Clinical and Experimental Health Sciences

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Nesfatin-1 Ameliorates Sepsis-Induced Remote Organ Injury: The Role of Oxidant-Antioxidant Status and Neutrophils

Nesfatin-1 Sepsisin Yol Açtığı Uzak Organ Yaralanmasını İyileştirir: Oksidan Antioksidan Dengesinin ve Nötrofillerin Rolü

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ABSTRACT

Purpose: Protective effects of nesfatin-1 was studied in sepsis-induced injury of remote organs.

Methods: Male rats were randomly divided as control and sepsis (cecal ligation-perforation) groups, treated with either saline or nesfatin-1 (10 µg/kg). At 16 h following surgery, samples of brain, kidney, liver and lung tissues were removed and myeloperoxidase (MPO) activity, glutathione (GSH), catalase (CAT), superoxide dismutase (SOD) and malondialdehyde (MDA) levels were measured in these tissues.

Results: In saline-treated septic rats, elevated MDA and MPO activities were accompanied with depleted CAT, SOD and GSH levels in the brain, kidney, liver and lung tissues, implicating extensive oxidative damage in all remote organs. Nesfatin-1 reduced MDA levels (brain, lung) and MPO activities (brain, kidney), and preserved antioxidant GSH (brain, lung), CAT (brain) and SOD (kidney) levels. Severe hepatocyte degeneration, neuronal damage, glomerulotubular degeneration and alveolar disturbance in saline-treated septic rats were replaced with regular tissue morphologies in nesfatin-1-treated rats.

Conclusion: Nesfatin-1 alleviates oxidative damage by enhancing endogenous antioxidant systems and inhibiting recruitment of neutrophils, suggesting that nesfatin-1 may be have a potential therapeutic impact on the treatment of septic shock to reduce subsequent remote organ failure.

Keywords: Sepsis, CLP, Nesfatin-1, oxidative stress

ÖZ

Sepsisin yol açtığı uzak organ hasarlanmalarında anti-inflamatuvar bir peptit olan nesfatin-1'in koruyucu etkilerinin incelenmesi hedeflendi.

Yöntem: Erkek Sprague-Dawley sıçanlar, taklit cerrahi kontrol, serum fizyolojik ile tedavi edilen sepsis (çekal bağlama-delme) ve nesfatin-1 (10 ug/kg) ile tedavi edilen sepsis gruplarına bölündü. Ameliyattan 16 saat sonra karaciğer, akciğer, böbrek ve beyin dokuları örnekleri histolojik analiz, miyeloperoksidaz (MPO) aktivitesi, malondialdehit (MDA), glutatyon (GSH), süperoksit dismutaz (SOD) ve katalaz (CAT) seviyeleri ölçümleri için alındı.

Bulgular: Serum fizyolojik ile tedavi edilen septik sıçanlarda, karaciğer, akciğer, böbrek ve beyin dokularındaki yükselmiş MDA ve MPO düzeylerine, tükenmiş GSH, SOD ve CAT seviyeleri eşlik etti ve tüm uzak organlarda geniş oksidatif hasar meydana geldi. Nesfatin-1 MDA düzeylerini (beyin, akciğer) ve MPO aktivitelerini (beyin, böbrek) azalttı ve antioksidan GSH (beyin, akciğer), CAT (beyin) ve SOD (böbrek) seviyelerini korudu. Serum fizyolojik ile tedavi edilen septik sıçanlarda şiddetli hepatosit dejenerasyonu, nöronal hasar, glomerulotubular dejenerasyon ve alveolar bozukluk şeklinde tespit edilen doku morfolojileri nesfatin-1 tedavisi ile düzenli hal aldı.

Sonuç: Nesfatin-1 endojen antioksidanların güçlendirilmesi ve nötrofil göçünün inhibisyonu ile oksidatif hasarı hafifletir ve bu etkileri ile septik şokun tedavisinde uzak organ yetmezliğini azaltmak için potansiyel bir terapötik madde olarak düşünülebilir.

