Antiepileptic effects of exogenous β-hydroxybutyrate on kainic acid-induced epilepsy

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Abstract. The aim of the present study was to explore the potential anticonvulsant effects of β-hydroxybutyrate (BHB) in a kainic acid (KA)-induced rat epilepsy model. The KA-induced rat seizure model was established and BHB was administrated intraperitoneally at a dose of 4 mmol/kg 30 min prior to KA injection. Hippocampal tissues were then obtained 1, 3 and 7 days following KA administration, following which the expression levels of neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP) were measured using a double immunofluorescence labeling method. In addition, the contents of glutathione (GSH), γ-aminobutyric acid (GABA) and ATP were measured using ELISA. Pretreatment with BHB markedly increased the expression of NSE after KA injection compared with that in the normal saline (NS) + KA group, suggesting that the application of BHB could alleviate neuronal damage in rats. The protective effect of BHB may be associated with suppressed inflammatory responses, which was indicated by the observed inhibition of GFAP expression in rats in the BHB + KA group compared with that in the NS + KA group. It was also found that GSH and GABA contents were notably increased after the rats were pretreated with BHB compared with those in the NS + KA group. To conclude, the application of exogenous BHB can serve as a novel therapeutic agent for epilepsy.

Introduction

Epilepsy is a group of neurological disorders that is characterized by epileptic seizures (1,2). As of 2015, ~39 million people were suffering from epilepsy (3), and it has been reported that ~80% of cases occur in the developing world (4). Epilepsy

resulted in 125,000 deaths in 2015, compared with 112,000 in 1990 (5,6). Specifically, children in the 5-9 years age group are particularly susceptible to morbidity associated with active epilepsy (7). Currently, seizures can be controlled with medication in ~70% patients (8). However, for the remaining ~30% patients with epilepsy, seizures cannot be controlled with drugs due to adverse reactions (9,10). Therefore, it remains essential to explore novel treatment strategies for epilepsy.

A number of studies have previously demonstrated that β-hydroxybutyrate (BHB) may serve an important role in epilepsy progression. Suzuki *et al* (11) found that BHB induced by a ketogenic diet (KD) may increase the concentration of γ-aminobutyric acid (GABA) in the epileptic brain by inhibiting astrocytic GABA degradation, which may account for its antiepileptic effects. Samoilova *et al* (12) showed that BHB is more suitable for treating epilepsy associated with metabolic disorders compared with that caused by KD. Additionally, BHB has been found to prevent neuronal injury induced by glutamate-mediated lipid oxidation and glycolysis inhibition (13). The administration of BHB improved glutamate transport in the brain and conferred an anticonvulsant effect (14). Abdelmalik *et al* (15) found that pretreatment with BHB reduced the frequency of seizures induced by acute hypoglycemia. BHB has also been reported to exhibit anticonvulsant effects on epileptic models induced by pilocarpine, flurothyl and 4-aminopyridine (16-19).

A previous study demonstrated that exogenous BHB administration at a dose of 4 mmol/kg served as an alternative to KD in exerting protective effects in a kainic acid (KA)-induced the epilepsy model (20). Therefore, in the present study, the potential antiepileptic effects of exogenous BHB on KA-induced epilepsy were explored further. The expression levels of neuro-specific enolase (NSE) and glial fibrillary acidic protein (GFAP) were evaluated using double immunofluorescence labeling, whilst the contents of glutathione (GSH), GABA and ATP were measured using ELISA.

Materials and methods

Animals. A total of 60 male Wistar rats (age, 3 weeks; weight, 60±10 g) were obtained from The Shandong University Animal Center. Rats had free access to food and tap water and were housed at a standard temperature (22±1˚C) and humidity (50±5%) under a 12-h light/dark cycle. The rats were

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maintained under standard housing conditions until the time of the experiment. The present study was approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine (Shanghai, China). All experimental procedures were conducted according to the National Institute of Health Guidelines (21).

