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ORIGINAL ARTICLE

Protective effects of Nesfatin-1 peptide on cerebral ischemia reperfusion injury via inhibition of neuronal cell death and enhancement of antioxidant defenses

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Abstract

Nesfatin-1 is a novel peptide with anorexigenic and anti-hyperglycemic properties. According to previous studies, this multi-functional peptide protects dopaminergic cells against neurotoxicity via anti-apoptotic effects. In addition, Nesfatin-1 protects myocardial tissue after myocardial infarction via anti-inflammatory and anti-apoptotic mechanisms. In this study, we investigated the neuroprotective effects of nesfatin-1 against cerebral ischemia reperfusion injury in the CA1 area of hippocampus in rats. 56 male Wistar rats (240-270 g) were randomly selected and allocated into four groups: (1) sham, (2) nesfatin-1, (3) ischemia/reperfusion, (4) ischemia/reperfusion+nesfatin-1. Cerebral ischemia induced by the occlusion of the common carotid arteries for 20 min was followed by reperfusion. Saline as a vehicle and nesfatin-1 (20 μg/kg) were injected intraperitoneally (IP) at the start of cerebral reperfusion. Apoptotic and necrotic cell death was detected by TUNEL and Nissl staining. Malondialdehyde (MDA) and antioxidant enzymes (GSH and SOD) levels were measured by the ELISA method. The results showed that cerebral ischemia increased the apoptotic and necrotic cell death in the CA1 area of hippocampus, while, treatment with nesfatin-1significantly reduced apoptotic and necrotic cell death. Moreover, the MDA levels of the hippocampus in ischemic rats were higher, whereas in nesfatin-1-treated rats the MDA levels were decreased. Furthermore, the SOD and GSH levels in the ischemic rats were decreased, whilst in ischemic rats treated with nesfatin-1, the SOD and GSH levels were increased. This study for the first time found that nesfatin-1 treatment improves CA1 hippocampus injuries after cerebral ischemia through preventing neuronal cell death and enhancement of antioxidant defenses.

Keywords Nesfatin-1 · Apoptosis · Necrosis · Brain ischemia · Hippocampus

Introduction

Nesfatin-1 is a novelty adipocytokine that was discovered in 2006by Oh-I et al. It is the 82-amino acid protein with anorexigenic and anti-hyperglycemic properties (Oh et al. 2006; Su et al. 2010). It has been shown that nesfatin-1is encoded by NEFA gene (also known as NUCB2) and it is widely distributed in peripheral tissues, including pancreatic islets, gastric glands, and duodenum. It is also expressed in many areas of central nervous system such as cortical areas, hypothalamus, and thalamus (Foo et al. 2008; Zegers et al. 2011; Zhang et al. 2010). Previous observations demonstrated that nesfatin-1 has a regulatory role in food intake and glucose homeostasis, and the underlying mechanisms are provided by the direct influence of nesfatin-1 on insulin secretion and modulation of insulin sensitivity through the modifying AKT phosphorylation and GLUT-4 membrane translocation (Li et al. 2013). It has to

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be noted that peptides have been applied as therapeutic strategies in an incremental form in recent years, which reveals that peptides have important roles in treatment of many diseases such as infectious and autoimmune diseases (Thundimadathil 2012; Xiao et al. 2015).

