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Safranal carried by nanostructured lipid vehicles inhibits generalized epilepsy in mice

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Safranal, a main component of *Crocus sativus*, is suggested to have neuroprotective effects. The aim of this study was to investigate the effect of safranal and nanostructured lipid vehicle (NLV) carried safranal in acute and chronic experimental mice models of epilepsy. In PILO acute seizure model, safranal dose-dependently extended latency to generalized seizure, decreased the highest seizure stages and the number of generalized seizures. Moreover, NLV carried safranal further enhanced the anti-seizure effect, which is comparable to the action of sodium valproate. Meanwhile, NLV carried safranal reduced and delayed the electroencephalogram spectra power after pilocarpine injection. In histological aspect, safranal dose-dependently reduced the loss of neurons induced by seizure and NLV system further improved this protection at the same dose. In MES acute model, safranal markedly increased the electroconvulsive threshold, where NLV further improved its effect. In PTZ chronic seizure model, NLV carried safranal significantly delayed the kindling rate of progress and the time it took to reach generalized seizures as compared to NLV control group. In conclusion, this study indicates that safranal inhibits generalized seizure in acute and chronic epilepsy models in mice and NLV can enhance this effect. So, NLV carried safranal may have potential value in treatment of generalized epilepsy.

1. Introduction

Epilepsy is a group of neurological diseases characterized by recurrent spontaneous seizures and temporary brain dysfunction (Engel, 1995). World Health Organization reported epilepsy as being the second most common nervous system disease which affects fifty million people with a morbidity rate of 7%. Current antiepileptic drugs (AEDs), such as sodium valproate and carbamazepine have been widely used in clinical treatment for epilepsy. However, these AEDs have serious side effects such as dizziness, liver, and kidney damage which limits its potential for treatment of seizures (Kwan and Brodie, 2001; Manning et al., 2003). Thus, it is imperative to find novel AEDs for the treatment of seizures.

Safranal (2, 6, 6-trimethyl-1, 3- cyclohexadiene-1-carboxaldehyde, C10H14O) is a monoterpene aldehyde and a main constituent of Crocus sativus, which is responsible for its characteristics of saffron aroma. It has been previously reported that safranal showed neuroprotection in spinal cord injury and cerebral ischemic injury, exerting anti-apoptotic and anti-oxidative action (Rezaee and Hosseinzadeh, 2013; Zhang et al., 2015). Moreover, it has shown to reduce seizure duration in pentylenetetrazol (PTZ)-induced acute seizure models (Hosseinzadeh and Talebzadeh, 2005). Since different types of epilepsy have different pathogenesis, it is worth further investigating in the effects of safranal in various seizure models.

Abbreviations: NLV, nanostructured lipid vehicle; EEG, electroencephalogram; AEDs, antiepileptic drugs; BBB, blood-brain barrier; CNS, central nervous system; PILO, pilocarpine; MES, maximal electroshock; PTZ, pentylenetetrazol; VPA, sodium alproate; OA, oleic acid; ICG, indocyanine green; FFT, Fast Fourier transform; TB, Toluidine Blue; LD50, lethal dose 50; GABA, gammer amino butyric acid.

Over the past decade, many different nanostructured lipid vehicle (NLV) systems have been developed and applied in the drug delivery for the therapy of cerebral diseases (Baek et al., 2015; Beloqui et al., 2016). Due to unsatisfactory stability as well as limited permeability to the blood brain barrier (BBB) of safranal, we developed a new NLV system to enclose safranal in this study. We investigate the effects of safranal carried by NLV in acute seizure models induced by pilocarpine (PILO) and maximal electroshock (MES), as well as in chronic seizure model induced by PTZ.

2. Investigations and results

2.1. Characteristics of NLV carried safranal

Particle size, polydispersity index and zeta potential of NLV carried safranal dispersion were measured. The mean particle size was 164±43.2 nm (Fig.1A) with polydispersity index of 0.137. It has been reported that nanoparticles with size between 100-200 nm were appropriately used in drug formulations, which can penetrate the BBB to deliver drugs to the central nervous system (CNS, Sun et al., 2016). Moreover, the polydispersity index lower than 0.3 was regarded as an optimal condition for lipid nanoparticle dispersions. Besides, zeta potential of NLV was -23.2±1.8 mv, so the nanoparticles were able to disperse stably by electrostatic repulsion between them (Vitorino et al., 2011). Therefore, NLV carried safranal is a lipid nanoparticle with proper size and stability that has potential to penetrate the BBB.