Establishment of the KA-induced rat epilepsy model. On post-natal day 21, 60 Wistar rats were randomly assigned into the following four groups (n=15 rats in each group): i) normal saline (NS); ii) NS + KA; iii) BHB + KA; and iv) BHB groups. Rats in the BHB and NS groups were injected with 4 mmol/kg BHB (1 mmol/ml, cat. no. H6501; Sigma-Aldrich, Merck KGaA) or 4 ml/kg NS, respectively. Rats in the BHB + KA group were pretreated with 4 mmol/kg BHB (1 mmol/ml) that was administered intraperitoneally 30 min prior to KA (10 mg/kg; cat. no. K0250; Sigma-Aldrich, Merck KGaA) injection intra-peritoneally. Rats in the NS + KA group were administered NS intraperitoneally 30 min prior to KA injection. Selection of the BHB dose was based upon a previous study (20). Seizure behavior of rats was analyzed for 2 h, 1 h after KA administra-tion according to the scale previously devised by Racine (22): i) stage I, facial clonus; ii) stage II, head nodding or wet dog shaking; iii) stage III, forelimbs clonus; iv) stage IV, rearing forelimbs; and v) stage V, rearing, jumping or falling. Rats which presented with seizure behaviors of stages ≥IV were considered to be epileptic. If the status epilepticus continued for >90 min, 10% chloral hydrate (400 mg/kg; Sigma-Aldrich; Merck KGaA) was injected intraperitoneally to stop seizure behavior. No rat exhibited any sign of peritonitis after chloral hydrate injection.

NSE and GFAP expression. At 1, 3 and 7 days after KA administration (n=5 rats at each time point), rats were anes-thetized with 10% chloral hydrate (400 mg/kg, intraperitoneal injection, n=5 rats at each time point in each group) and decapitated before their skulls were immediately cut open. No rats exhibited signs of pain after the administration of chloral hydrate. The left hemisphere of the brain was then obtained and immediately fixed in 4% paraformaldehyde for 24 h at 4°C, which was embedded in paraffin and 4-μm thick coronal paraffin sections were prepared for staining. The expression levels of NSE and GFAP in the hippocampal tissues were assessed using a double immunofluorescence labeling method. Coronal paraffin sections were dewaxed successively in xylene for 10 min twice and then rehydrated using a descending ethanol gradient before 0.3% Triton X-100 was added for 15 min at 37°C. After blocking with 5% bovine serum albumin (Beijing Zhongshan Jinqiao Biotechnology Co. Ltd; OriGene Technologies, Inc.) for 1 h at 37°C, the coronal paraffin sections were incubated with a rabbit NSE antibody (1:100 dilution; cat. no. ab79757; Abcam) and goat GFAP antibody (1:100 dilution; cat. no. ab53554; Abcam) overnight at 4°C. After washing three times, the sections were incubated with DyLight® 488-conjugated AffiniPure donkey anti-rabbit IgG H + L (1:1,000 dilution; cat. no. ab96919; Abcam) and Alexa Fluor 647-conjugated AffiniPure donkey anti-goat IgG H + L (1:1,000 dilution; cat. no. A21447; Life Technologies; Thermo Fisher Scientific, Inc.) secondary antibodies for 2 h at room temperature. DAPI (100 ng/ml; Beijing Solarbio

Science & Technology Co., Ltd.) was used to stain the nucleus for 15 min at room temperature. After washing for a further three times, the sections were observed under a fluorescence microscope at x400 magnification (Olympus Corporation), with three view fields of view taken per section. From the images, the mean optical density of NSE- and GFAP-positive fibers was measured using the ImageJ (version 1.49; National Institute of health) program to assess changes in neuron and astrocyte content in the rat brains, respectively.

