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Pretreatment with Methylene Blue Protects Against Acute Seizure and Oxidative Stress in a Kainic Acid-Induced Status Epilepticus Model

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Statistical Analysis C

Data Interpretation D

Manuscript Preparation E

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Background: The aim of the present study was to investigate the potential anticonvulsant effect of methylene blue (MB) in a kainic acid (KA)-induced status epilepticus (SE) model. The effects of MB on levels of oxidative stress and glutamate (Glu) also were explored.

Material/Methods: Sixty C57BL/6 mice were randomly divided into 5 equal-sized groups: (1) controls; (2) KA; (3) MB 0.5 mg/kg+KA; (4) MB 1 mg/kg+KA; and (5) vehicle+KA. The SE model was established by intra-amygdala microinjection of KA. Behavioral observations and simultaneous electroencephalographic records of the seizures in different groups were analyzed to determine the potential anticonvulsant effect of MB. The influences of MB on oxidative stress markers and glutamate were also detected to explore the possible mechanism.

Results: MB afforded clear protection against KA-induced acute seizure, as measured by the delayed latency of onset of generalized seizures and SE, decreased percentage of SE, and increased survival rate in mice with acute epilepsy. MB markedly increased the latency to first onset of epileptiform activity and decreased the average duration of epileptiform events, as well as the percentage of time during which the epileptiform activity occurred. Administration of MB prevented KA-induced deterioration of oxidative stress markers and Glu.

Conclusions: MB is protective against acute seizure in SE. This beneficial effect may be at least partially related to its potent antioxidant ability and influence on Glu level.

Keywords: **Kainic Acid • Oxidative Stress • Status Epilepticus**

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Background

Epilepsy is one of the most prevalent neurological disorders. Temporal lobe epilepsy (TLE), the most common form of epilepsy in adults, is characterized by recurrent seizures that arise in the medial structures of the temporal lobe, including the hippocampus and amygdala [1]. The International League Against Epilepsy defines status epilepticus (SE) as seizure activity that is clinical and/or seen on an electroencephalogram (EEG), or recurrent seizure activity without recovery to baseline that lasts ≥ 5 min [2]. SE is a common neurological emergency with a high mortality rate, and it can trigger epileptogenesis, the process of developing epilepsy. Although existing medications can suppress seizures associated with TLE or SE to a certain degree, in about one-third of patients, they lose effectiveness over time or result in adverse effects [3,4]. Thus, it is imperative to develop alternative therapies to control refractory epilepsy and to prevent epileptogenesis.

Accumulating evidence suggests that disordered excitatory and inhibitory neurotransmitter balance plays a vital role in the pathology of epilepsy. The development of SE is the combined result of excessive release of an excitatory neurotransmitter and/or insufficient release of an inhibitory neurotransmitter [5]. In the nervous system, glutamate (Glu) is the primary excitatory neurotransmitter and gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter. Kainic acid (KA) is an excitatory Glu analog which has been widely used to induce SE and TLE in rodents [6,7]. High levels of extracellular Glu during SE can overstimulate Glu receptors, leading to increased influx of calcium, and ultimately, to neuronal cell death, which is referred to as neuro-excitotoxicity. Glu-mediated excitotoxicity can induce severe oxidative stress, result in excessive accumulation of free radicals, and give rise to an imbalance in endogenous free radicals and

antioxidant enzymes, which further promotes epileptic seizures [8]. Therefore, alleviating injury associated with oxidative stress will be protective against epilepsy [9]. Malondialdehyde (MDA) is the final product of lipid peroxidation and its level reflects the state of free radicals. Free radical scavengers, such as superoxide dismutase (SOD) and reduced glutathione (GSH), could protect against oxidative stress injury [10]. Previous clinical research has demonstrated that patients with SE had significantly lower concentrations of SOD and GSH and higher concentrations of MDA compared to controls [11]. KA triggered a decrease in SOD activity and increase in MDA concentration [12]. Therefore, we speculate that a large amount of MDA is produced and the activities of SOD and GSH are weakened during SE induced by KA, resulting in a serious oxidative stress response, a process that may be alleviated or even reversed by antioxidants.

Methylene blue (MB) is a water-soluble thiophenazine compound that functions as a powerful antioxidant. **Figure 1** shows the chemical structure and redox balance of MB [13]. It can reduce oxidative damage to the body by removing reactive oxygen free radicals from tissue in the brain [14-17]. MB already has been shown to exert a nerve-protective effect in ischemic brain damage, Leber optic neuropathy, Alzheimer disease, Parkinson disease, and other neurodegenerative diseases [18-20]. Therefore, in recent years, the role of MB in treatment of various central nervous system (CNS) diseases has generated widespread concern. MB has been confirmed to prevent methylmalonate-induced seizures on EEG and oxidative damage in rat striatum [17]. However, to the best of our knowledge, there has been no definitive evidence of the anticonvulsive effects of MB in a KA-induced acute seizure model until now. Based on favorable outcomes in other CNS diseases, we hypothesized that MB would protect against acute behavioral seizure and oxidative stress during SE. Hence, the

