokines including TNF-α and IL-1β *via* activation and translocation of NF-Kβ [36]. IL-1β and TNF-α, which are produced by neutrophils and microglia, are the main components of cytokine storm [37, 38]. Elevated IL-1β and TNF-α levels were well established in LPS-induced experimental models [39–41]. In a study performed in aged rats, both markers were shown to be increased in blood and hippocampus [14]. Similar to this study, we found elevated levels of IL-1β and TNF-α in cortex, cerebellum, and hippocampal tissues. In western analyses, increased TNF-α levels in brain tissues were ameliorated by LCM. This finding was supported by immunohistochemistry in the brain tissues including hippocampus. Showing the increased expressions of these cytokines in brain tissues and cerebellum is important, as this increment can reflect the disruption of the BBB integrity and subsequent

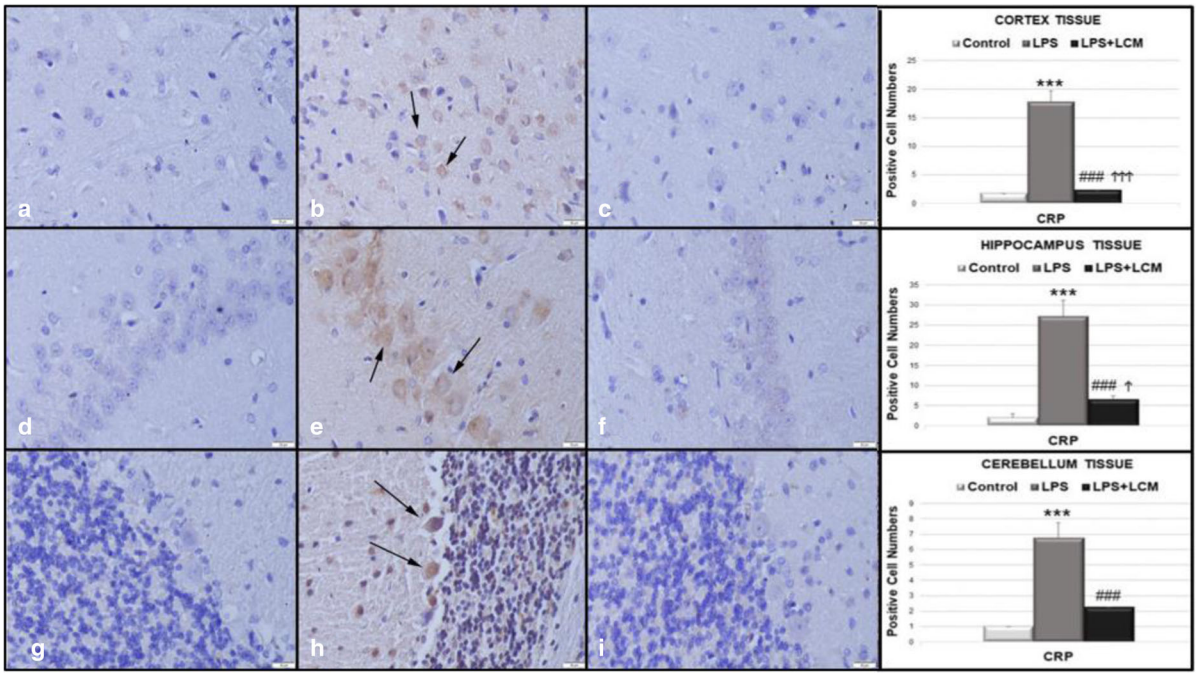

Fig. 5. CRP immunoreaction and statistical analysis of immunohistochemical positive cell numbers of cortex (A, B, C), hippocampus (D, E, F), and cerebellum (G, H, I) tissues. (A) No expression in control group in cortex. (B) Increased expression in neuronal cells (arrows) in LPS group in cortex. (C) Decreased immunoreaction in LPS + LCM group in cortex. (D) No expression in control group in hippocampus. (E) Increased expression in cells (arrows) in LPS group in hippocampus. (F) Decreased immunoreaction in LPS + LCM group, in hippocampus. (G) No expression in control group in cerebellum. (H) Increased expression in neuronal cells (arrows) in LPS group in cerebellum. (I) Negative immunoreaction in LPS + LCM group in cerebellum. Streptavidin biotin peroxidase method, bar = 20 μm. Values are presented as means ± SD. The relationships between groups and results of immunohistochemical parameters are assessed by one-way ANOVA (*post hoc* LDS test). $^{***}p < 0.001$, compared with control; $^{\#\#\#}p < 0.001$ LPS + LCM, compared with LPS; $p < 0.05$ LPS + LCM, compared with control; $p < 0.001$, LPS + LCM, compared with control.

