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Research Article

Metabolic Alterations of the Zebrafish Brain after Acute Alcohol Treatment by ^{1}H Nuclear Magnetic Resonance Spectroscopy

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The purpose of this study is to investigate the metabolic alterations associated with acute alcohol treatment in zebrafish by ^{1}H nuclear magnetic resonance spectroscopy (NMRS). The brain metabolism of zebrafish was investigated after acute alcohol treatment (one-hour long exposure of adult fish to 0.00%, 0.25%, 0.50%, or 1.00% ethyl alcohol) with whole brain extraction. The results of this study showed that glutamate (Glu) was significantly decreased, scyllo-inositol (sIns) showed a small apparent increase only in the highest acute treatment dose group, and myoinositol (mIns) showed a significant decrease. [Glu]/[tCr] and [mIns]/[tCr] levels were significantly reduced regardless of the alcohol dose, and [sIns]/[tCr] was increased in the highest alcohol treatment dose group. The present NMR study revealed that specific metabolites, such as Glu and mIns, were substantially decreased in case of acute alcohol exposed zebrafish brain.

1. Introduction

Alcohol (i.e., EtOH, ethyl alcohol, and ethanol) abuse and alcoholism are prevalent conditions that cause significant problems for both individual patients and society. Alcohol abuse and alcoholism involve neural processes distinct from other addictions [1]. Attempts to understand the common and unique aspects of alcohol addiction have spurred investigators to adopt new animal models and research methods that have not been widely used for the investigation of other addictions. Partially, alcohol in the brain reduces glutamate and it could be interpreted as a response to N-methyl-D-aspartate (NMDA) receptor blockade [2, 3]. However, the mechanisms associated with alcohol addiction have not been completely identified [1, 4–6], despite numerous reported studies on the topic during the past several decades.

Animal models, including zebrafish, mice, rats, and monkeys, have been used to study alcohol addiction and the physiology of alcoholism [1]. Zebrafish have become a popular subject for embryology studies in genetic research, as well as neurobiology over the past three decades [7]. Attempts at *in vivo* MR imaging (MRI) and MR spectroscopy (MRS) of zebrafish have recently been reported [8, 9].

NMRS is a common modality for research of neurological diseases, such as Alzheimer and Parkinson disease, as well as epilepsy, depression, and schizophrenia in both animals and humans [10–14]. Previous human NMRS studies in alcoholics have shown brain metabolic changes, particularly in

the hippocampus, frontal cortex, basal ganglia, and cerebellum [15–17]. The N-acetyl aspartate (NAA), choline (Cho), and glutamic acids were changed in the frontal lobe and cerebellum of alcohol dependents immediately after abstinence, which increased after a month of abstinence [16–22].

Although there have been several reports on alcohol related research using zebrafish, including behavioral tests [23, 24] and chromatography [4], in addition to *in vivo* MRS in both rats and humans, NMR study of the effects of alcohol in zebrafish has not yet been reported. However, NMRS information, which is included in the changes of various neurometabolites, can be useful to understand the neurobiology in the zebrafish brain. Furthermore, zebrafish and NMRS can be helpful to elucidate molecular mechanisms, such as NMDA in alcoholism. Therefore, the purpose of this study is to investigate the metabolic alterations associated with acute alcohol treatment in the zebrafish brain by *in vitro* NMRS.

2. Materials and Methods

2.1. Animals. Forty wild-type zebrafish naïve to ethanol (*Danio rerio*; 3~4 months old) were used in this study. In order to investigate the effects of alcohol in the zebrafish (males and females pooled), they were divided into four groups: EtOH 0.00% ($n = 10$), EtOH 0.25% ($n = 10$), EtOH 0.50% ($n = 10$), and EtOH 1.00% ($n = 10$), where the % EtOH represents the alcohol concentration (volume percentage; v/v) in the holding tank, where the experimental fish were held for one hour. In order to assess the potential facilitation (lower doses), as well as inhibition (higher doses) effects of alcohol, alcohol concentrations, and incubation time (experimental conditions) were chosen on the basis of data published previously, which acute alcohol treatment 1 h in different concentrations (0.00, 0.25, 0.5, and 1.00% v/v EtOH) led to zebrafish behavioral and molecular alteration [4, 24–27]. Additionally, previous studies have described that blood alcohol level in zebrafish reached a maximum within 1 hour [23, 25, 26]. All fish were exposed to alcohol concentrations for 60 min prior to brain removal. This period of time was expected to lead to a significant and stable blood alcohol level in the subjects.