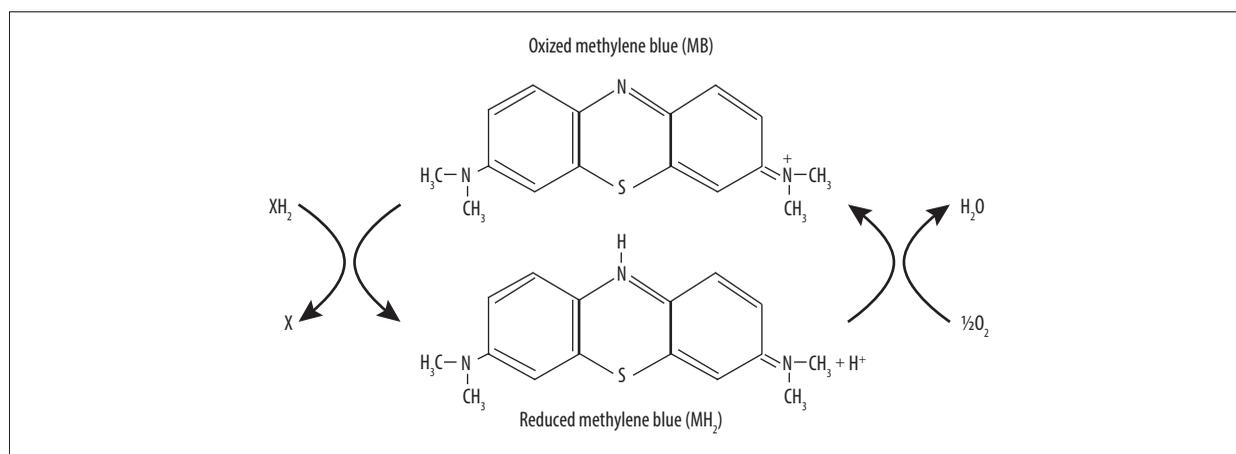


Figure 1. Chemical structure and redox balance of methylene blue (MB). MB has powerful autoxidizable properties. It can accept electrons from an electron donor in its oxidized form and acts as an electron donor in its reduced form. At low concentrations in vivo, the 2 forms of MB are at equilibrium, forming a reversible reduction-oxidation system.

Life Science, China) was implanted in the right amygdala. Three stainless-steel screws that touched the cortex and were used for EEG recording also were implanted. Two other screws were used to affix the dental cement. All the electrodes were connected to a miniature receptacle and attached to the skull of the mice with the guider cannula and screws using dental acrylic cement. After surgery, the animals were housed individually and were allowed to recover for at least 10 days. On the day of the experiment, the guider stylet was removed and a stainless-steel injection cannula was placed 1 mm below the tip of the guider cannula. KA, at a dose of 0.2 μL (1.5 $\mu\text{g}/\mu\text{L}$), was injected into the amygdala through the cannula. The injection, which was done using a 5- μL Hamilton microsyringe, continued for 1 min and the needle was kept in for another minute. The mice in the control group received an injection with an equal amount of saline following the same procedure.

Behavioral Evaluation of Seizure Progression

After administration of KA, mouse behavior was observed for 3 h. Seizure activity was assessed according to Racine's classification [21]: phase 1, facial clonus; phase 2, nodding; phase 3, forelimb clonus; phase 4, forelimb clonus with rearing; phase 5, rearing, jumping, and falling. Phases 1 to 3 and 4 to 5 were considered focal seizures and generalized seizures, respectively [22]. Mice that reached phases 4 to 5 and maintained that level for at least 30 min were considered to have experienced SE. The latent time to reach each seizure phase including SE, the proportion of occurrence of SE, and the survival rates of the mice were recorded and used to quantitatively analyze seizure progression.

EEG Recording and Data Analysis

The animals were continuously monitored for 2 h following administration of KA with a kg KA PowerLab video EEG system (AD instruments, Australia). EEG recordings were sampled at 1 kHz using a 1000 \times gain preamplifier (bandpass-filtered 1-500 Hz) and analyzed by 2 individuals who were blinded to the experimental conditions. As described in the literature [23], the beginning of a seizure was defined as the time at which the amplitude of paroxysmal activity exceeded at least twice the standard error of mean (SEM) baseline amplitude. Seizure latency was defined as the time elapsed from administration of KA to the beginning of the first seizure recorded on EEG. The average duration of epileptiform activity was the average of the duration of each episode of epileptiform activity, including both rhythmic spiking activity and seizures observed during the full recording period. The total time showing epileptiform activity (% epileptiform activity) was calculated as the cumulative time spent in seizures during a 120-min recording period divided by 120 min. The durations of separate EEG events were determined from the start of the repetitive EEG

pattern until the return to baseline. The average duration of the EEG events observed over 2 h was defined as the average seizure duration.