immune cell migration into the central nervous system. In addition, increased levels of these cytokines are important not only to show the inflammation but also to predict the prognosis. In a recent clinical study, increased cytokine levels including IL-1β and TNF-α were stated to be an indicator of poor prognosis and adverse outcomes in sepsis-induced brain dysfunction [42]. In an *in vitro* study, aging was shown as a factor that could change the glial response to LPS. These results imply that older age could induce a greater IL-1 response in the hippocampus more than in the cortex, with no difference in TNF-α, but proportionately less neurotoxicity because of decreased nitric oxide response to LPS [43]. In our results, mean levels of inflammatory parameters were higher in the hippocampus compared with levels in the cortex and the cerebellum. This difference may be due to the different vulnerability of hippocampal tissues against inflammatory stimuli. Therefore, the changes in the proinflammatory cytokines induced by LPS in response to age should be investigated in further studies.

Secretion of proinflammatory cytokines is regulated by NF-kβ in sepsis-associated multiple organ failure [44]. Impaired NF-kβ activation in diabetic rats was shown to be associated with relatively decreased acute lung injury compared with non-diabetics [45]. NF-kβ containing p50 and p65 subunits is activated by the phosphorylation of IkB and translocated to the nucleus to induce the targeted genes for cytokine release [41]. In our study, NF-kβ activation and phosphorylation by LPS as the main regulators of inflammation were manifest in the brain tissues. LCM, as a potent anti-inflammatory agent, significantly reversed these changes. Similar to our study, Fu et al. [14] showed increased hippocampal NF-kβ/p65 protein levels at day 1 through day 30 following LPS administration. Moreover, in our study, increased NF-kβ p65 activation was evident within 6 h after LPS exposure.

