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Nesfatin-1 ameliorates oxidative brain damage and memory impairment in rats induced with a single acute epileptic seizure

Sevil Arabacı Tamer [a,b], Türkan Koyuncuoğlu [a,c], Ayça Karagöz Köroğlu [d,e], Dilek Akakın [e], Meral Yüksel [f], Berrak Ç. Yeğen [a,*]

[a] Marmara University, School of Medicine, Department of Physiology, Istanbul, Turkey
[b] Sakarya University, School of Medicine, Department of Physiology, Sakarya, Turkey
[c] Biruni University, School of Medicine, Department of Physiology, Istanbul, Turkey
[d] İstinye University School of Medicine, Department of Histology & Embryology, Istanbul
[e] Marmara University, School of Medicine, Department of Histology & Embryology, Istanbul, Turkey
[f] Marmara University, Vocational School of Health-Related Professions, Department of Medical Laboratory, Istanbul, Turkey

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ABSTRACT

Aims: We aimed to investigate putative neuroprotective effects of nesfatin-1 on oxidative brain injury and memory dysfunction induced by a single epileptic seizure and to compare these effects with those of antiepileptic phenytoin.

Main methods: Wistar albino rats were randomly divided into a control group and pentylenetetrazole (PTZ)-seizure groups pretreated intraperitoneally (ip) with saline or nesfatin-1 (NES-1; 0.3, 1 or 3 μg/kg/day) or phenytoin (PHE; 40 mg/kg/day) or PHE + NES-1 (0.3 μg/kg/day) at 30 min before the single-dose PTZ injection (45 mg/kg; ip). All treatments were repeated at the 24th and 48th h of the provoked epileptic seizure. Passive-avoidance test was performed to assess memory function. The rats were decapitated at the 72nd hour of seizures and brain tissues were analyzed for histopathological changes and for measuring levels of malondialdehyde, glutathione, myeloperoxidase activity and reactive oxygen/nitrogen species.

Key findings: In parallel to the effects of phenytoin, NES-1 reduced seizure score, elevated antioxidant glutathione content, depressed generation of nitric oxide and protected against seizure-induced neuronal damage. Additionally, increased malondialdehyde levels and elevated glial fibrillary acidic protein immunoreactivity in the cortex and hippocampus were decreased and memory dysfunction was improved by NES-1. However, NES-1 had no impact on myeloperoxidase activity or production of reactive oxygen species in the brain.

Significance: The findings of the present study demonstrate that nesfatin-1 treatment provides neuroprotection against seizure-induced oxidative damage and memory dysfunction by inhibiting reactive nitrogen species and upregulating antioxidant capacity, indicating its potential in alleviating memory deficits and increasing the effectiveness of conventional anti-convulsant therapies.

1. Introduction

Generalized epilepsy is a chronic neurological disease characterized by recurrent seizures that occur due to neuronal hyperexcitability, which is exacerbated by glia-mediated excitation and inflammation [1]. Epileptic seizures affect the quality of life of millions of patients worldwide, who mostly suffer from deteriorated cognitive functions as an outstanding comorbidity of epilepsy [2]. Following the initial insult, early pathological processes that occur during the formation of an epileptic brain include the disruption of the blood-brain barrier (BBB), oxidative/nitrative injury and neuroinflammation [3,4]. It has been proposed that brief epileptic seizures result in oxidative stress with generation of excess reactive oxygen species (ROS) and mitochondrial DNA damage [5,6], all of which contribute to declined cognitive performance in the epileptic patients [7]. In parallel, GABA receptor antagonist pentylenetetrazole (PTZ)-evoked brief epileptic seizures in rodents, which have resulted in apoptotic neurodegeneration with depleted antioxidant capacity and increased lipid peroxidation, were also accompanied by impaired memory function [8–11]. Thus, considering that oxidative stress is described as a major underlying mechanism

* Corresponding author at: Marmara University School of Medicine, Basibüyük Mah. Maltepe Basibüyük Yolu No. 9/1, 34854 Maltepe Istanbul, Turkey.
E-mail address: byegen@marmara.edu.tr (B.Ç. Yeğen).

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in seizure-induced excitotoxic neuronal death and cognitive deficits [12,13], several antioxidants that scavenge ROS and increase endogenous antioxidant capacity have been used for their potential neuroprotective actions in epilepsy [14–20]. However, there is still need for alternative targeted therapies to prevent antioxidant-oxidant imbalance and cognitive comorbidities in epilepsy.

Numerous endogenous peptides were associated with the occurrence of seizures through their modulatory effects on the balance between the excitatory and inhibitory neurotransmitters and were considered for their therapeutic potencies [21]. Nesfatin-1 (1–82), originally defined as an anorexigenic peptide, is proteolytically cleaved from the precursor-NEFA/nucleobindin 2 (NUCB2) peptide in the central nervous system and several peripheral tissues [22]. The functions of nesfatin-2 (85–163) and nesfatin-3 (166–396), which are also synthesized from the NUCB2, are currently unknown [22]. Despite the extensive research on the expression and function of biologically active nesfatin-1, the receptor that is involved in its actions has not been identified yet [23]. It was reported that saliva and serum nesfatin-1 levels were extremely higher in non-treated patients with generalized epilepsy than those of the control group [24]. In parallel with that, plasma nesfatin-1 levels were elevated in rats induced with epileptic seizures [25]. However, the effect of exogeneously administered nesfatin-1 on epileptic seizures and seizure-related cognitive performance has not been determined yet. On the other hand, systemic administration of nesfatin-1 was reported to display anti-oxidant, anti-inflammatory and anti-apoptotic properties in several peripheral tissues [26–30]. Moreover, exogenous administration of nesfatin-1 was shown to upregulate antioxidant enzyme systems and exert neuroprotection in subarachnoid hemorrhage- or ischemia-reperfusion-induced cerebral injury in rats [31,32]. Based on the aforementioned studies, the present study conducted in rats was aimed to investigate the neuroprotective effects of nesfatin-1 treatment on oxidative brain injury and memory dysfunction due to a single epileptic seizure and to compare these effects with those of the commonly used antiepileptic drug, phenytoin.

2. Materials and methods

2.1. Animals and drugs

Wistar albino male rats (240–320 g) were supplied by the Marmara University Animal Center (DEHAMER) and were kept under laboratory conditions with controlled humidity, temperature (22 ± 2 °C), light/dark (12/12 h) cycles and free access to standard rat chow and water. The study was approved by the Marmara University (MU) Animal Care and Use Committee (approval code: 006.2016.mar) and the experiments were performed in compliance with the guidelines of the New York Academy of Sciences and the Turkish law on the use of animals in experiments. Nesfatin-1 (1–82; rat) was purchased from Phoenix Pharmaceuticals Inc., USA; (cat. no. 003-22B), PTZ was obtained from Sigma-Aldrich and phenytoin was obtained from Embil İlaç, Istanbul, Turkey.

2.2. Experimental design and procedures

Since the susceptibility to epileptic seizures was shown to be dependent on sex and estrogen receptor activity [9], only male rats were used. The rats ($n = 84$) were randomly divided into 7 groups with 12 rats in each group: as a control group and 6 groups of PTZ-seizure groups, which were pretreated intraperitoneally (ip) with saline or nesfatin-1 (NES-1; 0.3, 1 or 3 μg/kg/day) or phenytoin (PHE; 40 mg/kg/day) or PHE + NES-1 (0.3 μg/kg/day) at 30 min before the single-dose PTZ injection (45 mg/kg; ip), while the control group was injected with saline (Fig. 1). The rationale in selecting the doses of NES-1 was based on our previous studies, reporting the anti-oxidant and anti-inflammatory effects of NES-1 in various tissues [26–29,32,33]. All the treatments were repeated at the 24th and 48th h of the provoked epileptic seizure. Passive avoidance test (PAT) was performed initially before PTZ injection and repeated immediately before euthanasia. At the 72nd hour of the PTZ-seizure, all rats were anesthetized with ketamine and xylazine hydrochloride (100 mg/kg and 10 mg/kg, ip). Four rats in each group were then perfused transcardially with paraformaldehyde to obtain brain samples for histological analysis, while the remaining non-perfused rats were decapitated to determine brain tissue levels of malondialdehyde (MDA) and glutathione (GSH), myeloperoxidase (MPO) activity and chemiluminescence levels of luminol, lucigenin and nitric oxide.

2.3. Seizure induction and evaluation

Based on our previous studies, the dose of PTZ (45 mg/kg, ip) was chosen as an intermediate convulsive dose [9,34]. The epileptic seizures of the rats were recorded with a video camera for 30 min and then were evaluated using the Racine's scoring system: *0*: *no behavioral changes*; *1*: *facial movements with twitching of ears and whiskers*; *2*: *myoclonic jerks without rearing*; *3*: *myoclonic jerks with rearing*; *4*: *clonic convulsions accompanied with posture loss*; *5*: *generalized tonic-clonic seizures* [35].

