The study on estimation of time since death with proton magnetic resonance spectroscopy under different temperature

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That death has been correctly certified, and that justice has been served. Estimation of the time of death (post-mortem interval, PMI) is occasionally a critical issue in adjudication of a criminal or civil action. An inclusion or exclusion of possible suspects based on a trustworthy and early estimation of PMI can be decisive for further measures of tracing, collecting evidence, and directing a successful investigation. There are few areas of forensic medicine research that have received so much attention for so many years. There is currently no single accurate marker of the time of death with the exception of a credible witness or time-sensitive scene [1]. One reason for this disappointing outcome may be the fact that most of these methods attempted to characterize PMI by a few or even just one specific parameter's variable in constant ambient temperature [2]. It is crucial that we reduce or eliminate the effect of temperature variation in the estimation of PMI. Here we show the first results by using proton magnetic resonance

spectroscopy (^{1}H-MRS) technique to determine the order of degradation of certain substances, present in the brain tissue of a rabbit model, under different temperatures; our goal is to reduce or eliminate the temperature effect in the estimation of PMI.

More than 300 years ago, an Italian scholar once said that PMI was crucial for forensic medicine. Many scholars have done a lot of researches on the estimation of PMI over the years, but no objective and precise means has been derived for estimating PMI. If new parameters are studied, the following should be kept in mind: what is the nature of post-mortem interval. Methods on estimation of time drawn after biological processes, and any biological processes are mainly influenced by enzymatic changes, while the activity of enzymes is strongly influenced by ambient temperature. Among all the causes, the biggest problem is the impact of the ambient temperature on PMI [3]. After death, the body no longer maintains its internal metabolic homeostasis and different changes shall occur to the various metabolites inside corpses under different ambient temperatures. We are able to determine the variation law after death for some kind of substance under a certain temperature, but we are unable to be sure of the variation law after death under different temperatures. To determine the variation law for a substance restricted to a specific temperature shall not make a veracious estimation of PMI. Postmortem changes not only involve some kind of substance, but the synergistic effects of multiple substances or the whole body as the temperature changes. It is doubtful if there exists some relevant law of degradation, when two or multiple substances degrade under different temperatures; namely, all these substances are affected by the temperature without exception when analyzed independently, but if there is some degradation association among two or more substances due to the impact of a common temperature, thus the changing association between these substances is slightly or not influenced by the ambient temperature. That is to say, we no longer focus on the postmortem variation for an individual metabolite, but the postmortem relationship of

change among several substances. PMI is thereby deduced by this association with some kind of variation law. Surely, what is of the most importance is that the association is not sensitive to temperature changes. Proton magnetic resonance spectroscopy (^{1}H-MRS) is a neuroimaging modality that is widely used to examine regional alterations in tissue concentrations of certain neurochemicals that are indicative of brain metabolism [4].It may determine the content of multiple metabolites non-invasively by once[5, 6]. This technological feature of ^{1}H-MRS may avoid experimental errors during multiple detections for diversified substances in different experimental platforms and under different experimental techniques.

We adapted ^{1}H-MRS techniques in order to carry out studies of rabbits' brains as early as 24h post-mortem and to follow the developmental changes of the N-acetylasoartate, total choline and total creatine concentrations and studied their ratio relation at group A(10℃) and group B(30℃). **^{1}H-MRS manifestations at 0h:** As shown in **Figure. 1.**