Sample Processing for Assessment of MDA/SOD/GSH and Glu/GABA

Immediately after the behavioral evaluation, the animals were anesthetized and sacrificed. The left hippocampus was quickly collected and frozen in liquid nitrogen. Then, the tissues were stored at -80°C for subsequent detection. For all of the experimental procedures, the hippocampus tissue was prepared as a 10% tissue homogenate in an ice-cold 0.9% saline solution. After centrifugation (3500 rpm, 4°C , 15 min), the supernatant was collected to detect the content of MDA, SOD, and GSH using corresponding detection kits and following the manufacturer's instructions. As previously reported [24,25], spectrophotometric measurement was performed at 532 nm, 550 nm, and 412 nm, respectively.

The same supernatant also was used to detect levels of Glu and GABA with liquid chromatography-tandem mass spectrometry (LC-MS/MS). The LC-MS/MS consisted of an Agilent 1200 liquid chromatography system (United States) equipped with a quaternary solvent delivery system, an autosampler, and a column compartment and a 3200 QTRAP system (Applied Biosystems, Foster City, California, United States) with a hybrid triple quadrupole linear ion trap mass spectrometer equipped with Turbo V sources and a Turbolon Spray interface [26].

Statistical Analysis

Results from the experiment were presented as the mean \pm SEM. All statistical analyses were conducted using SPSS 22.0 Statistics Software for Windows (SPSS, Inc., Chicago, Illinois, United States). Means among multiple groups were compared with 1-way analysis of variance followed by Student-Newman-Keuls post hoc tests for different pair-wise comparisons. The number of animals that experienced SE and survived were calculated as percentages (percentage SE and percentage survival, respectively), and compared using a chi-square test. $P < 0.05$ was considered statistically significant.

Results

MB Pretreatment Relieves Behavior-Related Events of SE

Ten minutes before saline or MB administration, normal behavior was observed in every group. After saline solution/MB administration, observation continued for another 30 min and there was no significant change in behavior. As shown in **Table 1**, animals in the KA group had progressive convulsive

Table 1. Effect of MB on KA-induced acute seizure onset in mice. Data are mean \pm SEM for latency to different seizure phase.

Group	Latent time (min)			Percentage of incidence of SE (%)	Survival percentage (%)
	Phase 1-3	Phase 4-5	Phase SE		
Control	0	0	0	0	100
KA	9.6 \pm 0.65	21.1 \pm 1.05	55.1 \pm 8.88	83	92
MB 0.5 mg/Kg+KA	9.9 \pm 0.49	27.4 \pm 1.14*	112.1 \pm 5.35*	17*	100
MB 1 mg/Kg+KA	10.7 \pm 0.61	30.4 \pm 1.11*	117.3 \pm 2.67*	8*	100
Vehicle+KA	9.7 \pm 0.62	23.4 \pm 0.91	55.18 \pm 8.85	83	92

* $P < 0.05$ vs KA or Vehicle+KA group. (n=12 mice/group).

