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Ginsenoside Rg_1 Reduced Spontaneous Epileptiform Discharges and Behavioral Seizure in the Zebrafish

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Abstract : Epileptifrom discharges were induced in the telencephalon of the adult zebrafish via perfusion with pentylenetetrazole (PTZ), bicuculline methiodide, kainic acid-treated artificial cerebrospinal fluid (aCSF), and Mg^{2+}-free aCSF. Ginseng total saponin [GTS (50 μg/ml)] was shown to attenuate the occurrence rate of epilpetiform discharges by 50-75%, compared to the control. Ginsenoside Rg_1 (130 μM) reduced the epileptiform discharges in the isolated telenephalon and delayed the occurrence of behavioral seizures observed from the adult zebrafish placed in the PTZ (10 mM)-containing aquarium water. However, Re was not effective in the suppression of epileptiform discharges and behavioral seizures. These results indicate that Rg_1 may be useful in the control of epileptiform discharges and effective in controlling behavioral seizures, and that the zebrafish can be used as a model animal for the testing of potential anticonvulsant drugs.

Key words : *Panax* ginseng, ginseng total saponin, ginsenoside Rg_1, zebrafish telencephalon, anticonvulsant

INTRODUCTION

The zebrafish, a well-known model system in the field of developmental biology, appears to be a useful system for electrophysiological studies, as the entire brain can be readily isolated and recorded *in vitro*. Our previous study showed that the zebrafish harbors a GABAergic inhibitory system in the adult telencephalon, and bicuculline methiodide (BMI) treatment causes the transformation of electrically evoked field excitatory postsynaptic potentials (fEPSPs) into bursting discharges.[1)] It was further demonstrated that electrically evoked responses in the adult zebrafish telencephalon are mediated via the activity of glutamatergic receptors, and these are transformed into a bursting afterdischarge in the presence of Mg^{2+}-free artificial cerebrospinal fluid (aCSF).[2)]

Recently, extracellular recordings obtained from the optic tectum of the zebrafish larvae revealed ictal and interictal-like electrographic discharges following the application of pentylentetrazole (PTZ), a GABA receptor antagonist.[3)] Epileptiform discharges were suppressed by the commonly-used antiepileptic drugs, valproic acid and diazepam. In addition, the characteristic behavioral patterns of the larvae have been documented as three seizure stage categories in the presence of PTZ. The distinctive seizure behavior makes the zebrafish larvae suitable for the high throughput screening for the antiepileptic drugs[4)] and the seizure liability test for in-development drugs at an early stage in pre-clinical safety assessment.[5)]

In this study, we first attempted to determine whether a blockade of the GABAergic system or the activation of the glutamatergic system would induce an imbalance in synaptic transmission and, eventually, epileptiform discharges in the adult zebrafish. In order to block GABAergic synaptic transmission, GABA receptor antagonists, including PTZ and BMI, were applied to artificial cerebrospinal fluid (aCSF) and incubated with the isolated telencephalon tissue. To activate glutamatergic synaptic transmission, kainic acid was added to normal aCSF, or Mg^{2+} ion was not included in aCSF. We further assessed the anticonvulsant effects of ginsenoside Rg_1 on chemical-induced spontaneous epileptiform discharges in the zebrafish telencephalon.

The major biological effects of ginseng root are known to be mediated by ginseng total saponin, from which more than 30 different ginsenosides have been isolated and chemically identified. Ginsenosides have been shown to be effective in the protection of excitotoxic neural cell death, spinal cord injury, and brain ischemia.[6)] Rg_1 is one of the most extensively studied ginsenosides in the central nervous system, and has been shown to exert a variety of

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effects in the nervous system. In the present study, we demonstrated that ginsenoside Rg_1 suppressed spontaneous epileptiform discharges in the zebrafish brain and the seizure model of adult zebrafish is valid in testing potential anticonvulsants.