2.2. Tissue Extraction and Sample Preparation. For extraction of brain metabolites from a zebrafish, a modified procedure reported by Suhartono et al. [28] was used. All zebrafish were euthanized with 4.2% tricaine and their brains were carefully removed from the skull and immediately frozen in liquid nitrogen. Reagent-grade methanol and chloroform (4°C) were added in a ratio of 2 : 1 to frozen, ground each brain and to frozen cell pellets. The tissue-solvent mixture was allowed to thaw before being transferred to centrifuge tubes. The cell pellet-solvent mixture was then sonicated. After approximately 15 min in contact with the first solvents, chloroform and distilled water were added to the samples in a ratio of 1 : 1 to form an emulsion. The samples were then centrifuged at 13,000 rpm for 20 min. The upper phase (methanol and water) was separated from the lower phase using a glass syringe, and both fractions were dried in the freezing state. All dried extract samples were redissolved in 99.9% D_2O containing 0.05% trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) solvent (pH 7) for absolute internal quantification [9]. In this procedure, only one zebrafish brain was used for making one NMR sample.

2.3. Data Acquisition. *In vitro* MR spectroscopy was performed at 25°C with a Varian 500 MHz FT-NMR spectrometer. One-dimensional MRS parameters were as follows: proton resonance frequency = 500.384 MHz, Carr-Purcell-Meiboom-Gill (CPMG) spin echo pulse, relaxation/saturation delay/presaturation time = 2.0/2.0 s (effective TR = 4 s), interpurse delay = 1 ms (effective TE = 2 ms), complex number = 8192, spectral width = 8000 Hz, and number of scans = 256. The acquired spectra were analyzed using MestreNova, which included postprocessed experimental spectra of zero filling (64 k), phase correction, Gaussian apodization (2 Hz), and baseline correction.

2.4. Quantification. The final peak areas were imported into an Excel spreadsheet, and molal metabolite concentrations were calculated to three significant figures using the following:

$$C_m\,(\text{mol/kg}) = \frac{\text{Mass of solvent}\,(D_2O + \text{TSP}) \times 0.05\%\,(\text{mg})}{\text{Molecular weight of TSP}\,(172.23\,\text{g/mol})} \times \frac{\text{Area of metabolite}}{\text{Area of TSP}} \times \frac{9\,(\text{number of TSP proton})}{n\,(\text{number of metabolite proton})} \times \frac{1000\,(\text{g/kg})}{\text{Tissue mass}\,(\text{mg})}, \quad (1)$$

where 0.75% was treated as a constant, and the n^{1}H/metabolite as the number of protons per metabolite resonance or multiplet used for quantitation. Metabolites were quantified using selected Gaussian peaks as follows: NAA (2.02 ppm; $n = 3$), total creatine (creatine + phosphocreatine, tCr; 3.04 ppm and 3.94 ppm; $n = 6$), total choline (choline + phosphocholine + glycerophosphocholine, tCho; 3.19~3.23 ppm; $n = 9$), glutamine (Gln; 2.14, 2.44 and 3.76 ppm; $n = 4$), glutamate (Glu; 2.05, 2.35 and 3.74 ppm; $n = 4$), γ-aminobutyric acid (GABA; 1.89 and 3.01 ppm; $n = 4$), myo-inositol (mIns; 3.52, 4.05, 3.61, 3.27 ppm; $n = 6$), scyllo-inositol (sIns; 3.35 ppm; $n = 6$), alanine (Ala; 1.47 ppm; $n = 3$), taurine (Tau; 3.24 and 3.42 ppm; $n = 4$), Acetate (Acet; 1.91 ppm; $n = 3$), and N-acetyl aspartyl glutamate (NAAG; 2.04 ppm; $n = 3$).

2.5. Statistical Analysis. All of the data was analyzed using SPSS (Windows Version 13.0; SPSS, Chicago, IL, USA). One-way variance analysis (ANOVA) was performed to investigate the effect of different alcohol concentrations. P values of less than 0.05 were considered statistically significant.

