Metabolite quantitation in experimental demyelination model using in-vitro MRS: A pilot study

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Introduction: Multiple sclerosis (MS) is an inflammatory demyelinating disease wherein the pathological changes are associated with aletrations in the metabolic functioning of the tissues. MR techniques (MRI and MRS) serve as a potential tool to gain insight about the tissue metabolism at the molecular level [1, 2] and provide information about pre-morphological biochemical changes that indicate disease initiation. In MS, MRS enables evaluation of the severity of the disease, follow disease evolution and understand its pathogenesis. However, since such detailed studies are difficult in humans, experimental models of demyelination have been established wherein a complete evaluation of the disease pathology and biochemistry can be achieved beginning from the onset. In this study a comprehensive characterization and quantitation of the various metabolites present in control and demyelinated brain tissues (on day 3) were carried out using in vitro ^{1}H-MRS to monitor the changes in the metabolites levels during demyelination. In vitro MRS has an advantage that even metabolites present in very small concentration can be identified. To the best of our knowledge, this is the first in vitro study on quantitation of metabolites in animal model of demyelination.

Materials and Methods: 10 control and 8 Wistar rats (male, body wt = 200-250 g) with lysophosphatidyl choline (LPC)-induced demyelination (in the internal capsule (ic) area) were used. 5 out of the 8 rats were monitored on day 3 for the presence of demyelination lesion. These rats were sacrificed on day 3, their ic region of the brain were excised and snap frozen in liquid N_2. The tissues were subjected to perchloric acid extraction to study water soluble metabolites. The resulting sample was lyophilized and dissolved in D_2O then subjected to proton NMR spectroscopy on a 700 MHz NMR spectrometer (Varian). 3-Trimethyl silyl propionic acid (TSP) served both as a chemical shift reference and concentration standard. One-dimensional spectra with water suppression were acquired using over a spectral width of 7716 Hz using 32 K data points, 64 scans and a relaxation delay of 14 seconds. For assignments of the peaks observed in the 1D spectrum, two-dimensional correlated spectroscopy (COSY) and total correlation spectroscopy (TOCSY) wer carried out. The concentrations of metabolites were determined by comparing the integrated intensity of the isolated resonances with that of the TSP signal and the values were reported as mean ± standard deviation. Statistical analysis was carried out using Man Whitney U test to compare between means. Probability values of 95% ($p<0.05$) were reported as significant results.