Anahtar Kelimeler: Sepsis, CLP, Nesfatin-1, oksidatif stress

INTRODUCTION

Sepsis is a complex clinical syndrome with a high mortality rate and a huge economic burden (1-4). In addition to the presence of systemic inflammation, characterized by a sepsis-induced imbalance between pro-inflammatory and anti-inflammatory mediators (5), intravascular blood clots are formed and blood flow to vital organs is impaired, leading to varying degrees of multiple organ dysfunction (6). Accordingly, studies have focused on developing new therapies for ameliorating sepsis-induced multiorgan failure, but most of the agents that were shown to have a potential therapeutic capacity in animal models have failed in septic patients (7). Based on results obtained from animal models and clinical trials indicating that oxidative stress contributes to the pathogenesis of sepsis-induced organ dysfunction, antioxidant therapies including vitamins, selenium, melatonin and several peptides have been considered in developing additional therapeutic strategies for the management of sepsis (5, 8).

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We have previously shown that nesfatin-1, an anorexigenic peptide with 82 amino acids (9), exerts antioxidant, anti-inflammatory and anti-apoptotic actions on the skin (10), stomach (11) and brain (12). Although nesfatin-1 was originally isolated in the hypothalamus (9) recent immunohistochemical and autoradiographic studies have revealed that several tissues including brain, lung, liver and kidney possess expressions of nesfatin-1/NUCB2 protein and nesfatin-1receptor (13, 14), suggesting an extensive role of nesfatin-1 in regulating several homeostatic functions. By maintaining oxidant/antioxidant balance, exogenous administration of nesfatin-1 has protected against oxidative damage of intestines (15), kidneys (16) and heart (17) in ischemia/reperfusion models. It was reported that nesfatin-1-releasing neurons can be stimulated by inflammatory signals from the periphery (18), while exogenously administered nesfatin-1 passes the blood–brain barrier (19, 20), implicating that centrally or peripherally administered nesfatin-1 can exert its anti-inflammatory effects via its central and peripheral receptors. In the light of aforementioned studies, our aim was to evaluate the putative protective role of intraperitoneally administered nesfatin-1 against oxidative injury of multiple organs affected by sepsis.

MATERIALS AND METHODS

Animals

Male Wistar albino rats (230-300 g, n=30), obtained from the Marmara University (MU) Animal Center (DEHAMER), were kept under controlled conditions (65-70 % humidity and 22 ± 2°C temperature) and at standard cycles of light and dark (12 h/12 h). Rats had free access to standard rat chow and water. MU Animal Care and Use Committee has approved the experimental protocols in the study (92.2010.mar).

Experimental and Surgical Procedures

Under anesthesia accomplished with the combination of intraperitoneally administered ketamine (100 mg/kg) and xylazine (3 mg/kg), sepsis was induced by ligation and puncture (CLP) of the cecum (n=20). After a midline laparotomy, in the CLP groups 3 pinholes were made on the ligated segment, while the control group underwent a laparotomy without any ligatures or punctures (sham-operated; n=10). Following the closure of the abdominal incision and saline (30 ml/kg body weight, subcutaneously) injection for replacing fluid loss during the surgery. CLP model of sepsis, which involves focal infection, bacteremia and circulating bacterial products, is accepted to mimic the hemodynamic and inflammatory properties of polymicrobial sepsis, but not the whole spectrum of human sepsis (21). Immediately after surgery for CLP, saline (saline-treated CLP group) or nesfatin-1 (10 µg/kg; nesfatin-1-treated CLP group) was injected ip, while the sham-operated rats were injected with saline. Since the previous studies have indicated that the highest rate of mortality in CLP sepsis occurs within the first 3 days (22), rats were decapitated earlier at the 16 h following the surgery, during which they had no food but water. Samples of brain, kidney, liver and lung tissues were removed. Histological (in hematoxylene-eosin stained samples) and chemical (to measure myeloperoxidase activity, catalase, superoxide dismutase, glutathione and malondialdehyde levels) analyses were made.

Measurement of Malondialdehyde and Glutathione Levels in the Tissues

As lipid peroxidation indicators, malondialdehyde (MDA) levels were measured (23). Samples of brain, kidney, liver and lung tissues were homogenized and centrifuged in trichloracetic acid (10% TCA in ice; Sigma) at 2,000 g (at 4°C, 15 min). After removing supernatants, samples were recentrifuged for 8 min at 41,400 g. Expression of MDA levels was given as nmol MDA/g tissue.