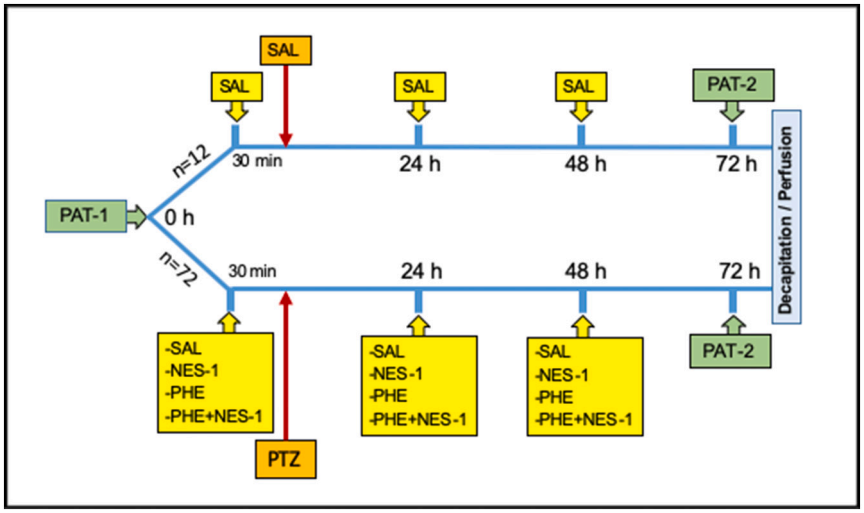

Fig. 1. Schematic representation of the experimental design. PAT: passive avoidance test; SAL: saline; NES-1: nesfatin-1 (0.3, 1 or 3 μg/kg/day); PHE: phenytoin (40 mg/kg/day); PHE + NES-1 (40 mg/kg/day +0.3 μg/kg/day); PTZ: pentylenetetrazole (45 mg/kg). All injections were made intraperitoneally.

2.4. Assessment of memory function

As the initial step of the experimental protocol (Fig. 1), rats were placed in the illuminated compartment of the passive avoidance apparatus (Northel, Istanbul), in which the rats are expected to move instinctively from the illuminated compartment to the dark compartment of the apparatus [36]. After the rat moves into the dark compartment, which is equipped with an electric grid floor, guillotine door closes and an electrical foot shock (0.3–0.6 mA) is applied for 5 s, which completes the acquisition phase of the passive avoidance test (PAT). Then, to evaluate the memory recall, the rats were placed again in the illuminated chamber at the 72nd hour of PTZ/saline injection and the duration of time to enter the dark compartment was recorded. Rats with a normal memory performance were expected to avoid entering the shock-associated dark compartment for 5 min (cut-off). However, entering the dark compartment in a shorter time was considered as an impairment in memory function [37].

2.5. Measurement of myeloperoxidase activity

Myeloperoxidase (MPO) activity was evaluated as an indicator of tissue neutrophil infiltration, and it correlates positively with the histochemically recorded neutrophil [38]. After the homogenization step with 50 mM potassium phosphate buffer, MPO activity in the brain tissues was determined using a spectrophotometer at 460 nm (T80 + UV/VIS; spectrophotometer, PG Instruments Ltd., UK) based on H_2O_2-dependent oxidation of o-dianisidine.2HCl, and MPO activity was presented as units per gram tissue [39].

2.6. Measurement of malondialdehyde and glutathione levels

Brain tissue samples were homogenized in trichloroacetic acid (10%, TCA) by an Ultra Turrax tissue homogenizer. As an indicator of the degree of lipid peroxidation due to oxidative damage, malondialdehyde (MDA) levels were measured spectrophotometrically at 535 nm wavelength by observing thiobarbituric acid reagent formation. The results were determined in nmol MDA/gram tissue. Using the modified Ellman procedure, glutathione (GSH) levels were measured spectrophotometrically at an absorbance value of 412 nm and the amount of GSH was given as μmol/g tissue [39].

2.7. Chemiluminescence assays

Chemiluminescence (CL) assay is a non-invasive and commonly utilized method to directly assess the levels of reactive oxygen species (ROS) and nitric oxide (NO). The probe for lucigenin (bis-N- methylacridinium nitrate; Sigma, St. Louis, MO) specifically detects $O_2^{\bullet -}$ radicals, while luminol probe (5-amino-2,3-dihydro-1,4-phthalazinedione) is frequently used for the detection of hydroxyl, hydrogen peroxide and hypochlorite radicals [40]. CL measurements of ROS in the brain samples were done by adding luminol or lucigenin probes at 0.2 mM concentration each, while NO was measured by adding K_2CO_3 (0.4 mM), desferrioxamine (60 mM), H_2O_2 (4 mM) and luminol-sodium salt (3.6 mM) to the tubes containing brain tissue samples [41]. Luminol, lucigenin and NO counts were recorded at room temperature by a luminometer (Junior LB 9509, EG&G, Berthold, Germany) and CL levels were calculated by linear approximation and expressed as the area under the curve (AUC) of relative light unit per mg of tissue. [42].

2.8. Histopathologic preparation and analyses

The brain tissues of rats (n = 4 from each group), which were transcardially perfused with 4% paraformaldehyde (dissolved in 0.1 M PBS; pH 7.4), were placed in 4% paraformaldehyde and kept at 4 °C overnight. Brain samples were later dehydrated through rising alcohol series (70% to 100%), cleared in xylene, kept in paraffin overnight at 60 °C and then embedded in paraffin. Sections (~4 μm) taken from paraffin blocks by a rotary microtome (Leica RM2125 RTS) were stained with Hematoxylin and Eosin (H&E) stain for morphological evaluation in the cortex and hippocampus (dentate gyrus -DG- and CA3). All sections were examined and photographs were taken with a digital camera (Olympus DP72, Tokyo, Japan) attached to a photomicroscope (Olympus BX51, Tokyo, Japan). Sections stained with H&E were scored semi-quantitatively regarding to neuronal damage (*0*: *no damage*, *1*: *mild damage*, *2*: *moderate damage*, *3*: *severe damage*). Immunohistochemical staining of glial fibrillary acidic protein (GFAP) was performed and the TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling) method was also applied.

For GFAP Immunohistochemistry, 4 μm-thick sections taken from the paraffin-embedded brain tissues were deparaffinized at 37 °C overnight and then incubated in xylene. Sections were processed through 96% ethanol and the endogenous peroxidase activity in the tissue was blocked with 3% hydrogen peroxide in methanol (10 min). Antigen retrieval was performed with citrate buffer solution (pH 6) in a microwave oven. Protein blockade (UltraTek Hrp Anti-Polyvalent, ScyTek) was applied to the tissues for 10 min to prevent non-specific staining. Then, sections were incubated with mouse anti-GFAP (1:1000, Merck Millipore, MAB360) at 4 °C, overnight. The sections were washed with phosphate buffered saline (PBS) for 5 min and incubated in biotinylated secondary antibody (UltraTek Hrp Anti-Polyvalent, ScyTek) for 20 min. After rewashing with PBS again, streptavidin peroxidase (UltraTek Hrp Anti-Polyvalent, ScyTek) was applied for 20 min. Then, 3,3′-diaminobenzidine chromogen was applied onto the sections for 5 min. Mayer's hematoxylin was used to counterstain sections before mounting. The immunostaining was scored using a semiquantitative method according to the density of GFAP-immunopositive cells (*0*: *baseline staining*; *1*: *mild immunoreactivity*; *2*: *moderate immunoreactivity*; *3*: *widespread severe immunoreactivity*) by considering the cell counts and the intensity of immunoreactivity in the astrocytes and their projections [43]. Sections were examined under a photomicroscope (BX51 Olympos, Japan) and average immunostaining scores for each group were statistical analyzed. For each animal, an average score for each region was calculated by taking three photomicrographs from the cortex and DG and CA3 regions of the hippocampus.

In situ DNA end-marking method was used for the determination of apoptosis and the method was applied in accordance with the manufacturer's instructions (ApopTag Plus, In Situ Apoptosis Detection Kit, S7101, Millipore). The tissue sections were incubated with proteinase K solution at room temperature followed by incubation with 3% H_2O_2 in PBS. After washing with PBS, sections were put in the equilibrium buffer and incubated in recombinant terminal transferase TdT enzyme at 37 °C. Sections were washed with PBS and then incubated with anti-digoxigenin conjugate. After washing with PBS, they were incubated with DAB (3,3-diaminobenzidine tetrahydrochloride dihydrate). Finally, the sections were counterstained with Mayer's hematoxylin, dehydrated with alcohol series and covered by using entellan. TUNEL-positive cells were counted as an average of cells observed in 4 different pictures (at x40 magnification) taken from the related brain regions of the sections.