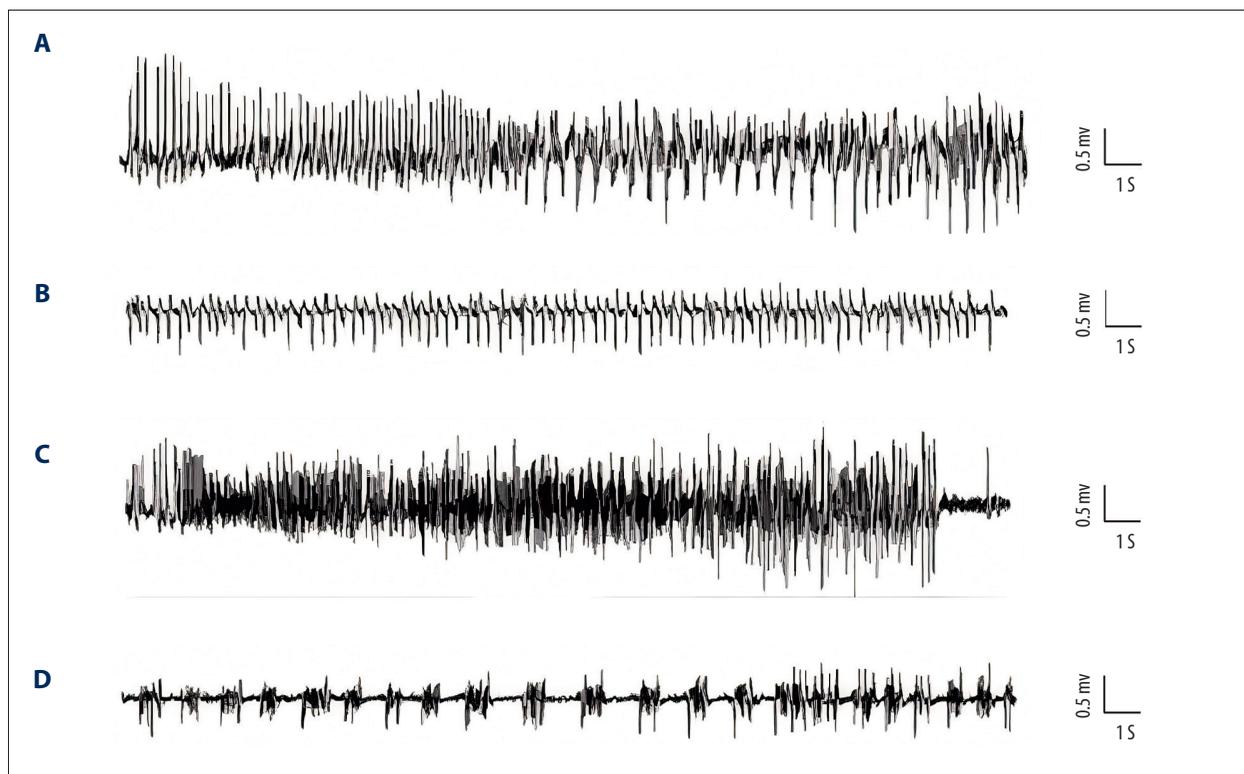


Figure 3. Representative kainic acid-induced seizure patterns on electroencephalogram in the present study. (A) Irregular high-amplitude spikes. (B) periodic epileptiform discharges. (C) High-frequency bursting. (D) A combination of periodic epileptiform discharges and high-frequency bursts of short duration. (Prism 8, version 8.3.0).

behavior and in 83% of them, it progressed to the SE phase; 92% survived 3 h after KA administration. Although there was a nonsignificant delay in the onset of focal seizure, MB pretreatment significantly delayed the onset of generalized seizures and SE ($P < 0.05$, compared with KA/vehicle+KA group). MB pretreatment also reduced the incidence of SE and improved the survival rate of the animals. The inhibition effect of 0.5 mg/kg MB on acute seizure progression was equal to the effect of 1 mg/kg. None of the mice in the control group had seizure activity and there was no difference in seizure onset between the KA and vehicle+KA groups.

MB Pretreatment Increases Latency to SE in EEG and Decreases Epileptiform Activity of SE

Representative EEG patterns during SE are shown in **Figure 3**. Pattern A EEG activity first emerged after KA administration (**Figure 3A**). As seizure severity increased, several other EEG patterns occurred (**Figure 3B-3D**), although patterns B to D were not temporally correlated with specific epileptic behavior. Complete representative traces from EEG recording results are shown in **Figure 4**. In vehicle-pretreated controls, KA administration resulted in uninterrupted seizures on EEG that