Based on the modified Ellman procedure (24), glutathione (GSH) levels in brain, kidney, liver and lung tissues were measured. Following the homogenization, and centrifugation (2,000 g, 10 min), obtained supernatants (0.75 ml) were added to $Na_2HPO_4 \cdot 2H_2O$ solution (1 ml) and then dithiobisnitrobenzoate (0.2 ml, in 1% sodium citrate) was added. Spectrophotometrically, absorbance measurements were made at 412 nm, and tissue GSH levels were calculated and given in µmol GSH/g tissue.

Measurement of Myeloperoxidase Activity in the Tissues

Tissue myeloperoxidase (MPO) activity is commonly used to express an estimation of tissue neutrophil accumulation in tissues with inflammation, because a positive correlation was shown to exist between the biochemically measured MPO activity and histochemically determined neutrophil infiltration. MPO activity in the brain, kidney, liver and lung tissues was measured (25). Following the homogenization of the tissue samples in potassium phosphate buffer (K_2HPO_4; 50 mM, at pH 6.0) that contains hexadecyltrimethylammonium bromide (HETAB; 0.5%), they were centrifuged (41,400 g at 4°C, 10 min). The pellets were re-homogenized in a solution containing K_2HPO_4 (50 mM) plus HETAB (0.5%; w/v) with EDTA (10 mM). In order to assess MPO activity, oxidation of o-dianizidine.2HCl was measured. Any change in absorbance (1.0/min) per gram of tissue weight measured at 460 nm was described as one unit of MPO activity.

Measurement of Superoxide Dismutase and Catalase Activities in the Tissues

Superoxide dismutase (SOD) activity in brain, kidney, liver and lung tissues (50-100 µL) was measured (26) in illuminated (20-W fluorescent tubes) and heated (37°C) cuvettes containing 50 mM potassium phosphate (2.8 ml) containing EDTA (0.1 mM), riboflavin (0.39 mM) in potassium phosphate (10 mM) and o-dianisidin.2 HCl (6 mM, 0.1 ml). At 460 nm, spectrophotometric absorbances were measured at the 0 and 8 min of illumination, and extrapolations for the absorbances were made and expressed (units per g tissue) using the curve that was obtained from the absorbance of standard bovine SOD.

Catalase (CAT) activity was determined based on the enzymatic activity of CAT in the decomposing H_2O_2. Briefly, the absorbances of

the brain, kidney, liver and lung homogenates containing H_2O_2 were read at 240 nm (20°C for 1 min) (27).

Light Microscopic Evaluation

After fixing in 10% formaldehyde, brain, kidney, liver and lung tissues were embedded in paraffin. For general histopathological evaluation, tissue sections of 4 µm were stained with hematoxylin and eosin (H&E), and examined under a photomicroscope (Olympus BX51, Tokyo, Japan) by an experienced histologist who was unaware of the treatment conditions. At least five microscopic areas were examined to evaluate the severity of degeneration and inflammation in lung (*alveolar structural disturbance, inflammatory cell infiltration, vascular congestion/interstitial edema*), liver (*dilation/vacuolization of hepatocytes, vascular congestion/dilation of sinusoids, Kupffer cell infiltration*), kidney (*degeneration of Bowman space and glomeruli, degeneration of proximal and distal tubules, vascular congestion/ interstitial edema, inflammatory cell infiltration*) and brain (*degeneration of neurons, vascular edema/ hemorrhage*).

Statistical analysis

Using GraphPad Prism Software 6.0 (La Jolla, CA, USA), analysis of data were made by Student's t-test or one-way ANOVA with post hoc Tukey multiple comparison tests. All data were presented as mean ± SE. When P values were less than 0.05, they were regarded as statistically significant.

RESULTS

Within the post-surgical 16 h period, no mortality was observed among the CLP-induced or sham-operated rats. MPO activity, measured as a marker of neutrophil infiltration at the 16th h of sepsis induction, was increased in all tissues that were studied, when compared with the respective MPO activities measured in the brain, kidney, liver and lung samples of the control rats ($p<0.05$-0.001; Fig.1). A single dose of nesfatin-1, when given immediately after sepsis induction, has significantly depressed sepsis-induced MPO elevations in both brain and kidney ($p<0.05$ and $p<0.001$), but nesfatin-1 treatment did not have a significant effect on hepatic or pulmonary MPO activity.