2.9. Statistics

Statistical analyses were performed using GraphPad Prism 9.2.0 (GraphPad Software, San Diego, CA, USA) program. For the analysis of the histological data, Mann Whitney *U* test was performed. The numbers of rats that exhibited stage 4–5 seizures were compared using chi-square test. All the other data were analyzed by one-way ANOVA followed by post-hoc Tukey test. All data are expressed as mean ± standard error of means (SEM). $p < 0.05$ was considered as statistically significant.

3. Results

Following PTZ injection, all the saline-pretreated rats exhibited tonic-clonic contractions and their average stage scores were the highest (Table 1). PHE-pretreatment reduced the number of rats that reached to stage 4 or 5 ($p < 0.01$) and significantly diminished the time stayed at stage 5 ($p < 0.05$). Similarly, administering PHE plus the lowest dose of NES-1 (0.3 μg/kg) before the injection of PTZ reduced the number of rats at stage 4/5 ($p < 0.01$), as well as the average of the stage scores ($p < 0.01$), while the single rat that exhibited stage 5 had a short duration similar to PHE alone. Pretreatment with NES-1 at the 1 μg/kg dose also significantly reduced the number of rats with tonic-clonic contractions ($p < 0.05$), but the reduction in the duration of stage 5 was not statistically significant. Neither of the 0.3 μg/kg or 3 μg/kg doses changed the stage duration or the number of rats scored at 4th and 5th stages.

Initial latency periods recorded at the acquisition phase of the PAT were not significantly different among the experimental groups, which were then randomly recruited to different treatment strategies (data not shown). During the recall phase of the test that was performed at the 72nd h of PTZ-seizure, all the rats in saline-treated PTZ group have entered the electrical shock-given dark chamber within a shorter period of time, indicating memory dysfunction ($p < 0.05$; Table 1). However, in NES-1-, PHE- or PHE + NES-1-treated groups, the latencies to enter the dark chamber were prolonged and were not different than that of the control group. On the other hand, only the highest dose of NES-1 treatment resulted in a significant delay in entering dark chamber as compared to saline-treated PTZ group ($p < 0.01$).

In saline-treated PTZ group, MDA levels ($p < 0.001$) and MPO activity ($p < 0.001$) in the brain tissues were elevated as compared to control group, suggesting the presence of PTZ-induced oxidative brain damage and enhanced neutrophil infiltration (Fig. 2). Despite a tendency to decrease, antioxidant GSH content in the saline-treated PTZ group was not significantly changed. The antiepileptic drug PHE, given before and after PTZ-injection, did not alter seizure-induced elevation in lipid peroxidation ($p < 0.001$), but PHE significantly depressed MPO activity and increased GSH levels were with respect to saline-treated PTZ group ($p < 0.001$). Neither of the NES-1 doses changed seizure-induced elevation in MPO activity, while MDA levels were suppressed by NES-1, reaching to statistical significance at its 1 and 3 μg/kg doses ($p < 0.05$–0.01). On the other hand, all the three doses of NES-1 significantly elevated brain GSH content with respect to control group ($p < 0.001$). When the lowest NES-1 dose was co-administered with PHE, MPO activity was depressed ($p < 0.001$) and GSH content was increased ($p < 0.01$) as compared to those of the saline-treated PTZ group, but MDA level was still elevated with respect to control group ($p < 0.05$).

In accordance with the MDA and MPO activity levels, provoking an epileptic seizure resulted in elevated CL levels of luminol, lucigenin and NO in the brain tissues of saline-treated PTZ group, showing the enhanced generation of ROS and reactive nitrogen species (RNS) (compared to control, $p < 0.05$–0.01; Fig. 3). Treatment with antiepileptic PHE, depressed the production of ROS and RNS ($p < 0.001$) and reversed all CL levels back to the control levels. Despite that NES-1 treatment had no significant effect on brain CL levels of luminol or lucigenin, NO levels were suppressed by both 0.3 and 3 μg/kg doses of NES-1 with respect to saline-treated PTZ group ($p < 0.01$). Co-administering the lowest dose of NES-1 with PHE did not have any additional effects on the already depressed ROS and RNS levels due to PHE treatment alone.

Our findings revealed the regular morphology of neurons in the cortices and hippocampal DG and CA3 regions of the control group determined by Hematoxylin and eosin staining, while a single seizure induced by PTZ resulted in severe neuronal degeneration in the cortex, hippocampal DG and CA3 regions (vs. control group, $p < 0.001$; Figs. 4 and 7–9). Marked neuronal degeneration, observed in the cortices and both hippocampal regions of rats, was decreased in all the treatment groups. As compared to saline-treated PTZ group, in both PHE- and PHE + NES-1 treated groups, a relatively milder damage was recorded with fewer degenerated neurons in all the three regions ($p < 0.05$–0.001). In the cortex, NES-1 only at its lowest dose also diminished the damage score in comparison to saline-treated PTZ group ($p < 0.05$). In the hippocampus, 0.3 and 1 μg/kg doses of NES-1 significantly reduced neuronal damage in the CA3 area ($p < 0.05$–0.01, vs. saline-treated PTZ group), but none of the NES-1 doses were effective on the neuronal degeneration of the hippocampal DG.

Brown-colored TUNEL-positive cells were observed in all groups. Increased number of TUNEL-positive cells observed in the cortices of saline-treated PTZ rats were decreased in 0.3 μg/kg NES-1 treated PTZ rats. The score of TUNEL-positive cells in the CA3 and the DG regions of the hippocampus of the saline-treated PTZ group tended to increase with respect to control group, but a statistically significant increase in TUNEL-positive cells was observed only in the cortex region ($p < 0.01$; Figs. 5 and 7–9). However, except for the 1 μg/kg dose of NES-1, all the treatment modalities reduced the TUNEL-positive cells in the cortex region to the levels that were not different than that observed in the control group. In the group treated with NES-1 plus PHE combination, TUNEL-positive cells in the DG were totally abolished, making it significantly different than that of the only PHE-treated PTZ group ($p < 0.05$). Similarly, combined treatment resulted in the lowest score of TUNEL-positive cells in the CA3 region, but a statistical difference was not reached.

Glial fibrillary acidic protein (GFAP) is the main protein of intermediate filaments of astroglial cells [44]. GFAP-positive staining was observed in all regions of the brain, while an increased GFAP-immunoreactivity was observed in the cortex and hippocampus of rats in the PTZ groups as compared to controls (Figs. 7–9). GFAP activity scores in the cortex, as well as in the DG and CA3 hippocampal regions of the saline-treated PTZ group were significantly higher than those of the control group ($p < 0.05$–0.01; Fig. 6). Treatment with the antiepileptic PHE slightly reduced the number of GFAP-positive cells in all the studied brain regions, but these reductions were not statistically significant. Similarly, in the DG area, none of the NES-1 doses resulted in a significant reduction. On the other hand, cortical GFAP activity score was reduced with both the 0.3 and 1 μg/kg doses of NES-1, as well as the PHE + NES-1 combination, as compared to saline-treated PTZ group ($p < 0.01$). GFAP activity scores, when compared to saline-treated PTZ group, were also reduced in the hippocampal CA3 area of the groups treated with 0.3 μg/kg dose of NES-1 and with PHE + NES-1 combination ($p < 0.05$), and this reduction in GFAP activity by PHE + NES-1 was significantly lower with respect to only PHE-treated group ($p < 0.05$).

4. Discussion

Our results verified that PTZ-induced seizure activity causes neuronal and astrocytic injury, apoptosis and memory dysfunction, which were accompanied by oxidative stress including enhanced lipid peroxidation, neutrophil infiltration and production of oxygen or nitrogen-derived radicals in the brain tissue. Similar to the effects of the antiepileptic drug phenytoin, nesfatin-1 reduced the seizure score, elevated antioxidant GSH content, depressed the generation of NO and protected against seizure-induced neuronal damage. Additionally, increased lipid peroxidation and elevated GFAP immunoreactivity in the cortex and hippocampus were decreased and memory dysfunction was improved by nesfatin-1, but these seizure-induced changes were not significantly affected by the administration of phenytoin. On the other hand, neutrophil infiltration to the brain tissue and production of ROS were reduced by phenytoin, but not by nesfatin-1-treatment. Thus, nesfatin-1 appears to provide neuroprotection and memory improvement by inhibiting RNS injury and upregulating antioxidant capacity without altering neutrophil- or ROS-associated inflammation.

The brain tissue has the highest level of oxygen utilization and an abundance of polyunsaturated fatty acids, but a relatively lower

Table 1
Seizure scores and the results of the passive avoidance test of the rats induced with pentylentetrazole (PTZ)-seizure as compared to control rats.