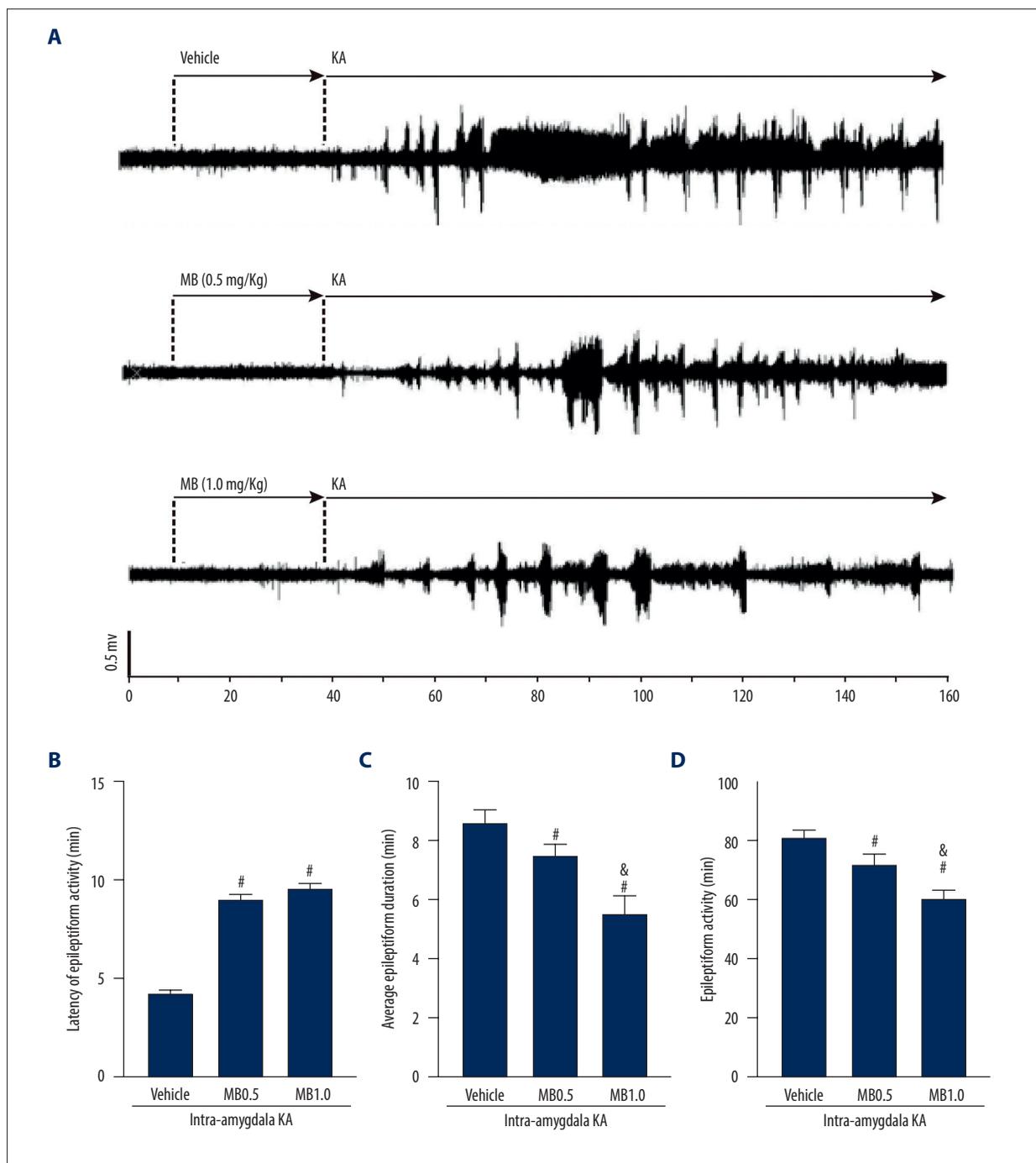


Figure 4. Effect of methylene blue (MB) on kainic acid (KA)-induced epileptiform activity in vivo. **(A)** Representative tracings showing KA-induced epileptiform activity for the full recording period in vivo in mice pretreated with vehicle, 0.5 mg/kg of MB, and 1 mg/kg of MB, respectively. Electroencephalogram tracings are labeled to indicate drug administration times (vehicle, MB, and KA) accordingly. Histograms showing the latency (minutes) **(B)**, average epileptiform duration (minutes) **(C)**, and epileptiform activity (percentage) **(D)** of epileptiform activity in KA-treated mice in different group. Values are expressed as means±standard error of the mean. (n=11 vehicle+KA group, n=12 MB 0.5 mg/kg or 1 mg/kg+KA group). [#] *P*<0.05 vs vehicle+KA group; [&] *P*<0.05 vs MB 0.5 mg/kg+KA group. (Prism 8, version 8.3.0).