In parallel with the elevated MPO activity in all tissues, MDA levels – indicative of lipid peroxidation – were also increased in the brain, kidney, liver and lung tissues when compared with those of the control rats ($p<0.001$; Fig.2). Nesfatin-1 treatment significantly depressed MDA levels in the brain and lung tissues ($p<0.01$), but the slight reductions in the hepatic and renal MDA levels did not reach statistical significance.

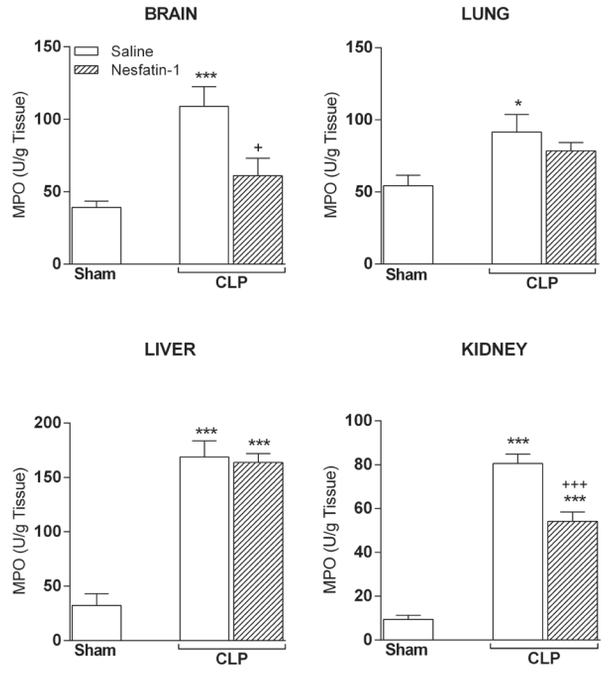

Figure 1. Myeleoproxidase (MPO) activities in the brain, lung, liver and kidney tissues of sham-operated control group and saline – or nesfatin-1-treated sepsis groups induced with cecal ligation and puncture (CLP). Each group consists of 8-10 rats. Values are represented as mean ± SEM. $^{*}p<0.05$, $^{***}p<0.001$ compared to control group;$^{+}p< 0.05$, $^{+++}p<0.001$ compared to saline-treated CLP group.

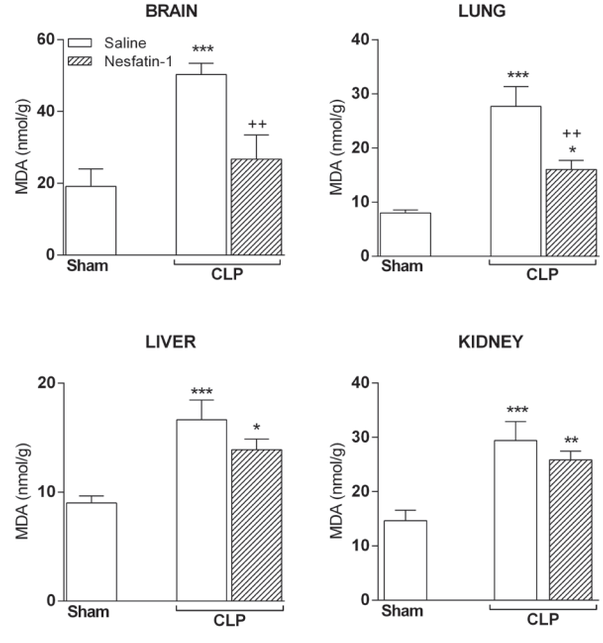

Figure 2. Malondialdehyde (MDA) levels in the brain, lung, liver and kidney tissues of sham-operated control group and saline – or nesfatin-1-treated sepsis groups induced with cecal ligation and puncture (CLP). Each group consists of 8-10 rats Values are represented as mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to control group; $^{++}p<0.01$ compared to saline-treated CLP group

At the 16th h of sepsis induction, intracellular antioxidants, GSH, SOD and CAT were all depleted in the studied tissues (p<0.05 – 0.001; Fig. 3-5). A single dose of nesfatin-1 treatment following CLP induction conserved the GSH levels in the brain and lung tissues (p<0.05, 0.01), while CAT activity in the brain (p<0.05) and renal SOD activity (p<0.05) were elevated in the nesfatin-treated CLP group. However, minor elevations in the antioxidant enzyme activities of the other tissues had no statistical significance.

