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Investigation of an Optimized Protocol for Brucine-Induced Seizure Model

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Abstract

Object:

Strychnine has been reported to develop seizure models. As its analogue, brucine may also have such property. The aim of the study was to create a chemoconvulsant model of seizures by fractionated administration of brucine.

Methods:

Healthy male Sprague-Dawley rats (n=140) were allocated randomly into three groups: experimental group, normal saline control group and alcohol control group. Rats in experimental group were then randomly divided into six groups during the kindling process: three of the six subgroups were single-dose subgroups, while another three were fractionated-dose subgroups. Rats from three single-dose subgroups (n=20 for each subgroup) received single injection of brucine at three various dose levels (91 mg/kg, 100 mg/kg and 110 mg/kg, respectively); rats from the other three fractionated-dose subgroups (n=20 for each subgroup) received fractionated injections of brucine by three times with one third of the total dose each time. Rats from normal saline and alcohol control group were given normal saline and solvent injection (n=10 for each group). Seizure frequency and intensity (rated as stage 1-5 according to Racine scale), duration and electroencephalographic activity were recorded. Seizures were observed in all single-dose and fractionated-dose subgroups.

Results:

As dose increased, higher frequency and intensity of seizures were observed. At the dose of 110mg/kg, all rats died after stage 5 seizures in both single and fractionated dose subgroups. At doses of 91mg/kg and 100mg/kg, in single-dose subgroups, 75.00-95.00% rats were observed stage 5 seizures, but the mortality was 75.00%-95.00%; In fractionated-dose subgroups, 50.00%-100.00% rats were observed stage 5 seizures, while the mortality was 0.00%-30.00%.

Conclusion:

The study has provided a novel chemoconvulsant model of seizures induced by brucine, and established an appropriate method to develop the model that had not been found in previous literature review.

Keywords: Strychnos nux-vomica; Brucine; seizures; Fractionated administration; Animal model

Introduction

Epilepsy is a common disorder characterized by outbreak of synchronous discharges in brain which causes spontaneous and recurrent seizures. According to World Health Organization (WHO), there are around 50 million people suffering from epilepsy globally [1]. Recurrent seizures often result in limitations in activities, anxiety, depression and impaired quality of life, and moreover, can increase the risk of death [2-5]. Nowadays, antiepileptic drugs (AEDs) are the primary treatment for epilepsy, whereas 20% of patients are resistant to the AEDs. Despite its existence for thousands of years, the pathogenesis and drug-resistant mechanism of epilepsy is still not well identified. This causes difficulty in developing more effective AEDs.

Hence, due to the restraints of sampling from human for experiments, animal models can be served as a valuable alternative in studying the underlying mechanisms of epilepsy.

Strychnos nux-vomica (Snv) is a widely used traditional medicine in China with abundant resources, as well as in other Asian countries such as Korea, Japan, and Cambodia, for the treatment of hemiparalysis, rheumatoid arthritis, amyotrophy, chronic bronchitis, bacterial infections and liver cancer. It also possesses analgesic and immunoregulatory effects [6]. In India, it is also an important constituent in various traditional Ayurvedic formulations [7]. Seeds of Snv are utilized in clinical practice after processed, which has been proved to be important in reducing toxicity. However, since the toxic

dose and therapeutic dose are very close, the poisoning is reported frequently [8]. Excessive use of Snv often leads to intoxication [9] with intense muscular convulsions [10].

Clinical cases of strychnos nux-vomica induced epilepsy

In China, Snv is a commonly used traditional medicine, to make medicinal liquor. By our clinical observation, some patients present seizures after excessive intake of medicinal liquor of brucine. In 2005, we observed 5 cases of Snv overdose related generalized epilepsy [11]. The patients consisted of three men and two women with ages ranging from 32 to 54 years old (46.8 years old on average). Dosages of Snv taken in these cases were from 6g to 35g (routine dosage: 0.3-0.6 g). Latent period of seizures was found varied in different cases. Three patients had seizures in 25-30 minutes after Snv was taken. Seizures occurred in one patient within 10 minutes, while the other one in one week after medicine was taken. No family history of seizures or other seizure causing factors were identified. Neither the physical examination nor CT scan revealed new lesions in the brain. Among these cases, generalized tonic-clonic seizure was the most common type of seizures being observed. Each patient had a sudden loss of consciousness and failed to recall what had happened after recovering. EEGs showed θ and δ waves, sharp waves and multiple slow-spike-waves complexes in frontal and temporal regions. After regular medical treatment, two patients achieved seizure free; in one patient, the seizure frequency decreased from 3-5 times every month to about once every few months; while it hadn't changed in the other two patients.