			Number of rats at stage 4–5 (n; %)	Average of stage scores (1–5)	In rats (n) that reached to stage 5; total time spent (*sec*)	Delay in entering dark compartment (sec)
Control						295.9 ± 3.71
P	Saline		8/8; 100.0%	4.75 ± 0.16	(6) 372.5 ± 78.9	**93.3 ± 45.5**$^{+}$
T	Phenytoin		**3/8; 37.5%****	3.62 ± 0.42	(3) **70.3 ± 20.9***	123.4 ± 51.9
Z	Nesfatin-1	(0.3 μg/kg)	6/8; 75.0%	4.25 ± 0.41	(5) 250.0 ± 71.5	228.8 ± 46.7
		(1 μg/kg)	**4/8; 50.0%***	3.75 ± 0.41	(3) 190.3 ± 77.8	206.5 ± 46.0
		(3 μg/kg)	7/8; 87.5%	4.37 ± 0.26	(4) 235.8 ± 116.9	**264.9 ± 35.1****
		(0.3 μg/kg) + Phenytoin	**2/8; 25.0%****	**2.75 ± 0.45****	**(1) 77.0 ± 0.0**	178.8 ± 51.1

*p < 0.05, **p < 0.01, compared to saline-treated PTZ group; +p < 0.05, compared to control group.

antioxidant capacity, making it particularly susceptible to oxidative damage [45,46]. Thereby, overproduction of ROS resulting in the oxidation of proteins, lipids and nucleotides easily causes oxidative neuronal damage and cell death [18,47]. Accordingly, seizure-induced oxidative stress, which occurs as a consequence of excessive ROS release and depletion of endogenous antioxidants, is implicated in the onset and progression of epilepsy [18,34,48–51]. In parallel with these findings, we observed that a single seizure induced by PTZ resulted in oxidative damage of the brain as observed with reduced GSH content and increased levels of MDA, NO and ROS. Our findings revealed that phenytoin-pretreatment increased the GSH content, inhibited the ROS and RNS generation and reduced the seizure score, but its successive administration before and after PTZ injection had no impact on the seizure-induced elevation in brain MDA level. Similarly, in an experimental post-traumatic epilepsy model induced with intracerebral injection of iron salts, phenytoin treatment prevented the occurrence of seizures, but had no impact on lipid peroxidation [52]. On the other hand, except the lowest dose used, nesfatin-1 treatment given within the first 3 days of post-seizure period, significantly decreased MDA levels, while all the used nesfatin-1 doses replenished the depleted GSH levels, demonstrating the antioxidant action of nesfatin-1. Moreover, nesfatin-1 either alone or along with phenytoin depressed the generation of NO in the cerebral tissue, but only the combination reduced the release of ROS. These results suggest that nesfatin-1 has a profound inhibitory effect on the amount of NO, which is postulated to behave as a neuromodulator having either anti-convulsant or pro-convulsant effects [53,54]. Previously, nesfatin-1 has been reported to have antioxidant and neuroprotective effects in traumatic brain injury [32,55] and ischemia-reperfusion injury [31,56]. In contrast to higher doses (10–20 μg/kg) of nesfatin-1 used in these studies showing its neuroprotective actions, the current study demonstrates that nesfatin-1 provides neuroprotection against seizure-induced injury at relatively lower doses (0.3–3 μg/kg). Using the similar lower doses, we have previously reported the antioxidant action of nesfatin-1 in the oxidative injury of testis and stomach [27–29]. Thus, the current findings showing the antioxidant actions of nesfatin-1 in seizure-induced neuronal injury further verify its potency in alleviating oxidative stress by maintaining the oxidant/antioxidant balance that includes the homeostasis of cerebral NO levels.

Recordings made using whole-cell current-clamp technique have revealed that nesfatin-1 causes hyperpolarization or depolarization of a large proportion of different subpopulations of neurons in the paraventricular nucleus, suggesting that nesfatin-1 may be involved in the regulation of neuronal activity [57]. In epileptic patients, serum nesfatin-1 levels were shown to be increased during the first 24 h following a seizure [58]. Moreover, in newly diagnosed patients, who have not received any antiepileptic treatments yet, nesfatin-1 levels in both saliva and serum were 160 folds higher than the levels measured in healthy controls [24]. On the other hand, serum nesfatin-1 levels in patients who have received antiepileptic drug treatments were depressed, but a 10-fold elevation with respect to control levels was still present. Thus, based on the dramatic increases in serum nesfatin-1 levels in relation to epilepsy, it was proposed that either excessive release of nesfatin-1 could be involved in the pathogenesis of seizure-induced excitotoxicity or epileptic seizure itself could cause nesfatin-1 release [24]. Since it was previously reported that inflammatory stimuli upregulate nesfatin-1-expressing neurons in the central nervous system [59] and nesfatin-1 can cross the blood-brain-barrier (BBB) in both directions [60], elevated serum levels of nesfatin-1 following an epileptic seizure could be attributed to seizure-induced neuroinflammation. Our results demonstrate that administration of nesfatin-1 in pharmacological doses, which are comparable to the elevated serum levels measured in epileptic patients [58], provides a neuroprotection against seizure-induced oxidative injury. Although we have not measured serum or cerebral levels of nesfatin-1 in our PTZ-seizure model, a previous study has reported that acute or chronic administration of PTZ itself causes elevated nesfatin-1 levels in the serum and brain tissues of rats [61]. Taken together with the aforementioned studies, it can be suggested that endogenous release of nesfatin-1 could be implicated as a compensatory mechanism in response to seizure-induced oxidative injury, and increasing nesfatin-1 level in the circulation via its exogenous administration could enhance the anti-oxidant action of nesfatin-1.

Our present data indicated that cerebral MPO activity of rats with PTZ-seizure was 4-folds higher than the non-seizure group when measured at the 72nd h. It has been hypothesized that the damage to the BBB plays a major role in the occurrence and recurrence of epileptic seizures by permitting the recruitment of leukocytes [3,62,63]. A recent study demonstrated that acute lung inflammation in mice was enhanced with the absence of NUCB2/nesfatin-1 and the elevations in MPO activity and the expressions of the pro-inflammatory cytokines and chemokines were exaggerated, suggesting the possible anti-inflammatory activity of endogenous NUCB2/nesfatin-1 in neutrophils [64]. On the other hand, the activity of MPO enzyme, which is considered as a vital enzyme in the neuroinflammation process of the ischemic brain, is not only found in the circulating or infiltrated neutrophils, but it is also abundant in activated microglial cells, neurons and astrocytes [65,66]. Our findings revealed that treatment with the anticonvulsant phenytoin, either in combination with or without nesfatin-1, depressed the elevation in seizure-induced MPO activity. However, none of the used doses of nesfatin-1 were solely effective in decreasing MPO activity. Although we have previously shown that the antioxidant effect of nesfatin-1 in gastric [27], dermal [28] or cerebral [32] injury models was accompanied by its inhibitory action on MPO activity, the neuroprotective effect of the currently used doses of nesfatin-1 on PTZ-induced injury appears not to be associated with the MPO activity arising from infiltrated neutrophils or innate neurons and astrocytes. Since a higher (10 μg/kg) dose of nesfatin-1, which has ameliorated subarachnoid hemorrhage-induced injury, has demonstrated an inhibitory effect on the MPO activity of the brain tissue [32], it appears that the suppressive effect of nesfatin-1 on infiltrated neutrophils or cerebral MPO activity could be a dose-dependent characteristics of the peptide.

Since oxidative injury has been proposed to be a major mechanism in the development and progress of epilepsy [67], it is also expected to account for seizure-related cognitive deficits [12], which significantly reduce the life quality of epileptic patients [2,7]. Furthermore,