MATERIALS AND METHOD

Field potential recording

For electrophysiological study, the brain was removed from the zebrafish under anesthesia, and immediately placed in ice-cold aCSF. The aCSF contained (in mM): 120 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgCl_2$, 2 $CaCl_2$, and 11 D-(+)-glucose. The telencephalon was carefully isolated and incubated in aCSF, which was bubbled continuously at room temperature with 95% O_2/ 5% CO_2 for more than 1 h prior to the recordings. The recording chamber was superfused continuously with aCSF at a flow rate of 1-2 ml/min. The recording electrode was filled with aCSF and placed in the dorsal telencephalon. Extracellular field potentials were amplified utilizing a DAM differential amplifier (World Precision Instruments, Sarasota, FL, USA) and the output was digitized online. The power spectral density of field potentials was calculated during 30 s of epileptiform discharges in 0.1-100 Hz frequency band by summing all spectral values in a given frequency range using pClamp 8.0 (Molecular Devices, Sunnyvale, CA, U.S.A).

Pentylentertazole (PTZ), kainic acid (KA), 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX), d-2-amino-5-phosphonovalerate (APV), bicuculline methiodide (BMI), and ginseng total saponin (GTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ginsenosides Rg_1 and Re were acquired from Btgin (Gumsan, Chungnam, Korea). All data were expressed as the means±standard errors unless otherwise specified. Statistical significance was evaluated via two-tailed Student's t test.

Behavioral monitoring

In order to induce the behavioral seizure, zebrafish were placed individually in 500 ml beakers containing 10 mM PTZ-treated aquarium water. For analysis of seizure stages, swimming behavior was monitored using a high-resolution video camera, recording sessions (60 min) were stored on standard alone DVR and later analyzed off-line.

RESULTS AND DISCUSSION

After stable fEPSPs were recorded for 10 min, GTS was added to the perfusing aCSF. Typical fEPSPs had a negative peak at a latency of 20-30 ms. Suppressive effect was shown 5-10 min after the treatment of GTS, and recovered within 20 min after washout. Effect of GTS was dose-dependent: the degree of reduction was 28.4±16.3%, 53.5±13.2%, and 68.5±19.0% at 10, 25, and 50 μg/ml, respectively (p<0.001, n=5) (Fig. 1A, B). To test whether the suppressive effect of GTS is dependent on the glutamatergic receptors subtypes, fEPSPs was induced in two different conditions. When electrically evoked fEPSPs was recorded in the presence of BMI, a $GABA_A$ receptors antagonist, and AP5, an NMDA receptors antagonist, the amplitude of fEPSPs was increased with bursting activities of late latency, which is mainly mediated non-NMDA receptors. Similarly, increased fEPSPs of late latency was recorded under the condition of Mg^{2+}-free aCSF and CNQX, a non-NMDA receptors antagonist, which is thought to be mediated through NMDA receptors. GTS (25 μg/ml) had a significant suppressive effect on both types of fEPSPs by 54.2% (n=8) and 34.8% (n=5) of the control (Fig. 1C, D).

The effects of several convulsants were studied with regard to their capacity to generate epileptiform discharges in the telencephalon of the adult zebrafish. Epileptiform discharges were induced via KA, BMI, and PTZ treatment, and when perfused with Mg^{2+}-free aCSF (Fig. 2). The discharges consisted of both interictal and ictal discharges. The ictal discharge involves an early large spike and the following bursting phase. The occurrence rate and duration varied slightly along experimental conditions when measured for 5 min after 10 min of drug treatment: in general, the interictal discharge was 150-250 ms in duration in KA (n=4), BMI (n=5), and PTZ (n=5)-treated groups. In Mg^{2+}-free aCSF, the interictal discharges occurred with duration of 250–300 ms. The ictal discharge durations were 2-20 s, and the occurrence rate was about 0.02/s in BMI, and PTZ groups. The duration and occurrence rates of the ictal discharges were 1-3 s and about 0.25/s, respectively, in KA and Mg^{2+}-free aCSF groups (n=5).

