Validation of the Zebrafish Pentylene Tetrazol Seizure Model: Behaviour Assay for Assessing Anti-Epileptic Drug Efficacy

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Abstract

The availability of zebrafish larvae model of epileptic seizure provides opportunities to identify novel anticonvulsants for treatment of people with epilepsy. However, the major parameters of zebrafish behavior assay for assessing antiepileptic drug efficacy existed disparity, which resulted in different results in previous studies. In this study, we chose the high, medium, slow-speed moved distances and the total distances moved for seizure-like activity quantification in individual wells of a 48-well plate in the dark phase and used this zebrafish seizure model to assess three commonly prescribed anti-epileptic drug’s efficacy and screen bioactive components from plant extract. Results showed that the high-speed moved distances were given a more reasonable and sensitive dose-response curve than the total distances in zebrafish larvae model exposed to 10 mM pentylene tetrazole. Besides, we also optimized the vehicle (dimethyl sulfoxide, DMSO) concentration used in epileptic behaviour assay. The three anti-epileptic drugs, phenytoin, valproate sodium and carbamazepine showed the same efficacy patterns in zebrafish seizure model as in mammalian epileptic models. We also found four positive hits from plant (Vaccinium spp.) extract in primary drug screening, two hits exhibited concentration-dependent inhibition of locomotor activity, confirming their anticonvulsant characteristics. These results indicated that this zebrafish larvae model could be useful for assessing anti-epileptic drug efficacy, facilitating the primary drug screening and evaluating of effective components in medicinal plants.

Keywords: Disease model; Epilepsy; Methods; Pentylene tetrazole; Seizure; Zebrafish larvae

Introduction

Epilepsy is a complex brain disorder marked by recurrent spontaneous epileptic seizures associated with typical neurobiological and behavioural alterations [1-3]. In the past few decades, rodent seizure models induced by a broad spectrum of mechanisms have contributed significantly to our knowledge on epilepsy and anti-epileptic drugs (AEDs) discovery [4-7]. Zebrafish larvae have recently emerged as a new species for chemoconvulsant-based models of epilepsy [8-10]. Because of the genetic similarity between zebrafish and humans, zebrafish larvae offer significant advantages for high-throughput drug screening [8,11-13]. Following on work first described by Baraban et al. in 2005 [14], several groups have confirmed the abnormal seizure-like behaviors, ictal and interictal like electrical activity and c-fos expression in brain regions when zebrafish larvae exposed to pentylene tetrazole (PTZ) [15]. With regard to anticonvulsant screens, medium-to-high-throughput screening is possible in 96-well format using an automated locomotor tracking system for the sensitivity to common antiepileptic drugs in this model [16]. Also, several groups developed a seizure model in zebrafish larvae and made antiepileptic drugs screening from nature plant [10,17]. However, the major parameters of zebrafish behavior assay for assessing antiepileptic drug efficacy existed disparity, which resulted in different results in previous studies [3]. Moreover, a question remained as to whether an AED-induced decrease in locomotion is truly indicative of anticonvulsant activity, as some drugs may impair larval movement through other mechanisms such as general toxicity or sedation. So, there still remained some uncertainties to clear before using PTZ-induced zebrafish larvae epileptic seizure model for anticonvulsant drug screening widely. In this study, we performed another behavioural seizure analysis of PTZ-induced seizures, refining parameters of the behavioural PTZ assay and assessing three different antiepileptic drug’s efficacy. We also used this PTZ-induced seizure model to discover anticonvulsant compound from plant extract. We have demonstrated that this model can be useful for AEDs primary screening. This will also provide an alternative method for test potential therapeutics of epilepsy.