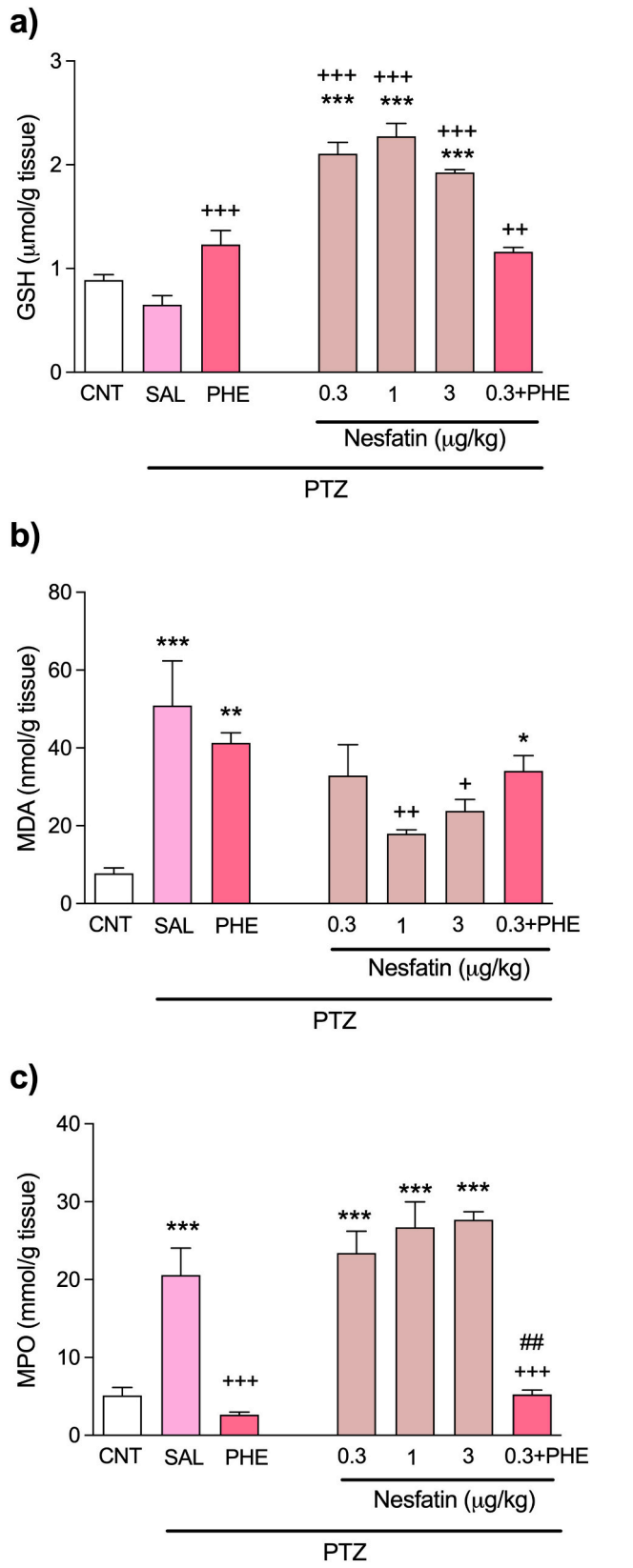

Fig. 2. Cerebral levels of glutathione (GSH) (a), malondialdehyde (MDA) (b), and myeloperoxidase (MPO) (c) activity in rats induced with pentylenetetrazole (PTZ)-seizure. Data are presented as mean ± SEM of 8 animals; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared to control group; +p < 0.05, ++p < 0.01, +++p < 0.001 compared to saline-treated PTZ group; ## p < 0.01 compared to phenytoin (PHE)-treated PTZ group.

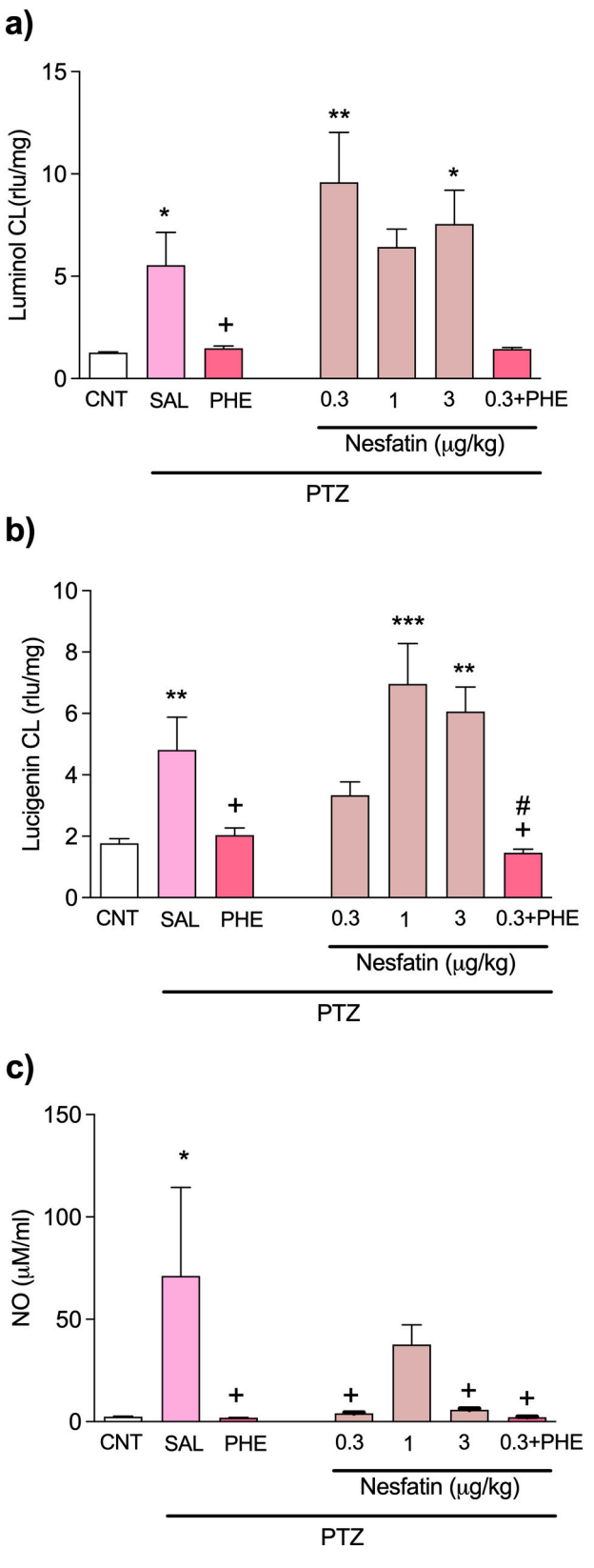

Fig. 3. Cerebral levels of luminol (a), lucigenin (b), and nitric oxide (NO) (c) chemiluminescence levels in rats induced with pentylenetetrazole (PTZ)-seizure. Data are presented as mean ± SEM of 8 animals; *$p < 0.05$, **$p < 0.01$, ***p < 0.001, compared to control groups +p < 0.05, ++p < 0.01, +++p < 0.001 compared to saline-treated PTZ group; # $p < 0.05$ compared to phenytoin (PHE)-treated PTZ group.

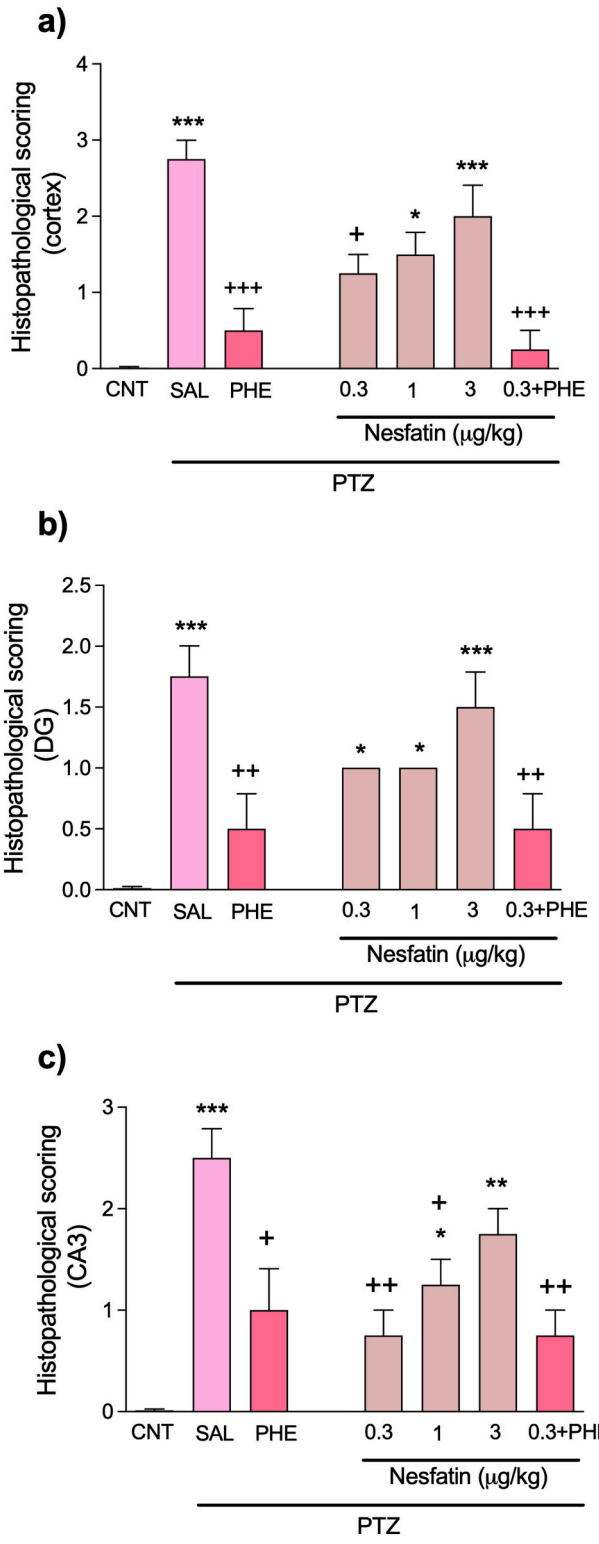

Fig. 4. Histopathological scores in the cortex (a), hippocampal CA3 (b), and dentate gyrus (c) regions of rats induced with pentylenetetrazole (PTZ)-seizure ($n = 4$).
*$p < 0.05$, **$p < 0.01$, ***p < 0.001, compared to control group; +p < 0.05, ++p < 0.01, +++p < 0.001 compared to saline-treated PTZ group.