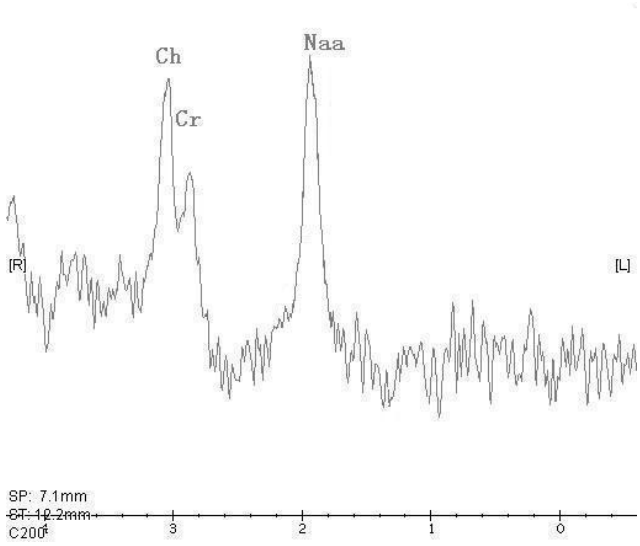

Figure 1 The following metabolites could be observed by ^{1}H-MRS at 0h: Oscillogram of ^{1}H-MRS at 0 hour. N-acetylasoartate (Naa, 2.0ppm), total choline (Cho,

3.2ppm), and total creatine (Cr, 3.0ppm). The ratios between relative quantitative were: Naa/Cr = 1.51±0.17, Naa/Cho = 1.31±0.14 and Cho/Cr = 1.15±0.13.

Changes in relative quantitative of Naa/Cr, Naa/Cho and Cho/Cr within the postmortem 24 h: Group A**:** Naa/Cr: continuous linear decline after execution, decreased to 1.33±0.13 at 0.5h, 1.03±0.13 at 6h and 0.64±0.13 at 24h; Naa/Cho: continuous linear decline after execution, decreased to 1.06±0.17 at 0.5h, 0.69±0.16 at 6h and 0.47±0.14 at 24h; Cho/Cr: continuous linear increase after execution, increased to 1.27±0.12 at 2h, 1.49±0.13 at 6h and 1.66±0.12 at 24h; Group B: Naa/Cr: continuous linear decline after execution,, decreased to 1.34±0.12 at 0.5h, 0.98±0.11 at 6h and 0.61±0.11 at 24h; Naa/Cho: continuous linear decline after execution, decreased to 1.05±0.17 at 0.5h, 0.56±0.12 at 6h and 0.26±0.12 at 24h; Cho/Cr: continuous linear increase after execution, increased to 1.28±0.16 at 0.5h, 1.74±0.12 at 6h and 2.38±0.13 at 24h. (Table 1, Figure 2)

PMI(h)	Naa/Cr		Naa/Cho		Cho/Cr	
	10℃	30℃	10℃	30℃	10℃	30℃
0	1.51±0.17	1.51±0.17	1.31±0.12	1.31±0.12	1.15±0.17	1.15±0.17
0.5	1.33±0.13	1.34±0.12	1.06±0.17	1.05±0.17	1.27±0.12	1.28±0.16
1	1.31±0.20 * *	1.32±0.11 * *	1.01±0.12 * *	0.94±0.11 * *	1.29±0.22	1.41±0.17 * *
2	1.31±0.12 * *	1.31±0.11 * *	1.01±0.12 * *	0.95±0.13 * *	1.29±0.15	1.37±0.13
4	1.28±0.13 * *	1.29±0.12 * *	0.92±0.13 * *	0.85±0.12 * *	1.40±0.14 * *	1.51±0.16 * *
6	1.03±0.13 * *	0.98±0.11 * *	0.69±0.16 * *	0.56±0.12 * *	1.49±0.13 * *	1.74±0.12 * *
8	0.90±0.16	0.88±0.12	0.58±0.11	0.43±0.12	1.55±0.11	2.02±0.32
12	0.74±0.12	0.73±0.10	0.49±0.16	0.31±0.17▲	1.63±0.17	2.36±1.29
16	0.72±0.13	0.68±0.12▲	0.47±0.12▲	0.28±0.22▲	1.64±0.11	2.37±0.10▲
24	0.64±0.13▲	0.61±0.11▲	0.47±0.14▲	0.26±0.12▲	1.66±0.12▲	2.38±0.13▲

Table 1 Variance of Naa/Cr, Naa/Cho and Cho/Cr in 24 hours(groupA & groupB): The

paired t test was applied for statistical analysis: Naa/Cr $P < 0.05$, no difference, Naa/Cho $P > 0.05$, with difference and Cho/Cr $P < 0.05$, no difference. * Indicates $P < 0.05$ between PMI and 0h , ** Indicates $P < 0.01$ between PMI and 0h, ^ Indicates $P < 0.01$ between PMI and 4h.