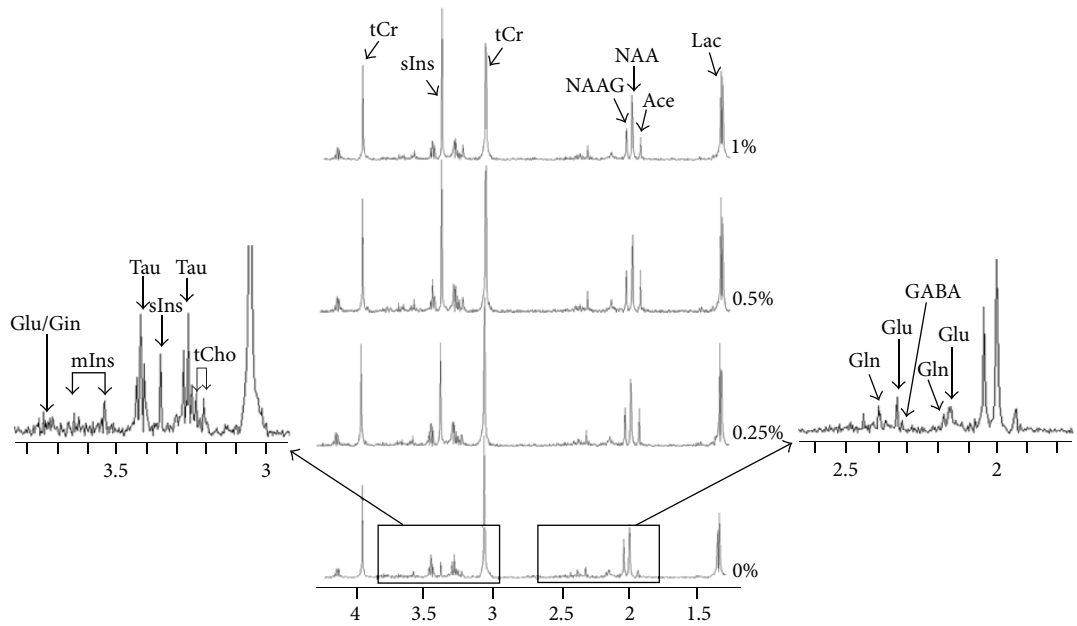

Figure 1: NMR spectra of zebrafish brain in each alcohol concentration group: 12 metabolites (Ace, GABA, Gln, Glu, Lac, mIns, NAA, NAAG, sIns, Tau, tCho, and tCr) were evaluated and quantified.

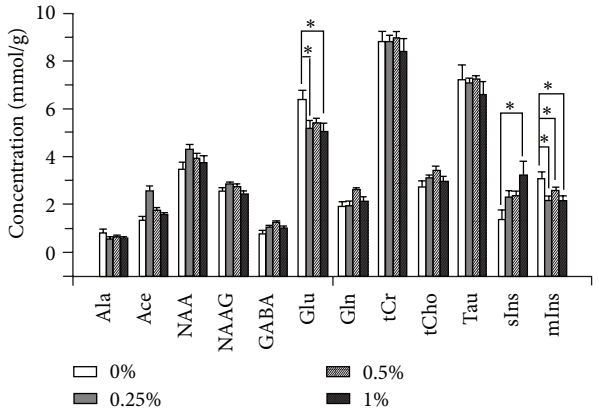

Figure 2: Absolute metabolite concentration in the whole brain of four zebrafish groups (0.00%, 0.25%, 0.50%, and 1.00%): Glu, mIns, and sIns were significantly different. Error bars = SEM. Significance level (ANOVA): $^{*}P < 0.05$.

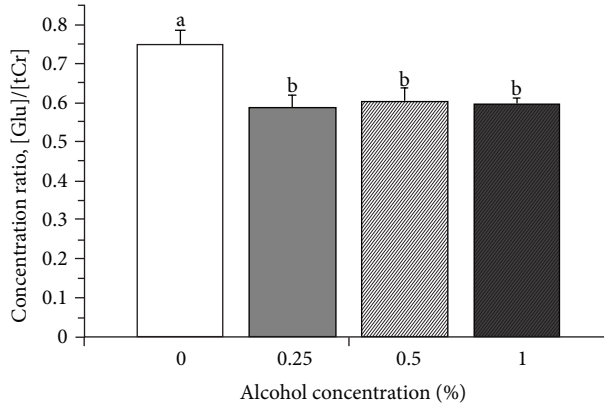

Figure 3: Acute, 1 h long, alcohol exposure increases glutamate to total creatine ratio ([Glu]/[tCr]) in zebrafish brain: mean ± SEM ($n = 10$). Bars not sharing a letter designation are significantly different from each other ($P < 0.05$). Observe the apparent decrease in [Glu]/[tCr] at all acute doses and note that the experimental groups significantly differ from the control.