Methods

Zebrafish-handling

Adult zebrafish (Danio rerio. AB strain) were housed in a light- and temperature-controlled aquaculture facility with a standard 14:10 hr light/dark photoperiod. Four to five pairs of zebrafish were set up for nature mating every time. On average, 200 - 300 embryos were generated. Embryos were collected and staged from multiple AB strain breeding pairs and pooled. Following 6 and 24 hpf (hours post fertilization) in an incubator in E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4) unfertilized embryos were removed and the remainder placed in a re-circulating Tecniplast aquatic system at 28°C (0.2% Instant Ocean Salt in deionized water, pH 6.9 - 7.2, conductivity 480–510 μS/cm and hardness 53.7 - 71.6 mg/L CaCO3) [18]. Also, embryos raised on the system displayed more consistent activity patterns than those reared in a petri-dish. After completing the experiments, all larvae were sacrificed through administration of an overdose of anesthetic (tricaine). The zebrafish facility at Hunter Biotechnology, Inc. is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAA LAC) International.

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Drug treatment

Pentylene tetrazole (PTZ, Sigma, CAS No. 54-95-5) was used to induce zebra-fish epileptic seizure. Three AEDs carbamazepine (CBZ, ACR ORGANICS, CAS No. 98-46-4), phenytoin (PHT, J&K, CAS No. 630-93-3), valproate sodium (VPA, Sigma, CAS No. 1069-66-5) and negative control drug aspirin (Sigma, CAS No. 50-78-2) were selected for the development and validation of zebra-fish epileptic seizure model. Stock solutions were prepared in 100% dimethyl sulfoxide (DMSO) and stored at -20°C; serial dilutions (1%, 2% and 3% DMSO) were made before each experiment. All compounds were dissolved in DMSO and diluted in embryo medium to achieve a final DMSO concentration of 1% w/v. For zebra-fish epilepsy model validation, PTZ was added to fish water to reach the desired concentrations ranging from 1.25 mM to 30 mM. Control embryos were raised in fish water without PTZ. For testing embryo response to anticonvulsants, AEDs and aspirin were added to the fish water to reach the indicated working concentrations 1 hour before the embryos were exposed to 10 mM PTZ for behavioral analysis and other examinations. For AEDs primary screening, the concentrations of plant extract were the maximal tolerable concentration for zebra-fish larvae.

Behavioural analysis

5 dpf (days post fertilization) zebra-fish larvae were pre-incubated in 100 μl of AED, aspirin or vehicle for 1 hr in individual wells of a multi-well plate at 28°C in the dark. In model optimization experiment, a 48-well and a 96-well plate was used respectively. For AEDs primary screening from plant extract, we performed in individual wells of a 48-well plate. At least eight larvae were used for each group and every experiment was performed in duplicate. The concentrations of AEDs and aspirin used were described in the results section. After the pre-incubation, 100 μl of embryo medium or 100 μl of PTZ solution was added. Larvae were allowed to habituate for 10 min in a dark chamber of an automated tracking device (ZebraBoxTM apparatus; Viewpoint, Lyon, France). Control groups were embryos of the same stages without any chemical treatment. The locomotor activity was then quantified using ZebraLab TM software (Viewpoint, Lyon, France) [19]. The system consists of an infrared light source, a high-resolution digital video camera to capture larval movements within a defined time period (60 min in our experimental set-up) and the software to analyze larval locomotor activity. The total and high-speed moved distances were chosen for subsequent efficacy assay. Efficacy of a test drug on epilepsy was calculated based on the formula below:

Drug effect on epileptic locomotor activity (%) = \[ \frac{[1- \text{Distance (vehicle)} \text{Distance (drug)}] \times 100}{\text{Total Distance}} \]

C-fos gene expression analysis

For RT-PCR, 5 dpf larvae were exposed to PTZ, PTZ + AEDs, or fish water, and then the total RNA was isolated from the whole larvae (n = 50 per group) using single TRI reagent (Sigma, catalog # T9424) and cDNA was prepared from 1 μg of the total RNA per group using RT-PCR kits (TOYOBO, catalog # FSK-100) in a 20 μl volume. Each PCR was carried out in triplicate in a 25 μl volume using Taq PCR MasterMix kits (Bioteke, catalog # PR 1701) on Lifepro Thermal Cycler System (BIOER, China) with standard parameters. C-fos cDNA was amplified with forward primer: 5'-AACGTCTACCGGGATCTCCTT-3' and reverse primer: 5'-GCCAGCTGTATGGTCCAGA-3'[14], the predicted size of the amplified cDNA fragment was 1030 bp. β-actin used as internal control were 5'-CATCAGCTAGCTCTGTC-3' and forward: 5'-GACGCGCATGAGGTACAGAGA-CACCCCT-3' (reverse). All the primers used for RT-PCR analysis were synthetized by Invitrogen.