GSH and GABA content. Hippocampal tissues were removed from the right hemisphere 1, 3 and 7 days after KA admin-istration and immediately stored at -80°C. The frozen hippocampal tissues were defrosted to room temperature and 9X weight of cold NS was added to the tissues and grind was done in ice-cold NS. After the cells were fragmented, 10% homogenized hippocampal tissue (the ratio of tissue:NS was 1:9) was centrifuged for 15 min at 510 x g at 4°C. The supernatant was then obtained for subsequent experimenta-tion. Using a Bio-Rad Model 450 microplate reader (Bio-Rad Laboratories, Inc.), GSH (cat. no. CEA294Ge) and GABA (cat. no. CEA900Ge) contents were measured using the corre-sponding ELISA kits (Cloud-Clone Corp.) according to the manufacturer's protocols.

Statistical analysis. Statistical analyses were performed using the SPSS software 20.0 (IBM Corp.). All data are presented as the mean ± standard error of the mean. Two-way analysis of variance was used to analyze the main effect of treatment, the main effect of time, and the interaction between treatment and time. Significant differences between specific groups were analyzed using Bonferroni corrections. In the present study, $P<0.05$ was considered to indicate a statistically significant difference. All experiments were performed in triplicate.

Results

NSE and GFAP expression. The expression levels of NSE and GFAP were evaluated using double immunofluorescence (Figs. 1-3). For NSE, the interaction between time and treat-ment revealed no statistically significant difference, whilst the main effect of the treatment factor was statistically significant ($P<0.01$). After KA administration, NSE expres-sion was found to be significantly lower in the NS + KA group compared with that in the NS group (D1, $P<0.01$; D3, $P<0.05$) and the BHB group (D1, D3, $P<0.01$; D7, $P<0.05$; Fig. 4A and Table SI). By contrast, the expression of NSE was revealed to be significantly higher in the BHB + KA group compared with that in the NS + KA group ($P<0.05$) after 1 day of KA administration (Fig. 4A and Table SI). No significant differences in NSE expression among different time points were observed, suggesting that time exerted little influence on NSE expression.