2.2. Ex vivo fluorescence imaging

Indocyanine green (ICG) is an amphiphilic dye and is generally excited by near-infrared light between 750 nm to 800 nm. The fluorescence intensity is directly proportional to ICG concentration in

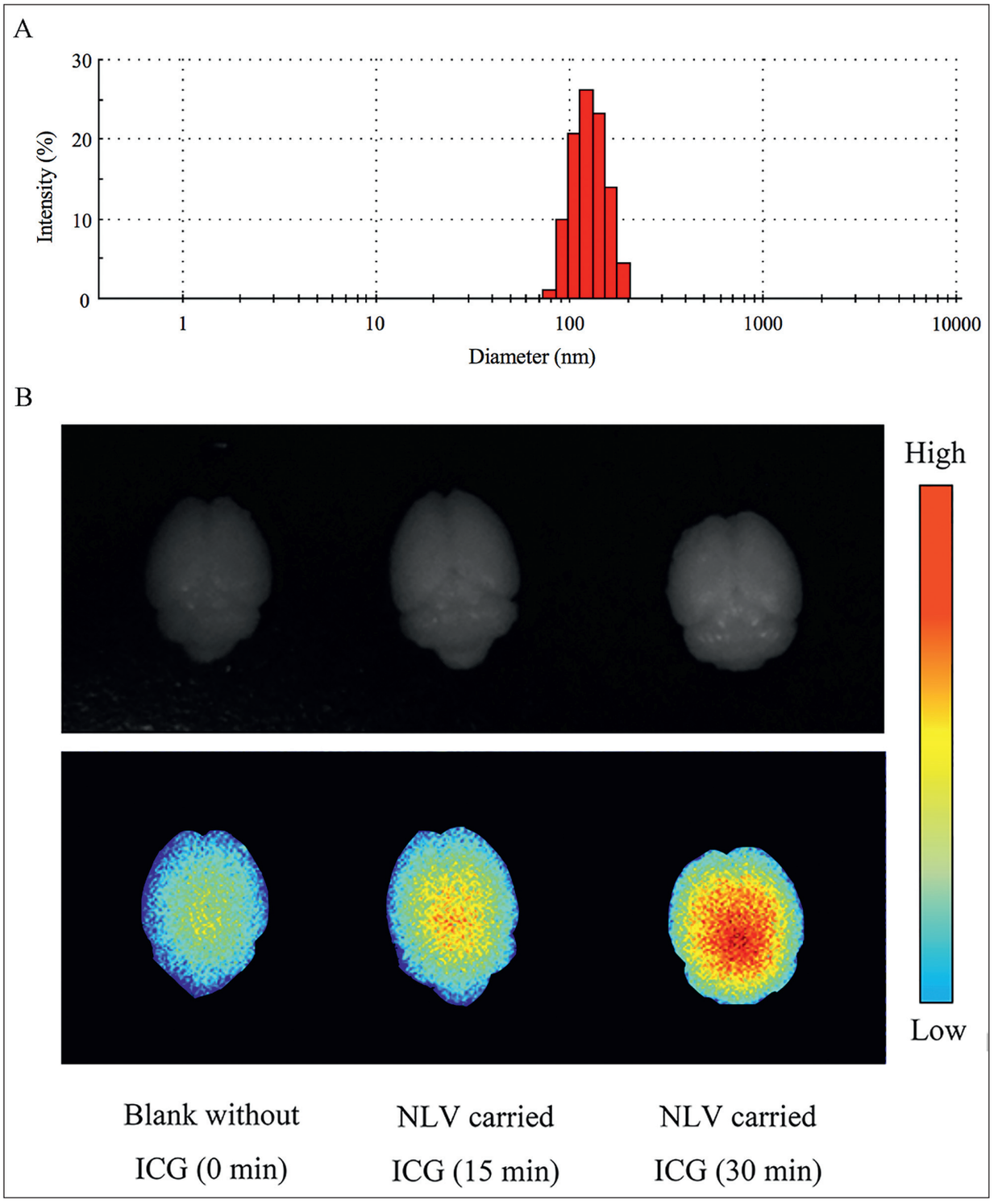

Fig. 1: (A) The particle size of NLV carried safranal dispersion measured by Zetasizer Nano-ZS90. (B) The ex vivo fluorescence images of the mouse brains after intraperitoneal injection of NLV carried ICG. At 15 min or 30 min after injection of NLV carried ICG, the fluorescence of the brains was examined using CRi Maestro Automated In-Vivo Imaging.

tissues (Reinhart et al., 2016). NLV carried ICG was injected into mice intraperitoneally. Fluorescence intensity of the brains became stronger under near-infrared light of 750 nm (Fig.1B) along with time, which suggested that NLV can penetrate into the brain as expected.

