Quantitative assessment of neurochemical changes in a rat model of long-term alcohol consumption as detected by in vivo and ex vivo proton nuclear magnetic resonance spectroscopy

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The aim of present study was to quantitatively investigate the neurochemical profile of the frontal cortex region in a rat model of long-term alcohol consumption, by using in vivo proton magnetic resonance spectroscopy ( 1H-MRS) at 4.7 T and ex vivo 1H high-resolution magic angle spinning (HR-MAS) technique at 11.7 T. Twenty male rats were divided into two groups and fed a liquid diet for 10 weeks. After 10 weeks, in vivo 1H MRS spectra were acquired from the frontal cortex brain region. After in vivo 1H MRS experiments, all animals were sacrificed and 20 frontal cortex tissue samples were harvested. All tissue examinations were performed with the 11.7 T HR-MAS spectrometer and high-resolution spectra were acquired. The in vivo and ex vivo spectra were quantified as absolute metabolite concentrations and normalized ratios of total signal-intensity (i.e., metabolitesnorm), respectively. The absolute quantifications of in vivo spectra showed significantly higher glycerophosphocholine plus phosphocholine (GPC + PCh) and lower myo-inositol (mlns) concentrations in ethanol-treated rats compared to controls. The quantifications of ex vivo spectra showed significantly higher PChnorm, Cho norm and tCho norm and lower GPCnorm and mlnsnorm ratio levels in ethanol-treated rats compared to controls. Our findings suggest that reduced mlns concentrations caused by the long-term alcohol consumption may lead to hypo-osmolarity syndrome and astrocyte hyponatremia. In addition, increased choline-containing compound concentrations may reflect an increased cell turnover rate of phosphatidylcholine and other phospholipids, indicating an adaptive mechanism. Therefore, these results might be utilized as key markers in chronic alcohol intoxication metabolism.

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1. Introduction

Alcohol is the most repeatedly abused substance in developing countries worldwide and ranks high as a cause of disability (Geiβbräsert et al., 2010; Lieber, 1995; Saraceno, 2002). Currently, long-term alcohol use disorders (i.e., alcohol abuse and dependence) are one of the most common health problems and a major cause of mortality (Jung et al., 2011).

Most current literature emphasizes that prolonged alcohol abuse can cause brain disorders, such as loss of brain volume, neurological dysfunction, functional abnormalities, and neurochemical alterations in the anterior frontal region (Dao-Castellana et al., 1998; Kril et al., 1997; Mosely et al., 2001). Previous studies have also reported that the anterior frontal region is especially

Abbreviations: MRS, magnetic resonance spectroscopy; NMRS, nuclear magnetic resonance spectroscopy; HR-MAS, high-resolution magic angle spinning; ppm, part per million; CP/MG, Carr-Purcell-Meiboom-Gill; CRLB, Cramér–Rao lower bound; LCM, Mathematical, Linear Combination Model; SSD, percentage standard deviation; Ala, Alanine; Act, acetate; Asp, aspartate; Cho, choline (free choline); Cr, creatine; Pcr, phosphocreatine; GABA, gamma-amino butyric acid; Gln, glutamine; Glg, glutamate; Glc, glucose; GPC, glycerophosphocholine; GSH, glutathione; Glc, glucose; GPC, glycerophosphocholine; GSH, glutathione; it, taurine; NAAC, N-acetylaspartate; NAAG, N-acetylaspartylglutamate; tNAA, NAA + NAAG; PCh, phosphocholine; tCho, Cho + PCh + GPC; Eth, ethanol; TAU, taurine; BALs, blood alcohol levels.

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vulnerable to the adverse effects of long-term alcohol abuse (Mosely et al., 2001; Pfefferbaum et al., 1997). Numerous proton magnetic resonance spectroscopy (1H MRS) studies have investigated the brain metabolites in chronic alcohol-dependent patients versus healthy subjects as well as light versus heavy drinkers (Bartsch et al., 2007; Bendszus et al., 2001; Biller et al., 2009; Bloomer et al., 2004; Meyerhoff et al., 2004).

