

Ghrelin improves pilocarpine-induced cerebral cortex inflammation in epileptic rats by inhibiting NF- κ B and TNF- α

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Abstract. Ghrelin has a protective function in the nervous system, including anti-inflammatory and antiapoptotic. The objective of the present study was to examine the anti-inflammatory effects of the ghrelin on nuclear factor- κ B (NF- κ B) and tumor necrosis factor- α (TNF- α) gene and protein expression in an epileptic seizure model. Epileptic seizures were induced in healthy male Wistar rats (~3 weeks old) with 300 mg/kg pilocarpine, and brains from rats with Racine stage IV or V seizures were investigated further in the present study. The effect of ghrelin treatment on TNF- α and NF- κ B protein and mRNA expression was assessed by immunohistochemistry and semi-quantitative reverse transcription polymerase chain reaction, respectively. TNF- α and NF- κ B protein and mRNA expression were significantly increased in the pilocarpine and the pilocarpine + saline groups compared with the control group. Ghrelin intervention significantly decreased TNF- α and NF- κ B protein and mRNA expression compared with the pilocarpine and the pilocarpine + saline groups, although it did not reduce expression levels to those seen in the normal control group. Ghrelin reduces inflammation in cortical neurons following epileptic seizure, and therefore may reduce necrosis and the loss of nerve cells, preserving the normal function of the cortex. Ghrelin may alleviate cortex inflammation reaction by adjusting the TNF- α and NF- κ B so as to reduce child epilepsy attack repeatedly. The findings of the present study may contribute to the clarification of the role of Ghrelin in the brain in seizure-induced immune system physiology and may also present novel approaches to the etiology and treatment of epileptic seizures.

Introduction

Li-chloride-pilocarpine induced inflammation in the brain cortex reaction by igniting model of temporal lobe and hippocampus of epileptic seizure. In order to achieve our experiment, we demand model rat must occur in the process of model making status epilepticus. Status epilepticus (SE) is a prolonged self-perpetuating seizure which requires prompt intervention to prevent its injury and mortality and is usually treated initially with a benzodiazepine, such as diazepam (1). However, if SE lasts >30-40 min, it becomes progressively more refractory to these agents (2). Therefore, applying novel efficient therapies for treating SE is required to reduce Li-pilocarpine-induced inflammation and suppress neuronal damage. In the present study, SE rat models in the present study were identified as demonstrating SE >10 min with good survival outcomes; epileptic seizures must achieve Racine stage IV-V using Racine's scale (3). In addition, Ghrelin, as an anti-inflammatory treatment, was administered prior to the administration of pilocarpine to induce SE and its efficacy on inflammation was observed.

Ghrelin is the endogenous ligand of the growth hormone secretagogue receptor (GHSR), discovered in 1999 by Kojima *et al* (4). In addition to its involvement in appetite regulation and glucose and lipid metabolism (5), ghrelin has a protective function in multiple systems, including the cardiovascular and immune systems (6). The association between ghrelin and inflammation has previously been examined: Dixit *et al* (7) proposed that ghrelin functions as an important anti-inflammatory factor, and is involved in endocrinal regulation of the immune system. However, ghrelin has also previously been reported to mediate nuclear factor (NF)- κ B to stimulate interleukin (IL)-8 production and function as a pro-inflammatory factor in the colon (8). This inconsistency across systems suggests that the function of ghrelin merits further study.

Epilepsy is a group of neurological diseases characterized by abnormal excessive discharge of neurons in the brain, bringing about a temporary impediment of brain function (9). Although the exact pathogenesis is unclear, previous studies have demonstrated that T lymphocytes are involved in the epileptic immune response and cell-mediated immunity disorders in patients with epilepsy (10,11). Based on the previous evidence supporting the involvement of ghrelin in the regulation of inflammation, the present study investigated the effect

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of ghrelin on NF- κ B and tumor necrosis factor (TNF)- α gene and protein expression levels in epileptic rats with pilocarpine-induced cerebral cortex inflammation, and explored the anti-inflammatory effect of ghrelin on the cerebral cortex, providing a novel target for the prevention and treatment of epilepsy in children.