2.3. Effect of safranal on acute seizures induced by PILO

In acute seizures induced by PILO, safranal dose-dependently extended latency to generalized seizure ($P<0.05$ for 100 mg/kg vs. control, and $P<0.01$ for 300 mg/kg vs. control, Fig.2A), decreased the highest seizure stages ($P<0.05$ for both 100 mg/kg and 300 mg/kg vs. control, Fig.2B) and the numbers of generalized seizures ($P<0.01$ for both 100mg/kg and 300mg/kg vs. control, Fig.2C) compared to control group, but had no effect on the positive incidence of status epilepticus and mortality. VPA (200 mg/kg), as a kind of AEDs, exhibited an inhibitive action in all above measurements, while the effects of safranal at 300 mg/kg were close to that of VPA in terms of latency to generalized seizure, the highest

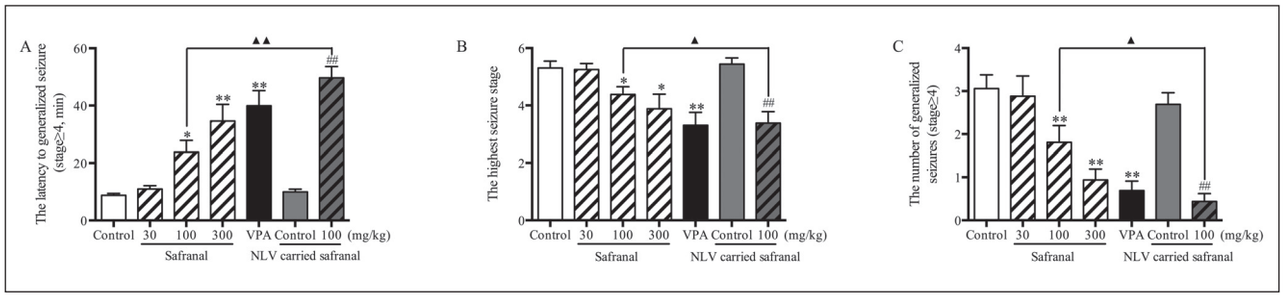

Fig. 2: (A) The latency to generalized seizure (stage≥4); (B) The highest seizure stage; (C) The number of generalized seizures (stage≥4); *:P<0.05, **:P<0.01 vs. control group, #:P<0.05, ##:P<0.01 vs. NLV control group, ▲:P<0.05, ▲▲:P<0.01 vs. Safranal (100mg/kg), n=16 for each group.

Table 1: *P<0.05 vs. Control group; ##P<0.01, #P<0.05 vs. NLV control group, n=16 for each group.

Treatment	Positive incidence of status epilepticus (%)	Mortality (%)
Control group	75.0	68.8
Safranal group (30 mg/kg)	68.8	50.0
Safranal group (100 mg/kg)	37.5	25.0
Safranal group (300 mg/kg)	43.8	31.3
Sodium Valproate group (200 mg/kg)	25.0*	18.8*
NLV control group	81.3	68.8
NLV carried safranal group (100 mg/kg)	25.0##	25.0#

seizure stages and the numbers of generalized seizures (Fig.2 and Table 1). To further study the ability of NLV system, we compared the effects of safranal with NLV carried safranal at the dose of 100 mg/kg. We found that NLV carried safranal group (100 mg/kg) showed a robust anti-seizure effect in all the seizure behaviors compared to NLV control group (Fig.2 and Table1), while NLV control group had no notable difference with control group in any measurement. Notably, NLV carried safranal (100 mg/kg) further significantly extended latency to generalized seizure (P<0.01, Fig.2A), reduced the highest seizure stages (P<0.05, Fig.2B) and decreased the numbers of generalized seizures (P<0.01, Fig.2C) compared to 100 mg/kg safranal group. Its anti-seizure action was even comparable to VPA group. Meanwhile, EEG was recorded continuously for 1 hr in NLV control group and NLV carried safranal (100 mg/kg) group. After PILO injection, EEG spectra power quickly increased in NLV control group, while it was reduced and delayed in NLV carried safranal (100 mg/kg) group (Fig.3).