The effect of GTS on chemical-induced spontaneous epileptiform discharges was assessed. GTS (50 μg/ml), which was added for 10 min to perfusing aCSF, suppressed the epileptiform discharges within 3-5 min after treatment, and this occurred in a reversible manner. In Mg^{2+}-free aCSF, GTS reduced the occurrence rate of interictal discharge by 72.2±14.9% (Mg^{2+}-free aCSF; 0.22 ±0.15/s, GTS; 0.06±0.02/s) and the occurrence rate of ictal discharge by 82.4±17.5% (Mg^{2+}-free aCSF; 0.12±0.05/s, GTS; 0.02±0.01/s, n=5, p<0.001) (Fig. 2A). In PTZ group,

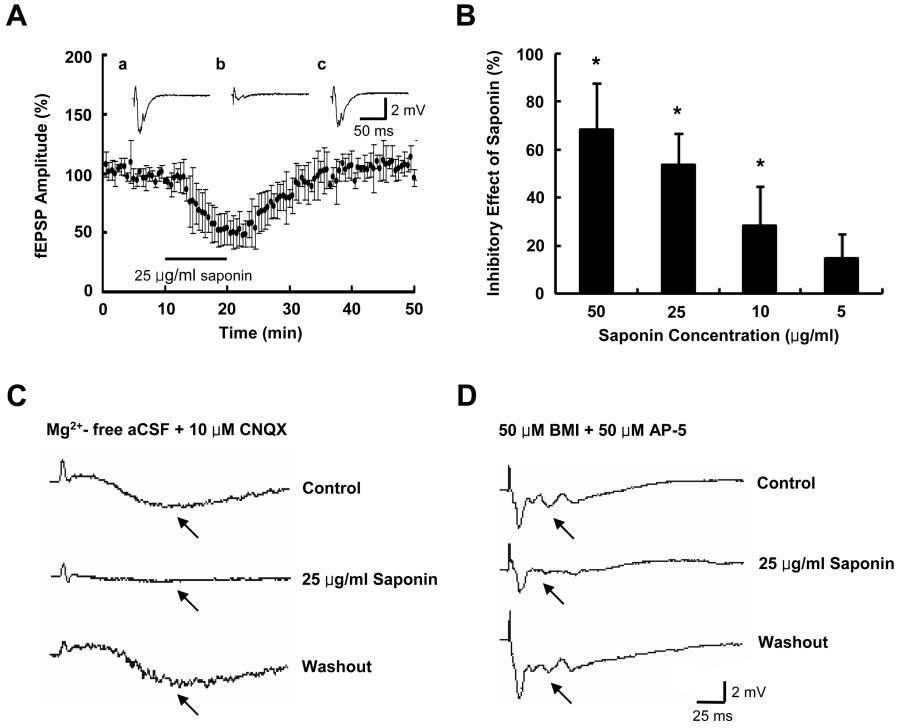

Fig. 1. Suppressive effects of GTS on fEPSPs in the telencephalon of the zebrafish.
Treatment with GTS (25 μg/ml) for 10 min reduced electrically evoked fEPSPs, which returned to the control level 20 min after washout (A). Representative traces are shown at the top. GTS effects were dose dependent: 5, 10, 25, and 50 μg/ml GTS reduced fEPSPs by 14.6±10.1%, 28.4±16.3%, 53.5±13.2%, and 68.5±19.0% of the control, respectively (B). Prolonged fEPSPs from brains perfused Mg^{2+}-free aCSF containing non-NMAD receptors antagonist, CNQX (10 μM), was completely blocked by 25 μg/ml GTS (n=8) (C). Electrically evoked bursting activity of late latency (indicated by arrow) in the presence of BMI (50 μM) and AP5 (50 μM) was also reduced by 25 μg/ml GTS (n=5).