Materials and methods

Materials. Pilocarpine hydrochloride was purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany); ghrelin was obtained from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA); NF- κ B, TNF- α and β -actin antibodies were purchased from Santa Cruz Biotechnology, Inc. [Dallas, TX, USA; cat. nos. NF- κ B P-65 (sc-109), TNF- α (sc-52791); TRIzol reagent was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA); reverse transcription kit (NF- κ B p65, sc-8008), *Taq* DNA Polymerase and DNA Marker DL2000 were purchased from Takara Bio. Inc. (Otsu, Japan); and SP-9000 Histostain-Plus kits were obtained from OriGene Technologies, Inc. (Rockville, MD, USA)].

Animals. Healthy male Wistar rats (60 in total; ~3 weeks old; 40–50 g) were obtained from Shengjing Hospital Laboratory Animal Centre of China Medical University (Shenyang, China). The present study was approved by the ethics committee Qingdao Municipal Hospital. Individuals were kept at 19 \pm 2°C in a quiet environment on a diurnal cycle, and were breast-fed. Unweaned rats and young rats were bred together with 12-h light/dark cycle. Rats were divided at random into the normal control group (n=8), pilocarpine group (n=8), pilocarpine + saline group (n=8) and pilocarpine + ghrelin group (n=8).

Administration method. At 9:00 a.m., 3 mg/kg lithium chloride was injected into the peritoneal cavity. The control group rats were administered with physiological saline only. The following day at 8:30 a.m., 80 μ g/kg ghrelin or saline was injected intraperitoneally, then 30 min later 30 mg/kg pilocarpine was injected to SE. The behavioral manifestations were observed and a resultant epileptic seizure of Racine stage IV or V was used as the inclusion criteria for further analysis (3). Seizure severity was ranked using Racine's scale: 1, seizure consisted of immobility and occasional facial clonus; 2, head nodding; 3, bilateral forelimb clonus; 4, rearing; 5, rearing and falling. In addition to the four groups, the experiments were performed with young mice, a group of young mice, and their mothers could continue to have mice. These mice as well as rats were kept at 19 \pm 2°C in a quiet environment on a diurnal cycle, and were breast-fed. Unweaned rats and young rats were kept together with 12-h light/dark cycle. Diazepam (10 mg/kg) was injected 60 min following the epileptic seizure. Epileptic rats were administered diazepam intraperitoneally to reduce the mortality of these rats when undergoing an epileptic seizure. Lithium chloride combined with pilocarpine was employed to produce the epileptic rat model.

Immunohistochemistry. After 24 h following the onset of epileptic seizure, under 2% lidocaine (~4.0–4.5 mg/kg, intraperitoneal injection) abdominal anesthesia, the chest was