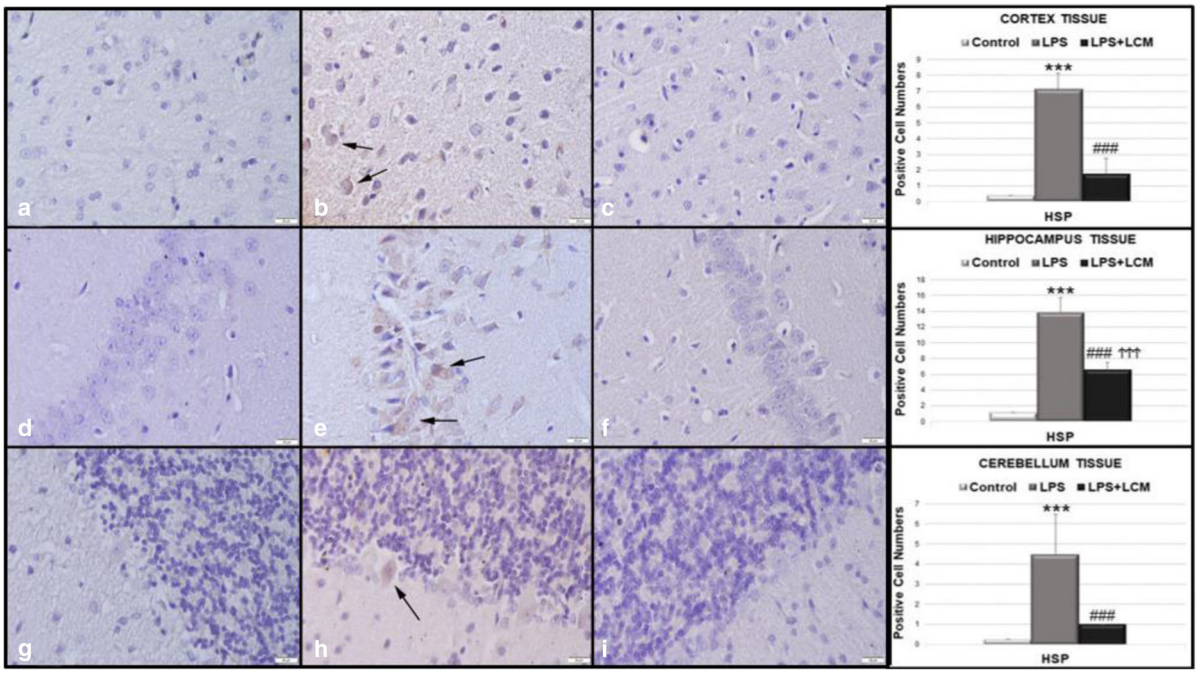

Fig. 6. HSP immunoreaction and statistical analysis of immunohistochemical positive cell numbers of cortex (A, B, C), hippocampus (D, E, F), and cerebellum (G, H, I) tissues. (A) No expression in control group in cortex. (B) Increased expression in neuronal cells (arrows) in LPS group in cortex. (C) Negative immunoreaction in LPS + LCM group in cortex. (D) No expression in control group in hippocampus. (E) Increased expression in cells (arrows) in LPS group in hippocampus. (F) Decreased immunoreaction in LPS + LCM group, in hippocampus. (G) No expression in control group in cerebellum. (H) Increased expression in neuronal cells (arrows) in LPS group in cerebellum. (I) Negative immunoreaction in LPS + LCM group in cerebellum. Streptavidin biotin peroxidase method, bar = 20 μm. Values are presented as means ± SD. The relationships between groups and results of immunohistochemical parameters are assessed by one-way ANOVA (*post hoc* LSD test). $^{***}p < 0.001$, compared with control; $^{\#\#\#}p < 0.001$ LPS + LCM, compared with LPS; $p < 0.001$, LPS + LCM, compared with control.

LPS-induced OS, and accompanying inflammation, leads to alteration in capillary permeability and circulation of vessels that supply nerves [22]. Besides, accumulation of xanthine oxidase in the capillary endothelium as a result of increased OS contributes to the breakdown of the BBB. This causes more OS at the BBB level with increased peroxidation that triggers proinflammatory cytokine release and further tissue damage [34]. In our design following LPS, histopathology revealed neuronal cell hyperemia, microhemorrhages, and degenerative changes that were ameliorated by LCM. This neuroprotective effect of LCM could be attributed to its antioxidant and anti-inflammatory properties. TNF-α is known to block the voltage-gated sodium channels [22]. Therefore, LCM could be protective by modulating voltage-gated sodium channels through decreasing TNF-α as well as direct anti-inflammatory effects *via* NF-Kβ inhibition.

HSPs are molecular chaperons involved in protein folding and maturation. As a protection mechanism of cells against stress including pathogens, some HSPs have been shown to be increased with LPS administration. This increment was suggested to be associated with cell damage as in lung injury [46, 47]. On the other hand, some HSPs have protective effects on organ damage in sepsis like HSPA12B and HSP70 [48, 49]. Metukuri et al. showed that HSP-60 increased significantly in the first 6 h following LPS administration [50]. Parallel to this study, HSP70 levels in cortex, hippocampus, and cerebellum tissues in our study were found to be elevated in LPS groups, whereas decreased immunoreactions were detected with LCM treatment. This can be due to the neuroprotective effects of LCM, revealed by decreased HSP70 expressions. Thus, LCM is a candidate neuroprotective molecule to deal with cellular stress *via* restoring OS, inflammation, and apoptosis.

In humans, CRP, the most used acute phase reactant, is increased in response to infection, inflammation, or tissue damage. Detection of elevated circulating levels