By our investigation, two in five of the cases were drug-resistant. However, the pathogenesis of Snv induced seizures remains unclear. Therefore it is urgent to establish experimental models of seizures to explain how Snv causes the clinical epilepsies, as the findings could assist in developing new AEDs.

Why did we establish brucine induced seizure model?

Alkaloids are the main bioactive ingredients in Snv [12]. Brucine is the predominant alkaloid present in the bark of the tree Snv [13], which is also one of the main bioactive ingredients in Snv. Another important alkaloid is strychnine. Brucine and strychnine are of the primary effects among all alkaloids. Their poisonous effects are associated with an inhibitory action of the glycine receptor [14,15]. Glycine is a major inhibitory neurotransmitter in adult spinal cord and brain stem [16], which controls both motor and sensory pathways [16]. Brucine and strychnine are considered as chemoconvulsants for long. Chemical seizure model induced by strychnine has been reported and proposed as models for drug-resistant epilepsy for more than a decade [17]. The toxicological property of brucine is akin to strychnine [13]. However, can brucine be used to establish a seizure model as well? In addition, inspired by some research finding that fractionated lower dose might be superior to single full dose in Pentylenetetrazol (PTZ) [18] and pilocarpine models [19], we sought to explore how to generate a chemoconvulsant model induced by fractionated administration of brucine in Sprague-Dawley (SD) rats and discussed the optimal dosing and timing for establishing the model, which had not been reported in previous literature. Although only one study compared effects of different chemical convulsants, including brucine, their aims and methods are different from ours [20]. In this study, we increased the sample size based on our preliminary test to further explore the feasibility to establish chemoconvulsant model of seizures induced by brucine.
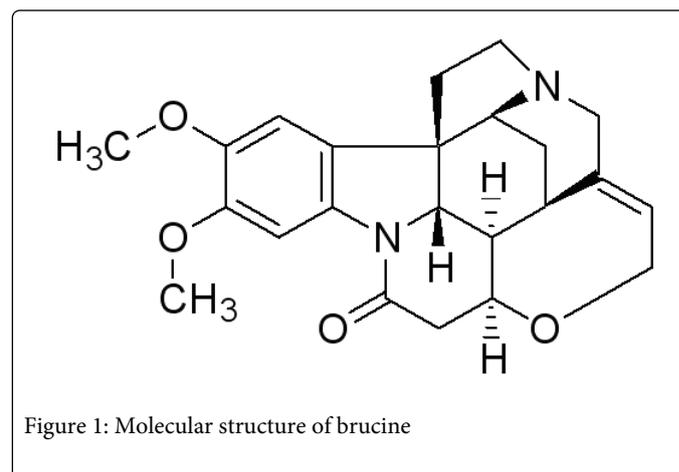
Methods and Materials

Animals

140 adult male SD rats, 6-8 weeks old, weighing 180-220 g, were used. All rats were provided by Animal Centre of Chongqing Medical University, China. The sample rats were kept in individual cages with controlled environment with constant temperature from 22℃ to 25℃, humidity of 50-60%, and 12 hrs light/dark cycle. Food and water were randomly allocated to all rats. All the procedures were performed in the morning to minimize circadian variations. Caring and handling of animals in this research was conducted in compliance with the Animal Welfare Act [21]. The protocol was approved by Chongqing Medical University Ethical Committee for Experimental Animals.

Drug used to induce kindling: Brucine

Brucines used in this study were sourced from Beijing Institute for The Control of Pharmaceutical and Biological Products. The brucine injection was extracted from Snv. The chemical name is 10, 11-Dimethoxystrychnie, and empirical formula $C_{23}H_{26}N_2O_4$. Chemical structure of brucine is shown in (figure 1). It is an antagonist of glycine-receptor [15]. The concentration of brucine used in this study was over 95%.