opened and the heart exposed. The ascending aorta of the left ventricle was intubated and 50 ml cold saline followed by 50 ml 4% paraformaldehyde PBS was perfused to fix the brain tissue at 4°C overnight. Fixed brain tissue was placed in 20% sucrose for 12 h, and 30% sucrose for a further 12 h. Following washing with PBS, structural parts of the cerebral cortex were cut into 5- μ m coronal sections, dried at room temperature for 6 min and then preserved at -20°C. A total of 9 sections were cut from each rat cortex in each group (n=8). Brain tissue fixed in 4% paraformaldehyde for 24 h, conventional materials and 20% sucrose solution for 48 h and then embedded in paraffin. Sections of samples were cleaned with 0.01 mol/l PBS 100 ml three times for 5 min. Paraffin-embedded sections were treated with a solution of methanol and 0.3% hydrogen peroxide (methanol + 0.01 mol/l PBS 100 ml 80 ml + 30% hydrogen peroxide) for 30 min at room temperature after three washes with 0.01 mol/l PBS for 5 min in room temperature. Subsequently, paraffin-embedded sections were treated with 0.3% Triton X-100 (30% Triton X-100 + 100 ml 0.01 mol/l PBS) at 20°C for 30 min, followed by three washes with 0.01 mol/l PBS for 5 min. Primary antibodies were used at the following dilutions: NF- κ B P-65 (cat. no. SC-372:1), 1:200 and TNF- α , (cat. no. SC-8301:1) 1:300 and sections were incubated in a wet box at room temperature for 1 h, then 4°C overnight; subsequently, the temperature was increased to 37°C after 45 min out of the fridge. Following incubation, the sections were incubated with secondary antibodies NF- κ B P-65 (cat. no. SC-8008:2), 1:250 and TNF- α , (cat. no. SC-358919:2), 1:300 at 37°C at room temperature for 2 h then washed with 0.01 mol/l PBS, three times, three min each time. Immunohistochemical staining reagent. (Vector Laboratories, Inc., Burlingame, CA, USA; cat. no. PK-6103) was used. Immunohistochemical staining reagent was added, incubated at room temperature for 30 min, and washed with PBS three times, each time for 3 min. Then washed with distilled water for three times of 2 min each. Dehydrate: Put the slices in 50, 70, 80, 90, 95 and, 100% ethanol for two min. Transparent: Place the slices in 100% xylene for 10 min. Neutral resin 50 μ l seal, room temperature preservation.

Primary and secondary antibodies were replaced with PBS for a blank control. NF- κ B and TNF- α immunohistochemically-positive cells were counted using dark-field microscopy (CX31-1v320; Olympus Corporation, Tokyo, Japan) and quantified using Image-Pro Plus image analysis software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA; Table I). A total of 5 views per field were analyzed.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Surgical dislocation was used to remove head and brain 24 h following the onset of epileptic seizure. Seizures following 24 h, with 2% lidocaine (~4–4.5 mg/kg) abdomen anesthesia, then the cerebral cortex of the brain was isolated. Total RNA was extracted using TRIzol reagent. Following measurement of RNA concentration with a UV spectrophotometer, a 25 μ l RT reaction was set up according to the kit instructions: 2 μ g RNA was added to 5 μ l 5x loading buffer, 2.5 μ l MgSO₄ (40 mmol/l), 2.5 μ l dNTP (2.5 mmol/l), 1 μ l oligo (dT) primer, 40 μ l RNase inhibitor, 30 μ l UAMV and Rnase-free H₂O to 25 μ l. Following mixing, cDNA was synthesized at 94°C for 60 sec, 37°C for 60 sec and 120 sec

Table I. Effect of ghrelin on TNF- α and NF- κ B protein expression levels in the cortex of a pilocarpine-induced epileptic rat model expressed as gray values via immunohistochemical analysis (n=8).

Group	TNF- α	NF- κ B
Normal control group	0.175 \pm 0.032	0.145 \pm 0.006
Pilocarpine group	0.606 \pm 0.042 ^a	0.420 \pm 0.012
Pilocarpine + saline group	0.595 \pm 0.150 ^a	0.474 \pm 0.056
Pilocarpine + ghrelin group	0.254 \pm 0.061 ^b	0.275 \pm 0.026 ^a

^aP<0.05 vs. control group; ^bP<0.05 vs. pilocarpine and pilocarpine + saline groups; n=8. TNF- α , tumor necrosis factor- α ; NF- κ B, nuclear factor- κ B.

at 72°C; 25 cycles. PCR primers were designed to amplify TNF- α , NF- κ B and β -actin cDNA by referring to previous literature (12) and are identified in Table II. Amplification products were electrophorised on 1.5% agarose gels (10 μ l per lane) containing 20 g/l TAE buffer. UV camera systems were used to acquire images and analysis software (SPSS version 19.0, IBM Corp., Armonk, NY, USA) was used to multiply the size and strength of the strips as PCR product content. TNF- α and NF- κ B mRNA expression levels were calculated relative to β -actin.