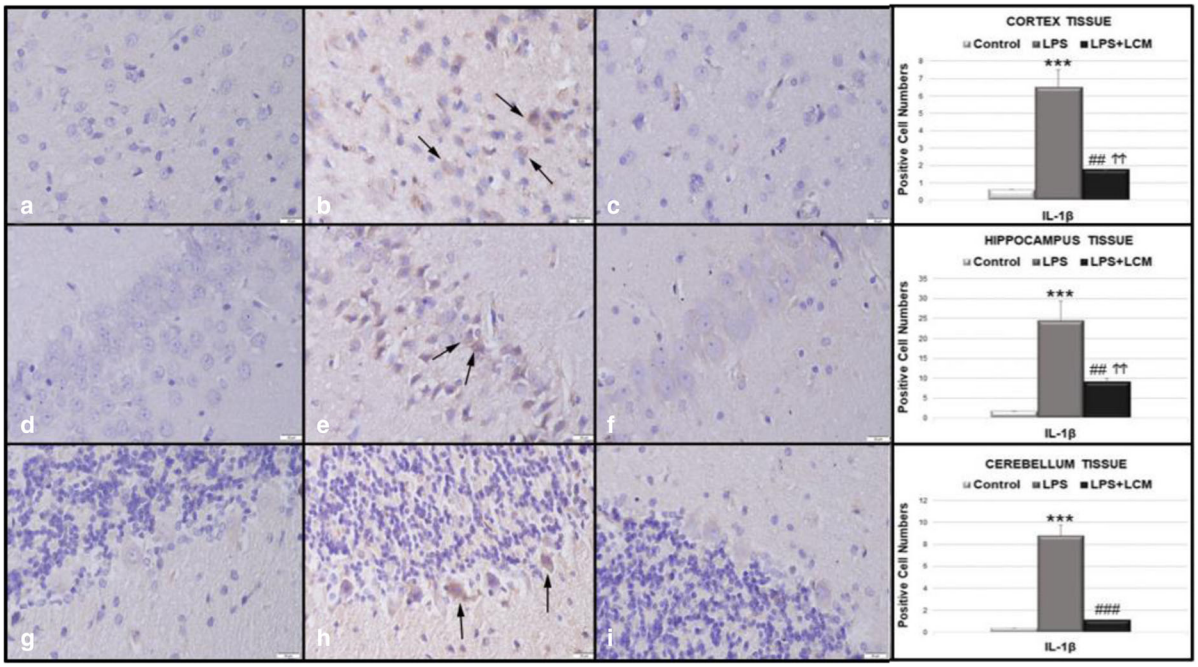

Fig. 7. IL-1β immunoreaction and statistical analysis of immunohistochemical positive cell numbers of cortex (A, B, C), hippocampus (D, E, F), and cerebellum (G, H, I) tissues. (A) No expression in control group in cortex. (B) Increased expression in neuronal cells (arrows) in LPS group in cortex. (C) Decreased immunoreaction in LPS + LCM group in cortex. (D) No expression in control group in hippocampus. (E) Increased expression in cells (arrows) in LPS group in hippocampus. (F) Decreased immunoreaction in LPS + LCM group in hippocampus. (G) No expression in control group in cerebellum. (H) Increased expression in neuronal cells (arrows) in LPS group in cerebellum. (I) Negative immunoreaction in LPS + LCM group in cerebellum. Streptavidin biotin peroxidase method, bar = 20 μm. Values are presented as means ± SD. The relationships between groups and results of immunohistochemical parameters are assessed by one-way ANOVA (*post hoc* LSD test). ***$p < 0.001$ compared with control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ LPS + LCM compared with LPS; $p < 0.01$ LPS + LCM compared with control.

within the 6 h makes CRP a useful biomarker in blood [51]. In clinical setting, CRP especially gains importance as it appears to be limited to inflammatory stimuli in aged patients [52]. Anti-inflammatory effect of LCM was previously shown by Kumar et al. in an experimental seizure model, and this effect was said to be continued up to 24 h [53]. In our study, CRP levels were not evaluated in blood, but expressions in cortex, hippocampus, and cerebellum tissues were found to be increased in the LPS group as an indicator of acute inflammation. LCM decreased CRP expressions, particularly by an anti-inflammatory mechanism.

OS is known to disturb mitochondrial membrane, leading to cell apoptosis [54]. In a study, decreased level of FLICE-inhibitor protein that is known to catalyze Cas-8 was shown in the brain of rats following CLP-induced NI [55]. Comim et al. [56] showed increased apoptosis and Cas-3 positive apoptotic cells in the hippocampus of CLP-induced septic rats. In our study, LCM decreased brain tissue apoptosis after LPS exposure, as shown by reduced Cas-3 levels. Overall, these data suggest that one of the neuroprotective mechanisms of LCM is its antiapoptotic potential.

In our study, we used LPS to induce the NI and prophylactic LCM treatment improved the LPS-induced changes. LCM, an approved antiepileptic drug with a known safety record, can be an appropriate candidate for conditions related to NI such as encephalitis. LCM can also be suggested as a first-line therapy for epileptic patients with a diagnosis of sepsis or patients with tumor-related epilepsy.

Overall, we conclude that LCM can be a novel alternative against NI, especially for the elderly patients, owing to its antioxidant, anti-inflammatory, and antiapoptotic properties. In the current study, we evaluated prophylactic use of LCM against LPS-induced