The interaction between treatment and time on GFAP expression showed significant differences ($P<0.01$), whilst the main effect of treatment on GFAP expression was also found to be significant ($P<0.01$). After 3 and 7 days of KA admin-istration, GFAP expression was significantly higher in the NS + KA group compared with that in the NS (D3, $P<0.05$; D7, $P<0.01$) and BHB groups (both $P<0.01$, Fig. 4B), whilst
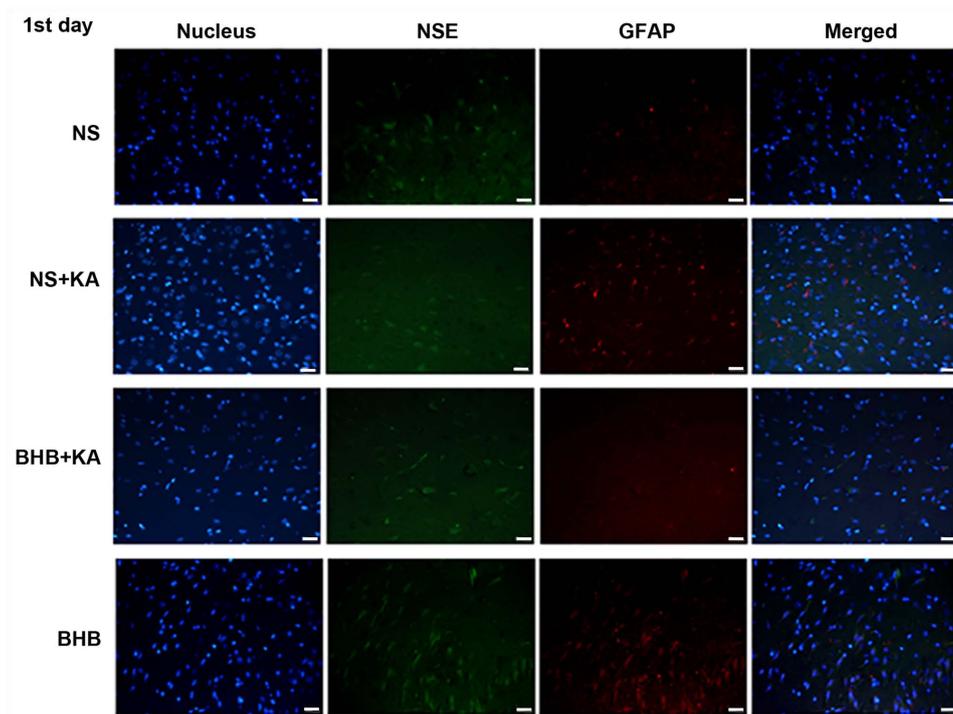
Figure 1. Immunofluorescence staining of NSE and GFAP in the hippocampus of rats 1 day after KA injection. Green signals represent NSE, red signals represent GFAP and the blue signals represent the cell nuclei stained with DAPI. Scale bar, 50 μm. NSE, neuron specific enolase; GFAP, glial fibrillary acidic protein; BHB, β-hydroxybutyrate; KA, kainic acid; NS, normal saline.
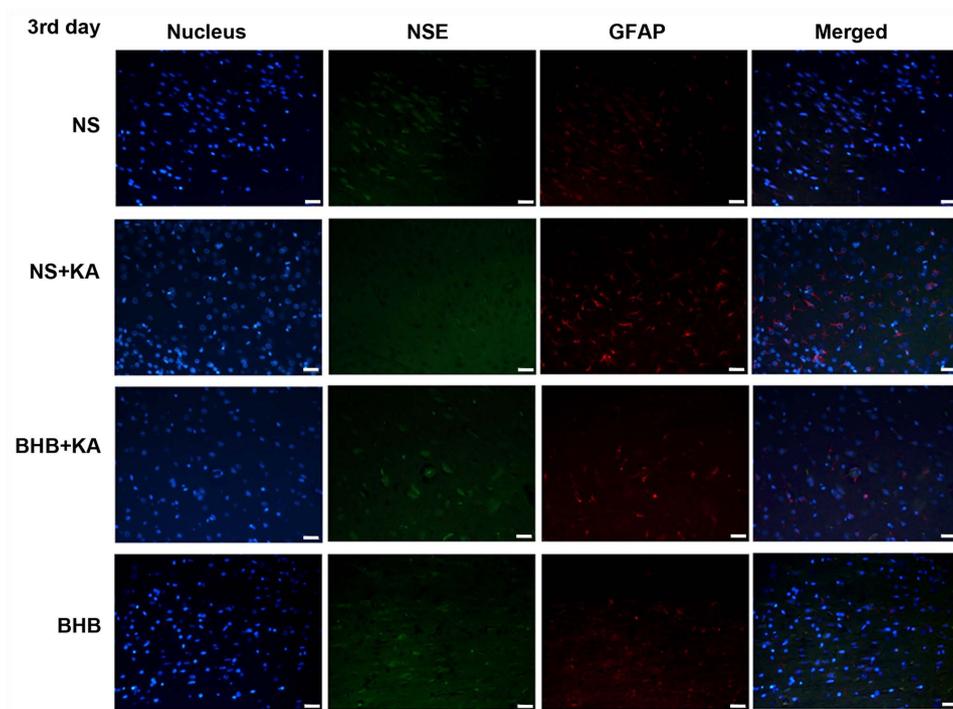
Figure 2. Immunofluorescence staining of NSE and GFAP in the hippocampus tissues of rats 3 days after KA injection. Green signals represent NSE, red signals represent GFAP and blue signals represent the cell nuclei stained with DAPI. Scale bar, 50 μm. NSE, neuron specific enolase; GFAP, glial fibrillary acidic protein; BHB, β-hydroxybutyrate; KA, kainic acid; NS, normal saline.