Statistical analysis. Data were analyzed using SPSS version 19.0 software (SPSS, Inc., Chicago, IL, USA). Each set of data obtained were the average values of 6 repeated experiments, and all data were expressed as the mean \pm standard deviation. One-way analysis of variance was used to compare parameters between the different groups, and Fisher's Least Significant Difference method was used for pair comparison. P<0.05 was considered to indicate a statistically significant difference.

Results

Ghrelin affects NF- κ B and TNF- α protein expression in the cerebral cortex of young rats with pilocarpine-induced epilepsy. Compared with the cerebral cortex of young rats in the normal control group (Fig. 1A and Table II), TNF- α protein expression was significantly increased 24 h subsequent to induction of pilocarpine-induced SE (P<0.05; Fig. 1B and Table III). TNF- α protein expression was also significantly increased (compared with control) 24 h subsequent to induction of pilocarpine-induced SE when an i.p. injection of saline was administered 30 min prior to pilocarpine (P<0.05; Fig. 1C and Table III). However, in rats who received an i.p. injection of ghrelin 30 min prior to pilocarpine induction of SE, significantly fewer TNF- α positive cells were observed in the cytoplasm of cerebral cortical neurons compared with the pilocarpine and pilocarpine + saline groups (both P<0.05; Fig. 1D and Table III).

In addition, compared with the cerebral cortex of young rats in the normal control group (Fig. 2A and Table II), NF- κ B protein expression was increased 24 h subsequent to induction

of pilocarpine-induced SE (P<0.05; Fig. 2B and Table III). NF- κ B protein expression was also increased (compared with control) 24 h subsequent to induction of pilocarpine-induced SE when an i.p. injection of saline was administered 30 min prior to pilocarpine (P<0.05; Fig. 2C and Table III). However, in rats who received an i.p. injection of ghrelin 30 min prior to pilocarpine induction of SE, significantly fewer NF- κ B positive cells were observed in the cytoplasm of cerebral cortical neurons compared with the pilocarpine and pilocarpine + saline groups (both P<0.05; Fig. 2D and Table III).

Ghrelin affects NF- κ B and TNF- α mRNA expression in the cerebral cortex of young rats with pilocarpine-induced epilepsy. A260/A280 nm of extracted RNA was 1.8-2.0, indicating that the total RNA purity was high, with no protein pollution. Compared with the cerebral cortex of young rats in the normal control group (Fig. 3, lane 1 and Table IV), TNF- α mRNA expression was significantly increased 24 h subsequent to induction of pilocarpine-induced SE (P<0.05; Fig. 3, lane 2 and Table IV). TNF- α mRNA expression was also significantly increased (compared with the control) 24 h subsequent to induction of pilocarpine-induced SE when an i.p. injection of saline was administered 30 min prior to pilocarpine (P<0.05; Fig. 3, lane 3 and Table IV). However, in rats who received an i.p. injection of ghrelin 30 min prior to pilocarpine induction of SE, TNF- α mRNA expression levels were significantly reduced in the cytoplasm of cerebral cortical neurons compared with the pilocarpine and pilocarpine + saline groups (P<0.05 and P<0.05, respectively; Fig. 3, lane 4 and Table IV).

In addition, compared with the cerebral cortex of young rats in the normal control group (Fig. 4, lane 1 and Table IV), NF- κ B mRNA expression was also significantly increased 24 h subsequent to induction of pilocarpine-induced SE (P<0.05; Fig. 4, lane 2 and Table IV). NF- κ B mRNA expression was also significantly increased (compared with control) 24 h subsequent to induction of pilocarpine-induced SE when an i.p. injection of saline was administered 30 min prior to pilocarpine (P<0.05; Fig. 4, lane 3 and Table IV). However, in rats who received an i.p. injection of ghrelin 30 min prior to pilocarpine induction of SE, NF- κ B mRNA expression levels were significantly reduced in the cytoplasm of cerebral cortical neurons compared with the pilocarpine and pilocarpine + saline groups (both P<0.05; Fig. 4, lane 4 and Table IV).