In humans, previous studies utilizing 1H MRS have identified changes in the metabolite concentrations of N-acetylaspartate (NAA), choline-containing compounds, total creatine (tCr, creatine + phosphocreatine), and myo-inositol in chronic alcohol-dependent patients (Biller et al., 2009). Among these metabolites, most studies have reported reduced NAA levels (Bartsch et al., 2007; Bendszus et al., 2001; Bloomer et al., 2004; Meyerhoff et al., 2004; Schweinsburg et al., 2003) and elevated choline-containing compound levels (Ende et al., 2006; Meyerhoff et al., 2004) in chronic alcohol-dependent patients, compared to healthy subjects. The myo-inositol levels have varied results, from reduction or elevation (Biller et al., 2009; Meyerhoff et al., 2004; Parks et al., 2002; Schweinsburg et al., 2001; Seitz et al., 1999), and creatine levels are referred to as stable (Biller et al., 2009; Parks et al., 2002; Seitz et al., 1999). However, several previous studies suggest that the brain metabolism from alcohol abuse has large variations among individuals, depending on the consumption pattern of alcohol, types of alcohol (relative dose of alcohol), and period of alcohol dependence (Geibprasert et al., 2010; Zahr et al., 2010). Thus, a study of long-term alcohol consumption using an animal model is necessary for more quantitative investigation.

In vivo 1H MRS provides a noninvasive method for quantification of specific brain biochemical markers and neurotransmitters that reflect molecular processes (Hong et al., 2011; Opstad et al., 2010; Zahr et al., 2010). However, quantification of the in vivo 1H MRS technique has been severely limited by overlapping peaks in the narrow chemical shift range (Kim et al., 2011; Tzika et al., 2002). Therefore, ex vivo proton high-resolution magic angle spinning (1H HR-MAS) nuclear magnetic resonance spectroscopy (NMRS) is also necessary for obtaining more detailed cerebral neurochemical information (Martinez-Bisbal et al., 2004). HR-MAS is a powerful technique for observing the cerebral biochemical profile and allowing high-resolution spectra to be harvested directly from biopsy tissues (Opstad et al., 2009, 2010). The HR-MAS technique can also provide the narrow line-widths of metabolite signals by reducing the line-broadening effect in semi-solid tissues through rapid sample spinning at a magic angle (54.7 degrees) against the magnetic field (Beckont et al., 2010).

We hypothesized that the specific neurochemical profile of the frontal cortex region would be significantly altered in the rat model of long-term alcohol consumption as compared to control rats. The aim of present study was to quantitatively characterize the neurochemical changes in frontal cortex of a rat model of long-term alcohol consumption, as detected by in vivo 1H MRS at 4.7 T (200 MHz) and by ex vivo 1H HR-MAS at 11.7 T (500 MHz).

2. Materials and methods

2.1. Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC Number: 2010-0030-04). The animals were maintained according to the “Guide for the Care and Use of Laboratory Animals” (NIH Publications No.80-23) issued by ILAR, USA.

Six-week-old male Sprague-Dawley rats (177.0 to 192.0 g, n = 20, Orient Bio Inc., Seongnam, Korea) were divided into two groups (control group: n = 9; ethanol-treated group: n = 11). All animals were individually housed in standard plastic cages and maintained on a 12-h light–dark cycle, in ambient temperature of 24–25 °C. Before the start of the experiments, rats were allowed free access to food and water for a week. After a week, all animals were fed a Lieber-DeCarli liquid diet (Control diet: #710027; Ethanol diet: #710260, Dyets Inc., Bethlehem, PA, USA) for 10 weeks. The ethanol and control liquid diets were nutritionally complete material containing 67 mL of 95% pure ethanol per 1 L of the liquid diet, and 1.0 kcal/mL, of which 35% fat, 11% carbohydrate, 18% protein, and 36% derived from pure ethanol. The control diet contained 1.0 kcal/mL, of which 35% fat, 47% carbohydrate, and 18% were derived from protein. An equivalent amount of maltose dextrin (ethanol liquid diet: 25.6 g/L, control liquid diet: 115.2 g/L) was added for caloric balance between the control group and ethanol group. The design of the liquid diet exposure studies has been previously described (Simonyi et al., 2002; Wills et al., 2008).