Figure 1: Molecular structure of brucine

Kindling procedure with Brucine

Our prior studies have demonstrated that at least a 110 mg/kg brucine dose can inevitably induce significant stage 5 (Racine scale) seizures in SD rats. Whereas if the dose was lower than 91 mg/kg, stage 4-5 seizures were not elicited (pilot study, unpublished). Therefore, we consider a dose of 91 mg/kg as a threshold dose of kindling, and a dose of 110 mg/kg a maximal dose. In order to achieve the optimal level of dosage with the highest kindling rate and lowest mortality, we evaluated the effects at three different dosing levels of 91 mg/kg, 110 mg/kg and their meanvalue 100 mg/kg, respectively. In addition, our studies have indicated that when the total dose (≥ 91 mg/kg) was given in single injection, most rats died after acute stage 5 seizures. However, if fractionated dosing was used, that is, a 1/3 of total dose was utilized by three times with an interval of 30 minutes (as proven in our prior study), the mortality rate would decrease dramatically. That is to say, injection of brucine in fractionated doses seems to be safer and more efficient for kindling than in a single dose. Accordingly, the kindling procedure directly commenced with the verified doses. The effects of a single dose and fractionated doses were compared.

Intraperitoneal injection (i.p.) of different does of brucine (91,100 and 110 mg/kg) was used to kindle six groups of rats. Three groups were given brucine in a single dose. Equal amounts of doses were administered to the other three groups but in three times, with one third of the total dose as the first three groups for each time. Due to its insolubility in NS, brucine was firstly dissolved in alcohol (70 mg brucine dissolved in 1 ml anhydrous alcohol as previous study confirmed). Then added NS to dilute and dissolve the alcohol-dissolved brucine for preparation. To eliminate the confounding factors from alcohol, the alcohol control group was designed. Samples were randomly assigned to experimental groups; NS control group and alcohol control group with a 12:1:1 allocation ratio. The experimental groups consisted of six subgroups: three subgroups received single injection (group a, b and c), and the other three subgroups received three injections (group A, B and C). The single-dose subgroups received brucine of 91 mg/kg (group a, n=20), 100 mg/kg (group b, n=20) and 110 mg/kg (group c, n=20) respectively; fractionated-dose subgroups received identical total amount of brucine of 91 mg/kg (group A, n=20), 100 mg/kg (group B, n=20) and 110 mg/kg (group C, n=20) in three injections respectively. Control groups consisted of NS group (group D, n=10) and alcohol group (group E, n=10). Different doses of brucine were administered with the same amount corresponding to each subgroup. NS group was given identical volume of NS. Alcohol group was given equal volume of anhydrous alcohol dissolved in same volume of NS as all corresponding groups above. In subgroups for three injections, doses were trisected equally, given at an interval of 30 minutes.

The seizure intensity was graded according to the criterion of Racine scale (1972) for mouse as follows: stage 1, mouth or facial movements, characterized by eye closure, sniffing, twitching of vibrissae or facial clonus; stage 2, head nodding accompanied with more severe facial clonus; stage 3, clonus of forelimb; stage 4, rearing, often accompanied with bilateral forelimb clonus; stage 5, all of those above with loss of balance and falling, characterized by generalized clonic seizures [22]. The interval of fractional injection of 30 minutes was determined in our preliminary test with lower mortality and higher kindling rate [23,24]. Behavioral and electrographical performance were recorded and compared. The present protocols were shown in Figure 2.
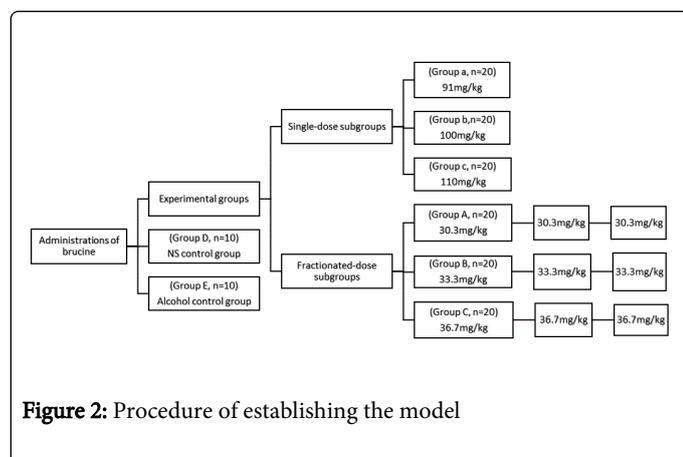

Figure 2: Procedure of establishing the model

Behavioral and electroencephalographic monitoring

Implantation of electrodes

Before injections, 30 minutes baseline electroencephalogram was recorded. After injections, all animals were randomly allocated into single-injection group, fractionated-injection group, NS control group and alcohol control group. EEGs were recorded constantly before and after injections.