the expression of GFAP was significantly decreased in the BHB + KA group compared with that in the NS + KA group (D3, P<0.05; D7, P<0.01; Fig. 4B and Table SII). However, after 7 days of KA administration, GFAP expression was significantly

higher in the BHB + KA compared with that in BHB group (P<0.01; Fig. 4B and Table SII). In addition, time was also found to significantly exert influence on GFAP expression (P<0.01). Within the NS + KA groups, GFAP expression increased along
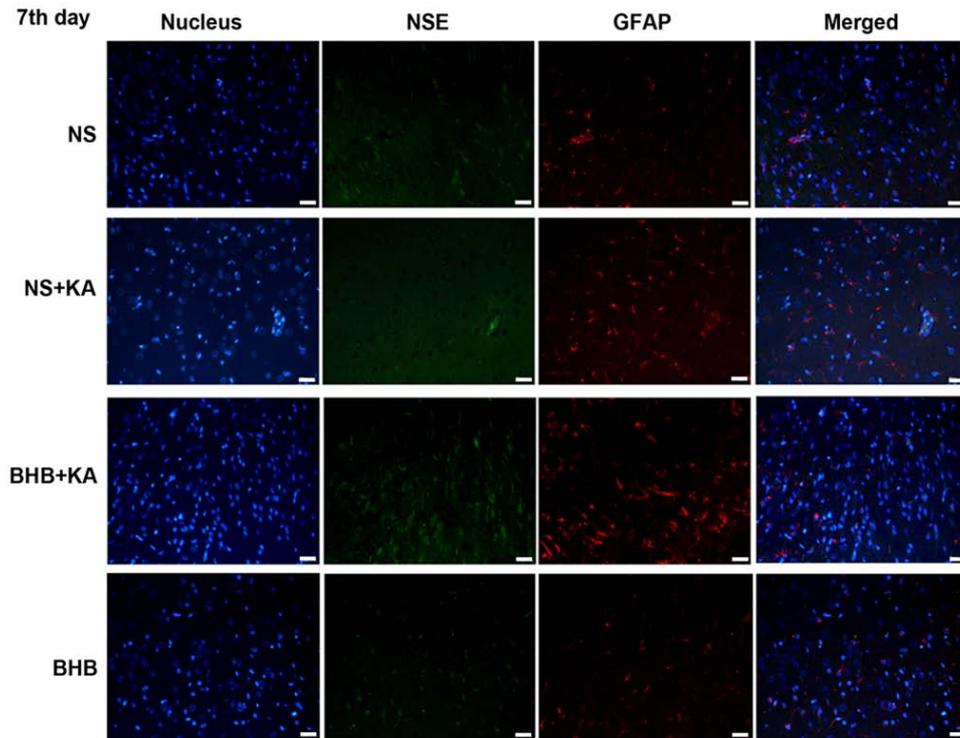
Figure 3. Immunofluorescence staining of NSE and GFAP in the hippocampus tissues of rats 7 days after KA injection. Green signals represent NSE, red signals represent GFAP and blue signals represent the cell nuclei stained with DAPI. Scale bar, 50 μm. NSE, neuron specific enolase; GFAP, glial fibrillary acidic protein; BHB, β-hydroxybutyrate; KA, kainic acid; NS, normal saline.
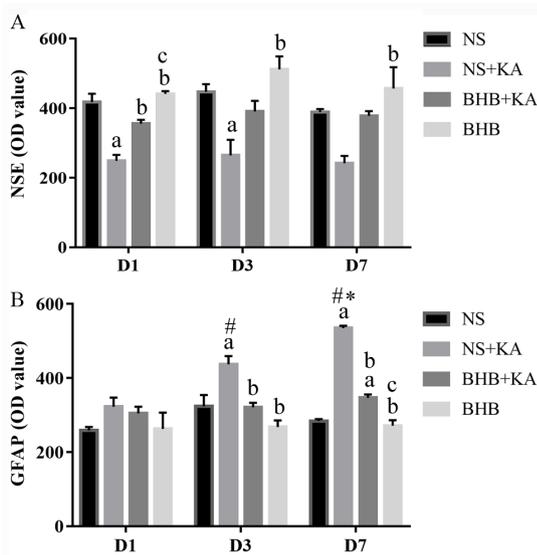
Figure 4. Expression levels of NSE and GFAP in the hippocampal tissue. (A) NSE expression 1, 3 and 7 days after different treatments. KA administration decreased the NSE expression (D1 and D3), whilst BHB alleviated this reduction (D1). (B) GFAP expression 1, 3 and 7 days after different treatments. KA administration increased the GFAP expression but pretreatment with BHB significantly reduced GFAP expression (D3 and D7). $^{a}P<0.05$ vs. NS; $^{b}P<0.05$ vs. NS+KA; $^{c}P<0.05$ vs. BHB+KA; $^{\#}P<0.05$ vs. D1 NS + KA; $^{*}P<0.05$ vs. D3 NS + KA; OD, optical density; NSE, neuron specific enolase; GFAP, glial fibrillary acidic protein; BHB, β-hydroxybutyrate; KA, kainic acid; NS, normal saline.