3. Results

NMR spectra among the four zebrafish groups were assessed and quantified (Figure 1). Figure 2 shows the concentrations of 12 brain metabolites in the four groups. ANOVA (Tukey HSD analysis) showed a significant alcohol dose effect for all neurochemicals tested: glutamate (Glu) $F(3, 39) = 4.624$, $P < 0.01$; scyllo-inositol (sIns) $F(3, 39) = 2.881$, $P < 0.05$; myo-Inositol (mIns) $F(3, 39) = 7.380$, $P < 0.005$. The concentrations relative to tCr of sIns, mIns, and Glu were significantly different among the four groups: [Glu]/[tCr] $F(3, 39) = 7.122$, $P < 0.005$ (Figure 3); [sIns]/[tCr] $F(3, 39) = 3.162$, $P < 0.05$ (Figure 4); and [mIns]/[tCr] $F(3, 39) = 6.887$, $P < 0.005$ (Figure 5). The concentration of Glu ([Glu]) and [Glu]/[tCr] was reduced after acute alcohol exposure; [sIns] and [sIns]/[tCr] increased, while [mIns] and [mIns]/[tCr] decreased.

In the metabolites except for Glu, sIns, and mIns, the significant changes of the concentrations and the metabolite to tCr ratios were not found ($P > 0.05$).

4. Discussion

Alcohol, that is, related in NMDA receptors affects glutamatergic change. Partially, Ferko [29] has reported the alteration

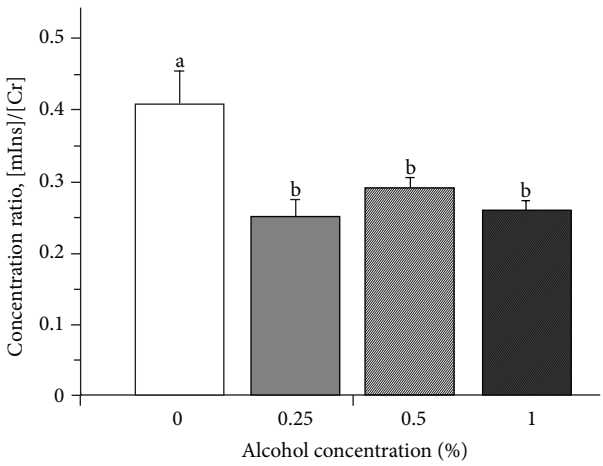

Figure 4: Acute, 1 h long, alcohol exposure increases myo-inositol to the total creatine ratio ([mIns]/[tCr]): mean ± SEM ($n = 10$). Bars not sharing a letter designation are significantly different from each other ($P < 0.05$). Observe the apparent decrease in [mIns]/[tCr] at all acute doses and that all experimental groups significantly differ from the control.

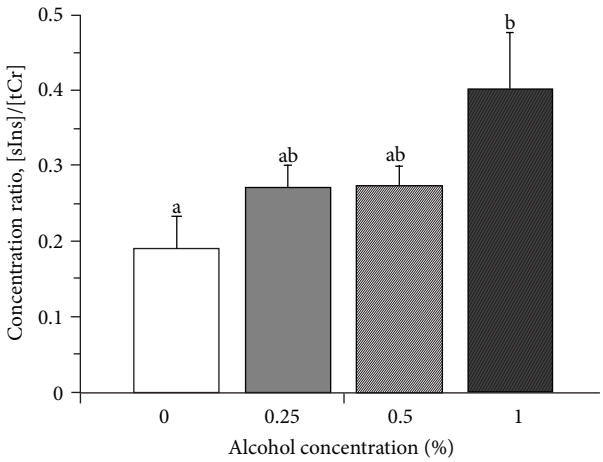

Figure 5: Acute, 1 h long, alcohol exposure increases scyllo-inositol to total creatine ratio ([sIns]/[tCr]): mean ± SEM ($n = 10$). Bars not sharing a letter designation are significantly different from each other ($P < 0.05$). Observe the apparent increases in [sIns]/[tCr] but note that only the highest dose group differs significantly from the control group.