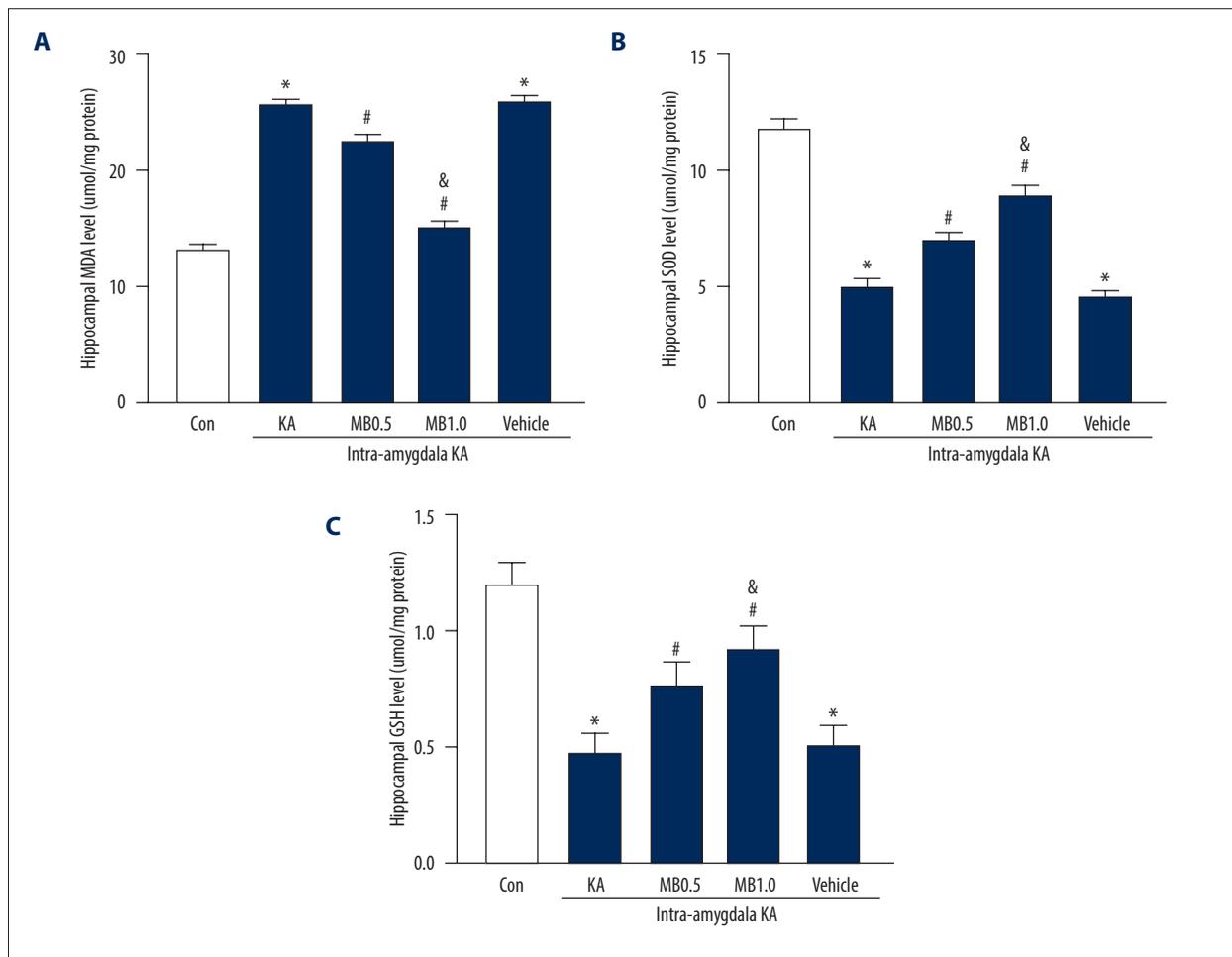


Figure 5. Effect of methylene blue (MB) on levels of hippocampus (A) malondialdehyde, (B) superoxide dismutase, and (C) glutathione 3 h after kainic acid (KA) administration. Values are expressed as means±standard error of the mean. (n=11 vehicle+KA group, n=12 MB 0.5 mg/kg or 1 mg/kg+KA group) * $P<0.05$ vs control group; # $P<0.05$ vs KA or vehicle+KA group; & $P<0.05$ vs MB 0.5 mg/kg+KA group. (Prism 8, version 8.3.0).

persisted throughout the recording period. However, i.p. injection of both 0.5 and 1 mg/kg MB (Figure 4A) was effective in reducing epileptiform activity. Statistical analyses revealed that MB pretreatment augmented the latency to the first onset of epileptiform activity compared with that in the vehicle-pretreated mice (Figure 4B, $P<0.05$). During the 2 h after KA administration, MB pretreatment also reduced the average duration of epileptiform events (Figure 4C, $P<0.05$) and the percentage of time exhibiting epileptiform activity (Figure 4D, $P<0.05$). These effects were dose-dependent and the prophylactic effect of 1 mg/kg MB was much better than the effect of 0.5 mg/kg (Figure 4C, 4D, $P<0.05$).

MB Pretreatment Alleviates KA-Induced Deterioration Trends in Oxidative Stress Markers

As shown in Figure 5, the level of MDA in the KA group increased significantly 3 h after KA administration compared with

the control group (Figure 5A, $P<0.05$), whereas pretreatment with MB significantly weakened the increase in MDA in acute epileptic mice ($P<0.05$). Unlike MDA, SOD activity decreased significantly 3 h after KA administration compared with the control group (Figure 5B, $P<0.05$), and pretreatment with MB inhibited the declining trend in SOD activity ($P<0.05$). The variation in the GSH trend was the same pattern as that for SOD, which decreased after KA administration, and pretreatment with MB prevented the decreasing trend (Figure 5C, $P<0.05$). In addition, the inhibitory effects of MB on changes in non-oxidative stress markers were dose-dependent. In acute epileptic mice, the effect was markedly greater with 1 mg/kg than with 0.5 mg/kg of MB (Figure 5A-5C). Regarding the levels of MDA, SOD, and GSH, there was no significant difference between the vehicle+KA and KA groups.