2.2. In vivo proton MR spectroscopy experiments

Designs of the in vivo 1H MRS studies were previously described (Kim et al., 2010, 2011). In vivo proton spectra were acquired from 20 animals using 4.7 T horizontal BIOSPEC MR system (Bruker Medical GmbH, Ettlingen, Germany) with a 400 mm bore magnet and 150 mT/m actively shielded gradient coils. Before the MR image and MR spectroscopy data acquisitions, rats were anesthetized using an inhalation isoflurane chamber at 4–6% concentrations, in a 5:5 mixture of N2O and O2 gas. The anesthesia concentrations of isoflurane were maintained at 1–2% during the MR scanning. Anesthetized rats were placed in the prone position with the head firmly fixed on a palate holder equipped with an adjustable nose cone. The volume of interest [Fig. 1 (A and B), VOI (4.0 × 1.6 × 3.0 mm3; volume: 19.2 μL)] was positioned in the frontal cortex based on multi-slice axial T2-weighted MR images [Rapid Acquisition with a Relaxation Enhancement (RARE), TR/TE = 5000/90 ms, number of acquisitions = 4, slice thickness = 1.0 mm, matrix = 256 × 256]. The VOI was adjusted to minimize intracranial lipid contamination. Water suppressed 1H MRS spectra were acquired using a point-resolved spectroscopy (PRESS) pulse sequence (TR/TE = 4000/20 ms, number of acquisitions = 384, number of data points = 2048, scan time = 25 min 36 s). The

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**Fig. 1.** Multislice T2-weighted RARE sagittal (A) and axial (B) images (TR/TE = 5000/90 ms, number of acquisitions = 4) of the rat brain with the volume of interest (VOI) centered in the frontal cortex region. The pink color illustrations on the images show the size of the rectangular volume of interest is 19.2 μL. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
unsuppressed water signal was also acquired (TR/TE = 4000/20 ms; number of acquisitions = 16, scan time = 1 min 4 s).

2.3. NMR sample preparation

After the in vivo 1H MRS experiments, all animals were sacrificed using CO2 gas. Twenty frontal cortex tissues were quickly and carefully harvested using the brain slicer matrix (Brain Slicer Matrix, 1.0-mm coronal section interval; Zivic Instruments Inc., Pittsburgh, PA, USA). All tissues were immediately stored in liquid nitrogen (at −196 °C) to prevent tissue decomposition and biochemical changes. The harvested tissue samples were dissected quickly and inserted in a 4 mm rotor at room temperature (18–20 °C). All prepared NMR rotors (Agilent Technologies Korea Ltd., Seoul, Korea) were tightly closed with a zirconium plug (Agilent Technologies Korea Ltd., Seoul, Korea). The ampoules (1.0 mL) of D2O containing 0.75 wt.% TSP were used for referencing and scaling. A few drops of D2O were added to the NMR rotor to provide a locking signal. The mass of brain tissue and D2O + TSP solvent were 13–19 mg and 10–14 mg, respectively.