It was also reported that nesfatin-1 peptide can penetrate the blood-brain barrier by a non-saturable mechanism, which provides the possible therapeutic permission for nesfatin-1 as a pharmacological transfer agent to the central nervous system (Price et al. 2007). Furthermore, it has been shown that nesfatin-1 can protect MES23.5 dopaminergic cells against rotenone-induced neurotoxicity by reversing mitochondrial dysfunction and its anti-apoptotic effects (Tan et al. 2015). Administration of Nesfatin-1 exerts a cardioprotective effect against isoproterenol (ISO)-induced myocardial infarction. This protection mechanism results from the diminution in the number of apoptotic and necrotic cells and also the reduction of the levels of proinflammatory cytokines includinginterleukin-1β, interleukin-6 and tumor necrosis factor-α in myocardial tissues (Tasatargil et al. 2017). Furthermore, it has recently been indicated that the application of nesfatin-1 has anti-apoptotic and anti-inflammatory effects on the model of subarachnoid hemorrhage (SAH) brain injury by reducing the levels of oxidative brain injury markers such as Myeloperoxidase (MPO), Malondialdehyde (MDA) and caspase-3 (proapoptotic protein). Moreover, it has been shown that nesfatin-1 causes the enhancement of antioxidant enzyme activities including superoxide dismutase (SOD), glutathione (GSH), and catalase in subarachnoid hemorrhage model (ÖzsavcÍ et al. 2011).

Cerebral ischemia-reperfusion (I/R) causes complex pathological conditions leading to the prevalence of mortality and disability worldwide (Wicha et al. 2017). In I/R, the blood supply to an organ is limited that is followed by a concurrent renewed perfusion and reoxygenation. During reperfusion, the returned blood can bring oxygen to the cells; therefore, there is the possibility of DNA, protein, and plasma membrane damage.

Interactions of complex pathophysiological processes including apoptosis, excitotoxicity, and inflammation, can induce cerebral ischemia-associated tissue damage (White et al. 2000). It has been stated that during blood flow limitation, the irreversible nerve cell necrosis occur in the ischemic core and apoptosis cell death happen in the ischemic penumbra that can be reversible (Hu et al. 2017). Consequently, the study of the apoptosis molecular pathways consider rescuing the apoptotic cells and developing neuroprotection strategies for treatment of cerebral ischemia (Wicha et al. 2017). Previous studies demonstrated that increasing the production of reactive oxygen species (ROS) has an important role in brain ischemic cascades and causing structural and functional cellular injuries, which lead to necrotic and apoptotic neuronal death (Chen et al. 2011; Coimbra-Costa et al. 2017). In physiological status, antioxidant system creates the balance between the elimination and the formation of ROS (Chen et al. 2011).

Certain brain regions such as pyramidal neurons of the hippocampus, have shown more susceptibility to cerebral ischemia (Erfani et al. 2015a). Considering the characteristics of nesfatin-1 and cerebral ischemia mechanisms, this study aims to examine the protective effects of nesfatin-1 in hippocampal CA1 area after the occurrence of cerebral ischemia reperfusion.

Materials and methods

Animals and drugs

Fifty-six Male Wistar rats (weighting 240-270 g) were purchased from Tehran Pasteur Institute and were kept in standard cages and controlled environment (22–24 °C, 45–50% humidity, and 12-h light/dark cycle). They also had free access to food and water. It is to be noted that all the experiments were conducted in accordance with the Helsinki Declaration. Nesfatin-1peptide (Sigma Aldrich, Germany) was dissolved in saline for injection.

Experimental design and protocols

Rats were randomly divided into four distinct groups: sham (n = 14), ischemia/reperfusion (n = 14), ischemia/reperfusion+Nesfatin-1 (n = 14), and Nesfatin-1 (n = 14). The ischemia/reperfusion+Nesfatin-1 and Nesfatin-1 groups received Nesfatin-1 (20 μg/kg) intraperitoneally (IP) injection at the start of the reperfusion period. In the ischemia/reperfusion groups, the rats underwent common carotid arteries occlusion (CCAO). Rats in the sham-operated group (treated as a control group) were exposed to the similar surgical method with the difference that their common carotid arteries were not occluded; however, Nesfatin-1 group was not subjected to the induction of ischemia surgical procedures. Seven days after the induction of ischemia, half of the rats in each group went under anesthesia and transcardiac perfusion carried out for tissue staining. The other half were decapitated and their hippocampal tissues were collected and stored at −80 °C for the measurement of MDA, GSH and SOD levels.