Prior to the implantation of the electrodes, 3.5% chloral hydrate (0.35 g/kg, i.p.) was used to anesthetize the animals. The electrodes were made of twisted bipolar nichrome wires (diameter=0.1 mm). The stereotaxic coordinates used in our study were derived from the rat brain atlas of Paxinos and Watson [24] and our preliminary experiments on SD rats. One electrode was implanted into a tiny pore, drilled on the skull aiming at the left hippocampus with the following coordinates: 3.6 mm posterior to bregma, 5. 0 mm lateral to midline, and 5.5 mm deep. Another electrode was symmetrically implanted into the skull, which was located 3.0 mm anterior to the bregma as the reference electrode. All electrodes were fixed to the skull with dental acrylate cement. Administration of brucine was performed one week after implantation of the electrodes.

EEG recording and behavior monitoring

The EEGs were recorded with an EEG recording system (BL-420F, Biological experimental system, Chengdu Taimeng science and technology, China). Rats had continuous electrographic recordings 30 minutes before administration of brucine until recovering from seizures.

Behavioral changes of each kindled rats were continuously monitored by Sony DCR-DVD92 video camera recorder from 30 minutes before each brucine injection to 2 hours after the injection. Seizure frequency, duration in 2 hours, and intensity as per the criterion of Racine were recorded to compare the changes under different doses and injection times. The seizure intensity was scaled from stage 1 to stage 5. Seizure duration was defined as the duration of limbic (stage 1-2, immobility or occasional facial clonus or head nodding) or motor seizures (stage 3-5) or both. Limbic seizure activity after termination of a motor seizure was not included in the seizure duration [25, 26].

Statistical analysis

The statistical processing system used in this study was SPSS for Windows (version 11.5). χ^2 tests was used to compare the differences of successful Kindling ratio in each group. A two-sided p value <0.05 was considered statistically significant.

Results

The rate of kindling

A total of 140 rats were enrolled in this study, and 120 of them received brucine injections. In the NS control group and alcohol control group, no clinical reactions to the NS and alcohol injections were observed.

After injections, at the dose of 110 mg/kg, either in single-dose group or fractionated-dose group, all rats were fully kindled and then died following severe convulsions (stage 5). Therefore the kindling rate

and mortality rate at 110 mg/kg were both 100.00% in two different groups (group c and C).

At the dose of 91 mg/kg and 100 mg/kg, in single-injection subgroups (group a and b), 75.00%-95.00% rats were fully kindled with stage 5 seizures, with a median latency of 10min, but all died after seizures. Stage 1-3 seizures were observed in five rats of group a, and one rat of group b which were considered not being kindled. So no rats were successfully kindled in single-injection subgroups. While in three-injection subgroups (group A and B), 50.00%-90.00% rats were observed kindled with stage 5 seizures, with a median latency period of 13min, and the mortality rate of 0.00%-30.00% (Table1 and Figure 3). Rats alive after fully kindled were considered successfully kindled. The intergroup differences of the successful kindling rate were significant between group a and A, group b and B. Ten rats in group A and two rats in group B were not kindled, and six rats in group B died after seizures.

Group	Kindled (Racine stage4-5)	Died after convulsion	Successfully kindled
Single-dose subgroups			
a (n=20)	15 (75.00%)	15 (75.00%)	0 (0.00%)
b (n=20)	19 (95.00%)	19 (95.00%)	0 (0.00%)
c (n=20)	20 (100.00%)	20 (100.00%)	0 (0.00%)
Fractionated-dose subgroups			
A (n=20)	10 (50.00%)	0 (0.00%)	10 (50.00%)
B (n=20)	18 (90.00%)	6 (30.00%)	12 (60.00%)
C (n=20)	20 (100.00%)	20 (100.00%)	0 (0.00%)

