Behavioural Brain Research 332 (2017) 40–49

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Time dependent effect of chronic embryonic exposure to ethanol on zebrafish: Morphology, biochemical and anxiety alterations

Nurul Farhana Ramlan[a], Nurul Syafida Asma Mohd Sata[a], Siti Norhidayah Hassan[a], Noraini Abu Bakar[a], Syahida Ahmad[b], Syaizwan Zahmir Zulkifli[a], Che Azurahanim Che Abdullah[c,d], Wan Norhamidah Wan Ibrahim[a,e,*]

[a] Department of Biology, Faculty of Science, Universiti Putra Malaysia 43400 Serdang, Selangor Malaysia
[b] Department of Biochemistry, Faculty of Biotechnology and Biomolecular, Universiti Putra Malaysia 43400 Serdang, Selangor Malaysia
[c] Department of Physics, Faculty of Science, Universiti Putra Malaysia 43400 Serdang, Selangor Malaysia
[d] Institute of Advanced Technology (ITMA), Seri Kembangan, 43400 Serdang, Selangor, Malaysia
[e] Institute of Bioscience (IBS), Universiti Putra Malaysia 43400 Serdang, Selangor, Malaysia

ARTICLE INFO

Keywords:
Ethanol
Anxiety
Withdrawal exposure
Continuous exposure
Zebrafish

ABSTRACT

Exposure to ethanol during critical period of development can cause severe impairments in the central nervous system (CNS). This study was conducted to assess the neurotoxic effects of chronic embryonic exposure to ethanol in the zebrafish, taking into consideration the time dependent effect. Two types of exposure regimen were applied in this study. Withdrawal exposure group received daily exposure starting from gastrulation until hatching, while continuous exposure group received daily exposure from gastrulation until behavioural assessment at 6 dpf (days post fertilization). Chronic embryonic exposure to ethanol decreased spontaneous tail coiling at 24 hpf (hour post fertilization), heart rate at 48 hpf and increased mortality rate at 72 hpf. The number of apoptotic cells in the embryos treated with ethanol was significantly increased as compared to the control. We also measured the morphological abnormalities and the most prominent effects can be observed in the treated embryos exposed to 1.50% and 2.00%. The treated embryos showed shorter body length, larger egg yolk, smaller eye diameter and heart edema as compared to the control. Larvae received 0.75% continuous ethanol exposure exhibited decreased swimming activity and increased anxiety related behavior, while withdrawal ethanol exposure showed increased swimming activity and decreased anxiety related behavior as compared to the respective control. Biochemical analysis exhibited that ethanol exposure for both exposure regimens altered proteins, lipids, carbohydrates and nucleic acids of the zebrafish larvae. Our results indicated that time dependent effect of ethanol exposure during development could target the biochemical processes thus leading to induction of apoptosis and neurobehavioral deficits in the zebrafish larvae. Thus it raised our concern about the safe limit of alcohol consumption for pregnant mother especially during critical periods of vulnerability for developing nervous system.

1. Introduction

Ethanol has been listed as neurotoxic to human [1], yet still not classified as a developmental neurotoxicant as this industrial chemical exposure is voluntary to human. Consumption of alcohol can lead to behavioral disinhibition and deficiency in cognitive control [2]. Nevertheless, the effects of alcohol to brain might be different within different stage of age. Large bodies of evidence suggest that the developing brain are more susceptible and sensitive to toxic chemicals than the adult brain [3]. The most prominent effect of alcohol exposure during human development is Fetal Alcohol Spectrum Disorder (FASD). Children with FASD will show distinct craniofacial anomalies together with cognitive, behavior and motor deficits [4]. However, beside FASD, psychiatric illnesses such as depression, stress and anxiety are also associated with ethanol exposure during the prenatal stage [5].

According to [6]; the time of ethanol exposure is an important determinant in the changes of central nervous system (CNS) and may affect functional endpoints later in life. A study by [7], have shown that prenatal exposure to ethanol affects the number of neuron in brainstem nuclei in a time dependent manner. The vulnerable effect of ethanol exposure during gastrulation mainly target the rostral rhombomeres, whereas exposure during neurogenesis, the vulnerable effect of ethanol,

* Corresponding author at: Department of Biology, Faculty of Science, Universiti Putra Malaysia 43400 Serdang, Selangor Malaysia.
E-mail address: wnwi@upm.edu.my (W.N.W. Ibrahim).

http://dx.doi.org/10.1016/j.bbr.2017.05.048
Received 1 February 2017; Received in revised form 4 May 2017; Accepted 20 May 2017
Available online 27 May 2017

can be seen at the basal plate. This study has proven that ethanol target specific cell population in a time-specific manner that may define different behavioral response at the later stage. The developing brain is complex, but developmental process has to be reached on schedule and in the accurate progression, thus it will give rise to the window of susceptibility to toxic interference. In a case where the correct programming of the brain development is disturbed due to exposure to neurotoxic insults even at minute concentration or short period of time, the consequences on the nervous system at later age can be permanent.

Similarities of the fundamental neural mechanism of diseases in rodents and zebrafish lead to their popularity in alcohol research. Due to their small, transparent and fast developing eggs that aids high throughput chemical screening [8], marks zebrafish as a model of choice. In addition, 70% of their genes homologous to human genes [9] and they are rich with quantified behavioural repertoire which makes them a valuable model organism for neuropsychiatric studies [10,11]. Zebrafish embryos exposed to ethanol during gastrulation stage (8–10 hpf) resulted in decreased of the anxiety related behavior when they reached adult stage [12]. Another study showed that early ethanol exposure to zebrafish embryos (8–24 hpf) also altered motor neuron and muscle fiber morphology which lead to deficits in locomotion [13]. In addition, zebrafish exposed to ethanol during development (1–9 dpf) exhibited anxiolytic effect not only in the juveniles but also in the adults after withdrawal from ethanol exposure [14]. While, zebrafish exposed to low concentration of ethanol from 2 dpf until 9 dpf showed a reduction in social approach, shoaling and increase anxiety like behavior when they reached adult stage [15]. These previous studies showed that exposure of the zebrafish to ethanol during the developmental process give rise to different responses, according to the window of sensitivity. Hence, determination of the adverse effects of different duration of ethanol exposure during the embryonic stage is important as an alteration might occur depending on the specific window of susceptibility.