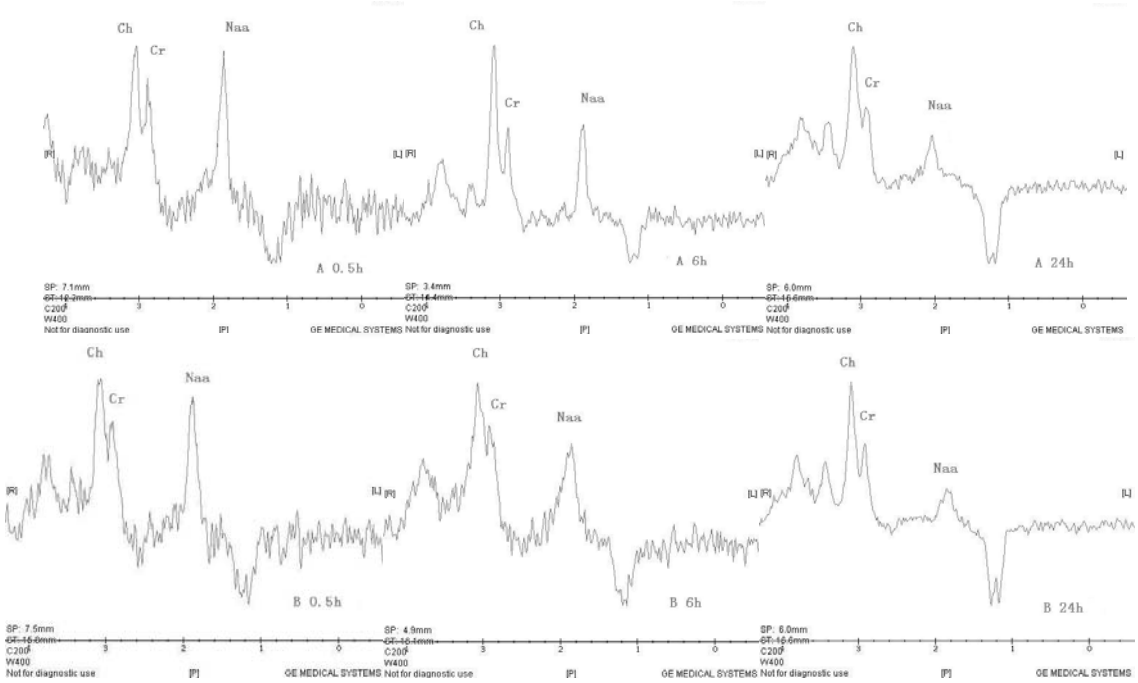

Fig 2 Oscillogram of ^{1}H-MRS in 0.5hours ~ 24 hours(groupA & groupB): There we show the apparent decrease of Naa and Cr in group A and B.

Statistical analysis of Naa/Cr and Naa/Cho within 24h postmortem: There was no significant difference in Naa/Cr and Cho/Cr between group A and B at different temperatures within 24h. Statist/ical analysis was executed for Naa/Cr and Cho/Cr in group A and B. Naa/Cr: continuous linear decline after execution of experimental animals, decreased to 1.34±0.13 at 0.5h, 0.99±0.12 at 6h and 0.63±0.12 at 24h; Cho/Cr: continuous linear increase after execution of experimental animals, increased to 1.28±0.14 at 0.5h, 1.62±0.13 at 6h and 2.02±0.13 at 24h **(Table 3, Figure 3).**