the occurrence rates of interictal and ictal discharge were significantly reduced during 10 min GTS treatment by 70.4±16.9% (PTZ; 0.30±0.12/s, GTS; 0.09±0.03/s) and 72.5±13.4% (PTZ; 0.12±0.08/s, GTS; 0.03±0.02/s, n=5, $p<0.001$), respectively (Fig. 2B). In KA group, GTS reduced the occurrence rates of interictal discharges by 77.1±14.6% (KA; 0.3±0.25/s, GTS; 0.07±0.03/s), and ictal discharges by 62.5±15.2% (KA; 0.10±0.09/s, GTS; 0.03±0.02/s, n=4, $p<0.001$) (Fig. 2C). Similarly, in BMI group, the occurrence rates of interictal and ictal discharges were also reduced following GTS treatment by 64.2±19.8% (BMI; 0.24±0.15/s, GTS; 0.09±0.03/s) and 57.7±20.5% (BMI; 0.15±0.10/s, GTS; 0.06±0.04/s, n=5, $p<0.001$), respectively (Fig. 2D).

Ginseng total saponin contains more than 30 ginsenosides that are classified into protopanaxadiol (PD) and protopanaxatriol (PT) GTS according to the number of hydroxyl residues attached to carbon rings of the aglycon structure. In our previous study, Rg_1, one of the most abundant PT GTS, inhibited fEPSPs in a dose-dependent manner, with an approximate 60% reduction at 100 μg/ml.[8] Meanwhile, Re, a PT GTS, was not effective in the suppression of fEPSPs. Therefore, the effects of ginsenosides Rg_1 and Re on spontaneous epileptiform discharges were assessed in Mg^{2+}-free aCSF. A high dose of Rg_1 (130 μM), added for 10 min to perfusing aCSF, suppressed the epileptiform discharges within 5 min after treatment in a reversible manner (Fig. 3A). In Mg^{2+}-free aCSF, Rg_1 significantly reduced the occurrence rate of interictal discharge by 89.6±11.6% of the control (n=5, $p<0.01$). The occurrence rate of ictal discharge was also reduced from 0.32/s to 0.06/s on average by 81.8±9.9% of the control during 10 min treatment with Rg_1 (n=5, $p<0.001$). The duration was 0.8±0.4s (n=5), as compared to the controls (3.1±1.4s, n=5, $p<0.001$). A low dose of Rg_1 (65 μM) also tended to reduce the occurrence rates of interictal and ictal discharges by 38.3±17.4% and 14.5±19.9% (n=6) of the control with no significance (data not shown). A high dose of Re (110 μM) did not show any suppressive effects (Fig. 3B). In addition, we analyzed the power of field