Statistical analysis

One-way ANOVA followed by the Dunnett’s multiple comparison tests was used to compare differences among groups. All statistical analyses were performed using SPSS 16.0 software (SPSS, USA) and P < 0.05 was considered statistically significant. For quantitative analysis, all data were presented as mean ± SEM and results were statistically compared between drug-treated and vehicle-treated zebra-fish groups.

Results

Pro-convulsant treatments

To induce seizures, PTZ (1.25 - 30 mM) was added to fish water. PTZ added to fish reliably elicited distinct seizure-like behaviors in a concentration-dependent manner. Zebra-fish larvae exhibited signs of agitation within seconds after exposed to PTZ. The larvae swam along the periphery of the multi-well plate, thus displaying stage I seizure-like behaviour as described previously [15]. This was succeeded by rapid ‘whirlpool-like’ movement, and then followed by a short pause before swimming in a rapid, jerky manner with occasional body-stiffening and loss of posture (larva turning onto its side or back). These events could be likened to tonic and clonic seizure phases in mammals (stages I - III) [20]. However, the results were obviously different when the locomotor activity of zebra-fish seizure model was quantified by different parameters; the high-speed (V >20 mm/s) moved distance was given a more reasonable and sensitive dose-response curve than the total distance (Figures 1A and 1B). PTZ at 10 mM was selected for following behaviour test, which induced the most significant increase in locomotor activity within 1 hr. In order to avoid the influence on the experimental results of improper use of DMSO, serial dilutions (1%, 2% and 3%) were added after PTZ-induced seizure model. A 48-west and a 96-west plate were also used respectively for behavioral analysis. Results showed that the high-speed moved distance decreased significantly in 2% and 3% (P < 0.01) DMSO groups when a 48-west plate was used. However, the high-speed moved distance decreased significantly (P < 0.05 or P < 0.01) in serial dilutions DMSO when a 96-west plate was used (Figures 2A and 2B). Therefore, our results demonstrated that if zebra-fish larvae were placed in individual well of 96-west microplate, there would be more false positive than in 48-west microplate, and even 1% DMSO treated alone, the most used vehicle, would give a significant positive effect on zebra-fish seizure-like movement.

Evaluation of the anticonvulsant activity of CBZ, PHT, VPA and aspirin in the zebrafish PTZ model

The capacity of the investigated drugs to reduce PTZ-induced
convulsions in zebrafish larvae was assessed through quantification of the high-speed moved distance. A sample movement plot was given in Figures 3A-3C. Individual drug concentration/response data were given in Figures 4A-4D. As expected, PHT and VPA respectively suppressed zebrafish larvae locomotor activity by 7.4 - 47.2% and 15.6 - 16.6% when comparing with PTZ treated alone group. Statistically significant positive effect on zebrafish larvae seizure-like motility was observed at 300 μM and 1000 μM for PHA (P < 0.01), 1000 μM and 3000 μM for VPA (P < 0.01). Whereas CBZ promoted zebrafish larvae movement by 26% at 30 μM and 116.7% at 100 μM (P < 0.01), and sharply suppressed zebrafish larvae motility about 94.8% at 300 μM (P < 0.01) when comparing with PTZ treated alone group. Aspirin had no significant effect on zebrafish larvae locomotor activity.

Assessment of changes in c-fos gene expression by RT-PCR

Using semi-quantitative approach, our experiment showed an obvious increased expression of c-fos mRNA during PTZ exposure (P < 0.01). CBZ at 30 μM and 300 μM could partially rescued PTZ-evoked abnormal increase of c-fos expression (P < 0.05 and P < 0.01 respectively). The results of c-fos gene expression were not well consist with the behaviour assay. Aspirin had no significant effect as expected (Figures 4A and 4B).