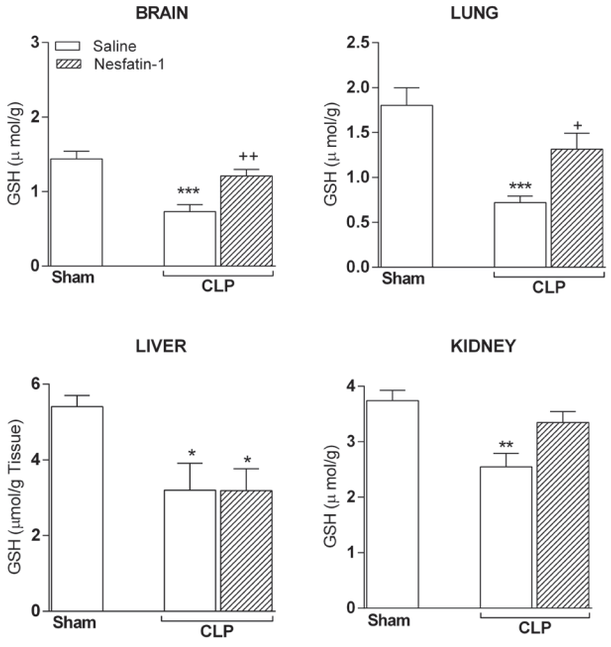

Figure 3. Glutathione (GSH) levels in the brain, lung, liver and kidney tissues of sham-operated control group and saline – or nesfatin-1-treated sepsis groups induced with cecal ligation and puncture (CLP). Each group consists of 8-10 rats. Values are represented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared to control group; +p<0.05, ++p<0.01 compared to saline-treated CLP group.

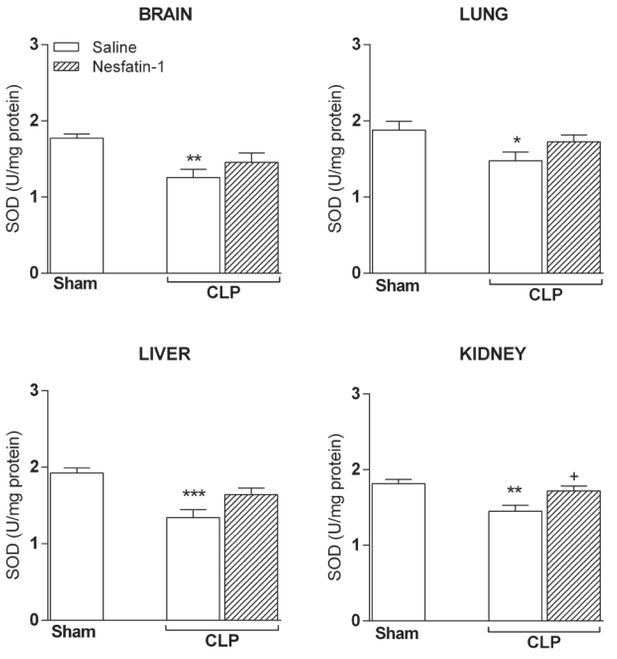

Figure 4. Superoxide dismutase (SOD) levels in the brain, lung, liver and kidney tissues of sham-operated control group and saline – or nesfatin-1-treated sepsis groups induced with cecal ligation and puncture (CLP). Each group consists of 8-10 rats. Values are represented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared to control group; +p<0.05 compared to saline-treated CLP group.