2.4. Ex vivo proton HR-MAS NMR spectroscopy experiments

1H HR-MAS NMR spectroscopy was performed on the VNMR-500 spectrometer [11.7 T (500.13 MHz); Agilent Technologies Korea Ltd., Seoul, Korea] with a quadruple nuclei 1H, 2H, 13C, 31P) HR-MAS NMR nano-probe. The samples were placed in 4-mm-diameter rotors and equipped on top of the nano-probe, and typically spun at 4–5 kHz and at 54.7 degrees. The design of the 1H HR-MAS NMR spectroscopic studies was previously described (Beckonert et al., 2010; Woo et al., 2010). All one-dimensional HR-MAS NMR spectra were acquired with the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence at 277.15 K (4.0°C) [complex data number = 16384, spectral width = 8012.8 Hz, acquisition time = 2.05 s, relaxation delay time = 10.0 s, pre-saturation time = 2.0 s, inter-pulse delay (τ) = 0.4 ms, big-tau (eighty 180-degree refocusing pulses) = 0.064 ms, number of acquisitions = 128, and a total scan time = 25 min 16 s].

2.5. In vivo and ex vivo spectral analyses

Acquired in vivo and ex vivo raw data were analyzed in a fully automated spectral process, using the Linear Combination Model software (LC Model, Version 6.2–1L and Copyright: Stephen W. Provencher, 2001). CRLBs have been used as acceptable reliability for estimates of fitting uncertainty.

2.6. Statistical analyses

All statistical analyses were performed with the PASW Statistics 18 software (SPSS Inc., IBM Company, Chicago, IL, USA). From the numeric CRLBs (% SD), we only used the range of acceptable reliability values (in vivo 1H-MRS CRLBs: below than 40% SD; ex vivo 1H NMRs CRLBs: below than 10% SD). An independent t-test of the in vivo 1H MRS experiment was used for comparison of the mean values of the absolute concentrations by internal water reference between the control and the ethanol-treated group. An independent t-test of the ex vivo 1H NMRs experiment was also used for comparison of the mean values of the normalized ratios by the total signal intensity between the control and the ethanol-treated group.

3. Results

3.1. In vivo and ex vivo proton NMR spectra

The in vivo and ex vivo spectra provide a large amount of biochemical information available from the absolute metabolite concentration and high-resolution spectrum. The in vivo spectra were assigned the brain metabolites NAA + NAAG (tNAA), Glu + Gln (Glx), Cr + Pcr (tCr), GPC + PCh, Tau, Asp, and mlns. In the ex vivo spectra, brain metabolite signal assignments are tCr, GABA, tNAA, Act, Ala, Glx, Gln, Asp, GSH, GPC + PCh + Cho (tCho), Tau, and mlns. Fig. 2 (panels A–D) shows the representative fitted in vivo MR spectra (A and B) and ex vivo HR-MAS NMR spectra (C and D) of the frontal cortex of the control and the ethanol-treated groups. The scale of the chemical shift range was equally matched (3.85 to 0.20 ppm) between the in vivo and the ex vivo spectra. The signal intensities are proportionally represented by the cerebral metabolite concentrations. The in vivo spectra [Fig. 2 (A and B)] show that the total choline-containing compound (tCho) signal intensities were higher in the ethanol-treated group compared to the control group. In our results of the 4.7 T in vivo 1H-MRS experiment, the 1CH (3.522 ppm), 3CH (3.522 ppm), 4CH (3.614 ppm), and 6CH (3.614 ppm) protons of mlns are mostly observed as a singlet or doublet peak at approximately 3.56 ppm and overlapped with glycine (3.55 ppm). In the 11.7 T ex vivo 1H HR-MAS NMRs spectra [Fig. 2 (C and D)], the mlns peak shows as a doublet of doublet shape at 3.522 and 3.614 ppm.

3.2. Quantification of in vivo proton MR spectra

Fig. 3A illustrates the metabolite concentrations (μmol/g) that were quantified from the twenty analyzed in vivo spectra of the frontal cortex region. The concentration of GPC + Pch (p < 0.01) was significantly higher in the ethanol-treated group compared to the control group. The GPC + Pch concentration of the ethanol-treated group was 15.79% higher than the control group. However, the mlns concentration (p < 0.05) of the ethanol-treated group was 15.91% lower than the control group. All processed in vivo CRLB levels except GABA were below 40% SD in the control group and ethanol-treated group [Fig. 3B].