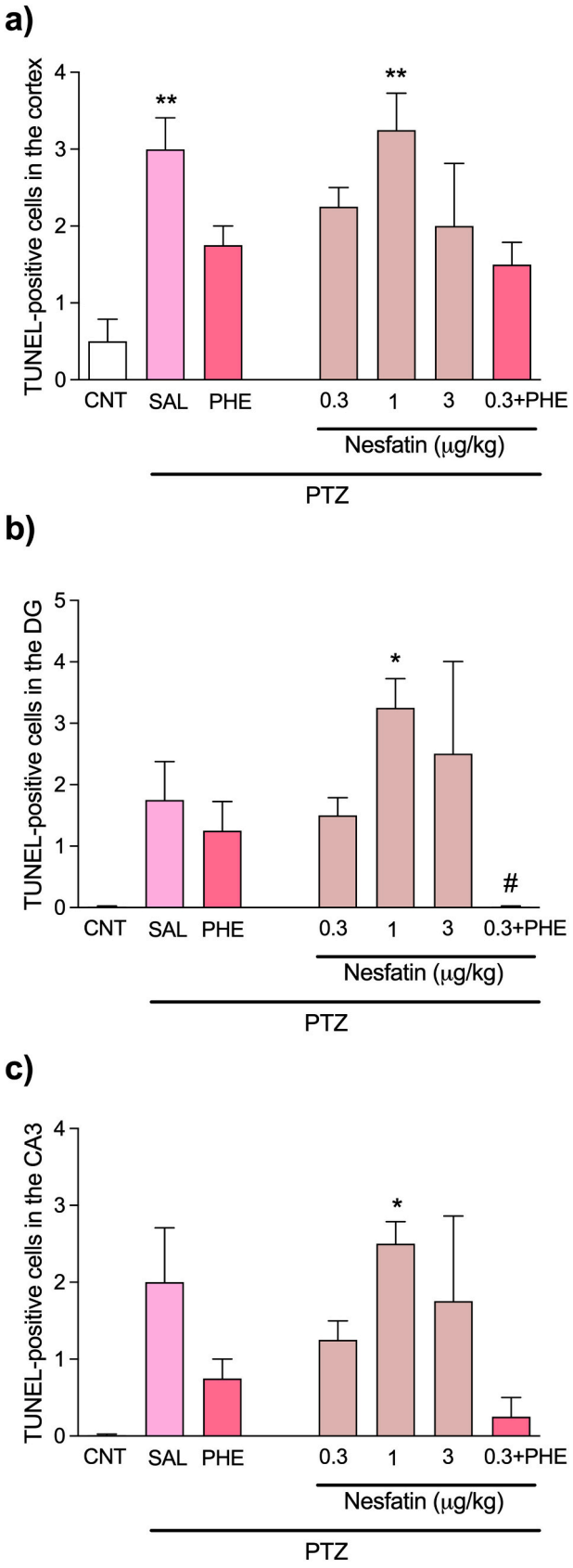

Fig. 5. TUNEL-positive cells in the cortex (a), hippocampal CA3 (b), and dentate gyrus (c) regions of rats induced with pentylenetetrazole (PTZ)-seizure (n = 4).
*p < 0.05, **p < 0.01, compared to control group; # p < 0.05 compared to phenytoin (PHE)-treated PTZ group.

antiepileptic drugs themselves were shown to cause impairment in cognitive function by suppressing neuronal excitability or increasing inhibitory neurotransmission [68,69]. In the present study, phenytoin did not worsen or ameliorate the memory dysfunction induced by a PTZ-

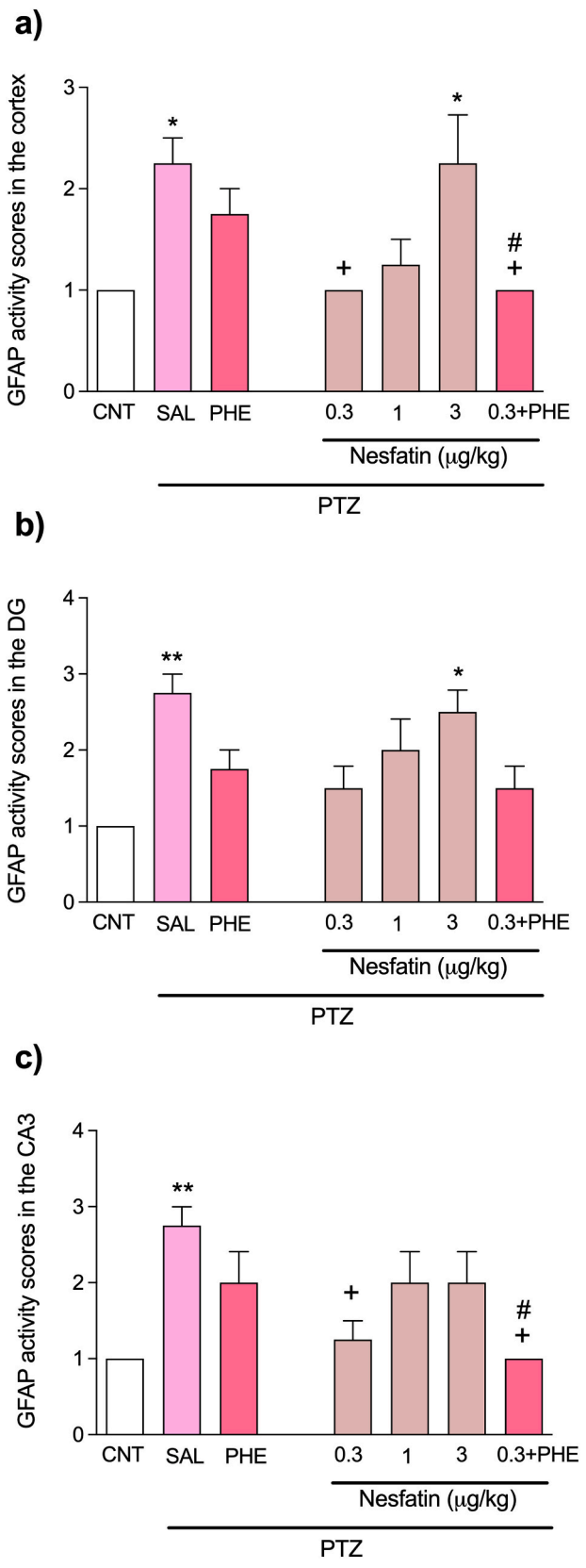

Fig. 6. GFAP-immunoreactive astroglial cells and immunoreactivity scores in the cortex (a), hippocampal CA3 (b), and dentate gyrus (c) regions of rats induced with pentylenetetrazole (PTZ)-seizure (n = 4).
*p < 0.05, **p < 0.01, compared to control group; +p < 0.05, ++p < 0.01, compared to saline-treated PTZ group. # p < 0.05 compared to phenytoin (PHE)-treated PTZ group.

seizure, but nesfatin-1 at its highest dose significantly improved memory. Therefore, it may be concluded that peripheral administration of nesfatin-1 supports the memory function, which appears to involve its antioxidant activity. Nesfatin-1 was demonstrated to cross BBB by non-saturable mechanisms [60,70], making it more advantageous in the prevention of oxidative injury-induced disruption of the microenvironment of the brain and its functional consequences. Since the use of antioxidant treatments is regarded as a possible therapeutic strategy in reducing epilepsy-related neurodegeneration [18], the present findings suggest that use of nesfatin-1 may be considered in adjunctive antiepileptic therapy for the improvement of cognitive functions.