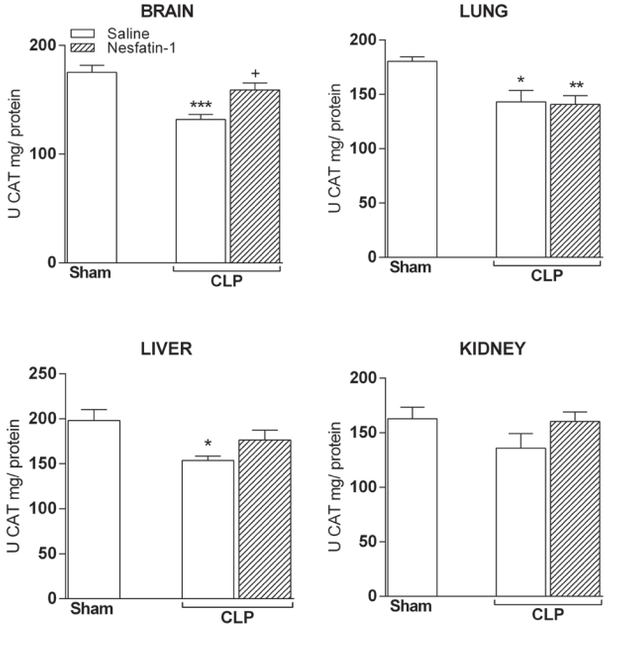

Figure 5. Catalase (CAT) levels in the brain, lung, liver and kidney tissues of sham-operated control group and saline – or nesfatin-1-treated sepsis groups induced with cecal ligation and puncture (CLP). Each group consists of 8-10 rats. Values are represented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared to control group; +p<0.05 compared to saline-treated CLP group.

In the control group, light microscopic evaluation revealed a regular morphology of cerebral cortex (Fig. 6A), lung parenchyme with alveolar organization (Fig. 6D), liver parenchyme with well-designated hepatocytes and sinusoids (Fig. 6G) and kidney with glomerular and tubular organization (Fig. 6J). In the saline-treated CLP group, perivascular and perineuronal edema and severe neuronal damage was observed in cerebral cortex (Fig. 6B), while disturbance in the alveoli, congestion in vessels and infiltration of inflammatory cells to the lung (Fig. 6E), severe degeneration of hepatocytes, vacuole formation and sinusoidal congestion in liver (Fig. 6H) and severe glomerular degeneration with vascular congestion, dilatation in Bowman's space, degeneration in tubules and inflammatory cell infiltration to the kidney (Fig. 6K) were evident. On the other hand, nesfatin-1-treated CLP group showed moderate damage of cerebral cortex (Fig. 6C), quite regular alveolar structure and decreased pulmonary vascular congestion (Fig. 6F), quite regular liver parenchyma with hepatocytes and mild sinusoidal congestion (Fig. 6I) and mild degeneration of renal corpuscles and proximal tubules with mild vascular congestion (Fig. 6L).

DISCUSSION

Our results demonstrated that induction of sepsis with CLP has generated oxidative stress in the target organs, which was evident with significant increases in lipid peroxidation and neutrophil infiltration in brain, lung, liver and kidney, accompanied by depleted antioxidant levels in all these tissues. In the brain tissue injured by sepsis, a single dose of nesfatin-1suppressed lipid peroxidation and neutrophil recruitment, while GSH, CAT and SOD levels were maintained. A single injection of nesfatin-1made immediately after sepsis induction diminished lipid peroxidation in the lung and prevented the depletion of pulmonary GSH level, while elevated MPO activity and depleted CAT level in the kidney were reversed by nesfatin-1. Although sepsis-induced oxidative changes in the hepatic tissue were not altered by nesfatin-1 treatment, histopathological findings revealed that all the studied tissues, including liver, demonstrated improved inflammatory changes when treated with nesfatin-1.