Seven days after PILO injection, TB staining was performed to observe the histopathological changes of hippocampal CA1 (Fig.4A1-H1) and CA3 regions (Fig.4A2-H2). The number of surviving cells were quantitatively analyzed in each group. Results showed that in control group and NLV control group, a large part of neurons in CA1 and CA3 regions of hippocampus disappeared (49.3±24.4 % and 57.6±28.5 % for CA1; 46.5±24.9 % and 56.5±28.6 % for CA3), which displayed large number of vacuole-like changes, while the remaining neurons were irregular (Fig.4A-H). Safranal group dose-dependently reduced the loss of neurons in CA1 (P<0.01, Fig.4I), but in CA3 the reduction was not significant (P>0.05, Fig.4J). However, VPA group and NLV carried safranal group (100 mg/kg) significantly lowered the loss of neurons to normal level both in CA1 (P<0.01, Fig.4I) and CA3 (P<0.05, Fig.4J). Moreover, neurons in experimental groups show a more intact morphology than that in their respective control groups (Fig.4I, J). The above results suggested that safranal attenuates generalized seizure symptoms and neuronal damage in PILO-induced model, while NLV system can enhance this therapeutic effect.

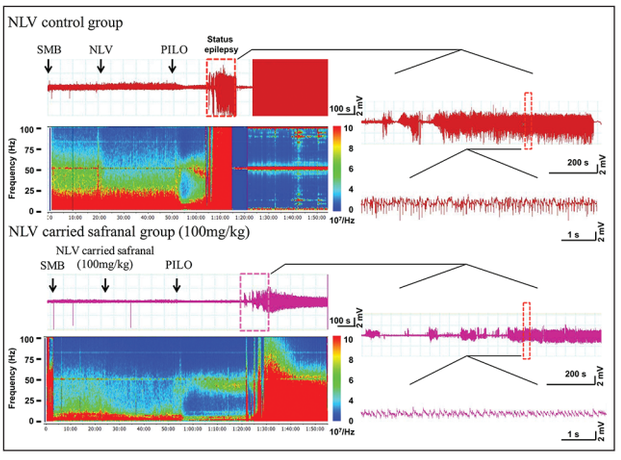

Fig. 3: The EEG was recorded continuously for 1 h in the NLV control group and NLV carried safranal (100 mg/kg) group. FFT was used for spectral analysis with a Hamming window. The FFT output provides the total power between 0 to 100 Hz.

2.4. *Effect of safranal on acute seizures induced by MES*

To further investigate the anti-seizure effect of safranal and NLV carried safranal in acute seizures, we employed another acute generalized seizure model induced by MES. In control group, ultimate electroconvulsive thresholds of most mice (71.4%) were similar as the initial electroconvulsive threshold. Safranal group (100 mg/kg and 300 mg/kg) and VPA group (200 mg/kg) displayed remarkable anticonvulsant effects with decreased percentage of convulsion at initial electroconvulsive threshold (P<0.05 for all compared with the control group, Table 2). Meanwhile, safranal group (100 mg/kg and 300 mg/kg) and VPA group (200 mg/kg) showed markedly increased electroconvulsive threshold (P<0.05 for all compared with the control group, Fig.5). Moreover, NLV carried safranal group exhibited a more remarkable anti-seizure effect compared to safranal group at the same dose (P<0.05, Fig.5). The results showed that safranal has a therapeutic effect in MES induced generalized seizure, while NLV could improve its effect.

Table 2: *P<0.05 vs. Control group; ##P<0.01 vs. Vehicle control group, n=14 for each group.

Treatment	Percentage of convulsion induced by MES (%)
Control group	71.4
Safranal group (30mg/kg)	35.7
Safranal group (100mg/kg)	14.3*
Safranal group (300mg/kg)	7.1*
Sodium Valproate group (200mg/kg)	14.3*
NLV control group	50.0
NLV carried safranal group (100mg/kg)	0.0##