It is well-described that any failure in the regulation of apoptosis, which is the physiologically programmed death of neurons, contributes to the pathogenesis of neurodegeneration [71]. Experimental studies have shown that seizure induced by PTZ administration has resulted in increased numbers of TUNEL-positive, caspase-3 and bax-positive cells in the cortex and the hippocampus, indicating an enhanced apoptotic neuronal cell death [72,73]. Similarly, our findings also showed that the numbers of TUNEL-positive cells were elevated in the brain tissues of rats induced with a single PTZ-seizure, reaching to statistical significance only in the cortices. Additionally, indicative of neural degeneration, cells showing increased activity of GFAP were also increased in the cortical and the hippocampal areas (CA3 or DG) after the PTZ-induced epileptic seizure. Although nesfatin-1 per se had no effect on seizure-related apoptosis, combination of the low-dose nesfatin-1 with phenytoin resulted in reduced number of TUNEL-positive cells in the hippocampal areas. On the other hand, nesfatin-1 with or without phenytoin depressed GFAP-positive cells in the cortex and hippocampal CA3 region. Tang et al. [55] have reported that administration of 10 or 20 μg/kg of nesfatin-1, but not its 5 μg/kg dose, reduced the number of apoptotic nerve cells and decreased caspase-3 activity after traumatic brain injury of rats. Moreover, nesfatin-1 was reported to attenuate the hippocampal apoptotic cells death and GFAP-positive cells following ethanol-induced neurotoxicity in early postnatal rats [74]. Consistent with our biochemical findings demonstrating the antioxidant action of nesfatin-1, our histopathological data also showed that neuronal damage observed in the hippocampal regions and cortex of PTZ-induced rats was alleviated by nesfatin-1.

5. Conclusions

The findings of the present study demonstrate for the first time that nesfatin-1 treatment provides neuroprotection against seizure-induced oxidative damage and memory dysfunction. Thus, nesfatin-1 requires further attention as a novel neuroprotective agent for its utility in alleviating memory deficits and increasing the effectiveness of conventional anti-convulsant therapies.

CRediT authorship contribution statement

All the experiments were performed at the Marmara University School of Medicine, Departments of Physiology & Histology and Marmara University Vocational School of Health Sciences.

All persons designated as authors qualify for authorship.

All persons who qualify for authorship are listed.

All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Design of work: Sevil Arabaci Tamer, Türkan Koyuncuoğlu, Berrak Ç.Yeğen.

Performing the experiments and data acquisition: Sevil Arabaci Tamer, Türkan Koyuncuoğlu, Ayça Karagöz.

Data interpretation and application of statistical analysis: Sevil Arabaci Tamer, Türkan Koyuncuoğlu, Ayça Karagöz, Meral Yüksel, Dilek Akakın, Berrak Ç. Yeğen (all authors).

Drafting of the manuscript: Sevil Arabaci Tamer, Türkan

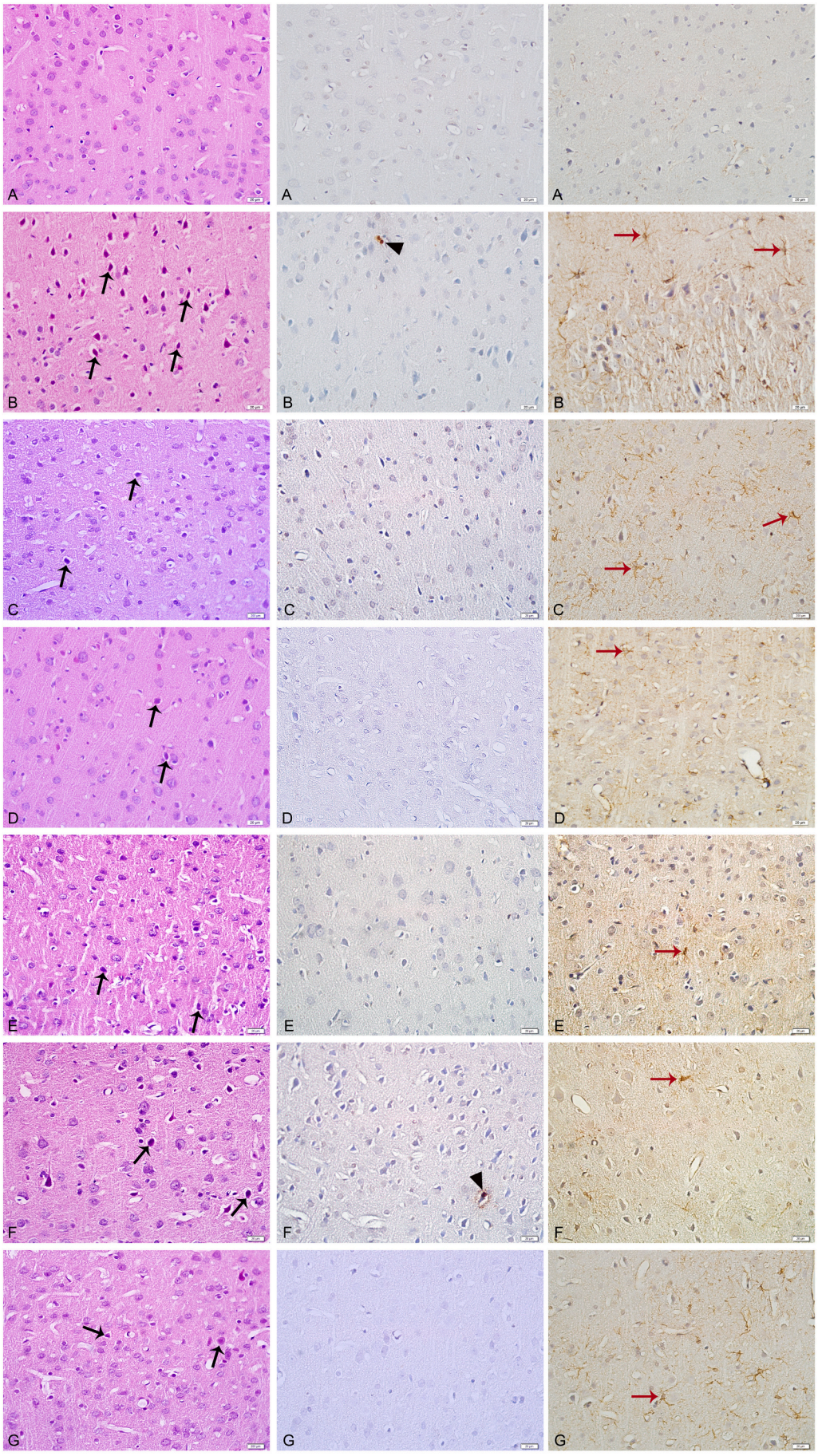

Fig. 7. Hematoxylin and eosin (H&E) staining (left), TUNEL staining (middle) and glial fibrillary acidic protein (GFAP) immunoreactivities (right) in the brain cortices of experimental groups. A: Control, B: saline-treated PTZ, C: Phenytoin-treated PTZ, D: 0.3 μg/kg NES-1-treated PTZ, E: 1 μg/kg NES-1-treated PTZ, F: 3 μg/kg NES-1-treated PTZ, G: Phenytoin+0.3 μg/kg NES-1-treated PTZ groups. Severely degenerated neurons (arrow) observed with shrunken cytoplasm and pyknotic nuclei in the saline-treated PTZ group were decreased in the 0.3 μg/kg NES-1-, phenytoin- and phenytoin+0.3 μg/kg NES-1-treated groups. Increased GFAP immunostaining observed in the saline-treated PTZ group was decreased in the 0.3 μg/kg NES-1-treated and phenytoin+0.3 μg/kg NES-1-treated PTZ groups. Arrows in the left represent degenerated neurons, arrowheads in the middle represent TUNEL-positive cells and arrows in the right represent GFAP immunoreactivity. Scale bars: 40 μm.