Given that period of exposure duration, age of the animal and dose of exposure are important determinants that influence different adverse effects of ethanol during brain development, thus, we interested to determine the effects of withdrawal and continuous ethanol exposure during development in the zebrafish larvae where gastrulation is considered as a starting point of exposure. During the development of embryo, we recorded the embryonic toxicity effects of ethanol such as mortality, heartbeat, morphological abnormalities and spontaneous tail coiling. At 6 dpf (day post fertilization), we assessed the behavioral alterations such as swimming activity and also anxiety like behavior. Lastly, we measured the biochemical changes in both withdrawal and continuous ethanol exposure treated larvae using Fourier Transform Infrared (FTIR) analysis.

2. Materials and method

2.1. Fish husbandry and egg collection

Adult wildtype zebrafish (shortfin phenotype) was purchased from the local supplier, Akuarium Fish Mart and were maintained in the Department of Biology, Universiti Putra Malaysia (UPM) in a recirculating system. They were kept in a mixed male and female with ratio 3 females:2 males on a 14 h light:10 h dark controlled photoperiod with temperature 28.5 °C [15] and were fed four times a day with brine shrimp (*Artemia salina*, San Francisco Bay Brand, San Francisco, CA) and with commercial dry flake food (Sera Vipan). Embryos were collected 1 h after the light onset [16] and were washed with distilled water and rinsed with embryo media (15 mM NaCl, 0.5 mM KCl, 1 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 1 mM $CaCl_2$, and 0.7 mM $NaHCO_3$, pH 7.0) once to eliminate fungal growth. Before further experiment, the embryos were observed under dissecting microscope to ensure fertilization and approximate age. All procedures were conducted according to the policies of Institutional Animal Care and Use Committee of Universiti Putra Malaysia (UPM) (IACUC/AUP-R024/2014).

2.2. Developmental ethanol exposure and anxiogenic drug exposure

Several concentrations of ethanol (0.00%, 0.25%, 0.50%, 0.75%, 1.50% and 2.00%) were prepared by diluting it with embryo media. Before the exposure, all the embryos were carefully staged using a dissecting microscope (SZX-12, Olympus). There were two types of exposure regimen where withdrawal exposure group received daily exposure starting from gastrulation until hatching and continuous exposure group received daily exposure from gastrulation until behavioural assessment. The exposure was conducted in a semi static condition where half of the media were renewed in every 24 h to avoid any possible effect of evaporation [17]. Caffeine was used as an anxiogenic drug to validate the anxiety related behavior in the zebrafish larvae. At 6 dpf, the larvae were exposed to 100 mg/L caffeine (Anhydrous, Nacalai tesque) 2 h prior to behavioral analysis and kept in the same solutions during the behavioral assays, which lasted for 2 h [18].

2.3. Evaluation of toxicity effect of ethanol

To investigate the toxicity effects of ethanol exposure during embryonic stage, we evaluated several endpoints such as the spontaneous tail coiling at 24 hpf, the heart rate at 48 hpf, the mortality rate at 72 hpf and the morphological deformities throughout the exposure time. The mortality rate and morphological deformities were observed daily until the end of the experiments. Dead embryos were removed during the observation time to avoid any contamination. The embryos were indicated as dead when it forms coagulation, non-detachment of the tail, lack of somite formation and heartbeat [19]. The first locomotor behavior observed in the zebrafish embryos is spontaneous tail coiling begin at 17 hpf Mckeown et al., 2009. However, the observation of the spontaneous tail coiling at 17 hpf was not performed as the spinal cord of the embryos at this stage is still immature yet [20]. Thus, it was suggested to use embryos between 24 hpf to 26 hpf to distinguish the touch response [21]. Embryos with no morphological malformations were randomly selected from each replicate and then spontaneous tail coiling was observed under dissecting microscope (n = 60). The number of tail coiling in the zebrafish embryos was counted within 1-min period. Heart rate of the embryos were evaluated at 48 hpf within 1-min period as normal developing zebrafish embryo, the heartbeat is visible after 48 hpf [19] (n = 60). Due to their transparent embryo, the heart rate can be observed clearly and easily using dissecting microscope.

2.4. Evaluation of morphological abnormalities

To assess the morphological development, the zebrafish embryos were collected and dechorionated at 24 hpf, 48 hpf and 72 hpf. The embryos were examined and the images were captured by using inverted microscope (Olympus IX73). The morphological abnormalities observed were the body length and diameter of egg yolk, the heart edema and the eyes. The measurement was taken by using CellSens Dimension 1.6 Software. Each treatment was recorded in triplicate (n = 30).

2.5. Quantification of apoptotic cell death in the zebrafish embryos

Detection of apoptotic cell death in the zebrafish embryos were conducted using acridine orange. The zebrafish embryos exposed to 0.75%, 1.5% and 2.0% ethanol were dechorionated at 24 hpf, 48 hpf and 72 hpf and then incubated in 5 μg/ml acridine orange solution for 30 min. The embryos were then rinsed thoroughly in embryo media two