Table 1: Kindling rate and mortality in six experimental groups. Table 1 shows the kindling rate and mortality in six groups with different injection technique at three dosing levels. Total doses were 91 mg/kg for group a and A, 100 mg/kg for group b and B, 110 mg/kg for group c and C. Group A and B had significantly higher successful kindling rates.
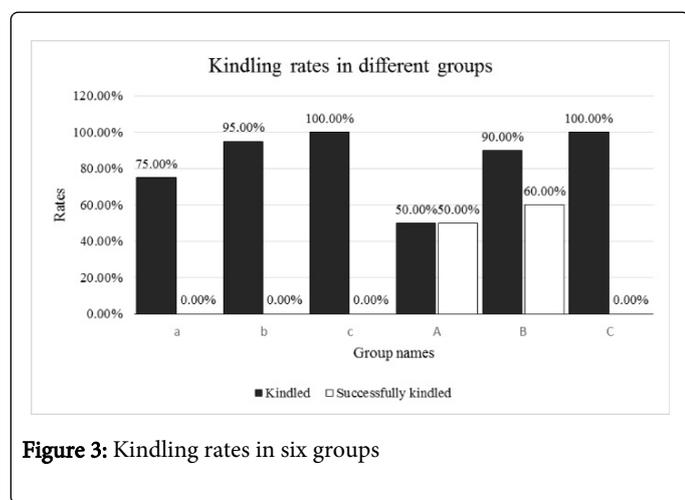

Figure 3: Kindling rates in six groups

Behavioral manifestations

Rats manifested typical behavioral changes 10min after injections. In single-injection subgroups, animals were soon observed seizures of stage 4 to 5, rearing, losing balance and collapsing, and followed by generalized tonic-clonic seizures (GTCS). GTCS often repeated several times with the duration between 3 and 12 minutes. After severe outbreak of seizures, most animals became dyspnea, soon dead. Five rats in group A and ten rats in group A were not observed stage 4 seizures. In three-injection subgroups, after staring, tachypea, piloerection and twitching of vibrissae, they showed a sudden general clonus. The seizure intensity was classified as seizures of stage 1 to 3. In fully kindled rats, rearing frequently recurred, often accompanied with bilateral forelimb clonus (stage 4), finally losing balance and falling over (stage 5).

Electroencephalography

Before injections, all rats showed normal baseline in EEG recording. The wave forms of burst focal spikes and polyspikes displayed during a seizure of stage 1-3. In stage 4-5 seizures, continuous high-amplitude discharges of epileptic seizure patterns, such as spike and sharp waves, poly-spike waves, were found. Reactions varied in the duration of these ictal electrographic episodes between 10 and 20 s. Afterwards, the rats gradually went into normal state with recovered EEG. In control groups, normal baseline EEG recording were observed consistently. (See figure 4)
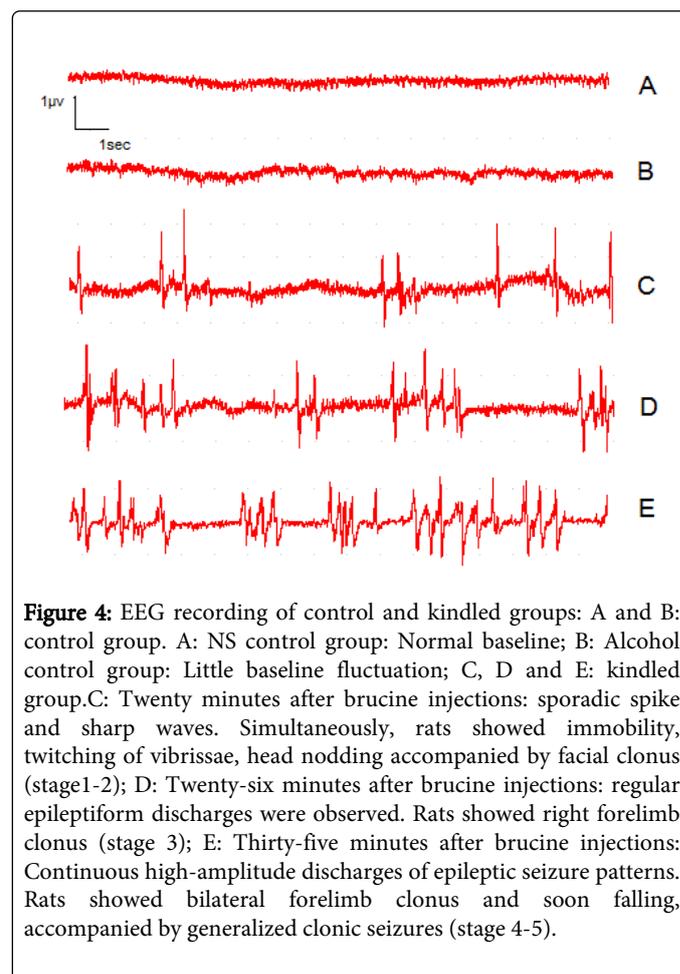