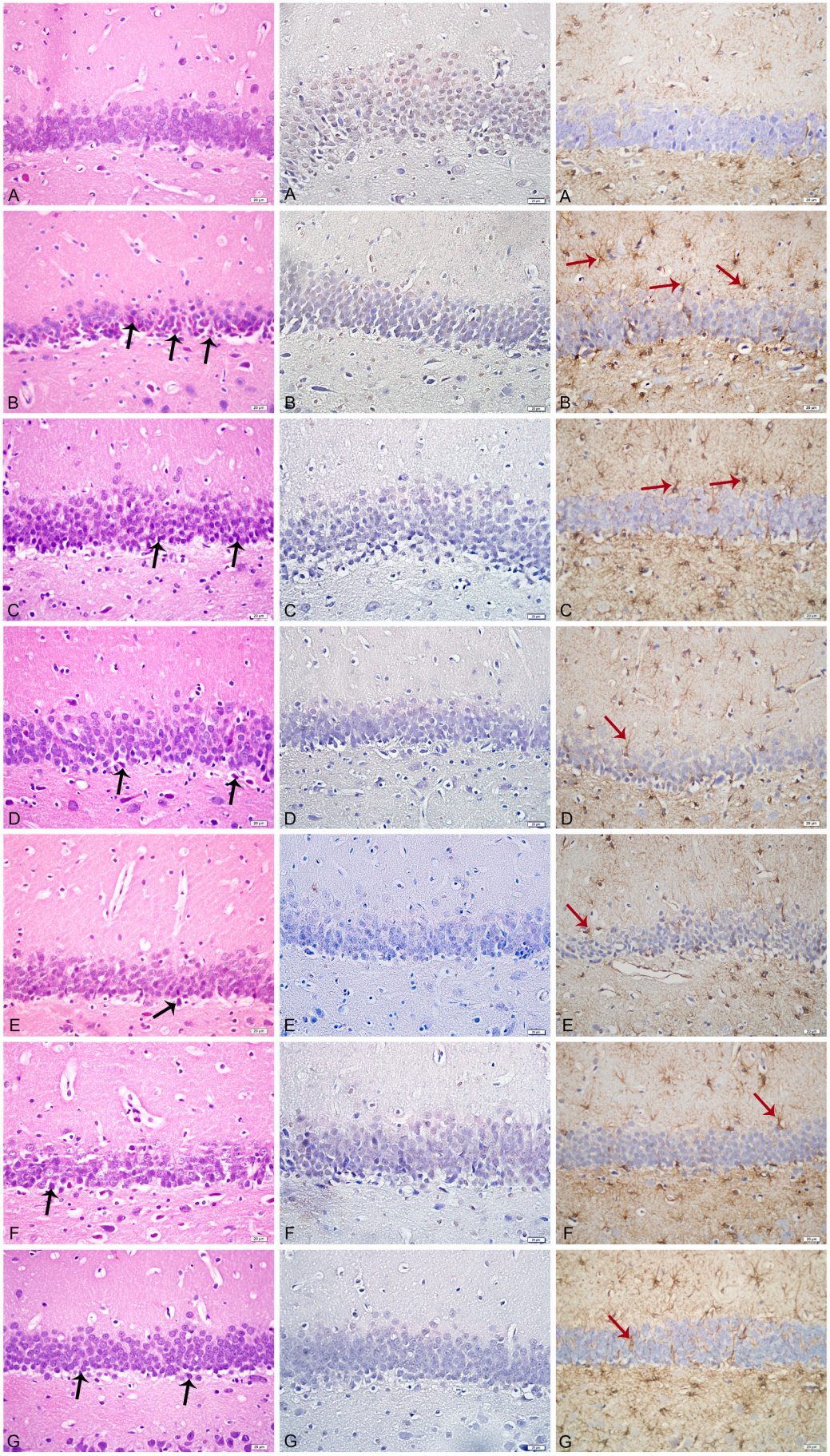

Fig. 8. Hematoxylin and eosin (H&E) staining (left), TUNEL staining (middle) and glial fibrillary acidic protein (GFAP) immunoreactivities (right) in the hippocampal dentate gyrus (DG) regions of the experimental groups. A: Control, B: saline-treated PTZ, C: Phenytoin-treated PTZ, D: 0.3 μg/kg NES-1-treated PTZ, E: 1 μg/kg NES-1-treated PTZ, F: 3 μg/kg NES-1-treated PTZ, G: Phenytoin+0.3 μg/kg NES-1-treated PTZ groups. Degenerated neurons (arrow) observed with shrunken cytoplasm and pyknotic nuclei in the saline-treated PTZ group were decreased in the phenytoin and phenytoin+0.3 μg/kg NES-1-treated PTZ. Increased GFAP immunostaining was evident in the saline-treated PTZ group compared to controls. Black arrows represent degenerated neurons and red arrows represent GFAP immunoreactivity. Scale bars: 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

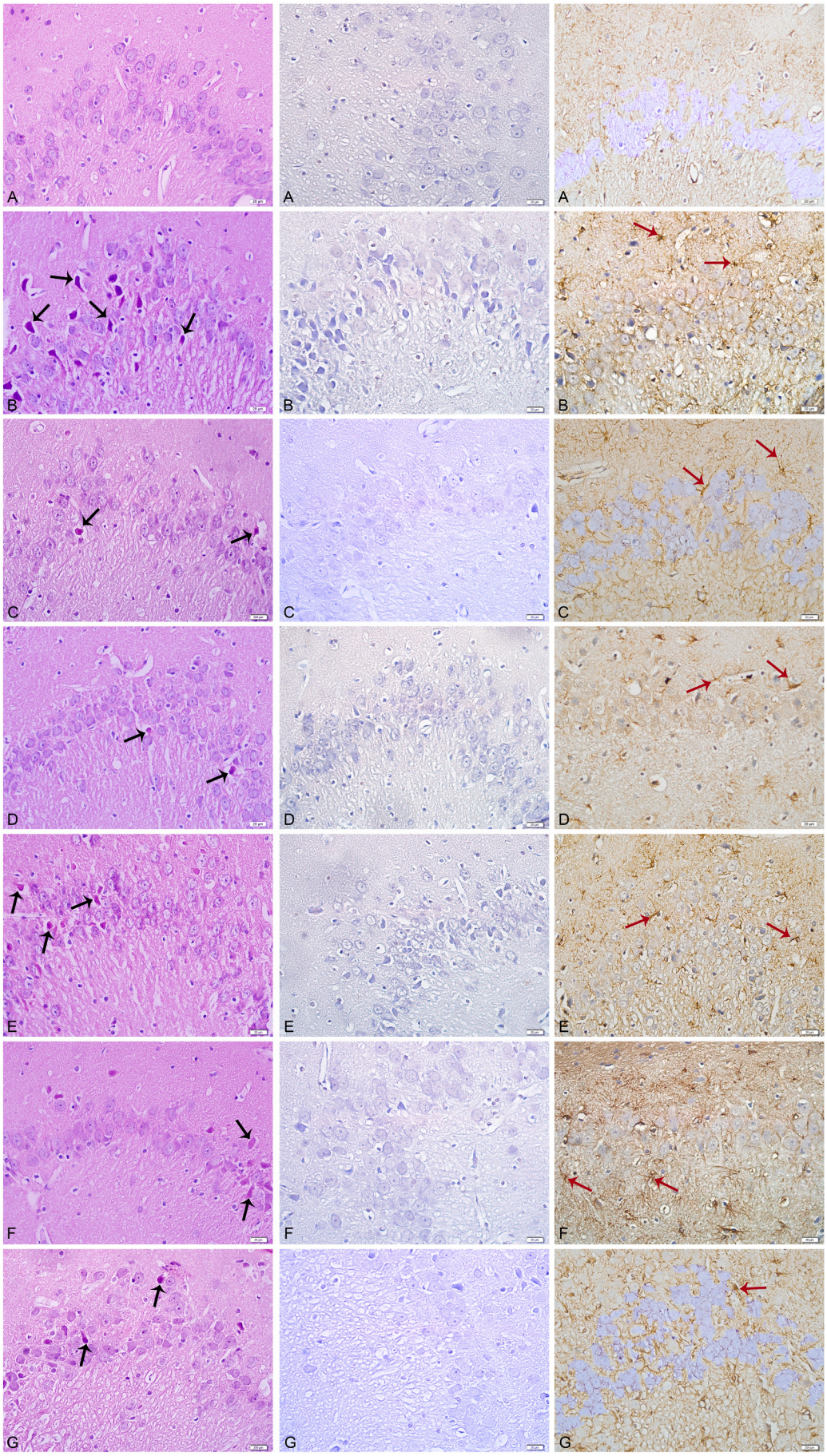

Fig. 9. Hematoxylin and eosin (H&E) staining (left), TUNEL staining (middle) and glial fibrillary acidic protein (GFAP) immunoreactivities (right) in the hippocampal CA3 regions of the experimental groups. A: Control, B: saline-treated PTZ, C: Phenytoin-treated PTZ, D: 0.3 μg/kg NES-1-treated PTZ, E: 1 μg/kg NES-1-treated PTZ, F: 3 μg/kg NES-1-treated PTZ, G: Phenytoin+0.3 μg/kg NES-1-treated PTZ groups. Degenerated neurons (arrow) observed in the saline-treated PTZ group were decreased in the 0.3 μg/kg NES-1-treated, 1 μg/kg NES-1-treated, phenytoin-treated and phenytoin+0.3 μg/kg NES-1-treated PTZ. Increased GFAP immunostaining observed in the saline-treated PTZ group was decreased in the 0.3 μg/kg NES-1-treated PTZ group. Black arrows represent degenerated neurons and red arrows represent GFAP immunoreactivity. Scale bars: 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Koyuncuoğlu, Ayça Karagöz, Meral Yüksel, Dilek Akakın, Berrak Ç. Yeğen (all authors).

Critical revision of the manuscript: Berrak Ç. Yeğen.

Approval of the final version of the manuscript: Sevil Arabaci Tamer, Türkan Koyuncuoğlu, Ayça Karagöz, Meral Yüksel, Dilek Akakın, Berrak Ç. Yeğen (all authors).

Data availability

Data will be made available on request.

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