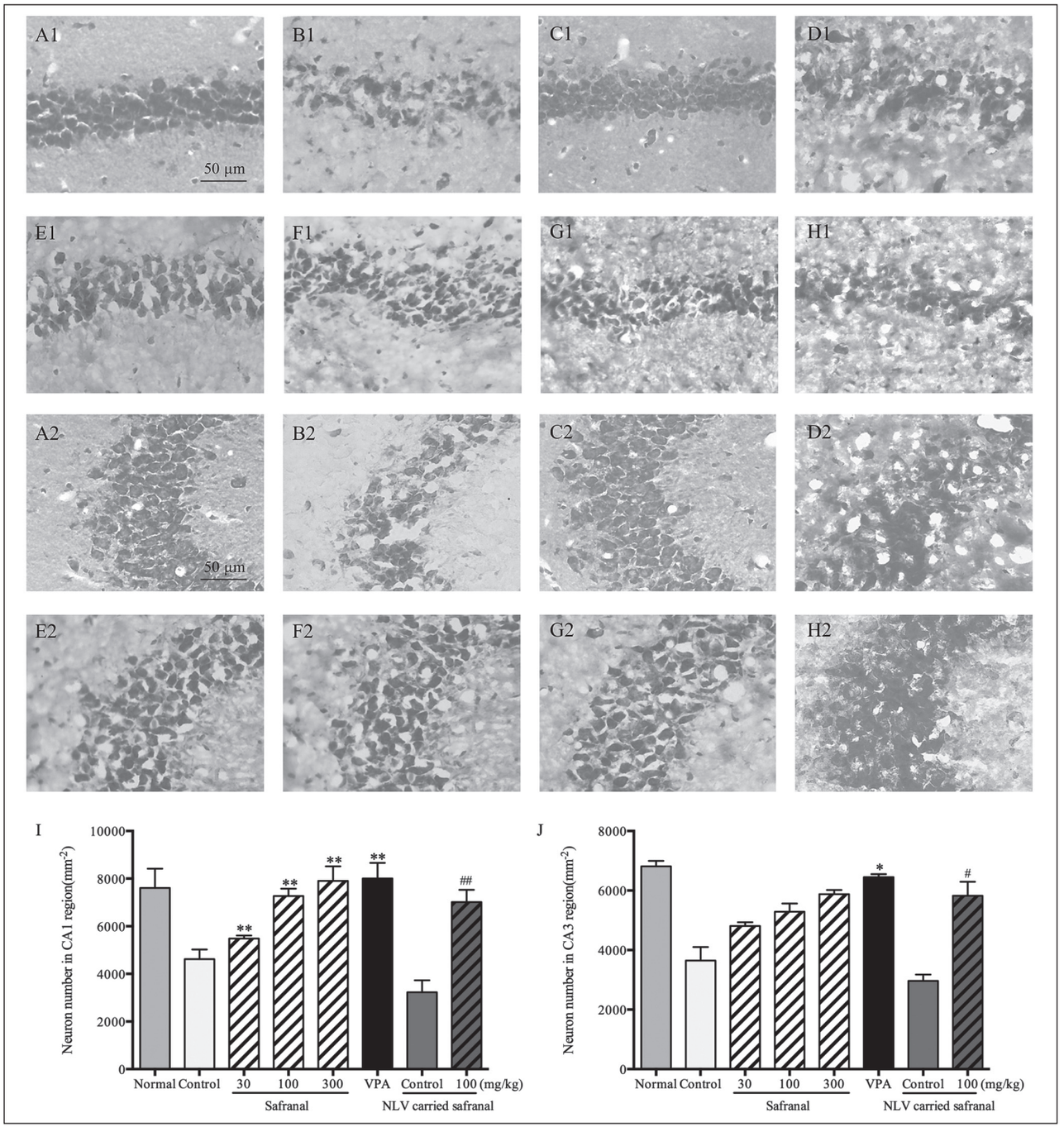

Fig. 4: (1) CA1 region; (2) CA3 region. (A) Normal (without PILO); (B) Control; (C) VPA 200 mg/kg; (D) NLV control; (E) Safranal 30 mg/kg; (F) Safranal 100 mg/kg; (G) Safranal 300mg/kg; (H) NLV carried safranal 100mg/kg; (J) Neuron numbers in CA1 region; (K) Neuron number in CA3 region; *:P<0.05, **:P<0.01 vs. Control group, #:P<0.05, ##:P<0.01 vs. NLV control group, n=4~6 for each group. The Fig.4J is updated to show the same scale of as Fig.4I without the change of data.

