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Research article

# Embryonic alcohol exposure promotes long-term effects on cerebral glutamate transport of adult zebrafish

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## HIGHLIGHTS

- Mild concentrations of ethanol in zebrafish embryo alter glutamate transporters.
- Alcohol consumption during development alters the glutamatergic system.
- Alcohol in zebrafish embryo alters glutamate transporters of adult brain.

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## ABSTRACT

Ethanol is a widely consumed substance throughout the world. During development it can substantially damage the human fetus, whereas the developing brain is particularly vulnerable. The brain damage induced by prenatal alcohol exposure may lead to a variety of long-lasting behavioral and neurochemical problems. However, there are no data concerning the effects of developmental ethanol exposure on the glutamatergic system, where extracellular glutamate acts as signaling molecule. Here we investigated the effect of ethanol exposure for 2 h (concentrations of 0.0%, 0.1%, 0.25%, 0.50%, and 1.00%) in embryos at 24 h post-fertilization (hpf) by measuring the functionality of glutamate transporters in the brain of adult (4 months) zebrafish. However, ethanol 0.1%, 0.25% and 0.50% decreased transport of glutamate to 81.96%, 60.65% and 45.91% respectively, when compared with the control group. Interestingly, 1.00% was able to inhibit the transport activity to 68.85%. In response to the embryonic alcohol exposure, we found impairment in the function of cerebral glutamate transport in adult fish, contributing to long-term alteration in the homeostasis glutamatergic signaling.



## 1. Introduction

Prenatal ethanol exposure implies significant economic costs to society. In United States, the lifetime cost for an individual suffering from FAS may be as high as U$2 million. Most of these costs could be required for special education, medical, and mental health treatment [26]. Prenatal ethanol exposure is the cause of FAS, a clinical condition characterized by several brain and physical malformations including microcephaly and microphtalmia. Thus, affected children exhibit a typical physical phenotype and CNS dysfunction at variable degree, ranging from mild cognitive disorders to deep intellectual disability [27]. On the other hand, last decade a growing number of studies have failed to detect adverse neurodevelopmental effects of mild-to moderate maternal drinking in exposed child and recent experimental study, mimicking conditions of mild drinking in prenatal period, provided powerful evidence that there are serious lifelong risks to fetus exposed to alcohol [10].

During the development, ethanol consumption affects a number of neurotransmitters and neuromodulators in the CNS, including dopamine, serotonin and GABA. Importantly, research findings of changes in glutamatergic neurotransmission induced by alcohol self- or experimental- administration have resulted in a focus on

*Abbreviations:* CNS, central nervous system; EAAT, excitatory amino acid transporters; FAS, fetal alcohol syndrome; FASD, fetal alcohol syndrome disorder; GABA, gamma-aminobutyric acid; hpf, hour post fertilization; PNEE, prenatal ethanol exposure.

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therapies targeting glutamatergic receptors and normalization of glutamatergic neurotransmission [33]. Glutamate is the major excitatory amino acid in the mammalian CNS, being implicated in several physiological processes. Termination of excitatory activity is mediated by high-affinity $Na^+$-dependent EAAT, principally located in glial cells surrounding synapses and in post-synaptic neurons. A family of $Na^+$-dependent transporters is of prominent importance for glutamate uptake and CNS homeostasis regulation [3,45]. The EAATs represent a protein family which displays considerable homology (50–60% at the amino acid level) [5]. To date, five structurally distinct subtypes of excitatory amino acid transporters have been identified and characterized in the mammalian brain: EAAT1 [42], EAAT2 [32], EAAT3 [21] EAAT4 [13] and EAAT5 [2]. In order to maintain extracellular glutamatergic tonus, the activity of EAAT plays a key role in the clearance of neurotransmitter from synaptic cleft [1,39].

Rat [20], mouse [28] and chicken [12] embryo experimental models have been used to characterize the nature of the alcohol-induced brain damage. However, the advances in translational research linking human beings and animal work are imperative in order to paint a vivid picture of damage caused by PNEE) [29]. Studies utilizing the zebrafish or clawed frog as models for PNEE have shown that ethanol exposure during development can cause growth retardation including reduced body length, microcephaly, skeletal deficits, and eye malformation [24,30]. Beyond these alterations, PNEE causes cognitive dysfunction in simple behavioral tasks such as visual acuity tests [6], associative learning [15], and social behavior [14], which were apparent even in the absence of physical malformations. Moreover, these deficits were also accompanied by changes in gene expression [30]. Reports of our group have described the use of zebrafish to study the reinforcing effects of abuse drugs. Concerning neurotransmitters signaling, ethanol acute and chronically inhibit gene expression and activities of enzymes responsible for purinergic signaling in zebrafish brain membranes [36,37]. Acetylcholinesterase activity after acute exposure to ethanol and the possible protective effect promoted by taurine was evaluated [35,38]. Concerning glutamatergic signaling parameters, our group has recently identified and characterized the presence and function of five glutamate transporter members in zebrafish brain [34]. Furthermore, we studied the differential toxicity *in vitro* of ethanol and acetaldehyde in zebrafish brain structures on glutamate uptake [47], suggesting the relevance of this species to assessing the toxicological actions of ethanol in CNS.