PMI(h)	n	Naa/Cr	Cho/Cr

0	24	1.51±0.17	1.15±0.17
0.5	24	1.34±0.13	1.28±0.14
1	24	1.32±0.15 *	1.30±0.20
2	24	1.31±0.12 *	1.32±0.14
4	24	1.29±0.12 *	1.46±0.15 *
6	24	0.99±0.12 *	1.62±0.13 *
8	24	0.89±0.14	1.79±0.20
12	24	0.74±0.11	2.00±1.18
16	24	0.70±0.13	2.01±0.10 ▵
24	24	0.63±0.12 ▵	2.02±0.13 ▵

Table 3 Variance of Naa/Cr and Cho/Cr in 24 hours: * Indicates $P < 0.01$ between PMI and 0h , ▵ Indicates $P < 0.01$ between PMI and 4h.

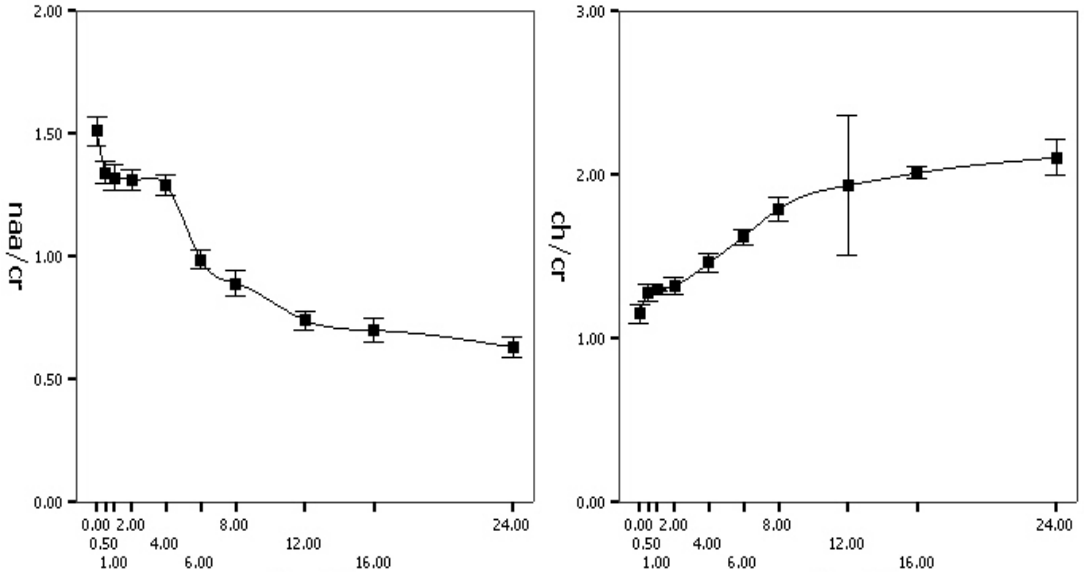

Figure 3 The extension of PMI of Naa/Cr and Cho/Cr in 24 hours

Regression analysis: having Naa/Cr as the independent variable, the quadratic polynomial regression equation is $y=0.0019x^2-0.803x+1.4498$ ($R^2 = 0.962$) (Equation 1); having Cho/Cr as the independent variable, the quadratic polynomial regression equation is $y=-0.0024x^2+0.926x+1.1777$ ($R^2 = 0.986$) (Equation 2). If we estimate PMI by Equation 1, the average deviations are about 0.03 ~ 0.63 h, and when we estimate PMI by Equation 1, the average deviations are about 0.03 ~ 0.68h.(Table 4, Figure 4, Figure 5)

x	方程（y 为 PMI）	R^2	F	P
Naa/Cr	$y=0.0019x^2-0.803x+1.4498$	0.962	88.91	<0.001
Cho/Cr	$y=-0.0024x^2+0.926x+1.1777$	0.986	248.23	<0.001

Table 4 The regression functions between PMI and Naa/Cr and Cho/Cr.