with time (P<0.05, D1 vs. D3, D3 vs. D7; P<0.01, D1 vs. D7). By contrast, there was no significant difference among the different time points in NS, BHB+KA and BHB groups.
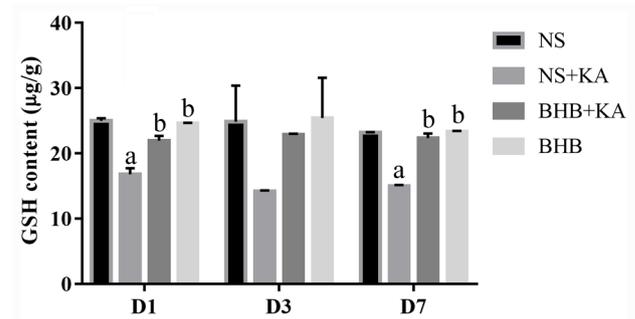
Figure 5. GSH contents in the hippocampal tissues as measured using ELISA after 1, 3 and 7 days of different treatments. GSH contents were decreased after KA administration whilst pretreatment with BHB alleviated this reduction (D1 and D7). $^{a}P<0.05$ vs. NS; $^{b}P<0.05$ vs. NS+KA; BHB, β-hydroxybutyrate; KA, kainic acid; NS, normal saline; GSH, glutathione.

These results suggested that KA can cause neuron damage and compensatory astrocyte hyperplasia, which can be reversed by BHB treatment. There were no differences in NSE and GFAP expression between the BHB and NS groups at any point in time, indicating that BHB did not exert toxic effects on the brain tissues.

GSH content. There was no difference in the interaction between time and treatment on GSH contents, where the main effect of time also did not reveal significant influence. At 1 and 7 days after KA administration, GSH content was found to be significantly lower in the NS + KA groups compared with that in the NS groups (both P<0.01) and the BHB groups (both P<0.01; Fig. 5 and Table SIII). GSH levels were also revealed to
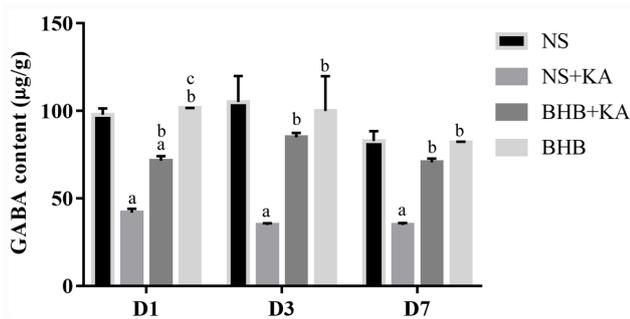
Figure 6. GABA contents in the hippocampal tissues as measured using ELISA after 1, 3 and 7 days of different treatments. GABA contents were reduced by KA administration whilst BHB pretreatment relieved this reduction (D1, D3 and D7). [a]P<0.05 vs. NS; [b]P<0.05 vs. NS + KA; [c]P<0.05 vs. BHB + KA; GABA, gamma-aminobutyric acid; BHB, β-hydroxybutyrate; KA, kainic acid; NS, normal saline.

be significantly higher in the BHB + KA group compared with those in the NS + KA groups after 1 and 7 days (both P<0.01; Fig. 5 and Table SIII). These results suggested that BHB can alleviate the reduction in GSH caused by KA administration in rats. In addition, no differences in the GSH contents were observed between the BHB and NS groups, implicating the safety of BHB.