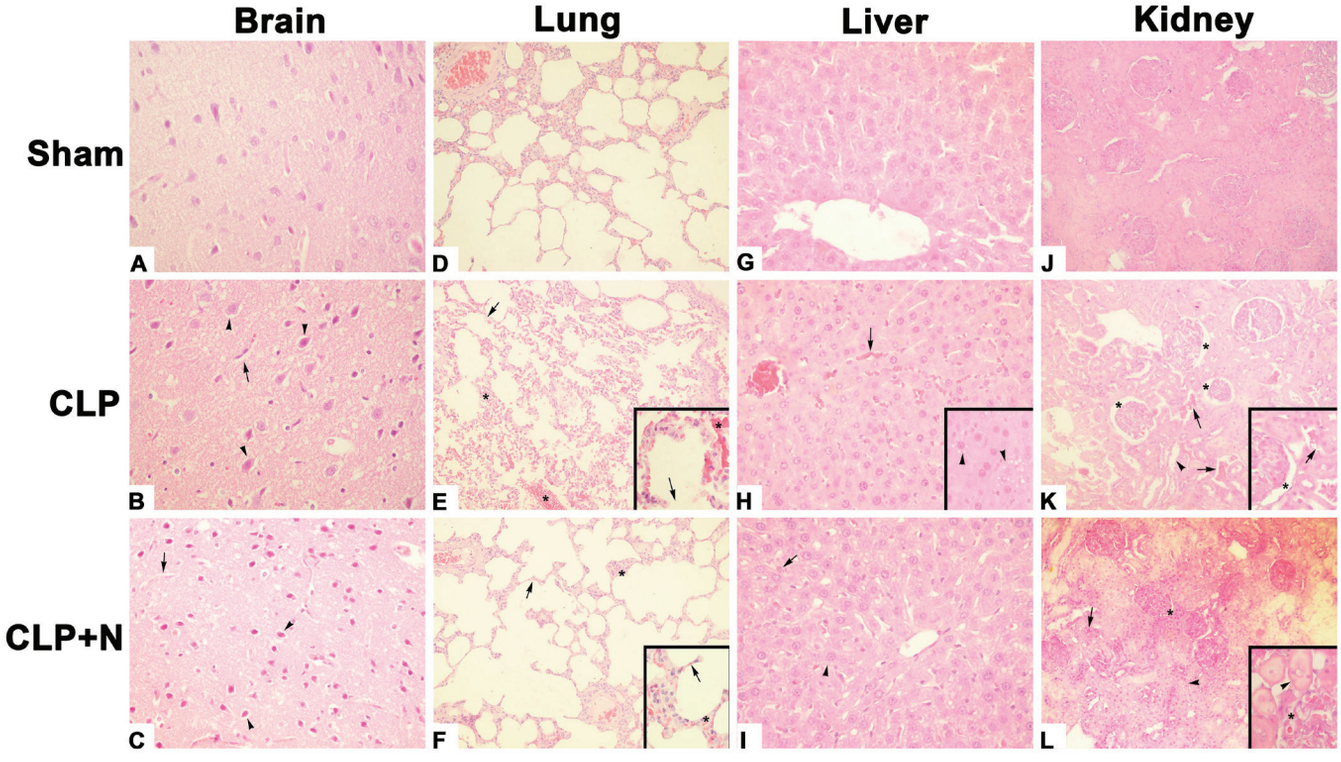

Figure 6. Representative photomicrographs of brain, lung, liver and kidney tissues of sham-operated control group and saline- or nesfatin-1-treated sepsis groups induced with cecal ligation and puncture (CLP). Regular morphologies of cerebral cortex (A), lung parenchyma with regular alveolar organization (D), liver parenchyma with well-organized hepatocytes and sinusoids (G) and renal cortex with renal corpuscle and tubular organization (J) are seen in sham-operated control group. Severe neuronal damage with perineuronal edema (arrow head) and vascular congestion (arrow) with perivascular edema in cerebral cortex (B), severe alveolar disturbance (arrow) and vascular congestion (*) in lung paranchyma (E); increased hepatocyte degeneration (arrowhead) with vacuole formation and dilatation in sinusoids (arrow) in liver parenchyma (I); severe glomerular degeneration with vascular congestion and dilatation in Bowman's space (*), degeneration in tubules (arrowhead) and vascular congestion (arrow) in kidney (K) are seen in saline-treated CLP group. Decreased neuronal damage with perineuronal edema (arrow) and vascular congestion (arrow) with perivascular edema in cerebral cortex (C); quite regular alveolar structure (arrow) and decreased vascular congestion (*) in lung parenchyma (F); quite regular hepatocytes (arrow) and decreased sinusoidal congestion (arrow) in liver (I); quite regular renal corpuscles (*), proximal tubules (arrowhead) and decreased vascular congestion (arrow) in kidney (L) are seen in nesfatin-1 treated CLP group. H&E staining, original magnifications: 200X, insets and liver micrographs: 400X