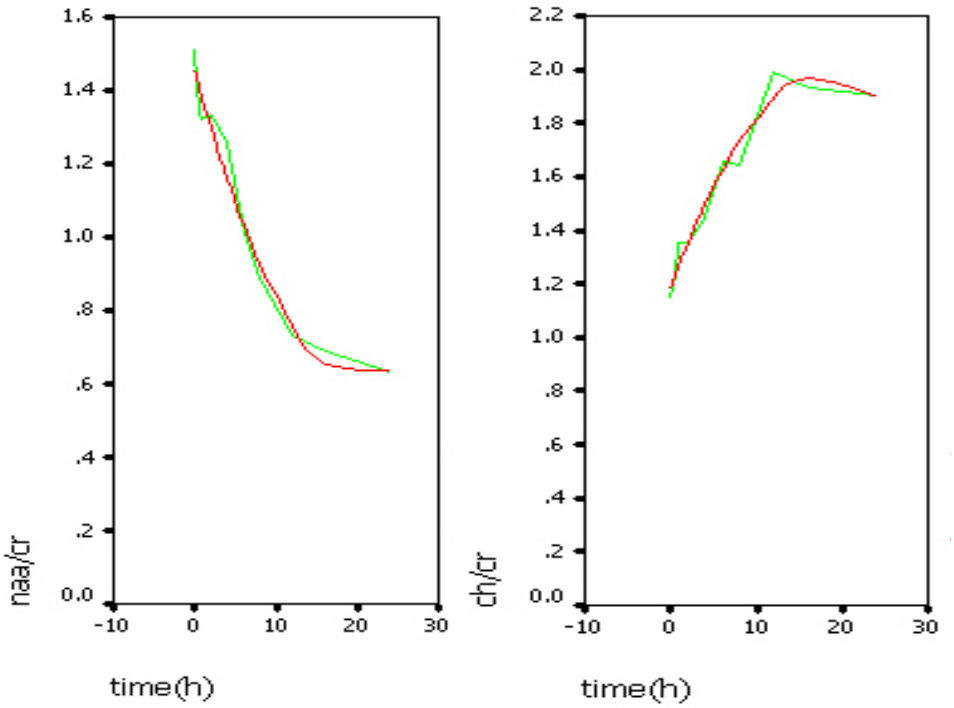

Fig 6 regression curve of Naa/Cr and Cho/Cr in 24h, — : the reference quadratic polynomial regression curve; — : the naa/cho and cho/cr reference quadratic polynomial regression curve.

PMI (h)	Naa/Cr			Cho/Cr		
	mean	residual error	average deviations (h)	mean	residual error	average deviations (h)
0.5	1.34	0.07016	0.03	1.28	0.05658	0.03
1	1.31	0.05147	0.51	1.30	0.03210	0.03
2	1.29	0.01301	0.03	1.32	0.03327	0.06
4	1.32	0.13037	0.52	1.46	0.04963	0.19
6	0.99	0.04775	0.28	1.62	0.02680	0.16
8	0.89	0.04135	0.33	1.79	0.02521	0.20
12	0.74	0.02498	0.29	2.00	0.05676	0.68
16	0.70	0.03948	0.63	2.01	0.03496	0.56
24	0.63	0.00732	0.17	2.02	0.00175	0.04

Table5 The respective average deviations of Naa/Cr and Cho/Cr equantion in PMI eatimation