Figure 4: EEG recording of control and kindled groups: A and B: control group. A: NS control group: Normal baseline; B: Alcohol control group: Little baseline fluctuation; C, D and E: kindled group. C: Twenty minutes after brucine injections: sporadic spike and sharp waves. Simultaneously, rats showed immobility, twitching of vibrissae, head nodding accompanied by facial clonus (stage1-2); D: Twenty-six minutes after brucine injections: regular epileptiform discharges were observed. Rats showed right forelimb clonus (stage 3); E: Thirty-five minutes after brucine injections: Continuous high-amplitude discharges of epileptic seizure patterns. Rats showed bilateral forelimb clonus and soon falling, accompanied by generalized clonic seizures (stage 4-5).

Discussion

About this Protocol

We draw the inspiration of developing this animal model from our observation of clinical cases. On the premise of these characteristics of Snv induced epilepsy, we tried to figure out whether a similar seizure animal model was able to be established. Even though effects of inferior olive lesion among different chemical convulsants with fixed doses were compared by Anderson et al in 1987, varied doses and effects were not studied [20]. Besides, the dose of brucine in their study (40 mg/kg) was comparatively lower than that of our finding, which may contribute to different outcomes.

This study found that brucine could be used to establish chemoconvulsant model that was consistent with clinical neurophysiological and behavioral features in human general tonic-clonic epilepsy. We also investigated the optimal dose and timing for establishing this model that multiple low-dose regimen via intraperitoneal injections minimized the mortality rate when compared to single high-dose injections.

In previous study, we have proved that the minimum single dose for brucine-kindled acute seizure was 91 mg/kg. With this dosing, stage 4-5 seizures were elicited repeatedly. However, more rats died with an increasing mortality rate of 75.00% followed by stage 5 seizures. Moreover, a 100 mg/kg dose with single injection resulted in 100.00% mortality. Researches on pilocarpine rats suggested that repeated administration of pilocarpine at low doses effectively induced status epilepticus (SE) and chronic epilepsy with much lower mortality rates, compared with the single-dose pilocarpine in lithium-pretreated rats, while the development of spontaneous recurrent seizures did not differ between the two groups [19]. They suggested that multiple low-dose intraperitoneal injections minimized the mortality rate compared with single high-dose injections [27].

Inspired by the evidence above, we developed a rat model with a protocol of multiple low-dose administration at an interval of 30 minutes instead of single high-dose injection. We discovered that with the identical amount of total dose, three times low-dose injections could effectively reduce mortality rate without decreasing the kindling rate compared with single-injection of high-dose groups. Only when the total dose increased to 110 mg/kg, the mortality ran up to 100.00%. Successful kindling rates were significantly higher in three-injection subgroups at doses of 91 mg/kg and 100 mg/kg. Our findings suggested that the total dose of 91 mg/kg-100 mg/kg was the appropriate dosing range of brucine for kindling, with low mortality and high kindling rate, which should be given by fractionated dose technique with one third dose and an interval of 30minutes. The outbreak of stage 4-5 seizures in kindled rats, accompanied with continuous discharges of high-amplitude spike, sharp and poly-spike waves in EEG, was identical with those of human epilepsy.