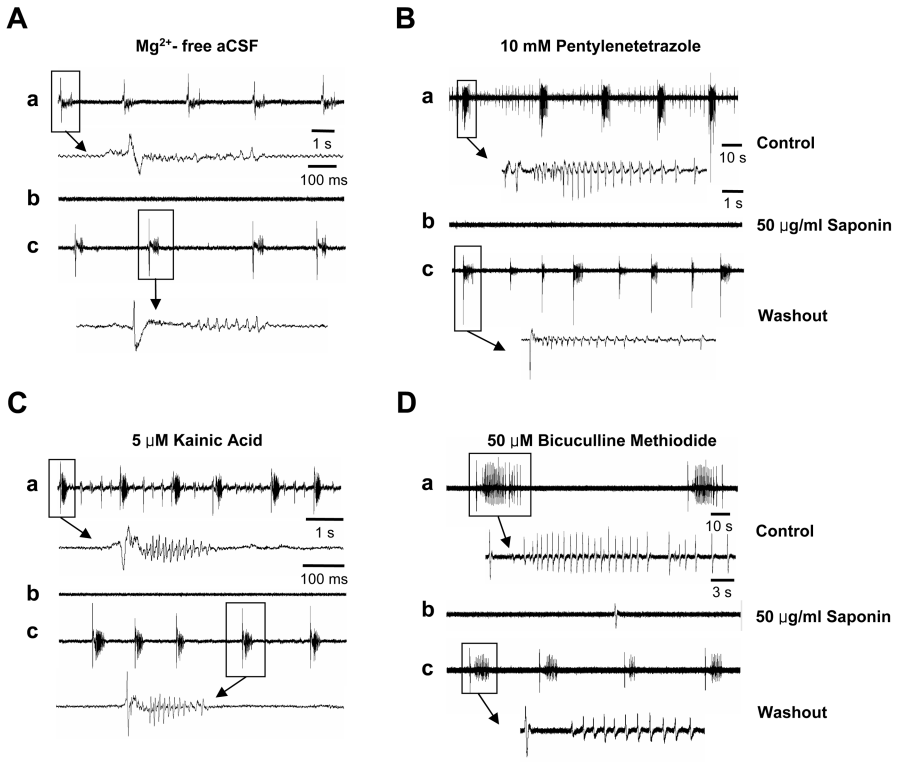

Fig. 2. Suppressive effects of GTS on spontaneous epileptiform discharges in the telencephalon
Spontaneous epileptiform discharges were induced via perfusion with Mg^{2+}-free aCSF or via the addition of normal aCSF with KA (5 μM, n=4), PTZ (10 mM, n=5), or BMI (50 μM, n=5). The occurrence rate of ictal discharges was about 0.25/s in KA and Mg^{2+}-free aCSF group, and about 0.02/s in PTZ and BMI groups. The duration varied in a range of 1-20 s. Under all conditions, the spontaneous epileptiform discharges were significantly suppressed during 10 min treatment with GTS (50 μg/ml).

potential during epileptiform discharges recorded under conditions of Mg^{2+}-free aCSF treatment in the 0.1-100 Hz frequency band. Following the application of a high dose of Rg_1, the power of epileptiform discharges is reduced to 86.8±11.5% of the control values in a reversible manner (n=5, p<0.001), with no specific band (Fig. 3C). Treatment with 110 μM Re did not suppress epileptiform discharges, as assessed via power spectrum analysis (Fig. 3D). These differential effects of Rg_1 and Re rule out the possibility that the suppression of epileptiform discharges by Rg_1 was the result of non-specific action. The differential effects of Rg_1 and Re have been also observed in studies of the neuroprotective effect on oxidative stress by H_2O_2 and excitotoxic damages by KA in primary spinal cord neurons; Rg_1 was effective, but Re was not.[9] It seems premature to categorize the suppressive action of ginsenosides according to the hydroxyl residues, as Rb_1, a PD GTS, suppressed fEPSPs to a substantial degree in the rat hippocampus. Furthermore, intraperitoneal injection of Rb_1 and Rb_3 mixtures has been demonstrated to exert anticonvulsant effects in the rat, which delayed seizure onset and severity in kainic acid or pilocarpine-injected rats.[10]

Furthermore, the effects of Rg_1 and Re were tested on behavioral seizure in the adult zebrafish. Stock solutions of Rg_1 and Re were made in 40% ethanol and the final content of ethanol in the medium was less than 0.05%. The behavioral seizure was most consistent and reliable in the zebrafish placed in the aquarium water containing 10 mM PTZ for 1 h, which showed initial small twitch at the tail and trunk, and abrupt fast random movement, and eventually, complete loss of balance. The seizure behavior started to occur 1-3 min following PTZ treatment and reached the stage of loss of balance in 5-10 min. The prolonged presence of the fish in the bath caused repetitive occurrence of behavioral seizure. The time to show the first loss of balance and the number of loss of balance during 10 min after occurring of first seizure were 104.5±45.1 and 42.7±22.3 s in PTZ-treated zebrafish, respectively (n=16) (Fig. 4). The time to show the first loss of balance was delayed by addition of Rg_1 (130 μM, 294.0±78.4 s, P<0.001) and valproic acid (100 μM, 265.3± 89.8 s, p<0.05). The number of balance loss observed dur-