GABA contents. The interaction between time and treatment showed no significant influence on GABA levels, where the main effect of time also did not reveal statistical influence. After 1, 3 and 7 days of KA administration, GABA levels were significantly reduced in the NS + KA groups compared with those in the NS groups (all P<0.01) and the BHB groups (all P<0.01; Fig. 6 and Table SIV). At all three time points, following pretreatment with BHB, the GABA contents were found to be significantly higher in the BHB + KA group compared with those in the NS + KA group (D1 and D7, P<0.01; D3, P<0.05; Fig. 6 and Table SIV). However, in the BHB + KA group, GABA contents remained significantly decreased compared with those in NS (P<0.01) and BHB groups (P<0.01) 1 day after KA administration (Fig. 6 and Table SIV). These results demonstrated that KA administration reduced GABA levels whilst BHB alleviated this decrease in GABA caused by KA treatment in rats. No differences were found between BHB and NS groups in terms of GABA levels.

Discussion

Epilepsy is one of the most prevalent serious neurological disorders, for which it is important to develop novel effective therapies. Previous studies have documented exogenous BHB to be an anticonvulsant that exerts neuroprotective effects both *in vitro* and *in vivo* (12,23). In the present study, the antiepileptic effects of BHB in a KA-induced epilepsy rat model were explored. Neuronal damage in the hippocampus was demonstrated to be alleviated after rats were pretreated with BHB. Additionally, the present study revealed that BHB was capable of blocking the activation of astrocytes whilst preserving the expression of GSH and GABA after KA administration.

NSE levels have been previously reported to be applicable for determining seizure durations and to estimate the

prognosis of brain injuries (24). The NSE contents were found to be significantly higher in the serum of children with epilepsy compared with those in unaffected children, suggesting that elevated serum NSE after epileptic seizures may be associated with brain damage (25,26). GFAP is a glial cell marker in the development of the central nervous system that is mainly expressed in activated astrocytes (27,28). After epileptic seizure attacks, GFAP expression was previously revealed to be significantly elevated in astrocytes (29). In the present study, NSE and GFAP were used to stain neurons and activated astrocytes respectively, where KA injection resulted in an inflammatory environment in rat brains, as indicated by the extensive activation of glial cells. In addition, the number of neurons was found to be increased in the BHB + KA group compared with that in the NS + KA group whilst the degree of astrocyte activation was reduced. These results indicated that neuronal damage induced by KA was alleviated after the rats were pretreated with BHB, which may be due in part to its ability to inhibit the activation of glial cells.

It has been previously shown that oxidative stress is one of the main pathological mechanisms of epilepsy (30,31). During the progression of epilepsy, reactive oxygen species (ROS) can damage the cell membrane, proteins, enzymes and DNA components within the nucleus and the mitochondria (32). GSH is a part of the main antioxidant system that neutralizes the excessive ROS. GSH is capable of preventing damage to important cellular components caused by ROS, including free radicals, peroxides, lipid peroxides and heavy metals (33). Previous studies have demonstrated that the elimination of GSH is closely associated with a number of human diseases, including neurodegenerative diseases, diabetes and acquired immune deficiency syndrome (34,35). The present study showed that GSH levels in the hippocampal tissues were significantly reduced after rats were treated with KA, suggesting that the ability to eliminate free radicals is reduced in epilepsy. Results from the present study also revealed that administration of BHB reversed the reduction in GSH caused by KA administration in the rat hippocampus. Therefore, it can be potentially concluded that BHB can diminish ROS damage caused by KA by preserving GSH levels. This is in accordance with a previous study that also showed that BHB treatment can reduce the overproduction of ROS and activate GSH further in the epileptic hippocampus (36).