Induction of transient global cerebral ischemia model

Transient global cerebral I/R model was accomplished by a method that was previously described (Shamsaei et al. 2015). The rats were put under anesthesia with ketamine/xylazine (40 mg/kg, IP injection) and then underwent brain ischemia surgery. At first bilateral common carotid arteries were seen and separated from their carotid sheet and vagus nerves carefully. The blockage of the common carotid arteries was done by the Yashargil Aneurism micro clips for a 20-min period. At

the end of the operation, the clips were removed and reperfusion was established immediately. The return of blood flow was confirmed visually. The feedback-regulated heating system maintained the rats' rectal temperature at 36.5 ± 0.5 °C during the experiment. After the surgery, the rats were placed in their cages with free access to food and water and they were kept separately for seven days. At the beginning of the reperfusion, Nesfatin-1 (20 μg/kg) was dissolved in 1 ml saline and was injected intraperitoneally to each rat.

Tissue preparation for staining

Seven days after ischemia, the rats were deeply anesthetized, and .9% saline and then 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) were used for transcardiac perfusion process. Then the brain tissues were extracted and postfixed in the similar fixative for three days, and then they were embedded in paraffin. Afterwards, for doing the Nissl and TUNEL staining, coronal sections were cut using a microtome in accordance with the Paxinos atlas (between 3.3 mm and 4.2 mm posterior to bregma) at 7 μm thicknesses (Aboutaleb et al. 2016).

Nissl staining

Nissl staining is usually utilized to identify necrotic cell death according to morphological changes in the brain. For Nissl staining, coronal sections (three sections per rat) were immediately mounted onto gelatinized slides. The slides were stained with 0.1% cresyl violet (Sigma Aldrich), dehydrated with ethanol, and cover-slipped with Entellan. Three photomicrographs were taken from each rat by a light microscope (LABOMED USA, 400 × magnification). Image analysis was done using the "Image tool-2 software". The number of necrotic cells was counted along the CA1 area of the right hippocampus fields (Khaksari et al. 2017).

TUNEL staining

TUNEL assay was applied for the detection of labeling DNA fragmentation in the apoptotic cell death by using "In Situ Cell Death Detection Kit" (Roche, Mannheim, Germany) based on the manufacturer's protocol (Aboutaleb et al. 2015). In brief, after deparaffinization with xylol, the sections (three sections per rat) rehydrated using descending series of alcohol and incubated with proteinase K at room temperature. Then, incubation with 3% H_2O_2 in methanol was performed for blocking endogenous peroxidase in the dark. Afterwards, the TUNEL reaction mixture was added in humidified atmosphere at 37 °C. After the washing step, converter-POD was used for 30 min in the dark for the visualization. Subsequently, the slides were rinsed with PBS, and DAB substrate (3,3-diaminobenzidine) was utilized for 10 min as a chromogen. In addition, hematoxylin was used for counterstaining. Eventually TUNEL positive cells were quantified using a light microscope. Counts was performed during transect of 400 μm length of CA1 area of the right hippocampus. The number of TUNEL-positive cells was counted along the CA1 area of the right hippocampus fields.

Measurement of MDA levels

For the indication of lipid peroxidation products, malondialdehyde (MDA) levels were evaluated in the hippocampal samples by using MDA assay kit (ZellBio GmbH, Germany). The collected tissues were homogenized in ice-cold 1.15% KCl for two minutes. Furthermore, MDA levels were measured based on the thiobarbituric acid reactive substance (TBARS) formation method. Cold 10% trichloroacetic acid (1 ml) and 10% thiobarbituric acid (2 ml) were mixed with homogenized tissues and were heated at 100 °C for 1 h. After the solution is cooled to room temperature and centrifuged, the precipitate was removed and the pink supernatant was transferred to the microplate. The measurement of reaction mixture absorbance was performed at 535 nm by microplate readers (ELx800, BioTek, USA). Finally, the concentration of MDA (μM) was calculated according to the standard curve.