3.3. Quantification of ex vivo proton HR-MAS NMR spectra

Fig. 4A shows the levels of normalized metabolites (total signal intensity ratios) that were quantified from 18 frontal cortex tissue samples in the control group and ethanol-treated group. The mean values and standard deviations of the normalized metabolic ratio
**Fig. 2.** The in vivo spectra (A and B) acquired at 4.7 T from the long-term ethanol treated group and control group, in the frontal cortex (PRESS sequence, TR/TE = 4000/20 ms, number of acquisitions = 384, number of data points = 2048). Ex vivo HR-MAS NMR spectra (C and D) acquired at 11.7 T from the tissue samples of the frontal cortex region (CPMG sequence, number of acquisitions = 128, number of data points = 16384, number of data points = 2048, $\tau = 0.4$ ms, relaxation delay time = 10.0 s, pre-saturation time = 2.0 s). The fitted LC Model spectra are represented in bold red. The residues are positioned under the fitted spectra. The chemical shift range is from 0.20 to 3.85 ppm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Fig. 3.** Concentrations of the brain metabolites (A) and corresponding Cramer-Rao lower bound levels (B) quantified by LC Model software in the frontal cortex (control group: n = 9; ethanol-treated group: n = 11). Metabolite concentration and CRLB level units are expressed as $\mu$mol/g and percentage standard deviation (%SD), respectively. Vertical lines on each of the bars indicate the (+) standard deviation of the mean values. Significance levels (independent t-test): *: $p < 0.05$, **: $p < 0.01$.

**Fig. 4.** Normalized metabolic ratio levels (A) and corresponding Cramer-Rao lower bound levels (B) quantified by LC Model software in the frontal cortex (control group: n = 9; ethanol-treated group: n = 9). Normalized metabolite levels analyzed by the total signal intensity ratios of the 1-D ex vivo proton NMR spectra. Vertical lines on each of the bars indicate the (+) standard deviation of the mean values. Significance levels (independent t-test): *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$. 

levels are shown. The GPCNorm (p < 0.05) and mnlsNorm (p < 0.05) ratio levels were significantly lower in the ethanol-treated group compared to the control group. However, the PChNorm (p < 0.05), ChoNorm (p < 0.005), and tChoNorm (p < 0.01) ratio levels were significantly higher in the ethanol-treated group than controls. Table 1 indicates the significant differences in 11 of the metabolites between the 2 groups in the in vivo and ex vivo experiments. Fig. 4B also shows the mean CRLB levels for estimation of the fitting error in the 1H HR-MAS spectra. The processed ex vivo CRLB levels, except GPC and PCh, were below than 10% SD in the control and ethanol-treated group.

4. Discussion

The present study conducted 1H MRS and HR-MAS NMR spectroscopy in a rat model for long-term alcohol exposure using the liquid diet technique in order to assess the neurochemical changes in the frontal cortex region. To the best of our knowledge, this study is the first to use in vivo 1H MRS in combination with ex vivo 1H HR-MAS NMR spectroscopy techniques in the frontal cortex region of long-term ethanol exposed rats. Moreover, we quantitatively assessed the neurochemical alterations (particularly the mnls and GPC + PCh metabolic levels) induced by long-term ethanol consumption. In this study, acquired in vivo MRS results are in good agreement with ex vivo HR-MAS results as well as with other previous studies, such as chronic alcohol dependent patients and long-term ethanol exposed rats (Braunová et al., 2000; Lee et al., 2003; Schweinsburg et al., 2000; Zahr et al., 2009).