Primary drug screening from plant (Vaccinium spp.) extract in PTZ-induced seizure model

For AEDs primary screening, ten different concentrations of plant (Vaccinium spp.) extract compounds were the maximal tolerable concentration for zebrafish larvae. 1% DMSO was made before each experiment. In 10 mM PTZ-induced seizure model, compound 2 (at 1 M), 5 (at 300 μM), 6 (at 1 M) and 7 (at 1 M) could partially rescued PTZ-evoked abnormal behavior (P < 0.05 and P < 0.01 respectively) in individual well of 96-well microplate (Figure 6A). The capacity of the four compounds to reduce PTZ-induced convulsions in zebrafish larvae was also assessed through quantification of the high-speed moved distance in individual well of 48-well microplate. Results showed that seizure-like swimming pattern was alleviated by the addition of either compound 5 (Cmpd 5) or compound 7 (Cmpd 7). Results also revealed that they exhibited concentration-dependent inhibition of both locomotor activity (Figures 6B and 6C).

Discussion

Zebrafish (Danio rerio) has emerged over the last decade as an attractive model for genetic studies and drug screening [5,21]. Numerous studies have showed that adult or zebrafish larvae exposed to PTZ exhibit increased locomotor activity, seizure-like behavior, and epileptiform electrographic activity [15,19] [22-24], which have a good correlation between zebrafish and rodent data [15,17,21,25]. Also, many researchers have used zebrafish seizure model for anti-
Figure 4: Quantitative analyses of behavioral seizures induced by PTZ and of response to AEDs values are given as Means ± SEM (n = 8). Asterisks (**) indicate values that are significantly different (P < 0.01) using one-way ANOVA with Dunnett’s post-test.
epileptic compound discovery, and proved that zebra-fish can be a very useful model for anticonvulsant drug screening and early efficacy in vivo assessment [15,19,26,27]. These models are currently in existence that subserves particular roles in achieving these aims, but all have their limitations [26]. Moreover, the major parameters of zebra-fish behavior assay for assessing anti-convulsant efficacy exist disparity, including drug treatment period, pre- or post-treatment, light condition, microplate format, and assessment criteria, which result in different results in different studies. So, there still remain some uncertainties to clear before using PTZ-induced zebra-fish larvae epileptic seizure model for anti-convulsant drug screening widely. In the present study, zebra-fish larvae were pre-treated for 1 hr before being evoked by PTZ, and then were kept at 28°C in the dark phase for another 1 hr, the total and the high-speed moved distances were chosen for subsequent efficacy assay. Also, our results showed that if zebra-fish larvae were placed in individual well of 96-well microplate, there would be more false positive than in 48-well microplate, and even 1% DMSO treated alone, the most used vehicle, would give a significant positive effect on zebra-fish seizure-like movement. Therefore, the 48-well microplate was used in this research, just as Baxendale et al. [25] did. Also, we

**Figure 5:** C-fos expression in zebrafish larvae after exposed to different AEDs (A) RT-PCR analyses of c-fos expression in zebrafish larvae. (B) Semi-quantification of c-fos mRNA by comparing with vehicle treated group. Values are given as Means ± SEM (n = 50). Asterisks indicate values that are significantly different (*P < 0.05, **P < 0.01) using one-way ANOVA with Dunnett’s post-test.