Measurement of glutathione (GSH) and superoxide dismutase (SOD) levels

Assessment of glutathione (GSH) and superoxide dismutase (SOD) levels was performed based on the instructions of GSH and SOD assay kit (ZellBio GmbH, Germany). After adding a certain amount of PBS (100 mM, pH 7.4), the hippocampal samples were homogenized and centrifuged. Subsequently, the supernatants were carefully collected; then after SOD and GSH interacted with chromogen reagent, the absorbances were read with a microplate reader (ELx800, BioTek, USA) at 420 nm and 412 nm to calculate SOD level (U/ml) and GSH level (mM), respectively, based on the defined formula.

Statistical analysis

Descriptive statistics were used to summarize the characteristics of the data set including mean and standard deviation. Various tests were utilized to analyze the data. The Kolmogorov–Smirnov test was used as the normality test of this study, which showed that the data was normally distributed. Moreover, one-way analysis of variance (ANOVA) test was used to compare the differences between the groups, and when there was a significant difference, a post hoc test (the Scheffe's or Dunnett's T3) was used to determine where the difference occurred. The significance level was set at $P \leq 0.05$. All data were analyzed using SPSS software (SPSS for Windows, Version16).

Results

Treatment with Nesfatin-1 attenuated ischemia-reperfusion-induced necrotic cell death

The results of Nissl staining showed that transient cerebral ischemia increased the percentage of necrotic cell death in the ischemia group (45.8% ± 18.84) compared to the sham (13.72% ± 2.71) and nesfatin-1 (18.7% ± 2.1) groups ($P < 0.001$). In nesfatin-1 treatment group (26.6% ± 3.39), necrotic cell death was decreased compared to the ischemia group ($P < 0.05$), (Fig. 1).

Treatment with Nesfatin-1 reduces ischemia-reperfusion-induced apoptotic cell death

The results of TUNEL staining showed that the percentage of TUNEL-positive cells in the hippocampal CA1 area in ischemia group (74.8% ± 3.63) was significantly increased, compared to the sham (12.2% ± 1.92) and nesfatin-1 (15.6% ± 1.81) groups ($P < 0.001$). Also, in ischemic rats treated with nesfatin-1, the percentage of TUNEL-positive cells (53.8% ± 3.27) was decreased, compared to the ischemia group ($P < 0.001$), (Fig. 2).

Treatment with Nesfatin-1 decreased the concentration of MDA following cerebral ischemia

In biochemical analyses, the MDA levels of the hippocampus in the ischemia group (52.35 μM ± 7.06) were higher than the sham (10.85 μM ± 7.08) and nesfatin-1 (25.42 μM ± 5.91) groups ($P < 0.001$). Additionally, treatment with nesfatin-1 decreased the MDA levels (37.59 μM ± 7.62) compared to the ischemia group ($P < 0.05$), (Fig. 3).

Treatment with Nesfatin-1 increased the levels of SOD and GSH following cerebral ischemia

There was a noticeable reduction in SOD levels in the ischemia group (25.41 U/ml ± 7.34) compared to the sham (68.02 U/ml ± 14.59) and nesfatin-1 (62.57 U/ml ± 7.99) groups ($P < 0.001$). In ischemic rats treated by nesfatin-1, SOD levels were increased (50.34 U/ml ± 13.68) compared to the ischemia group rats ($P < 0.05$). Also, GSH levels in the ischemia group (0.09 mM ± 0.01) were decreased compared to the sham (0.49 mM ± 0.1) and nesfatin-1 (0.35 mM ± 0.01) groups ($P < 0.001$). In ischemic rats treated with nesfatin-1, the GSH levels were increased (0.22 mM ± 0.06) compared to the ischemia group rats ($P < 0.05$), (Fig. 4).