Therefore, considering that (i) ethanol mediates its actions through several excitatory or inhibitory neurotransmitter systems in development; (ii) glutamatergic receptors play a role in mediating cellular and behavioral effects of ethanol; (iii) ethanol activates signal transduction pathways during the development, leading to changes in neuronal function in adult, in the present study we evaluated glutamate transport in cerebral tissue of adulthood zebrafish submitted previously with ethanol in embryonic period.

### 1.1. Material and methods

#### 1.1.1. Animals

Adult zebrafish (*Danio rerio)*; 10 to 12 months old, from a heterogeneous wild-type stock (standard short-fin phenotype) were obtained from a local commercial supplier (Delphis, RS, Brazil). The fish (mixed male/female) were housed in a re-circulating system maintained with mechanical and biological filtration at 28 °C, pH 7.4 and a conductivity of 500 μS (system water). Breeding arrays were used to obtain fertilized eggs. The room was illuminated by ceiling-mounted fluorescent lamps on a 14/10 light/dark photoperiod (lights on at 8:00 a.m.). The animals were fed four times a day with a commercial flake fish food (Alcon BASIC, Alcon, Brazil) and nauplii of brine shrimp (*Artemia salina*), and maintained according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2011). All procedures with animal subjects were approved by the Ethics Commission for the Use of Animals-CEUA (number 27725) from the Universidade Federal do Rio Grande do Sul.

#### 1.1.2. Experimental design

Eggs of zebrafish short fin were collected 2.5 hpf (Fig. 1). Approximately 500 fertilized eggs were randomly selected and were divided into 5 equal rearing tanks. At 24 hpf, each group of zebrafish embryos received one of the following concentrations of alcohol (Absolute Ethanol Merck® (CAS # 64-17-5)) solution: 0.00%, 0.1%, 0.25%, 0.50%, or 1.00% (v/v). The developmental stage of alcohol exposure was chosen to be 24 hpf, because of prior studies showed significant behavioral effects on fish exposed at this stage, characterized by neural tube development [4,18].

After 2 h of alcohol exposure, the embryos were washed twice with system water. With the above alcohol treatment procedure, we were hoping to induce only mild developmental abnormalities resulting in lack of increased mortality or gross structural aberrations but leading to minor changes detectable at the behavioral level [14]. Furthermore, it was established that ethanol equilibration across the chorion and between the embryo and its external environment (about 30% of media), was rapid and achieved steady-state well under 15 min over a wide range of ethanol concentrations [16,25].

The embryos were maintained on B.O.D. Incubator in system water at 28 °C and, once free swimming, were fed twice a day with paramecium during their larval stage. Three weeks later, the fish were moved into 2.8–l rearing tanks (20 fish per tank) of a high-density rack system, which had a multistage filtration and they were fed four times a day with a commercial flake fish food (Alcon BASIC, Alcon, Brazil) and nauplii artemia. Zebrafish remained in these holding tanks until behavioral experiments, which were conducted after the fish reached 4 months of age (mature young adults, 50 to 50% male–female). The sample sizes of treated fish were of at least eight animals per group.

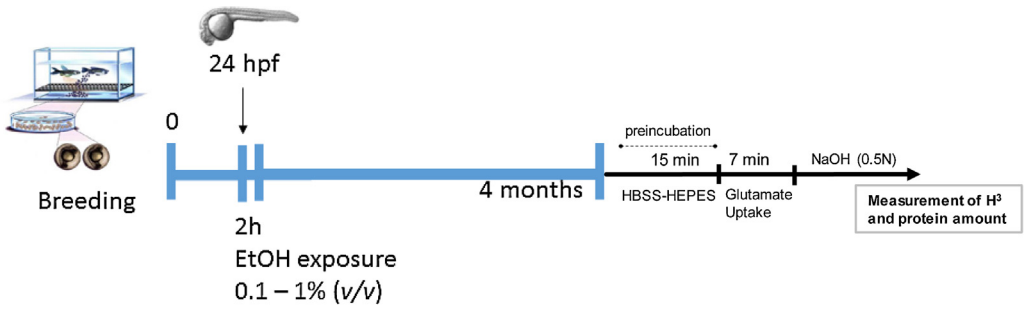


**Fig. 1.** The experimental protocol consisted of one exposure of embryos 24 h hpf during 2 h in ethanol concentrations: 0.00%, 0.1%, 0.25%, 0.50%, or 1.00% (v/v). After 4 months, brain were dissected out and further transferred to a 24-well microplate to assay Na+-dependent glutamate uptake.