Discussion

Inflammation is an important contributor to the pathophysiological mechanisms of epileptogenesis (13). Reducing inflammation helps to reduce the extent and scope of epilepsy, reduce neuronal necrosis and improve nerve cell recovery (10). Ghrelin is a peptide discovered in the gastric tissue of rats by Kojima *et al* (4), and is a biologically active endogenous ligand of GHSR, which promotes the secretion of growth hormone. A further previous study revealed that NF- κ B is a core component of the inflammatory response, and ghrelin acts in an anti-inflammatory manner by inhibiting TNF- α -inducing NF- κ B pathways (14). It has previously been reported that 50% of newborn rats suffer from temporal lobe hippocampal neuron loss and glial cell hyperplasia following lasting epileptic

Table II. TNF- α , NF- κ B and β -actin primer sequences.

Gene	Forward primers (5'-3')	Reverse primers (5'-3')	Product size (bp)
NF- κ B	TGCCGAGTGAACCGAAAC	TGGAGACACGCACAGGAGC	318
TNF- α	GGTGCCTATGTCTCAGCCTCTT	GCTCCTCCACTTGGTGGTTT	326
β -actin	AAATCGTGCGTGACATTA	CTCGTCATACTCCTGCTTG	473

TNF- α , tumor necrosis factor- α ; NF- κ B, nuclear factor- κ B.

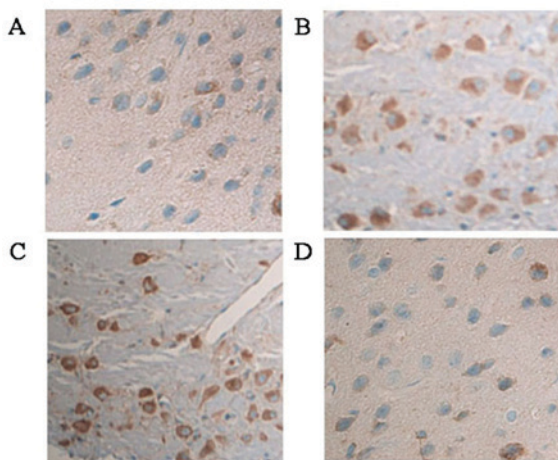


Figure 1. Tumor necrosis factor- α protein expression levels in the cortex of a rat pilocarpine-induced epilepsy model. (A) Normal control group. (B) Pilocarpine group. (C) Pilocarpine + saline group. (D) Pilocarpine + ghrelin treatment group (magnification, x400).

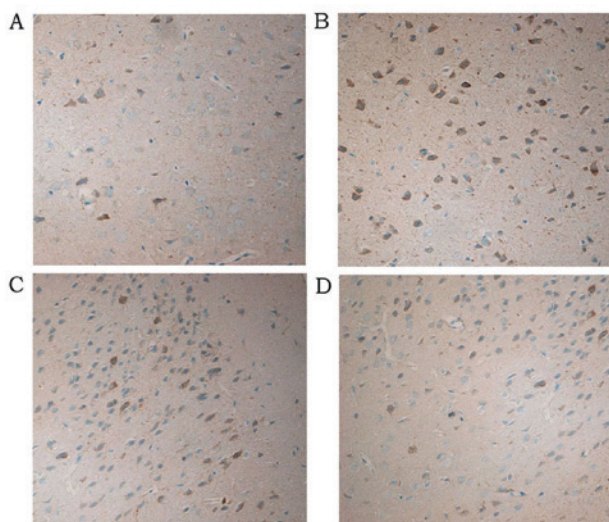


Figure 2. Nuclear factor- κ B protein expression levels in the cortex of a rat pilocarpine-induced epilepsy model. (A) Normal control group. (B) Pilocarpine group. (C) Pilocarpine + saline group. (D) Pilocarpine + ghrelin treatment group (magnification, x100).

seizure (15). Therefore, inhibition of the inflammatory reaction following seizures, to reduce neuronal death and fibrous tissue regeneration, may be important to prevent excessive damage caused by epilepsy in children and adults. The present study observed the effect of ghrelin on inflammatory factors

Table III. Effect of ghrelin on TNF- α and NF- κ B protein expression levels in the cortex of a pilocarpine-induced epileptic rat model, expressed as optical density values.