2.5. Effect of safranal on chronic seizure induced by PTZ

To further explore whether NLV carried safranal has similar anti-seizure effect on chronic seizure, we adopted PTZ-induced kindling model. In this chronic seizure model, repetitive administration of a sub-convulsive dose of PTZ (50 mg/kg, i.p. every day) leads to a gradual increase in the seizure stage. We found that the treatment with NLV carried safranal (100 mg/kg, i.p.) significantly delayed the kindling rate of progress and the time it took to reach generalized seizures as compared to NLV control group (P<0.05, Fig.6). Our results suggested that NLV carried safranal displays therapeutic effect in PTZ-induced kindling chronic model, especially inhibiting the development of generalized seizure.

3. Discussion

It is widely acknowledged that exploring new effective AEDs is of vital clinical significance for epilepsy control. In this study, the results indicated that safranal dose-dependently inhibits the generalized seizure both in the PILO-induced and MES acute seizure models. Meanwhile, NLV system significantly enhances the anti-seizure effect of safranal. NLV carried safranal exerted a

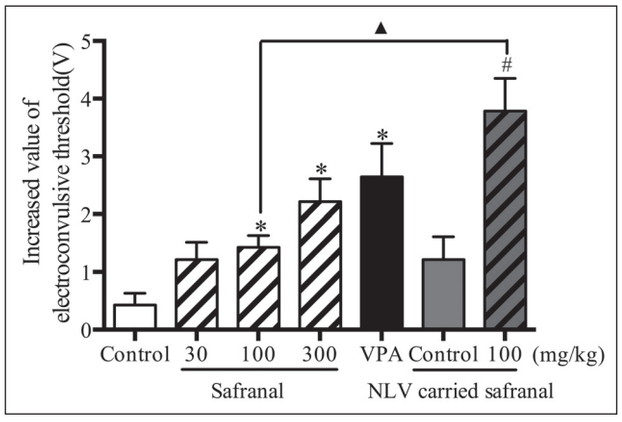

Fig. 5: *:P<0.05 vs. Control group, #:P<0.05, vs. Vehicle control group, ▲:P<0.05 vs. Safranal (100 mg/kg), n=14 for each group.

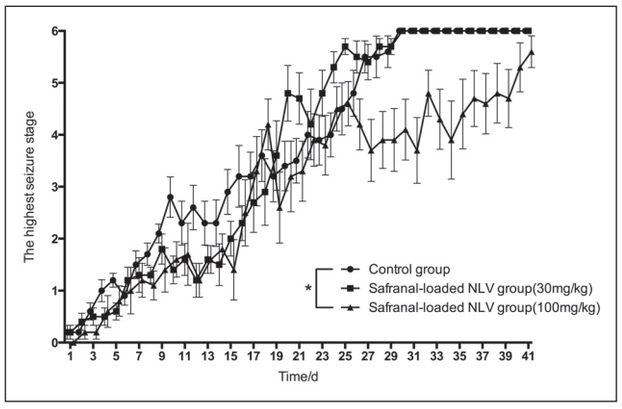

Fig. 6: *:P<0.05 vs. Control group, n=10 for each group.

comparable anti-seizure effect to a clinical AED: VPA, and also inhibits generalized seizure in PTZ-induced kindling model. Taken together, NLV carried safranal may have potential value in treatment of generalized epilepsy.

Epilepsy encompasses a large spectrum of syndromes and diseases with different pathogenesis, including partial seizure, generalized seizure, and status epilepticus. In acute epilepsy models, safranal showed positive effect on latency to generalized seizure, seizure stages, the frequency of generalized seizures, and the change in electroconvulsive threshold, all of which are main indexes of generalized epilepsy. In chronic epilepsy model, there was no difference between NLV carried safranal groups (30mg/kg, 100mg/kg) and NLV control group when the highest seizure level was under stage 4. After the seizure has reached stage 4 or above, NLV carried safranal significantly retarded the kindling acquisition and delayed the development of generalized epilepsy. Therefore, the results mentioned above suggested that NLV carried safranal inhibits generalized epilepsy in mice.

Numerous studies have developed nanostructured drug delivery carriers to enhance the permeability of AEDs through BBB (Bennewitz and Saltzman, 2009; Darius et al., 2000; Yusuf et al., 2012). Safranal is a main constituent of Crocus sativus essential oil which is usually dissolved in organic solvents such as ethanol and methanol (Rezaee and Hosseinzadeh, 2013). In our study, we dissolved it in 10% ethanol and 10% Tween 80, however it stratified in 1 hr. Safranal was found to have insufficient stability as well as limited permeability to the BBB. Thus, we developed a NLV system to successfully deliver safranal into the CNS with great stability according to the measurement results of mean particle size, polydispersity index, and zeta potential of NLV carried safranal.