In vivo 1H MRS is a clinically useful diagnostic tool with promising contributions to noninvasive evaluations based on its ability to discriminate between various biochemical characteristics in humans (Martínez-Bisbal et al., 2004). To understand the spectral characteristics of brain disorders using MR spectroscopy, the peaks of the biochemical compounds in in vivo spectra must be clearly resolved in narrow chemical shift range (0.0–4.0 ppm) (Tzika et al., 2002). For this, high-resolution spectra are required. However, at the magnetic field strengths typically used for clinical MRI system (~3.0 T), numerous metabolite peaks and macromolecule signals strongly overlap, and may cause inaccurate quantification of the biochemical information (Wilson et al., 2009). Ex vivo HR-MAS data improves the spectral resolution, thus clearly separating peaks in the narrow chemical shift range than those for in vivo MRS (Wilson et al., 2009). Previous studies suggest that the generally good agreement between in vivo MRS and ex vivo HR-MAS results can be used to reliably estimate the metabolite quantities in various brain disorders (Tzika et al., 2002; Wilson et al., 2009). Importantly, the choline-containing compound signals shown as GPC combined with PCh for in vivo spectra due to small chemical shift difference between the metabolites are clearly separated as the free Cho, GPC, and PCh signals in ex vivo HR-MAS spectra. The significantly lower mnls and higher GPC + PCh concentrations in long-term ethanol-intoxicated rats compared to controls for in vivo experiments were also observed as significantly lower mnlsNorm and GPCNorm, and higher PChNorm and ChoNorm levels in ex vivo HR-MAS experiments, supporting in vivo MRS findings. Thus, ex vivo HR-MAS could be used as complementary methodology to support the in vivo MRS data and improve analytical accuracy.

The choline containing compound signals are composed of ace-tylcholine (Ach), free-choline (Cho), phosphocholine (Pch), and glycerophosphocholine (GPC) (Ende et al., 2005). Most of the choline peak signal arises from the PCh and GPC signals (Ende et al., 2005, 2006; Parks et al., 2002). Previous studies reported that altered choline-containing compound levels were observed in the brain of the ethanol-intoxicated rodent models as well as alcohol-dependent patients (Lee et al., 2003; Seitz et al., 1999). Similarly, we found that the GPC + PCh concentration was significantly higher in the ethanol-treated rats compared to controls. We additionally studied the ex vivo 1H HR-MAS NMR spectra from the frontal cortex tissue samples after termination of the in vivo experiment. Interestingly, from our ex vivo 1H HR-MAS NMR results, the GPCNorm level was significantly lower in the ethanol-treated rats than the controls. Unlike the significantly higher GPC + PCh concentrations in the ethanol-treated rats in the in vivo study, the significantly lower GPCNorm levels may suggest that the GPC concentration might be reduced by long-term alcohol consumption in the ethanol-treated rats compared to controls. Nevertheless, we consider the possibility that the significantly higher GPC + PCh concentrations may reflect the substantially increased PCh concentrations. In fact, the PChNorm and tChoNorm ratios were significantly higher in the ethanol treated rats than controls. Moreover, we also found that the quantified ChoNorm was significantly higher. Thus, significantly higher GPC + PCh concentrations and tChoNorm ratios may contribute to substantially increased PCh signals.

Previous studies conducted in ethanol-intoxicated rodent models and alcohol-dependent patients reported increased choline-containing compound signals in the brain (Lee et al., 2003; Seitz et al., 1999; Zahr et al., 2009, 2010). Lee and colleagues reported initially increased choline-containing compound signals in rats chronically exposed to alcohol at 16 weeks. However, choline-containing compound signals significantly decreased with increased duration of alcohol consumption over 44 weeks (Lee et al., 2003). Zahr et al. investigated the brain of rats chronically exposed to ethanol using in vivo MRS (Scan 1: at the 0th [baseline], scan 2: at the 16th week, and scan 3: at the 24th week [Zahr et al., 2009]). Similar to a Lee et al. study (Lee et al., 2003), chronic ethanol-treated rats showed significantly increased choline signals with increased duration of alcohol consumption over 24 weeks (Zahr et al., 2003).