Group	TNF- α	NF- κ B
Normal control group	0.175 \pm 0.032	0.145 \pm 0.006
Pilocarpine group	1.098 \pm 0.043 ^a	0.920 \pm 0.512
Pilocarpine + saline group	1.305 \pm 0.050 ^a	0.850 \pm 0.086
Pilocarpine + ghrelin group	0.855 \pm 0.081 ^b	0.265 \pm 0.023 ^b

^aP<0.05 vs. control group; ^bP<0.05 vs. pilocarpine and pilocarpine + saline groups; n=8. TNF- α , tumor necrosis factor- α ; NF- κ B, nuclear factor- κ B.

following pilocarpine-induced seizure in immature rats with epilepsy.

Seizures result in inflammation of the central nervous system, and in the rodent brain NF- κ B, cytokines, chemokines, cell adhesion molecules and inflammatory molecules, including complement molecules, are expressed (16). When ghrelin was incubated with mononuclear cells or T cells in the presence of inflammatory stimuli, the inflammatory stimuli-induced secretion and expression of IL-6 and TNF- α , two pro-inflammatory cytokines, was reduced (17). This inhibition of inflammatory cytokines has been confirmed in a murine model of LPS-induced endotoxemia (7). TNF- α enhances the expression of endothelial adhesion molecules and increases capillary permeability, resulting in the infiltration of inflammatory cells to the site of infection and eventual tissue necrosis (18). TNF- α also stimulates and facilitates the release of other cytokines, including IL-1, IL-6 and IL-8. IL-6 is a glycoprotein with a molecular weight of 21-26 kDa and is primarily derived from monocytes, macrophages and endothelial cells, which are an important source of pro-inflammatory cytokines. Upon entering the circulatory system IL-6 initiates the hepatic synthesis of acute phase proteins, stimulates thousands of bone marrow cells, B cell generation and conversion, and promotes the activation of inflammatory cells, among other inflammatory responses (19). Using a pilocarpine-induced epileptic rat model, mRNA and protein expression levels of NF- κ B and TNF- α expression were measured using immunohistochemistry and semi-quantitative RT-PCR in the present study. NF- κ B and TNF- α expression was inhibited by ghrelin treatment, and this result indicates that ghrelin reduces seizure-induced inflammation in young rats with pilocarpine-induced epilepsy by reducing NF- κ B and TNF- α . Further studies are required to determine whether reduction of

Table IV. Effect of ghrelin on TNF- α and NF- κ B mRNA expression levels in the cortex of a pilocarpine-induced epileptic rat model, expressed as optical density values.

Group	TNF- α	NF- κ B
Normal control group	0.907 \pm 0.023	0.747 \pm 0.069
Pilocarpine group	1.667 \pm 0.016 ^a	1.907 \pm 0.075 ^a
Pilocarpine + saline group	1.684 \pm 0.032 ^a	1.658 \pm 0.113 ^a
Pilocarpine + ghrelin group	0.787 \pm 0.014 ^b	0.622 \pm 0.024 ^b

^aP<0.05 vs. control group; ^bP<0.05 vs. pilocarpine and pilocarpine + saline groups; n=8. TNF- α , tumor necrosis factor- α ; NF- κ B, nuclear factor- κ B.