Additionally, it is essential to relieve the side effects of safranal, since safranal can cause pathological changes both in kidney and lung with the intraperitoneal LD50 values of 1.48 mL/kg in male mice, 1.88 mL/kg in female mice (Hosseinzadeh et al., 2013). According to the previous studies of i.v. and i.p. deliveries of brain-targeted drugs, the NLV system achieves sustained releasing of carried drugs, as well as enhances effectiveness of drugs both in bio-distribution and pharmacokinetics, which may result from various endocytic pathways of endothelial or neuronal cells (Devkar et al., 2014; Peng et al., 2017; Xie et al., 2012). Therefore, when NLV enhances anti-seizure effect of safranal at a moderate concentration, it may also reduce toxicity of safranal to other non-target organs or tissues.

Previous studies reported that safranal can promote the recovery of neuronal functions after spinal cord injury in rats, relating to its anti-apoptotic, anti-inflammatory, and edema-attenuating functions, which suggested that safranal may have therapeutic potentials for various nervous system diseases (Zhang and Wang, 2015). In our study, we found that safranal also displays a neuroprotection with reduced neuronal damage after PILO induced seizure. Moreover, some previous researches reported that monoterpenoids such as linalool exerted depressant effects via competitively inhibiting glutamate receptors. This may contribute to the anti-seizure effect of safranal since it is a monoterpenoid (Atanassova and Roussinov, 1973; Brum et al., 2001). Additionally, it has been reported that upon pretreatment with flumazenil, a blocker of the GABAA-benzodiazepine receptor complex, the anti-seizure effect of safranal could be counteracted (Hosseinzadeh and Sadeghnia, 2007). Consequently, it might be assumed that safranal had an interaction with GABAA-benzodiazepine receptor complex or glutamate receptors, which lead to its anti-seizure effects in both acute and chronic epilepsy models, but these hypotheses still await further investigation.

4. Experimental

4.1. NLV carried safranal formulations

NLV was prepared by stirring and hot-melted method to enhance encapsulation of safranal in nanoliposome. OA as liquid matrix, glycerin monostearate as solid lipid matrix, mixed with safranal, then dissolved in acetone (the weight ratio was 3:7:10, 10% (w/v)) to compose a lipid phase. Tween 80 utilized as surfactant was diluted by deionized water to form a 1% (w/v) aqueous phase. Two phases were separately heated at 40°C under agitation at 600rpm for 30 min. Then, the lipid phase was added drop by drop into the aqueous phase using injectors. Mixture was conducted for 1 hr at 65°C under magnetic agitation at 800 rpm. After agitating for 20 min with sonicator and quickly cooled to 4°C, NLV carried safranal dispersion was obtained after restoring to room temperature. Besides, NLV without safranal could be obtained by the same method mentioned above, while without any safranal added into.

4.2. Determination of particle size and zeta potential

NLV carried safranal was diluted for twenty times with deionized water. Measurements of particle size and zeta potential were determined by Zetasizer Nano-ZS90 (Malvern Instruments, Malvern, UK) at 25°C. All data were measured in triplicate.

4.3. Ex vivo bioimaging

NLV carried ICG (the weight ratio of ICG to polymer was 1:100) was prepared according to the same method applied on NLV carried safranal. After mice were anesthetized with pentobarbital sodium (45 mg/kg), NLV carried ICG was injected (5 ml/kg, i.p.) into mice. At 15 min and 30 min after injection, one of the mice was transcardially perfused with saline and 4% paraformaldehyde. Their brains were separated and fluorescence in the brains was examined using CRi Maestro Automated In-Vivo Imaging.