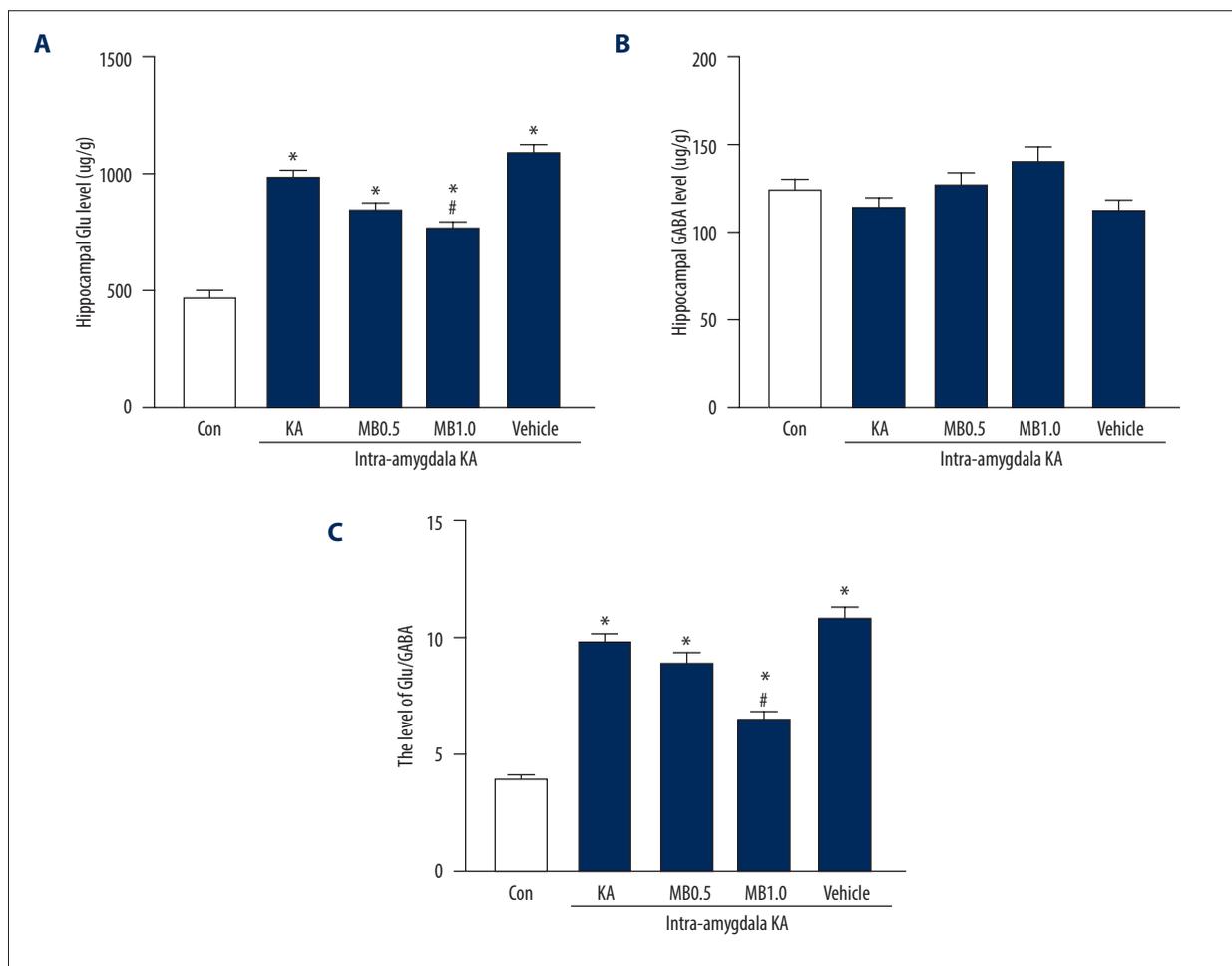


Figure 6. Effect of methylene blue (MB) on levels of (A) glutamate (Glu), (B) gamma-aminobutyric acid (GABA), and (C) Glu/GABA in kainic acid (KA)-treated mice. Values are expressed as means±standard error of the mean. (n=11 vehicle+KA group, n=12 MB 0.5 or 1 mg/kg+KA group) * $P<0.05$ vs control group; # $P<0.05$ vs KA group or MB 0.5 mg/kg+KA group or vehicle+KA group. (Prism 8, version 8.3.0).

MB Pretreatment Attenuates Excessive Release of Glu and Upward Trending of Glu/GABA During SE

As shown in **Figure 6A**, compared with the control group, the levels of Glu ($\mu\text{g/g}$ wet tissue) were increased significantly in the other 4 groups 3 h after treatment with KA ($P<0.05$). There was no difference in Glu between the KA and vehicle+KA groups ($P>0.05$). Compared with the KA and vehicle+KA groups, the level of Glu in the KA+MB 0.5 mg/kg group showed a declining trend, but there were no significant differences between them ($P>0.05$). However, the level of Glu in the KA+MB 1 mg/kg group was much lower than that in the other 3 KA-treated groups ($P<0.05$). The results suggested that MB pretreatment significantly lowered the level of Glu 3 h after KA administration. As shown in **Figure 6B**, there was no significant difference in the levels of GABA ($\mu\text{g/g}$ wet tissue) in the groups ($P>0.05$), although the level of GABA in the KA+MB 1 mg/kg group showed an upward trend compared with the other 4

groups. Of note is that the level of Glu/GABA had a change in pattern similar to that for Glu (**Figure 6A, 6C**) and it was much lower in the KA+MB 1 mg/kg group than in the other 3 KA-treated groups (**Figure 6C**, $P<0.05$). These results indicate that MB pretreatment could attenuate excessive release of Glu and upward trending of Glu/GABA during an acute seizure.