Possible mechanisms of convulsive effect of Brucine

Snv exhibits cytotoxic effects of four alkaloids- brucine, brucine N-oxide, strychnine and isostrychnine. Brucine and strychnine, accounting for 80% of alkaloids in Snv, are the chief pharmacological active principles [23, 28, 29]. They share the similar molecular structure, whereas strychnine is known to be more toxic. They inhibit the glycine-gated chloride currents, improve the excitability of neurons and enhance their contact [30-33]. Strychnine is the prototypic competitive antagonist of glycine receptors, which are often referred to as 'strychnine-sensitive glycine receptors' [34]. It blocks the uptake of inhibitory neurotransmitter glycine. This process occurs at the postsynaptic receptor in the motor neurons of neural horn in the spinal cord, which gives rise to very powerful tonic contractions. Chemical seizure model induced by strychnine has been used for developing rat models of drug-resistant epilepsy [17]. Although brucine is an analogue of strychnine with equal amount in Snv, there is currently no reports found that elaborate its use to create seizure models.

Brucine was originally isolated from the dry ripe seeds of Snv L by Pelletier and Caventou in 1819. Brucine was originally isolated from the dry ripe seeds of Snv L by Pelletier and Caventou in 1819. Glycine serves as an inhibitory neurotransmitter. Glycinergic synapses are particularly abundant in the spinal cord, brain stem and caudal brain, where the amino acid contributes to the control of reflex responses, process of sensory signals and motor rhythm generation via the family of strychnine-sensitive glycine receptors. Recent studies reveal that glycine receptors are also expressed in adult hippocampal neurons [30, 32], which is known as "windstorm" area of epileptogenesis. The imbalance between neuronal inhibition and excitation, which contributes to epileptogenesis in the CNS, is mainly mediated by γ-aminobutyric acid (GABA) and glycine. The antagonistic effect on glycine receptors induced by brucine impairs neuronal inhibition, which consequently enhances tendency to hyperexcitability and epileptogenesis. Glycine-induced chloride current was mediated by strychnine-sensitive glycine receptors [35].

Strychnine-sensitive glycine-gated chloride channels (GlyRs) are functionally expressed by CA1 pyramidal cells and GABAergic interneurons in mature rat hippocampal slices. Chloride current in CNS has been considered as the inhibitory current leading to hyperpolarization on neural membrane. Brucine may suppress chloride current via strychnine-sensitive glycine receptors and boost epileptogenesis.

Cytotoxic activity is another significant function of brucine. Necrosis and apoptosis are both revolved in the development of epilepsy. In an anti-tumor research on human hepatoma cells (HepG$_2$), brucine caused typical apoptotic programmed cell death, such as cell shrinkage, membrane blebbing and apoptotic body formation. Research found that cell apoptosis induced by brucine was likely mediated by multiple pathways. Recently emerging evidences suggest that intracellular [Ca^{2+}] played an important role in apoptosis, probably through the direct activation of caspases or collapse of the mitochondrial membrane potential. Furthermore, brucine induced a rapid and sustained elevation of intracellular [Ca^{2+}], which composed the mitochondrial membrane potential and then triggered the process of HepG$_2$ cell apoptosis. Bcl-2 was found to predominantly control the whole process of brucine-induced cell apoptosis. Therefore it is concluded that Ca^{2+} and Bcl-2 mediated mitochondrial pathway involves in brucine-induced HepG$_2$ cell apoptosis. The effect of brucine on HepG$_2$ cell apoptosis is a specific toxic effect, and is dose-dependent [29].

Advantages of Brucine-induced seizure model

In China, Snv is a commonly used traditional medicine, to make medicinal liquor, which is easily acquired. By our clinical observation, after excessive intake of medicinal liquor of Snv, some patients present seizures, particularly drug refractory. Given that brucine is the principle component in Snv, we speculate that brucine may have refractory-convulsive property, which could probably become a

potential chemoconvulsant for refractory seizure model. As the most common seizure model, PTZ-induced seizure model exerts its convulsive effect on animals, and not refractory, but no studies report such effect on human. Moreover, PTZ was reported to decrease frequency of spontaneous seizures in patients with epilepsy in the 1930s and 1940s (cited by Nilesh [36]).Therefore, brucine may be a better simulation for epileptogenesis in human.

Conclusion

This study and previous clinical cases suggest a strong link between brucine and the genesis of epilepsy. In the present study, we reported and described an optimal protocol for establishing the brucine-induced acute seizure model and its possible mechanism. Nevertheless, what is the chronic effect of brucine administration and does brucine induce refractory epilepsy just as some of the patients? These will be investigated further in our future studies.

Acknowledgment

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