**Figure 6:** Quantitative analysis of response to VPA and of compounds from plant (Vaccinium spp.) extract (A) A 96-well plate was used for primary drug screening in ten different concentrations compounds. (B) The capacity of the compound 5 to reduce PTZ-induced convulsions in zebrafish larvae in individual well of 48-well micro plate. (C) The capacity of the compound 7 to reduce PTZ-induced convulsions in zebrafish larvae in individual well of 48-well microplate. Values are given as Means ± SEM (n = 50). Asterisks indicate values that are significantly different (*P < 0.05, **P < 0.01) using one-way ANOVA with Dunnett’s post-test.
used the high-speed moved distances as criteria for AEDs efficacy assessment, not the total moved distances used by other researchers. Because we think that the high-speed moved distances were given a more reasonable and sensitive dose-response curve than the total distances in the pro-convulsant treatment experiment. Previous study have proved that when PTZ evoked clonus-like convulsion (stage III) seizure behavior in the presence of high concentrations, zebrafish exhibited stage III and the moved distance reduced when comparing with stage I and stage II [14,16,17,28], whereas zebrafish-kept in high-speed movement in these two earlier stages. Therefore, the high-speed moved distance is more typical than the total distance as an index for anticonvulsant efficacy assessment. Moreover, both the behavioral and RT-PCR analysis data showed that drugs were absorbed well by zebrafish larvae within a defined time period (1 hr pre-treated and 1 hr co-treated with PTZ, 2 hrs totally), and most false negative results could be avoided if maximum tolerant concentration was used. These results were consistent with Orellana-Paucaur et al. [17] and Baxendale et al. [25] data. Therefore, for anticonvulsant drug screening, it does not need to treat zebrafish-larvae more than 2 hrs, especially in high-throughput screening. We kept zebrafish-in the dark phase during the whole experiment, as Berghmans et al. [20] had done. It has been proved that the shift of light and dark could affect zebrafish-locomotor activity, and zebrafish being pre-treated with PTZ showed a reversal of the normal response to cycles of light and dark [9]. So, in order to minimize disruptive factors and the risk of compound photodegradation, it is preferred that the experiment should be performed in the dark phase. The results of the present study were consistent with mammalian experiments and clinical data. VPA acts on a number of intracellular targets including GLUT1, glutamine, VSCN sodium channels and calcium channels [27,28]. CBZ primarily acts as an inhibitor of voltage-gated sodium channels (VGS Cs), and likely opposes PTZ-induced activity through a general, non-specific decrease in neural activity, as found in a rodent model of mania [29]. Numerous studies have used the expression of the immediate early genes (IEGs) c-fos and c-Jun as indirect markers of central nervous system (CNS) neuronal activation, particularly in response to acute seizure [30-32]. In this present study, results showed that CBZ promoted zebrafish-larvae movement at 30 μM and 100 μM, and sharply suppressed zebrafish-larvae motility at 300 μM. Also, CBZ at 30 μM and 300 μM could partially rescued PTZ-evoked abnormal increase of c-fos expression; meanwhile CBZ at 100 μM had no significant effect. It has been reported that CBZ intoxication has been associated with aggravation of seizures; this may be due to increased levels of the CBZ-10, 11-epoxide metabolite [33]. Our results firstly revealed that CBZ could promote seizure-like motility in a zebrafish-larvae anticonvulsant activity assay. Overall, PHT and VPA appeared to provide a stronger opposition to the PTZ-induced changes in activity than CBZ. This would seem to reflect their previously defined therapeutic potential, as PHT and VPA are recommended as first-line treatments of bipolar disorder, while CBZ is only recommended as a second line treatment [34]. In the present study, we used this PTZ-induced zebrafish-seizure model and firstly obtained two anticonvulsant compounds from plant (Vaccinium spp.) extract in AEDs primary screening. Vaccinium spp. is a kind of perennial deciduous or evergreen shrub. It grows in many regions including the Europe, Russia, North America, the Alps Mountain and parts of China. It is used to treat neurological diseases in China, but its mechanism is not clear. Many natural plant products, possessing large molecular weight, will have biological activity when going through in vivo metabolism. Therefore, the traditional method of drug screening in vitro sometimes does not apply to the activities of natural plant products. Zebrafish-as an in vivo model, possessing high speed screening, short design cycle and small dose drug usage; it will provide a new research method for further research and development of the medicinal plants. The popularization and application of the zebrafish model will greatly facilitate the screening and evaluation of effective components in medicinal plants.

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References