Discussion

One of the major obstacles to management of SE is the unsatisfactory effect of the antiepileptic drugs currently being used. Therefore, there is an urgent need to identify alternative treatments that can efficiently control acute seizure and degeneration of neurons. In the present study, we investigated the possible anticonvulsant effects of MB on behavioral, EEG, and neurochemical alterations elicited by KA. We have confirmed for the first time that: (1) MB pretreatment affords

clear protection against KA-induced acute seizure, which was measured by the delayed latency to onset of generalized seizures and SE, decreased percentage of SE, and increased the rate of in mice with acute epilepsy; (2) MB pretreatment markedly increased the latency to first onset of epileptiform activity and decreased the average duration of epileptiform events as well as the percentage of time exhibiting epileptiform activity; and (3) KA-induced trends in deterioration of oxidative stress markers and Glu can be prevented by pretreatment with MB.

Animal models of epilepsy have shown significant value in exploring basic mechanisms underlying epileptogenesis and the development of therapeutic substitution [27]. One of the most mature models is for KA. In the present study, we studied the anticonvulsant effect of KA in a KA-induced SE model. We used both behavioral observations and simultaneous EEG records of seizures because behavioral observations alone are not a reliable way to evaluate the efficacy of anticonvulsant treatments [28]. The combination of behavioral observations and simultaneous EEG records of seizures more accurately reflects the anticonvulsant effect of MB. Our previous study found that MB had a protective effect in a model of SE induced by prolonged stimulation of the basolateral amygdala [29]. These findings, combined with results from the present study, clearly show that MB pretreatment exerts anticonvulsant effects on acute seizure induced by prolonged stimulus or KA injection into the amygdala. It should be noted that the results do not mean that MB is effective for other types of epileptic seizures, such as those induced by 4-aminopyridine or bicuculine.

In clinical practice, MB has been used for many years to facilitate memory [30-33] and promote neuroprotection because of its potency in enhancing metabolism and as an antioxidant [34]. Because MB has strong lipophilic properties, it can rapidly cross the blood-brain barrier and easily accumulate in brain tissue to exert a direct neuroprotective effect [16,18,35]. The potential antioxidant effect of MB has been confirmed in several clinical and preclinical studies [17,36,37]. In a model of cerebral ischemia reperfusion injury, MB was shown to reduce reactive oxygen species (ROS) and oxidative stress injury by altering transmission of electrons in the respiratory chain [38]. The mechanism for electron transfer to oxygen is due to a pivotal chemical property of MB, that is, the capacity for autooxidation (Figure 1). MB can accept electrons from an electron transport donor and, in turn, transfer electrons to oxygen to form water. Evidence from several studies suggests that MB may be a scavenger of free radicals by acting as an alternative electron acceptor for tissue oxidases [39,40]. In KA-induced SE, Glu-mediated excitotoxicity causes overproduction of ROS and lipid peroxidation and decreases the activity of antioxidant enzymes [41]. Consistent with previous research, our results also showed that KA-treated mice had an increased level of MDA

and a decreased level of SOD [10]. MB alters trends in deterioration of these biomarkers during SE, which may be related to its forceful antioxidative ability. Compared with low doses of MB, high doses result in better effects, possibly because targets more sensitive to oxidative damage require additional antioxidant protection [17]. Certainly, inflammatory processes and injuries are known to occur in KA-induced seizure [42,43], but whether MB is capable of reducing inflammation needs to be verified in our future research.

MB also may influence the glutamatergic system. In rat hippocampal slices, Glu-mediated synaptic transmission was inhibited by relatively high levels of MB [44]. In patients with Alzheimer disease, a favorable effect on cognitive functions was observed after treatment with MB, partly due to the influence of MB on the glutamatergic system [45]. In addition, MB has been suggested to effectively reduce S-nitroso-N-acetyl penicillamine-mediated Glu release. In our study, we found that MB pretreatment led to decreased levels of Glu and Glu/GABA, which were accompanied by a significant reduction in severity of KA-induced SE. Therefore, we believe that reduced production of Glu is involved in the anticonvulsant effect of MB, although it is difficult to determine whether that is a specific effect of MB or a non-specific effect of attenuated seizure activity. The potential mechanism accounting for it still needs to be explored.

One of the potential limitations of the present study may be the short period of EEG recording and relatively short period of behavioral observation after administration of KA. In future studies, telemetry could be used to extend EEG recording and explore longer-term outcomes related to the anticonvulsant and neuroprotective effects of MB. Another limitation is that previous studies demonstrated that a 4-mg/kg dose of MB was the most reliable for enhancing memory and it significantly improved both long-term behavioral habituation and object memory recognition [32]. However, we used 2 doses of MB in the present study, which were both lower than 4 mg/kg. Thus, it would be meaningful to perform a dose-response curve with different non-neurotoxic concentrations of MB to determine whether MB at higher concentrations would produce a better anticonvulsant effect in acute seizures.

Conclusions

Our results suggest that MB is able to protect against acute seizure induced by KA, but not all seizures. At least in part, this beneficial effect may be related to its potent antioxidant ability and influence on the Glu level. These results suggest that further studies are warranted to determine whether MB is a viable adjuvant therapy for preventing and/or treating epilepsy in humans.

Declaration of Figures' Authenticity

All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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