^{1}H-MRS technology may detect a variety of organs and substances. This article shall investigate the law of degradation for N-acetylasoartate, choline, phosphocreatine and creatine under different temperatures (10℃ and 30℃), and explore the relationship between the law and PMI. Among these substances, Naa is important in several neurobiological regulatory processes, such as neuronal protein synthesis, lipid production, and osmotic stress protection; it also acts as the primary storage pool for the excitatory amino acid aspartate [7, 8]. A decrease of Naa has been shown to be equivalent to a neuronal loss, cellular dysfunction, lowered neuronal viability, or impaired function of neurons, although the physiological role of such a loss is still unknown[5, 9], Choline is an organic cation that plays an essential role in plasma membrane formation and cell growth. In the brain, choline also contributes to neurotransmission as a precursor of acetylcholine [10]. Cho acts as a precursor to the neurotransmitter acetylcholine and is incorporated into two major neuronal membrane phospholipids: phosphatidylcholine and sphingomyelin [11], which could be associated with changes in local neuronal metabolism involved in the incorporation of cytosolic choline compounds into phospholipids [12]. Cho availability in the adult brain depends on the supply of Cho by transport through the blood-brain barrier. Newly taken up Cho is largely stored in bound form, mainly in the form of phosphatidylcholine from which it can be released by phospholipases [13]. A cho increase can be as a marker of new or increased synaptic plasticity, activity of cell differentiation, proliferation and abnormal increase in cell membrane metabolism, and then decline in Cho represents cell loss or injury [14, 15, 16]. Cr is an endogenous amino acid produced from glycine, methionine and arginine in the liver, kidney and pancreas [17]. Recent experimental findings have demonstrated that creatine affords significant neuroprotection against hypoxia, amyotrophic lateral sclerosis, ischemia, oxidative insults and excitotoxicity [18]. Cr represent the main energy

buffer system sustaining adenosine diphosphate and adenosine triphosphate in the cell [19], Cr, including phosphocreatine and creatine, are related with energy metabolism [20].

In this experiment, Naa/Cr and Naa/Cho of group A and B declined continuously within 24h and Cho/Cr increased consistently within 24h. There was no significant difference in Naa/Cr and Cho/Cr between group A and B, indicating that postmortem changes of Naa/Cr and Cho/Cr were hardly influenced by the ambient temperature. After death, essential causes of changes to all metabolites are ischemia, hypoxia and energy barrier. Therefore, all the postmortem changes are centered by energy consumption. In this experiment, the neuron marker Naa and cell membrane marker Cho all changed correspondingly centering on the change of Cr, the energy marker, and thus the ratios among them were not sensitive to temperature changes. Quadratic polynomial equations of one variable for postmortem Naa/Cr and Cho/Cr within 24h were respectively: Naa/Cr, $y=0.0020x^2 - 0.0815x + 1.4532$, $R^2 = 0.971$; Cho/Cr, $y= -0.0024x^2 + 0.0870x + 1.1876$, $R^2 = 0.962$. The Naa/Cr equation applied for PMI estimation, residual analysis showed that the average deviation time was between $0.03 \sim 0.63$ h when PMI<24h; the Cho/Cr equation applied for PMI estimation, residual analysis showed that the average deviation time was $0.03 \sim 0.68$h. These indicated that the degradation law of Naa/Cr and Cho/Cr within the postmortem 24h tallied comparatively well with the distribution of regression curves and errors of deviation time were relatively low. It should be pointed out specially that according to related literatures, degradation of Naa happened relatively rapidly within the postmortem 24h, to the utmost after 24h. Therefore, Naa/Cr might be applicable for estimation of early PMI, but not for estimation of late PMI. In addition, there was no evident statistical difference in Cho within the postmortem 24h, but with the alterations of the cell tissue structure, Cho, as a cell membrane marker, would change correspondingly, possibly lasting for a rather long period of time. The corresponding correlation between Cho and Cr may be suitable for late estimation of PMI.