4.4. PILO-induced acute seizure model

Mice were divided into eight groups, namely normal group without PILO injection, control group (mixture of 10% ethanol and 10% Tween 80), sodium valproate group (200 mg/kg), safranal groups (30 mg/kg, 100 mg/kg, 300 mg/kg; safranal was dissolved in 10% ethanol and 10% Tween 80), NLV control group (NLV without safranal) and NLV carried safranal group (100 mg/kg). Mice were injected with scopolamine methylbromide (1 mg/kg, i.p.) aiming to reduce peripheral cholinergic effects, followed by each group's drug (i.p.) treatment 5 min later. All mice except for those in normal group were injected with PILO (300 mg/kg, i.p.) 25 min later. Seizure stages were recorded during 1 hr after PILO administration, on the basis of Racine scale: stage 0, no abnormality; stage 1, mouth and facial movements; stage 2, head nodding; stage 3, forelimb clonus; stage 4, rearing; stage 5, rearing and falling; stage 6, death (Racine, 1972). Latency to generalized seizure (stage>=4), the highest seizure stages, numbers of generalized seizures (stage>=4), positive incidence of status epilepticus (a state with transient symptoms above grade IV followed by symp-

toms above grade III lasting for at least 5 min) and mortality were recorded. All mice were rescued with diazepam (1 mg/kg, i.p.) at 1 hr after PILO administration.

4.5. Electroencephalogram (EEG) recording and analysis

Mice were mounted in a stereotaxic apparatus with pentobarbital sodium anesthesia (1 mg/kg, i.p.). Electrodes were implanted into right ventral hippocampal CA3 for EEG recording (AP: -2.9 mm, L: -3.0 mm, V: -3.0 mm). Electrodes were connected to mini-receptacles which concatenated skull with dental cement. Mice were allowed 7 days for recovery after the implantation surgery. Then, mice were injected with NLV carried safranal (100 mg/kg, i.p.) and blank NLV respectively 30 min prior to PILO (300 mg/kg, i.p.) injection. EEG was recorded after PILO injection for 1 hr by digital amplifier.

EEG was analyzed by the Neuronscan System as our previous study: each EEG time-series was digitally band-pass filtered from 0.3 to 100 Hz. Fast Fourier transform (FFT) was used for spectral analysis with a Hamming window. FFT output provides the total power between 0 to 100 Hz (Wang et al., 2016).

4.6. Toluidine Blue (TB) staining

On the seventh day after PILO injection, mice were transcardially perfused with saline and 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde at 4 °C for 24 hr, and then in 30% sucrose in 1 mM PBS for 2 d. Frozen brain sections were cut at 10 mM on a cryostat (Leica, Germany). Slide-mounted brain sections were stained in 1% Toluidine Blue solution (pH = 7.5) for 10 min after washed with 1 mM PBS, rinsed by ddH_2O and then decolorized in 75% ethyl alcohol for 5 min and dehydrated in 85%, 90%, 95%, 100% ethyl alcohol for 2 to 3 min. Sections were then cleared in xylene for 2 to 3 min and mounted in a fume hood. Neuronal morphology in hippocampal CA1 and CA3 regions of each section was observed and the number of neurons was counted using ImageJ software.

4.7. MES-induced acute seizure model

Mice were divided into seven groups including control group, sodium valproate group (200 mg/kg), safranal groups (30 mg/kg, 100 mg/kg, 300 mg/kg), NLV control group and NLV carried safranal group (100 mg/kg). In MES model, mice received an electrical stimulus of sufficient intensity to induce maximal seizures of the hind limbs, with tonic extension as endpoint of the test (Toman et al., 1946; Swinyard and Kupferberg, 1985). Initial parameter was a 16 V, 0.5 s, single stimulus. Each mouse was allowed 10 min rest interval until the next electrical stimulation, and then voltage gradually increased in units of 1 V until the presence of tonic extension. The current voltage was the initial electroconvulsive threshold. Mice were then injected with experimental drugs. The ultimate electroconvulsive threshold was determined again 30 min after drug treatment by the same method mentioned above.

4.8. PTZ-induced kindling model

Mice were divided into three groups including NLV control group, NLV carried safranal group (30 mg/kg and 100 mg/kg). Mice were administered with corresponding agents, respectively, followed by the administration of PTZ (50 mg/kg, i.p.) 30 min later every day. Then seizure stage was recorded for 1 hr until the mice reach stage 6 based on Racine scale. Mortality was also recorded during the experiments.

4.9. Statistical analysis

All data was collected in a blind fashion, expressed as means ± SD. Mann-Whitney test was used to analyze difference among the highest seizure stages in PILO-induced acute model. Chi-square test was used for statistical evaluation of incidence of status epilepticus mortality in PILO-induced acute model and percentage of convulsion in MES model. A general linear model was used to analyze the difference in stages among different treatment groups in PTZ-induced kindling model, with consideration of all test days. One-way ANOVA was used to analyze the rest of data. The p-values less than 0.05 were considered to be statistically significant.

Conflict of interest statement: Nothing declared.

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