Discussion

This study for the first time demonstrated that the novel peptide, nesfatin-1, significantly attenuated apoptotic and necrotic cell death in CA1 pyramidal area after cerebral I/R in the rat hippocampus. Moreover, our findings revealed that nesfatin-1 showed effective results in the prevention of lipid

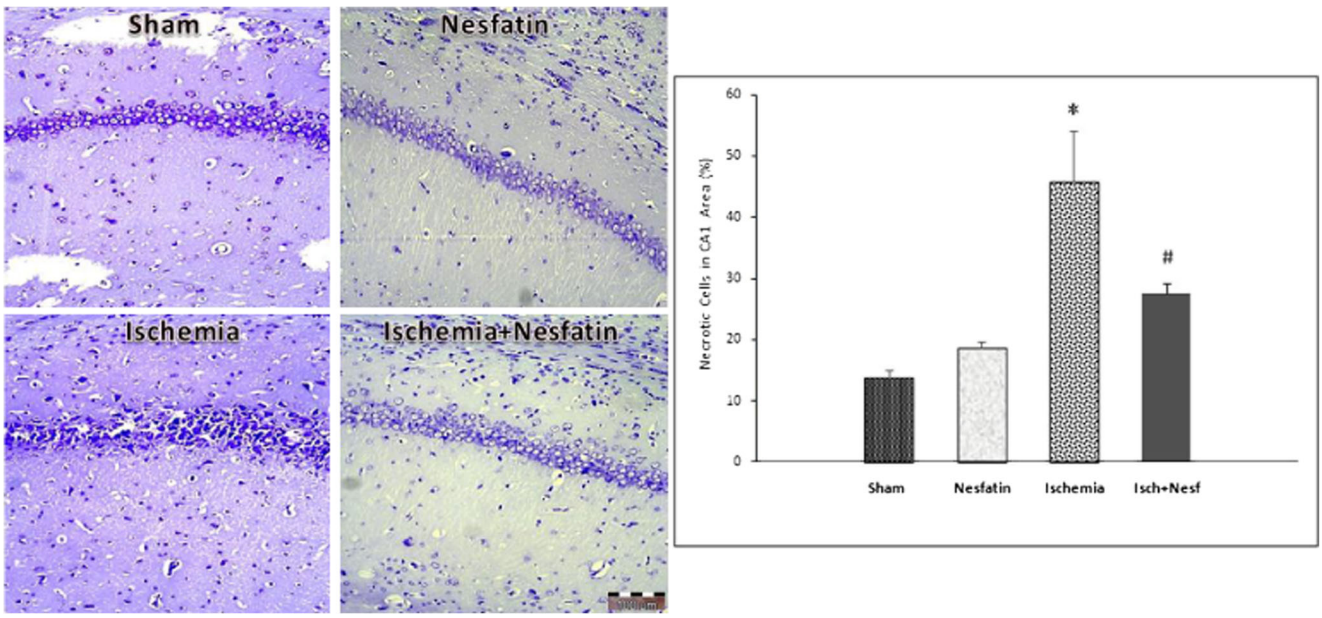

Fig. 1 **Left**: Photomicrographs of Nissl staining (by cresyl violet staining) in hippocampal CA1 area after transient global cerebral ischemia reperfusion (induced by CCAO method), (400× Magnifications). **Right**: Effects of nesfatin-1 treatment (20 μg/kg, IP injection) on ischemia reperfusion-induced necrotic cell death in the hippocampal CA1 area following the transient cerebral ischemia reperfusion ($N = 7$ per groups). * Significantly different compared with sham ($P < 0.001$) and nesfatin-1 ($P < 0.01$) groups. # Significantly different compared with ischemia group ($P < 0.05$)