To sum up, there are definite relationships between Naa/Cr, Cho/Cr and PMI, which may be used for estimation of time since death. The estimation of PMI by the degradation correlation among different substances under different temperatures may pave the new road for PMI estimation. The brains of the rabbits included in this experiment are our testing- specimen. Though it has certain limitations and the intracranial substance metabolism of human differs from that of rabbits, there is no doubt that if insensitivity of the substance metabolism to temperature exists in rabbits' brains, it will certainly exist in human brains. Proton magnetic resonance spectroscopy shall have broad prospects in PMI estimation through investigating the correlation of postmortem changes among different substances.

METHODS

24 healthy New Zealand white rabbits of either sex with weight 2.5kg-3.0kg were provided by the Animal Center of Tongji Medical College. To avoid the complete degradation or rapid degradation of the metabolites prepared for detection due to too low or high degradation temperature, and to leave some discrepancy interval for the degradation temperature in each group, the 24 experimental animals were randomly divided into group A (10℃) and group B (30℃), n=12 for each. The experimental animals were deprived of food and water within 8h before the experiment, in order to reduce the impact of blood glucose on intracranial metabolites [21]. Before operation, rabbits were anesthetized with Ketamine Hydrochloride (100mg/kg) intramuscularly. After the experimental animals were anesthetized, 10ml of air was injected into their auricular veins within 10s and later on these animals were put to death by air embolism. Their brains were removed as quickly as possible and were then tightly packaged into airtight plastic films to separate them with the outside environment, mimicking the intracranial state of the brains. The above brains of group A and B were respectively imbedded in homogeneous silica gel cases preheated under the temperature of 10℃ and 30℃, so as to enhance the signal noise ratio (SNR) in detection [22], and then the silica

gel cases loaded with rabbit brains were respectively put into the thermo flasks preheated under the temperature of 10℃ and 30℃, to ensure a constant temperature for degradation of specimens, making preparations for detection at the next time point.

GE Signa Highspeed MR/I 1.5T superconductive magnetic resonance imaging-spectrometer and its corresponding workstation version 4.0 Sun Workstation were applied for Single-voxel ^{1}H-MRS examinations. Rabbit brains were located in the center of surface coils and the multi-voxel ^{1}H-MRS examination was performed at 0h, 0.5h, 1h, 2h, 4h, 6h, 8h, 10h, 12h, 16h and 24h after the establishment of models. Coronal T2-weighted images (T2WI) and axial and sagittal T1-weighted images (T1WI) were obtained using spin echo (SE) sequences; The volume of interest (VOI) was placed in the parietooccipital region (7mm×7mm×7mm) of the rabbit brain, yielding a mix of gray (GM) and white matter (WM) of GM/WM≈ 1. The Probe SI scanning sequence used the sequence of point-resolved surface coil spectroscopy (PRESS) and chemical-shift selective saturation (CHESS) was used to perform water suppression. TR/TE = 1500ms/135ms, with the slice thickness of 3mm and slice gap of 1mm. The saturated zone was added surrounding VOI during positioning, to suppress the interfering constituents around VOI. Receival and launch gain adjustment, intra-voxel shimming and central frequency settings were all completed by auto prescan. The effect of shimming was < 10Hz while reaching the full width at half maximum (FWHM), water suppression > 96%. Func Tool software was used at Sun workstation (4.0) for analyzing; the software automatically performed metabolism spectrum reconstruction, baseline correction, signal averaging and metabolite identification; the peak area ratio between various metabolites was regarded as relative quantitative.

All data are expressed as mean ± standard deviation (SD). All statistical analysis and residual analysis was performed with SPSS 11.5. Paired-samples *t*-test used for comparisons between two sets of continuous data. $P < 0.05$ was regarded as significant difference.

1. *Handbook of forensic pathology* (eds Richard, C. & Froede, MD.) 69(College of American Pathologist. Press, Northfield, 2003)

2. Claus, H. & Burkhard, M. Estimation of the time since death. *J.* Forensic Sci. Int 165, 182-184 (2007).

3. C, Henssge. & B, Madea. Estimation of the time since death in the early post-mortem period. *J*. Forensic Sci. Int 144, 167-175 (2004).