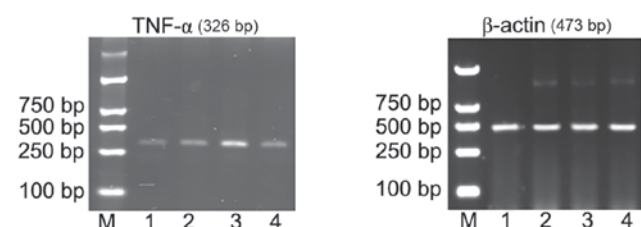


Figure 3. TNF- α mRNA expression levels in the cortex of a rat pilocarpine-induced epilepsy model. M, marker. 1, normal control group; 2, pilocarpine group; 3, pilocarpine + saline group; 4, pilocarpine + ghrelin group. TNF- α , tumor necrosis factor- α .

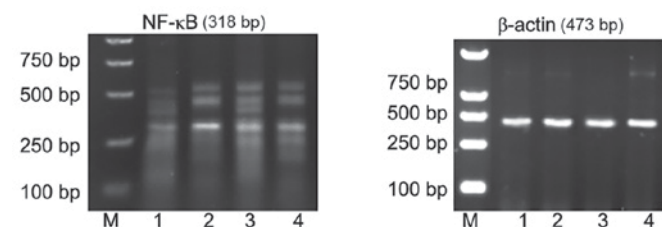


Figure 4. NF- κ B mRNA expression levels in the cortex of a rat pilocarpine-induced epilepsy model. M, marker (β -actin); 1, normal control group; 2, pilocarpine group; 3, pilocarpine + saline group; 4, pilocarpine + ghrelin group. NF- κ B, nuclear factor- κ B.

seizure-induced cortical damage results in improved disease prognosis.

Two major types of feedback adjustment apply to the NF- κ B activation process *in vivo*, one of which occurs via extracellular positive feedback: TNF- α and IL-1 β expression induces the activation of NF- κ B, while activation of NF- κ B increases TNF- α and IL-1 β gene transcription, resulting in increased TNF- α and IL-1 β production and release which continues to activate NF- κ B (20). In the central nervous system, NF- κ B is expressed in cell types including neurons, astrocytes, microglia, oligodendrocytes and brain vascular endothelial cells (21). Activated NF- κ B combines with appropriate target sequences in the nucleus, regulating transcription of target gene activity, including genes involved in inflammation, the immune response and the cell apoptosis process (22). NF- κ B in brain

vascular endothelial cells and glial cells is activated during brain ischemia, and promotes the transcription of TNF- α , IL-6 and intercellular adhesion molecule-1 (ICAM-1) pro-inflammatory genes (23).

Following brain injury, NF- κ B, pro-inflammatory cytokines and the inflammatory response form a complex network that increases the severity of brain injury (24). NF- κ B activation in the central nervous system stimulates the expression of inflammatory cytokines including TNF- α and IL-6, and acts on ICAM-1 expression to promote leukocyte adhesion and migration to the brain, causing long-term pathological changes following traumatic brain injury (25). The pilocarpine-induced epilepsy model used in the present study, which demonstrated increases in TNF- α and NF- κ B expression 24 h following epileptic seizure, embodied this response well. Despite not entirely suppressing this increase, ghrelin intervention significantly reduced the increase of these three indicators. The anti-inflammatory effect of ghrelin is apparent in these results.

To summarize, ghrelin treatment reduced levels of pro-inflammatory indicators, TNF- α , IL-6 and NF- κ B, in immature rats with pilocarpine-induced epilepsy 24 h following seizure. This effect may inhibit seizures caused by inflammatory necrosis of cortical neurons following brain injury, reducing overall brain damage. Further ghrelin-focused research will provide a deeper understanding of its protective effect against seizure-induced brain injury, and may open up a novel therapeutic approach for the treatment of epilepsy in children.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KH was involved in the design of the experiment, drafted and revised the manuscript, collected and processed the specimens, was responsible for the collection and analysis of the data and supervised QYW, RYZ, CXW, SYL, YW and WPT. QYW, RYZ, CXW, SYL, YW and WPT were involved in the design of the experiment, revised the important technical and theoretical content and provided final approval of the version to be published. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committee of Qingdao Municipal Hospital (Qingdao, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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