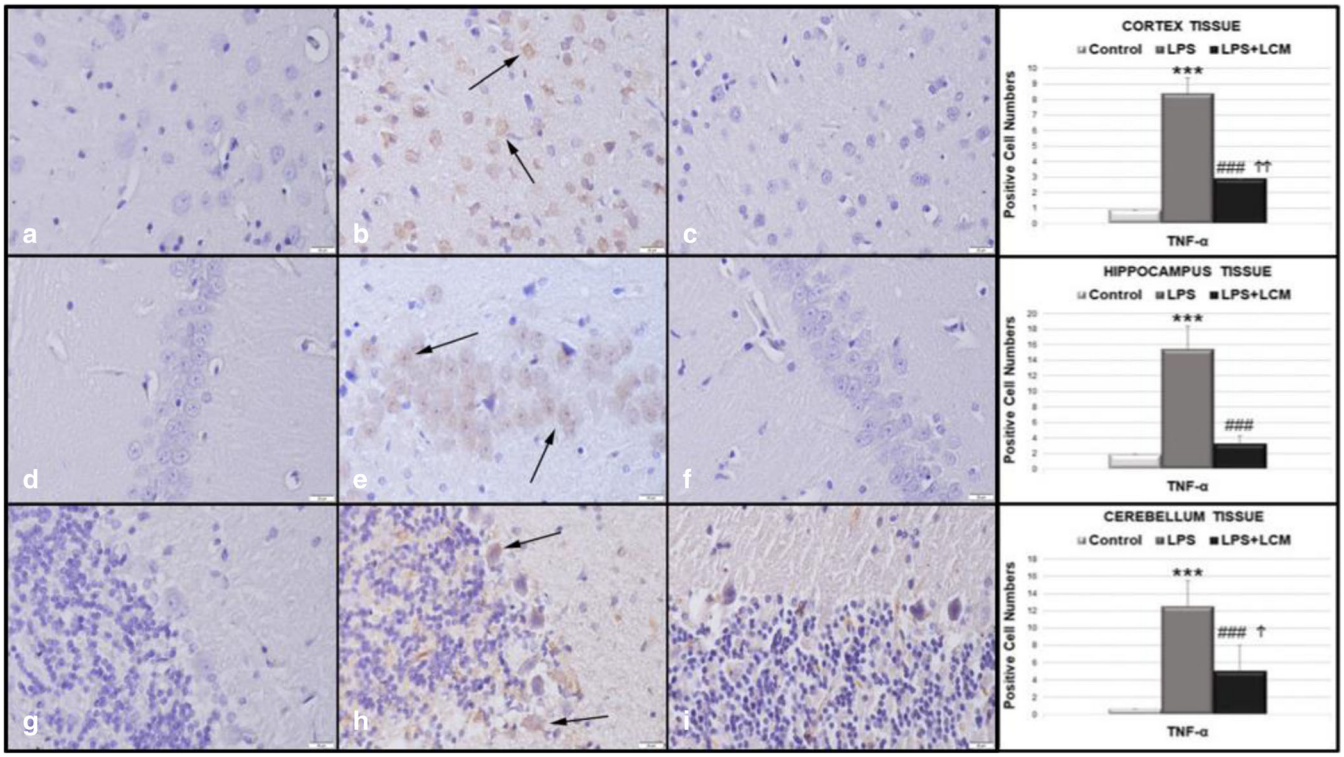

Fig. 8. TNF-α immunoreaction and statistical analysis of immunohistochemical positive cell numbers of cortex (A, B, C), hippocampus (D, E, F), and cerebellum (G, H, I) tissues. (A) No expression in control group in cortex. (B) Increased expression in neuronal cells (arrows) in LPS group in cortex. (C) Decreased immunoreaction in LPS + LCM group in cortex. (D) No expression in control group in hippocampus. (E) Increased expression in cells (arrows) in LPS group in hippocampus. (F) Negative immunoreaction in LPS + LCM group in hippocampus. (G) No expression in control group in cerebellum. (H) Increased expression in neuronal cells (arrows) in LPS group in cerebellum. (I) Decreased immunoreaction in LPS + LCM group in cerebellum. Streptavidin biotin peroxidase method, bar = 20 μm. Values are presented as means ± SD. The relationships between groups and results of immunohistochemical parameters are assessed by one-way ANOVA (*post hoc* LSD test). $^{***}p < 0.001$ compared with control; $^{\#\#\#}p < 0.001$ LPS + LCM, compared with LPS; $p < 0.05$, $p < 0.01$, LPS + LCM, compared with control.

acute NI. For this purpose, we used a pretreatment design to properly assess the prophylactic effect. As a limitation, the translational significance of our data and usefulness of LCM in combating the symptoms and causes of inflammation already present may be decreased. However, to our knowledge, LCM has not been evaluated before as a neuroprotective agent against inflammation-mediated brain pathology. Studies that evaluate post-treatment effects of LCM for longer duration of therapy with longer follow-up periods are needed to generalize the current results to clinical practice.

COMPLIANCE WITH ETHICAL STANDARDS

All experiments were performed in accordance with the guidelines for animal research from the National Institutes of Health, and approved by the Committee on Animal Research of Mehmet Akif Ersoy University, Burdur (No. 02.08.2017-308).

Conflict of Interest. The authors declare that they have no conflict of interest.

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