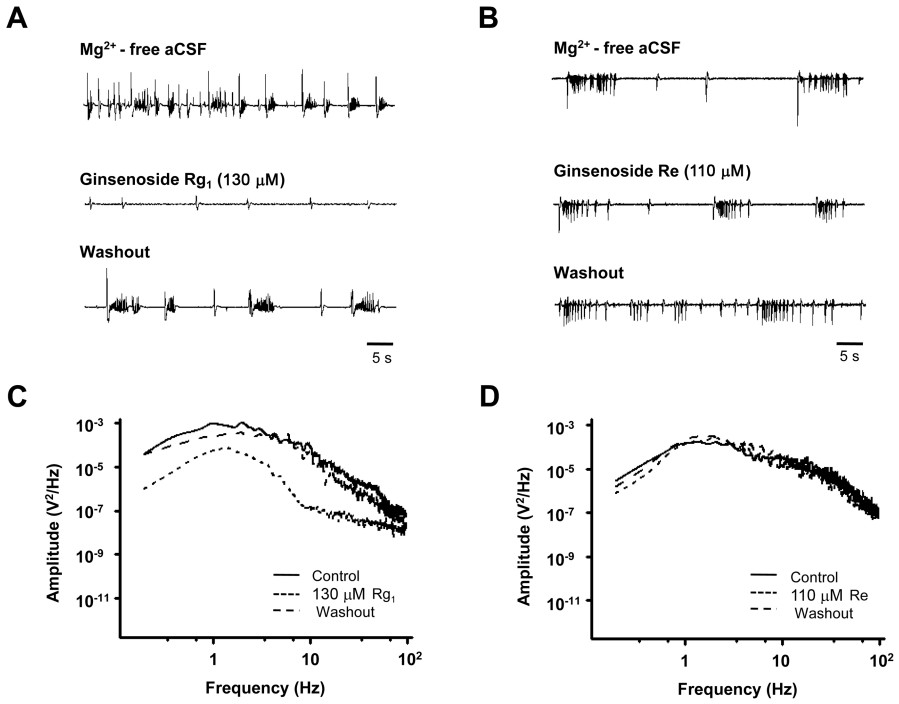

Fig. 3. Suppressive effects of Rg_1 on spontaneous epileptiform discharges in the telencephalon in Mg^{2+}-free aCSF
(A) Treatment with Rg_1 (130 μM) for 10 min reduced epileptiform discharges in terms of occurrence rate and duration, both of which returned to control levels 20 min after washout. The occurrence rate of ictal discharge was reduced to 0.06±0.02/s, as compared to the controls (0.32±0.14/s, n=5, p<0.001). The duration was also reduced to 0.8±0.4 s, as compared to the controls (3.1±1.4 s, p<0.001). (B) Power spectra showed Rg_1-mediated attenuation of epileptiform discharges in a reversible manner. (C) Treatment with Re (110 μM) did not affect epileptiform discharges. The occurrence rate was 0.08±0.04/s, as compared to the control (0.07±0.02/s, n=6). The duration was 3.4±2.1 s (n=6), as compared to the control (6.0±4.5 s). (D) Power spectra of epileptiform discharges before, during, after Re treatment. Three traces were overlapped, showing no decreases in epileptiform discharges.