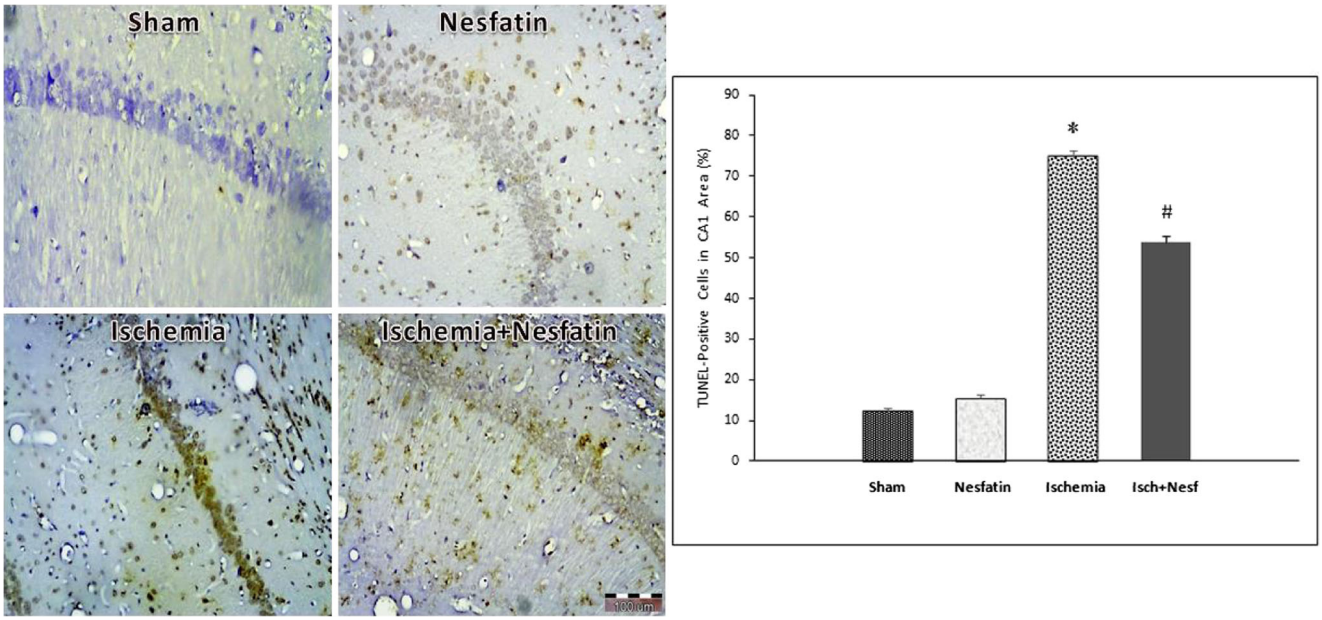

Fig. 2 **Left**: Photomicrographs of TUNEL staining (by using an "In Situ Cell Death Detection Kit") in hippocampal CA1 area after transient global cerebral ischemia reperfusion (induced by CCAO method), (400× Magnifications). **Right**: Effect of nesfatin-1 treatment (20 μg/kg, IP injection) on the percentage of TUNEL-positive cells in hippocampal CA1 area following the transient cerebral ischemia reperfusion (N = 7 per groups). * Significantly different compared with sham and nesfatin-1 groups ($P < 0.001$). # Significantly different compared with ischemia group ($P < 0.001$)

peroxidation and the significant elevation of antioxidant enzymes (SOD and GSH) capacity in cerebral ischemia.

Many complex pathological mechanisms happen following I/R injury, which are not clearly understood. However, necrosis, oxidative stress, apoptosis, calcium deregulation, ATP depletion, and excitotoxicity are defined as mechanisms that contribute to the brain I/R injury (Erfani et al. 2015b; White et al. 2000). Therefore, any factors suppressing these occurrences can be applied for the treatment of cerebral ischemia. It appears that nesfatin-1 is the factor that can exert those effects and reduce the ischemia damages, as a result.

The antioxidant capacity of nesfatin-1 has been proposed as a possible mechanism for its neuroprotective potential. Brain I/R injury is associated with the increased production of reactive oxygen species (ROS) in the reperfusion phase (Granger and Kvietys 2015). The generation and accumulation of ROS in the mitochondria can open mitochondrial permeability transition pores and promote mitochondrial