4. Lyoo, IK. & Renshaw, PF. Magnetic resonance spectroscopy: current and future applications in psychiatric research. *J*. Biol Psychiatry Feb, 51(3), 195-207 (2002).

5. Akira, M. & Tomonori, I. Non-invasive quantification of lactate by proton MR spectroscopy and its clinical applications. *J*. Clinical Neurology and Neurosurgery 107, 379-384 (2005).

6. Franz, R. & Michal, B. Decrease of NAA with aging outside the seizure focus in mesial temporal lobe epilepsy – A proton-MRS study at 3 Tesle. *J.* Brain Research 1179, 131-139 (2007).

7. Baslow, MH. N-acetylasoartate in the vertebrate brain: metabolism and funchtion. *J*. Neurochem Res Jun, 28(6), 941-953 (2003).

8. Demougeot, C. & Garnier, P. N-Acetylaspartate, a marker of both cellular dysfunction and neuronal loss: its relevance to studies of acute brain injury. *J*. Neurochem Apr, 77(2), 408-415 (2001).

9. Chang, K. & Adleman, N. Decreased N-acelaspartate in children with familial bipolar disorder. *J*. Biol Psychiatry Jun, 53(11), 1059-1065 (2003)

10. Hirohisa, T. & Masato, I. Functional characterization of choline transport in rat astuocyte. Neurosciencn Research 58S, 43 (2007).

11. Sung, T. & Chi, BC. Variation of the choline signal intensity in the dorsolateral prefrontal cortex of rats exposed to the forced swimming test as detected by in vivo ^{1}H-MRS spectroscopy. *J*. Journal of Neuroscience Methods 165, 89-94 (2007).

12. Duc, CO. & Weber, AH. Recyciling the cholines. *J*. Int Soc Reson Med 6,1210 (1997).

13. Klein, J. Functions and pathophysiological roles of phospholipase D in the brain. *J*. Neurochem 94, 1473-1487(2005).

14. Akira, M. & Tomonori, I. Correlation between choline and MIB-1 index in human glimas: A quantitative in proton MR spectroscopy study. *J*. Journal of Clinical Neuroscience May, 416-420(2005).

15. Derik, H. & Alexander, S. Dorsolateral Prefrontal Cortex N-Acetylaspartate/Total Creatine (NAA/tCr) Loss in Male Recreational Cannabis Users. *J*. Biol Psychiatry 61, 1281-1289(2007).

16. Thomas, E. & Ruth, S. Low N-acetyl-aspartate and high choline in the anterior cingulum of recently abstinent methamphetamine-dependent subjects: a preliminary proton MRS study. *J*. Psychiatry Research Neuroimaging 116, 43-52 (2002).

17. Wyss, M. & Kaddurah, DR. Creatine and creatinine metabolism. *J*. Physiol Rev 80, 1107-1213(2000).

18. Malcon, C. & Kaddurah, DR. Neuroprotective effects of creatine administration against NMDA and malonate toxicity. *J*. Brain Res 860, 195-198(2000).

19. Sartorius, A. & Vollmayr, B. Specific creatine rise in learned helplessness induced by electroconvulsive shock treatment. *J*. Neuoreport 14, 2199-2201(2003).

20. Reduced levels of creatine in the right medial temporal lobe region of panic disorder patients detected with ^{1}H- magnetic resonance spectroscopy. *J*. Neuroimage 16, 836-842 (2002).

21. Seapuist, ER. & Tkac, I. Brain glucose concentrations in poorly controlled diabetes mellitus as measured by high-field magnetic resonance spectroscopy. *J*. Metabolism Aug, 54(8), 1008-1013 (2005).

22. Tomohisa, O. & Setsu, S. Reproducibility of magnetic resonance spectroscopy in correlation with signal-to-noise ratio. *J*. Psychiatry research: Neuroimaging 156, 169-174 (2007).

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