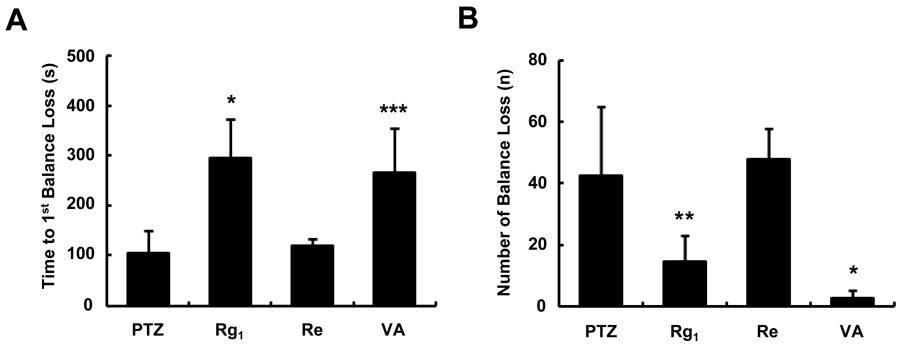

Fig. 4. Effects of Rg_1, Re, and valproic acid on PTZ-induced behavioral seizure in the zebrafish
(A) The time to show the first loss of balance was delayed by addition of Rg_1 (130 μM, 294.0±78.4 s) and valproic acid (100 μM, 265.3±89.8 s), but not by Re (110 μM, 119.3±12.5 s), compared to the control (104.5±45.1 s). (B) The number of balance loss observed during 10 min after the first balance loss was reduced by Rg_1 (130 μM, 14.6±8.3, n=7) and valproic acid (100 μM, 2.7±2.6, n=10), but not Re (110 μM, 48.0±9.9, n=3), compared to the control (42.7±22.3, n=16). When 200 μM valproic acid was treated, no loss of balance was observed (n=6). Drugs were pretreated 2 h before PTZ treatment and present throughout the experiment.

ing 10 min was also reduced by addition of Rg_1 (14.6± 8.3, n=7, p<0.01) and valproic acid (2.7±2.6, n=10, p< 0.001). More significantly, when 200 μM valproic acid was treated in the PTZ-contained water, no loss of balance

was observed at all during 1 h experimental period (n=6), suggesting that the seizure model of adult zebrafish is valid in testing potential anticonvulsants. Re (110 μM) appeared to increase behavioral seizure with no significance; the time to show the first loss of balance was not delayed (119.3±12.5 s) and the number of balance loss tended to be increased (48.0±9.9, n=3).

Consistent with the suppressive effect on epileptiform discharges, Rg_1 also attenuated the behavioral seizure, measured from loss of balance of the swimming zebrafish. The effect of Rg_1 on the behavioral seizure was less prominent than that on the spontaneous epileptiform discharges. However, this discrepancy is unlikely due to the inadequacy of zebrafish model, as valproic acid successfully blocked PTZ-induced behavioral seizure. The possible explanation is the presence of blood-barrier of brain (BBB) in the zebrafish, which may be partially permeable to ginsenosides. The epithelial tight junction-based BBB of the zebrafish has been reported to appear as early as 3 days post fertilization and mature with age.[11] The degree of BBB permeability of Rg_1 is still unknown and difficult to be determined; because ginsenosides may undergo metabolic modification such as deglycosylation[12] and their metabolites may have a high BBB permeability. To our knowledge, few studies have been reported on the comparison of Rg_1 effects *in vivo* and *in vitro*. In a recent study, intracerebroventricular infusion of Rb_1 (60 or 600 ng/day), a PD GTS, was shown to prevent apoptosis and upregulate antiapoptotic factor Bcl-xL expression in the ischemic hippocampus of the gerbil.[13] In the same study, similar effects of intravenously injected Rb_1 were also shown on apoptosis and Bcl-xL expression in the cortex of the middle cerebral artery-occluded rat, but at much higher dose of 6 or 60 mg/day in comparison with intracerebroventriclular infusion. Antiapoptotic effects of Rb_1 were also demonstrated, along with upregulation of Bcl-xL expression, in sodium nitroprusside-treated cultured cortical neurons at dose of 1 or 100 fg/mL.[13] These results are supportive of our results that the effective doses of Rg_1 were different in suppression of epileptiform discharges and behavioral seizure. Therefore, it is postulated that only partial portion of Rg_1 in the aquarium water is transported into the zebrafish brain. Further efforts are essential to produce derivatives of Rg_1 with higher transportability to the brain and to determine the structural moiety responsible for the suppression of neural activity, particularly given that ginsenoside Rg_1 is one of major components of ginseng, a traditional herbal medicine of which no serious side effects are documented.

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