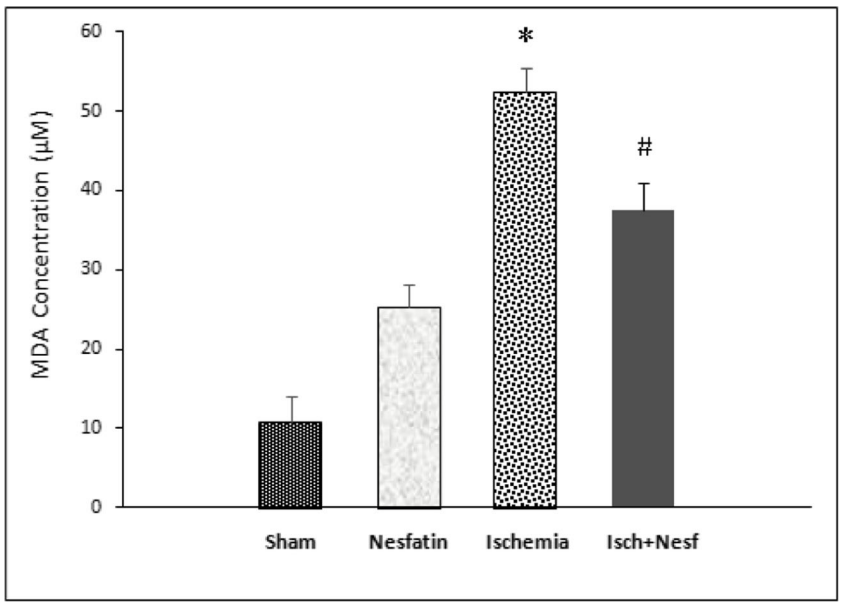

Fig. 3 Effect of treatment with nesfatin-1 (20 μg/kg, IP injection) on MDA concentration (measured by the ELISA method) in the hippocampus following cerebral ischemia reperfusion (induced by CCAO method), (N = 7 per groups). * Significantly different compared with sham and nesfatin-1 groups ($P < 0.001$). # Significantly different compared with ischemia group ($P < 0.05$)

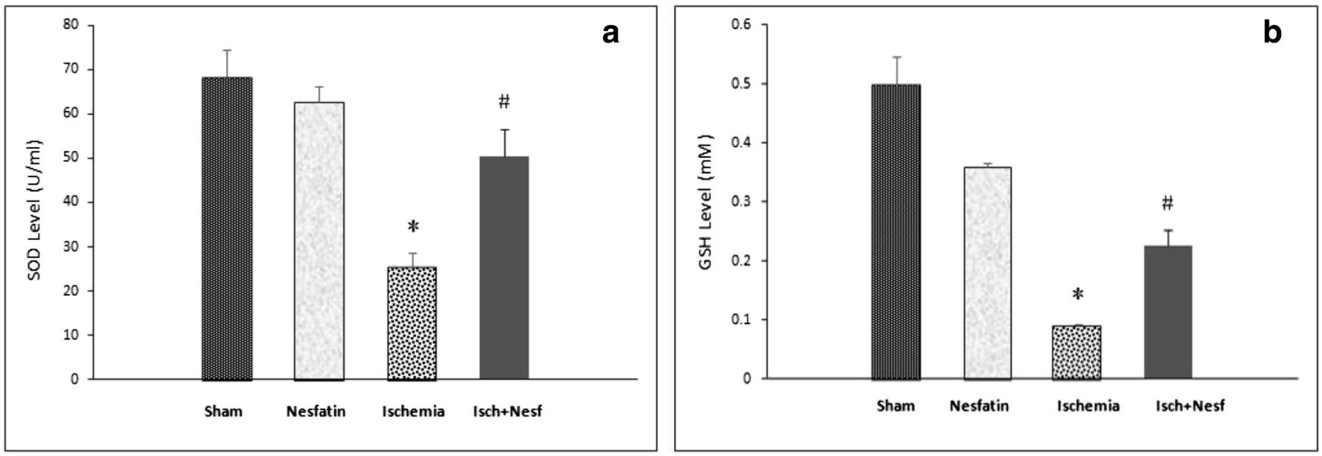

Fig. 4 Effect of treatment with nesfatin-1 (20 μg/kg, IP injection) on SOD (**a**) and GSH (**b**) levels (measured by the ELISA method) in the hippocampus following cerebral ischemia reperfusion (induced by CCAO method), (N = 7 per groups). * Significantly different compared with sham and nesfatin-1 groups ($P < 0.001$). # Significantly different compared with ischemia group ($P < 0.05$)

membrane hyperpolarization. At the damaged mitochondrial membrane, the proapoptotic proteins and cytochrome C leak from the mitochondria into the cytoplasm. Leakage of cytochrome C may lead to the activation of caspase-3 and nuclear fragmentation, as well as caspase-3-dependent apoptosis (Kowaltowski et al. 2001).