GABA is the main inhibitory neurotransmitter in the central nervous system that serves a critical role in the development of epilepsy (37,38). Increased GABA synaptic activity can reduce the excitability of neurons (39), whilst a reduced GABA level can enhance the excitability of neurons (37). GABA$_A$ receptors are ligand-gated ion channels that hyperpolarize neurons by increasing inward chloride conductance (38). Since the activation of these receptors results in a rapid inhibitory effect, they serve a principal role in nerve transmission processes in the central nervous system (38). GABA$_B$ receptors can reduce calcium entry and mainly mediate slow synaptic inhibition, which is involved with numerous types of epilepsy and cognitive impairment (37,38). It was demonstrated that ketones can alter glutamate metabolism by increasing GABA synthesis, which would in turn dampen seizure activity (40). It has also been previously demonstrated that BHB can reduce

the incidence of seizure-like activity in a $GABA_B$-dependent manner (41). In the present study, GABA levels in the hippocampal tissues were markedly reduced after rats were treated with KA. This reduction in GABA can increase the excitability of the neurons, thereby reducing the threshold of epileptic seizures. GABA levels in the hippocampus tissue were significantly increased after the rats were pretreated with BHB. These results suggest that elevations in the levels of GABA following the application of BHB can dampen seizure activity. In the present study, a correlation analysis between the GABA content and the number of neurons and astrocytes was not performed, which would be of significance for understanding the mechanism of BHB further. This is a limitation of the present study.

It has been previously demonstrated that BHB is a more efficient energy source compared with glucose and that the presence of BHB can reduce ATP production from glycolysis (42,43). Glycolytic ATP is the primary source of energy that supports plasma membrane functions, including ATP-sensitive potassium (K_{ATP}) channels. Lower glycolytic ATP levels would lead to higher K_{ATP} channel opening probability, which would cause membrane hyperpolarization and reduce the influx of calcium via voltage-gated calcium channels. This would in turn reduce the release of excitatory amino acids and decreased neuron excitability (44-46). K_{ATP} channels, which are widely distributed in the hippocampus, would open with higher probability in the presence of BHB, which may underlie the anticonvulsive effects of ketone bodies.

In the present study, only the ATP contents in the BHB group were found to be greater compared with that of the detection threshold on day 7 (46.26±0.81 ng/g). This experiment could not detect ATP in other experimental groups. Considering the rapid degradation of ATP during the tissue preparation process, the frozen hippocampus tissues might have been the main cause of this. Due to the significant elevations in ATP production, ATP could still be detected in the BHB group despite its rapid degradation.

Recently, several studies have demonstrated that BHB confers neuroprotective effects on the central nervous system against oxygen toxicity, Alzheimer's and Parkinson's disease (47-49). Although a series of studies have demonstrated that BHB has protective effects in various epileptic models (9,20,36,50), it remains necessary to verify the effects of BHB in other epileptic models. Additionally, it is difficult to maintain stable BHB concentrations in the blood, which limits the efficacy of BHB administration for clinical application (45). Therefore, further studies focused on BHB treatment for antiepileptic therapy are required to confirm its efficacy and explore the underlying mechanisms.

Taken together, the similarity between the results mediated by BHB and KD in epileptic models suggest that exogenous BHB could replace KD as an anticonvulsant treatment for epilepsy. In particular, there are some limitations of KD applications, including nausea, constipation and abdominal pain (51). By contrast, BHB administration has not been reported to cause adverse effects, which may improve the patients' quality of life. Therefore, the application of exogenous BHB may serve as a novel therapeutic technique in treating epilepsy. However, it is essential to explore the therapeutic effect of exogenous BHB further in the future.

Availability of data and materials

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

Authors' contributions

JW designed the study and supervised the project. JS performed the experiments and completed the manuscript. YW contributed to the acquisition, analysis and interpretation of data for the study. JX performed the statistical analyses, helped with supervising the whole project and was accountable for revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine (Shanghai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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