of glutamate by alcohol induced the loss of righting reflex in mice. Considering the antagonist properties of ethanol on NMDA receptors [5, 30–34], the reduction in extraneuronal glutamate by an ethanol challenge could be interpreted as a response to NMDA receptor blockade, which is correlated with detoxification of the blood [7]. Also, NMDA receptors antagonists can make confusing alcohol-induced conditioned place preference, as well as prevent alcohol self-administration in a free-choice operant task which was attenuated by microinjection [35, 36]. These results suggest that glutamate neurotransmission in the NAC may modulate alcohol self-administration and its reinforcing effects [37]. The first finding of this study was a decrease of glutamate in the brains of zebrafish after acute exposure to alcohol. This finding may demonstrate that the reduction of glutamate is associated with changes in the NMDA receptor. In addition, glutamate was decreased in zebrafish exposed alcohol regardless of the alcohol concentration.

Carboni et al. [2] suggested that the alcohol suppresses Glu release through an inhibition of NMDA glutamate receptors in the brain. In addition, Krystal et al. [38] explained that the modulation of glutamatergic function by alcohol contributes to both euphoric and dysphoric consequences of alcohol intoxication. Then, adaptations within glutamatergic systems appear to contribute to alcohol tolerance and dependence, and to both acute and protracted features of ethanol withdrawal [39]. GABA, a product of Glu metabolism, is synthesized from astroglial Gln and contributes to total Glu/Gln neurotransmitter cycling. The contribution of GABAergic neurons and inhibition to cortical energy metabolism has broad implications for the interpretation of functional imaging signals. Therefore, the role of alcohol in the glutamatergic/GABAergic neurotransmission has been studied using a combination of ^{1}H and ^{13}C NMR [40–42].

Several articles have reported that the acute alcohol effect reduces the mIns level in the animal brains [17, 43]. In this study, the [mIns]/[tCr] decreased regardless of the alcohol dose, although the absolute concentration of mIns was significantly reduced for all concentrations except for the 0.50% alcohol group. These findings are consistent with the previous reports [6, 17, 43]. In addition, Viola et al. [6] reported that sIns, a new marker in the brain, increased in patients with chronic alcoholism. They reported that high levels of cerebral sIns were correlated with altered glial and neuronal metabolism due to the sIns. The results of this study also showed that the sIns level of zebrafish exposed to alcohol was sharply increased.

Brain sIns comes from blood delivery or *de novo* synthesis [44–47] from glucose 6-phosphate, that is, converted into myoinositol 1-phosphate, which in turn is hydrolyzed into mIns and epimerized to sIns. The possibility that elevated brain sIns, in zebrafish after acute alcohol exposure, results from a higher blood uptake is unlikely considering the limited increase in blood serum sIns. Brain mIns, the precursor of sIns, can originate from the blood supply or be formed *in situ* from glucose-6 phosphate [6]. Therefore, the results of this study, which showed increased sIns and decreased mIns in zebrafish after acute alcohol treatment, support that sIns was synthesized from mIns within the brain.

A significantly elevated lactate peak intensity associated with anaerobic glycolysis occurred postmortem, prior to freezing the tissue (Figure 1). Because the warm ischemic period was variable, we did not carry out an analysis of the lactate levels. However, previously, this lab identified lactate as a contaminant from the plastic ware used during the preparation of extracts [48, 49].

It has been reported that other metabolites, such as NAA, Cho, and Cr, are changed in the frontal lobe and cerebellum of alcoholic patients [6, 17, 18, 50–52]. However, the results of this study showed that the metabolites did not differ significantly except for glutamate, mIns and sIns; this was likely

because the whole brain of zebrafish was studied. In this study, the zebrafish brain was so small (~3 μL) that metabolites in each brain region could not be quantified using NMR spectroscopy. However, NMR spectroscopy was a good method for the neurochemistry of the zebrafish brain. In addition, an accurate regional resection of the zebrafish brain and highly sensitive NMR equipment, such as the ultrahigh field NMR spectrometer (>900 MHz), may allow for the investigation of regional metabolite concentrations.

5. Conclusion

In this work, we have investigated metabolic alterations of acute alcohol which exposed zebrafish brains using NMRS, and the results showed that acute alcohol exposure decreased Glu and mIns, and increased sIns in the zebrafish brain. These results support the prior findings that alcohol exposure in the brain reduces the Glu level associated with detoxification of the blood and disturbs the sIns to mIns pathway. Most of all, this work shows that the combination of zebrafish and NMRS can be a useful method to study neurobiology/neuroscience.

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