It was also established that the product of ROS and the disruption of the metabolic balance are the main causes of necrosis (Northington et al. 2011). It is well-stated that the proapoptotic family proteins including Bax, Bak and caspase-3 play an important role in triggering apoptosis (Chen et al. 2016). On the other hand, there is a correlation between Bcl-2 expression, a key antiapoptotic protein, and resistance to apoptosis that results from Bcl-2 properties; for example, Bcl-2 sensitivity to redox changes and its antioxidant functions during calcium stress. Thus, Bcl-2can lead to attenuate cell death (Doyle et al. 2008). A recent study has reported that nesfatin-1 increases the activity of the selected antioxidative enzymes (catalase and superoxide dismutase) and decreases malondialdehyde (MDA) level. It also makes less apoptotic tubular cells by caspase-3 activity diminution and improves Bcl-2/Bax ratio in a renal ischemia reperfusion injury model (Jiang et al. 2015). In addition, it has antioxidant effects on hyperglycemia-induced skin injury by the depression of the MDA and myeloperoxidase (MPO) activities and the enhancement of GSH levels (Solmaz et al. 2016).

Following ischemic damage, several cytokines includinginterleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor α (TNF-α) are increased which are associated with some pathways involved in apoptotic neuronal death (Wang et al. 2008). Moreover, the toxic effects of IL-1β enhancement are in a synergistic manner due to the increased level of TNF-α. Therefore, neuroinflammation process and subsequent brain ischemia damage are induced by these inflammatory cytokines (Arvin et al. 1996). As a result, the inhibition of inflammatory response activation causes the diminution of the infarct size and the reduction of the neuronal ischemia injury (Zhao et al. 2016).

In addition, it is also shown that nesfatin-1 has anti-inflammatory effects and it decreases TNF-α and IL-6 levels in gastric injury (Wang et al. 2008). Moreover, previous studies demonstrated that nesfatin-1 can protect the brain against SAH-induced and traumatic injury in rats by suppressing TNF-α, IL-1β, and IL-6 (Özsavcí et al., 2011; Tang et al. 2012).

Cellular responses to various extracellular stimuli are regulated by extracellular signal-regulated kinases 1 and 2 (ERK1/2). ERK1/2 in the nervous system has a vital function for neuronal differentiation, plasticity, and probably the modulation of neuron survival (Hetman and Gozdz 2004); thus, the activation of ERK1/2 regulates the protective effects of different factors, which can improve the survival of neurons in hypoxia/ischemia models (Zhu et al. 2013). The neuroprotective effects of different pharmacological agents are essentially dependent on ERK1/2 activation. The recent study of Shen et al. (2017) showed that activation of C-Raf-ERK1/2 signaling by nesfatin-1 protects dopaminergic neurons against neurotoxicity-MPTP induced apoptosis in Parkinson's disease mouse model (Shen et al. 2017).

The findings of the current study suggest that treatment with nesfatin-1 can significantly decrease the neuronal damage in CA1 area of rat hippocampus that is induced by the cerebral ischemia. It seems that the neuroprotective effects of nesfatin-1 may arise from many mechanisms such as the inhibition of apoptosis and necrosis, and the improvement of the antioxidant system. In conclusion, if the protective effects of nesfatin-1on pathological conditions are taken into consideration, this peptide can be introduced as a therapeutic agent for various diseases, such as cerebral I/R; however, further research is required to identify more cellular mechanisms in this realm.

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