Table 1
The mean values of the in vivo metabolite concentrations (µmol/g ± SD) and ex vivo normalized metabolite ratio levels (MetaboliteNorm ± SD) in the frontal cortex of the rat brain with the probability values (p-value). Significance levels of the p-values as follows: 'p < 0.05; **p < 0.01; ***p < 0.005.'
Our results are in good agreement with the spectroscopic findings of Lee et al. and Zahir et al. (Lee et al., 2003; Zahir et al., 2009). The increased choline-containing compound signals were observed in the non-abstinent chronic heavy drinkers (Ende et al., 2005), as well as in the chronic ethanol exposed rodent model (Lee et al., 2003). The authors emphasized that alcohol consumption can lead to an increased choline signals. Other previous studies have also reported that significantly decreased choline and choline-containing compound signals were observed in detoxified alcoholic patients (Bendszus et al., 2001; Ende et al., 2005; Parks et al., 2002). The authors interpreted that increased choline signals may reflect an increased turnover of phosphatidylcholine and other phospholipids and thus an adaptive brain mechanism (Ende et al., 2006; Lee et al., 2003).

Previous studies have reported that phosphatidylcholine and ganglioside actions are most interesting connections with the alcohol mechanism in the brain, and these actions are potential markers of synaptic membrane development and alteration in chronic alcohol intoxication (Harris et al., 1984; Klemm, 1998). Moreover, Harris and colleagues interpreted that some of the changes in the physical properties of synaptic membranes produced by long-term alcohol exposure may be due to subtle alterations of ganglioside arrangement or synaptic membrane metabolism (Harris et al., 1984; Klemm, 1998). Thus, from our results and previous studies, significantly increased choline-containing compound signals might indicate that long-term intoxication of alcohol may lead to changes in synaptic membrane component turnover of the phosphatidylcholine and phospholipids, possibly due to subtle alterations in ganglioside arrangements or synaptic membrane metabolism (Ende et al., 2006; Lee et al., 2003).

Our results showed that mIns concentrations were significantly lower (approximately 15.9%) in the ethanol-treated group compared to the control group. In the ex vivo 1H HR-MAS NMR results, the mlnsNorm ratio levels were also significantly lower. Braunová and colleagues have reported a similar result in the chronic ethanol-treated rat model (Braunová et al., 2000). The mlns/tCr ratios decreased significantly (approximately 19%) in the central brain region of the rats exposed to ethanol for 8 weeks, compared to controls (Braunová et al., 2000). The myo-inositol is mainly found in glial cells, functions in osmoregulation, and its concentration is altered in many brain disorders (Brand et al., 1993; Haris et al., 2011; Schweinsburg et al., 2000; Thurston et al., 1989). The release of myo-inositol from brain cells serves to counteract cell swelling (Häussinger et al., 1994). Accordingly, Häussinger and colleagues suggested that myo-inositol concentrations may be reduced under conditions of hypo-osmolality (Häussinger et al., 1994). It has also been emphasized that chronic alcohol dependence can cause a hypo-osmolality syndrome with severe hyponatremia (Edwards and Mosher, 1980). Previous studies have reported that hyponatremia is caused by the hypo-osmolality of the extracellular fluid, leading to cell swelling (Putterman et al., 1993; Trimarchi et al., 1999). In a clinical study, it was reported that myo-inositol levels were significantly increased in recently detoxified alcoholics (mean abstinence period: 41.5 days) compared to healthy subjects (Schweinsburg et al., 2000). Schweinsburg and colleagues found that increased myo-inositol levels might indicate proliferation or activation of glial cells (Schweinsburg et al., 2000). However, decreased myo-inositol levels were reflected in reduced osmolar stability in glial cell activation (Schweinsburg et al., 2000). Thus, the results of the present study might indicate that decreased myo-inositol levels reflect the hypo-osmolality and hyponatremia of the astrocytes.