Despite the improvements in life-sustaining technologies developed for the management of patients in intensive care, sepsis accompanied with organ dysfunction still continues to result in significant morbidity and mortality mainly due to hepatorenal dysfunction (28), which is an independent prognostic factor indicative of high mortality (29-31). Since experimental and clinical studies have demonstrated that multiple organ dysfunctions in sepsis is associated with oxidative stress (32), several antioxidants, including pomegranate, α-lipoic acid and curcumin, were shown to improve survival by attenuating hepatorenal and pulmonary injury (33-35). Similar to recent studies that have focused on the potential antioxidant and anti-inflammatory effects of nesfatin-1 (10-12, 17), nesfatin-1 improved renal and cerebral damage induced by sepsis, through limiting the infiltration of neutrophils and conserving the antioxidant capacity. In accordance with our results, nesfatin-1 treatment improved acute renal ischemia-reperfusion injury in rats via the elevation of SOD and CAT activities (16). In addition, protective effect of nesfatin-1 on gastric (11), intestinal (15), cardiac (17), cerebral (12) and dermal (10) damage was attributed to its role in supporting antioxidant capacity of the related tissues. However, in a cholestatic liver injury model, 10-day nesfatin-1 treatment has decreased hepatic MDA levels and hepatocyte necrosis without significantly altering the oxidative DNA damage (36). In parallel to their results, our data demonstrate that histopathologically verified alleviation of sepsis-induced hepatic injury by nesfatin-1does not appear to involve an antioxidant action, suggesting the presence of other anti-inflammatory mechanisms operating in the hepatic cells, which require further investigation.

Autoradiographic analyses confirmed the presence of nesfatin-1 receptors in several brain regions (cerebellum, cortex, area postrema, dorsal motor nucleus of vagus and hypothalamic paraventricular nucleus), several peripheral organs (kidney, lung, and liver), including the endocrine tissues (adrenal gland, pancreas, pituitary, testis and adipose tissue) (14). Nesfatin-1 administration immediately after sepsis induction exerted a widespread protective effect on brain, lung, liver and kidney, which appear to express receptors for nesfatin-1. One of the targets during the early phase of sepsis is the brain tissue (37, 38), which is characterized by high demand of oxygen and high content of antioxidant enzymes making it more vulnerable to oxidative stress (39) and cognitive dysfunction (40). Current data also showed that sepsis resulted in oxidative neuronal damage along with the depletion of cellular antioxidants. Nesfatin-1 administration at the time of sepsis induction had an inhibitory impact on oxidative parameters, while the levels of antioxidants were maintained in a more comprehensive manner than all the other tissues. Previous studies have shown that nesfatin-1, which is colocalized with corticotropin releasing hormone, is involved in stress response (41); while nesfatin-1 neurons are stimulated with stress in rats (42), increasing the concentration of nesfatin-1 in plasma and its mRNA expression in the hypothalamus (43). On the other hand, nesfatin-1 and nesfatin-1-activated hypothalamic neurons are known to contribute to the regulation of food intake (44), and to reduced food intake in systemic inflammation and endotoxemia (18) during which the levels of catecholamines increase (45). It can be postulated that central nesfatin-1 response to sepsis, when augmented with its exogeneous administration, may support the systemic anti-inflammatory and hypometabolic activities, as well as the protection of neural tissue against oxidative stress.

Our findings demonstrate that the protective effect of nesfatin-1 on CLP-induced tissue injury was associated with the attenuation of inflammatory cell migration to cerebral and renal tissues. Previously we have shown that nesfatin-1, when administered peripherally, depressed subarachnoid hemorrhage-induced oxidative stress that has involved the inhibition of neutrophil accumulation to brain tissue (12). Similarly, indomethacin-induced gastric injury (11) and surgery-induced skin injury (10) were improved via the suppression of neutrophil recruitment to the respective tissues. Thus, taken with our aforementioned results, present data suggest that the anti-inflammatory effect of nesfatin-1 involves the inhibition of neutrophil accumulation to the inflamed tissue. It can be postulated that nesfatin-1 may have additional receptors on the neutrophils or endothelial cells, which may block the migration of inflammatory cells. However, further studies are required to test the mechanisms underlying the inhibitory effect of nesfatin-1 on recruitment of neutrophils to the tissues induced with various inflammatory challenges, including sepsis.

In an experimental model of sepsis induced with cecal ligation and puncture, nesfatin-1 has effectively improved oxidative organ damage of the target organs by inhibiting neutrophil-mediated inflammation and keeping the oxidant and antioxidant balance. These results suggest that further experimental and clinical studies are warranted to further assess the adjuvant therapeutic use of nesfatin-1 in ameliorating sepsis-related target organ injury for improving patient outcomes during multiple organ failure.

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