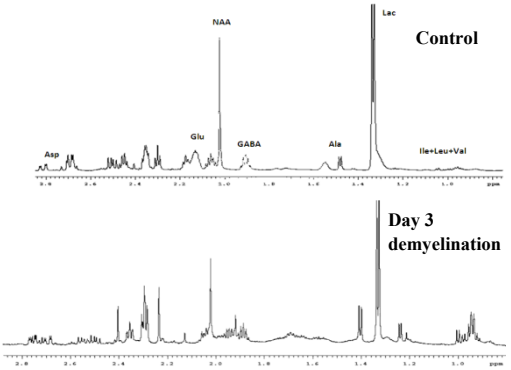

Figure 1: *1-D spectrum from (a) control (b) day 3 demyelination*

Results: 28 metabolites were assigned unambiguously in the extract of the rat brain tissues. The methyl protons of isoleucine (Ile), leucine (Leu) and valine (Val) appeared between 0.92-1.04 ppm. Resonances due to Lac and Ala were observed at 1.33 and 1.45 ppm respectively (Fig 1). The β-CH_2 resonances of glutamate (Glu)/glutamine (Gln) were identified as multiplets between 2.04-2.08 ppm in the 1-D spectra (Fig 1 and 2). Singlets observed at 2.02 ppm, 2.41ppm and 8.46 ppm were assigned to N-acetyl aspartate (NAA), succinate and formate (For) respectively. Resonances due to γ-amino butyric acid (GABA) were observed at 1.89, 3.02 and 2.30 ppm. Resonances of aspartate (Asp) were observed at 2.68 and 2.80 ppm (Fig 2). The resonance of the H1' proton of α-and β-anomer of glucose were assigned at 5.23 ppm and 4.64 ppm respectively. Resonances of myo-inositol (mI) were observed at 3.61 ppm and 3.27 ppm. N(CH_3) of glycerophosphorylcholine (GPC) and phosphorylcholine resonances were observed at 3.23 ppm and choline was observed at 3.20 ppm. The N(CH_3) of creatine and phosphocreatine were assigned at 3.02 ppm. Resonances of adenosine and ATP were assigned at 8.20 ppm and 8.27 ppm respectively. Significant decrease was observed in the concentration of NAA while significant increase in the concentrations of Ala, Tau and For was observed in demyelinated tissues when compared with controls ($p \leq 0.05$) (Table 1).No significant differences were observed in the concentration of Cho, mI and Glu and GABA.

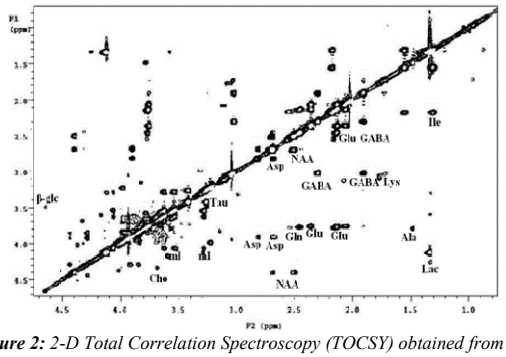

Figure 2: *2-D Total Correlation Spectroscopy (TOCSY) obtained from ic region of control rat brain*

Discussion: The results revealed a significant decrease in the concentration of NAA and increase in Ala, Tau and For in day 3 demyelinated tissues. NAA, which is primarily localized in neurons, is considered a marker for neuronal density as well as a regulator of myelin synthesis. Reduced levels of NAA indicate neuronal damage which is observed during demyelination due to the breakdown of myelin sheath. No significant difference was found in Cho although the values were observed to be higher in demyelinated tissues compared to controls. A possible hypothesis for this observation could be that since the tissues were obtained on day 3 which is the pre-acute stage of demyelination, changes in Cho had not yet started at this early stage. In addition, our results also revealed higher amount of Ala and Tau. Ala is suggested to serve as a nitrogen donor between glutamatergic neurons and surrounding astrocytes [3]. Increased levels of Ala could be attributed to damage to these neurons leading to its accumulation. Tau has been implicated in many biological functions including inhibitory neurotransmission, CNS development, membrane stabilization, and blood pressure regulation [4]. Increased Tau has been observed in pathophysiological conditions in which cerebral cells swell, such as ischemia-hypoxia, hypoglycemia, or epilepsy [5]. Hence, elevated Tau could be related to the decreased signal transmission across the neurons due to demyelination. Thus our study suggests that absolute quantitation of the metabolites enables a better understanding and detection of biochemical changes right from the pre-acute stage of the disease.

Metabolite	Concentration (mm/Kg wet wt)		
	Control (n = 10)	Day 3 (Lesion) (n=5)	P values
Ala	0.58±0.65	1.02±0.36	0.05
NAA	13.51±10.40	5.92±2.16	0.05
Tau	1.74±1.17	3.59±0.63	0.01
For	0.18±0.13	0.99±0.30	0.00

Table 1: *Concentration of metabolites in control and demyelinated tissue*

References: [1] Brindle JT et al. Nat Med. 2002; 8: 14439-44. [2] Kamlesh P et al. Spine 2005; 30: 68-72 [3] Waagepetersen HS et al. J Neurochem. 2000;75 (2):471-9. [4] Huxtable RJ et al. Neurochem Int. 1989; 15: 233-8. [5] Shimada N et al J Neurochem. 1993; 60: 66-71.