Demant and coworkers emphasized that hypo-osmolality and hyponatremia can be induced by severe neurological disorders and biological anomalies (Demant et al., 1971). One study showed that hyponatremia and hypo-osmolality were likely due to water intoxication (i.e., overhydration), low sodium content of alcohol, and poor dietary intake of sodium and potassium (Karthigasu, 1977). From these previous studies (Demant et al., 1971; Karthigasu, 1977), the factors may lead to hypo-osmolality and hyponatremia, which result in significantly lower levels of myo-inositol than that in the control rats, due to long-term alcohol intoxication. Other studies have also shown hyperosmolality condition in which acute alcohol intoxicated patients present elevated osmolarity levels with increased blood alcohol levels (BALs) (Champion et al., 1975; Vonghia et al., 2008). And, they have reported that the elevated serum osmolarity conditions can be influenced by various factors, including dehydration, renal impairment, vomiting and diarrhoea (Champion et al., 1975; Vonghia et al., 2008). Thus, serum osmolarity levels (hypo-osmolality and hyperosmolality) could be affected by several factors, such as subjects (alcoholic patients and alcohol-intoxicated animals) as well as various health conditions.

Some limitations of our methodologic approach should be discussed. First, we did not quantitate BALs because the ethanol liquid diet cannot control the self-administration timing in rodent models (Gil-Mohapel et al., 2010). Thus, the ethanol liquid diet has low and stable BALs (Gil-Mohapel et al., 2010). Nonetheless, the ethanol liquid diet has proven to be a very effective technique for self-administration of ethanol in rodent models (Gil-Mohapel et al., 2010). Although the ethanol liquid diet has low stable BALs, it has provided advantages including continuous delivery of both ethanol and essential nutrients without triggering aversion in animals (Gil-Mohapel et al., 2010; Lee et al., 2012). In addition, one important advantage of the liquid diet is the absence of side effects caused by poor nutrition (Lee et al., 2012). Hence, further study is needed to acquire more stable BALs in long-term ethanol-intoxicated rats using a quantitatively ethanol exposure method, such as vaporized ethanol inhalation technique. Second, previous studies showed different behavior patterns like the decreased locomotor activity (in open-field test), seizure susceptibility (in seizure thresholds test), and anxiety-related behavior (elevated plus maze test) in long-term ethanol-treated rats compared to controls (Kampov-Polevoy et al., 2000; Li et al., 2008). Because we focused on quantifying neurochemical changes induced by long-term ethanol consumption, we did not conduct behavior test using previously described methods. However, we could observe abnormal behavioral patterns over the time (i.e., loss of balance and no self-grooming) that commonly seen in alcohol-intoxicated rodent model (van Erp and Miczek, 1997; Kampov-Polevoy et al., 2000). Third, ethanol-treated rats have a relatively short period of ethanol intake. Previous studies have investigated longer periods of ethanol intake, up to 60 and 24 weeks (Lee et al., 2003; Zahir et al., 2009). These studies were likely long enough to observe significant changes of cerebral metabolism in the chronic ethanol exposed rats compared with control rats. Therefore, further study with longer period of ethanol intake is required to strengthen our findings. Finally, the number of experimental animals is too small for any definite conclusions. Hence, additional study on a larger population is necessary for more quantitative assessments.

5. Conclusion

In summary, the present study demonstrated that in vivo MRS and ex vivo HR-MAS spectra provide valuable information to interpret brain metabolism in the long-term ethanol-exposed rats. Decreased mlns concentrations in long-term alcohol exposed rats may reflect hypo-osmolality and hyponatremia of astrocytes. Moreover, an increase in choline-containing compounds may indicate an increased turnover rate of phosphatidylcholine and other phospholipids, reflecting an adaptive mechanism of the brain.
Thus, long-term alcohol consumption may cause functional and metabolic dysfunction in the frontal cortex region of the rat brain. Therefore, altered mlns and choline-containing compound levels might be utilized as key markers in chronic alcohol intoxication and provide useful neurochemical information about human chronic alcoholism-related brain damage.

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