The Pivotal Role of the Glutamate - glutamine Cycle in Minimal Hepatic Encephalopathy

An experimental magnetic resonance spectroscopy study

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The glutamate-glutamine cycle is essential for sustaining the neuronal secretion of the excitatory neurotransmitter glutamate. Hepatic encephalopathy, even in its most discreet form, minimal hepatic encephalopathy (MHE), interferes with the glutamate and glutamine balance due to the increase in ammonia levels in the central nervous system (CNS), induced by a combination of liver dysfunction, increased blood-brain barrier permeability and many other factors. An experimental study on 21 patients with chronic liver disease and 11 healthy volunteers was performed. Magnetic resonance spectroscopy demonstrated an increase of the glutamate-glutamine complex peak, with high predictive value for MHE, especially when the metabolites are referenced to creatine, a stabile metabolite in the CNS. This paper also explores the pathophysiology of MHE with emphasis on the involvement of the glutamate-glutamine cycle in the development of this complication.

Keywords: glutamate, glutamine, creatine, minimal hepatic encephalopathy, magnetic resonance spectroscopy

Glutamate is the most abundant excitatory neurotransmitter in the central nervous system, and is obtained in the neurons via glutaminase from the glutamine produced by the astrocytes [1]. The glutamate-glutamine cycle transfers precursors for glutamate and carries the released transmitter back to astrocytes, where it is reused and a minor fraction is degraded [2]. This process involves a coordinated activity of astrocytes and neurons and high affinity transporter proteins that are selectively distributed on these cells [3].

Hepatic encephalopathy is a brain dysfunction caused by liver insufficiency and/or porto-systemic shunting, and is characterized by a wide spectrum of neurological or psychiatric abnormalities ranging from subclinical alterations to coma [4]. The liver dysfunction leads to hyperammonemia, which alters astrocytes function, interferes with the glutamate-dependent neurotransmission and leads to brain edema due to glutamine retention in the astrocytes [5]. Minimal hepatic encephalopathy (MHE) is the mildest form of hepatic encephalopathy and refers to the subtle changes in cognitive function, electrophysiological parameters, cerebral neurochemical/neurotransmitter homeostasis, cerebral blood flow, metabolism and fluid homeostasis that are observed in patients with cirrhosis, who have no clinical evidence of hepatic encephalopathy [6].

Both glutamate and glutamine may be investigated by Magnetic Resonance Spectroscopy (MRS), which is a non-invasive imaging method that quantifies a short series of substances within a designated scanning volume [7]. Magnetic Resonance Spectroscopy may certify the diagnosis of MHE with significant specificity and sensibility by demonstrating the highly-specific pattern of metabolites ratio identified in this condition [8].

Experimental part

A prospective study was conducted on 21 patients (age range 32 to 69 years old, 12 male and 9 female) with liver cirrhosis and 11 healthy volunteers for control (aged 29 to 63, 6 male and 5 female). The patients suffered from liver cirrhosis with viral etiology, type B and C, matched between lots. The patients were subjected to a neuropsychiatric test and divided into two groups based on the results: with and without minimal hepatic encephalopathy. The group with MHE (labeled MHE+) comprised of 10 patients, while 11 were assigned to the group without MHE (MHE-).

The psychometric hepatic encephalopathy score (PHES) was used, employing the number connection test A and B, the line drawing test, the serial doting test and the digit symbol test [9]. The test was performed in the same day as the MRS investigation. The patients could score between +7 and -21 points, and assignation to the MHE lot was made according to the protocol, when the score was equal to, or lower than -5 points. Also, venous blood ammonia was measured in the morning of the test.

The cerebral magnetic resonance imaging investigation of the study lot was performed on a 1.5T Siemens Avanto machine with a dedicated workstation. Following a routine cerebral examination, the magnetic resonance spectroscopy imaging sequence was performed using a 20x20x20 mm voxel located in the parieto-occipital white matter of the dominant hemisphere (fig. 1). A short echo-time of 30 ms was used, with a single voxel spectroscopy spin echo technique. The post processing included an analysis with quantitative detailing. Creatine was used as a reference metabolite.

Since glutamate, glutamine and gamma-aminobutyric acid (GABA) resonate at similar frequencies that are possible to delineate only at high-field Magnetic Resonance

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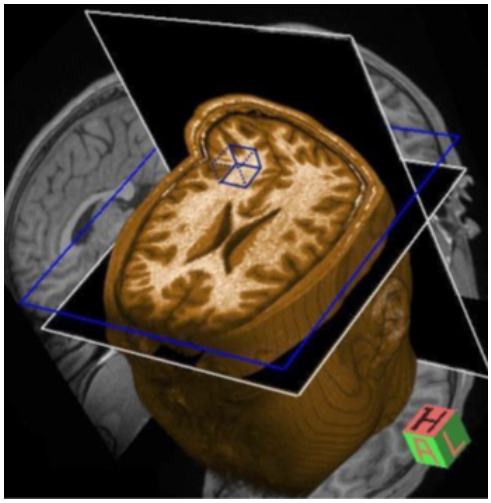

Fig. 1. MR spectroscopy voxel placement in the study group - within the parieto-occipital white matter of the dominant hemisphere

Imaging (in vivo or in vitro), the metabolites values were expressed as the complex labeled Glx. All secondary peaks of the complex were noted and analyzed.

The statistical analysis was performed using MedCalc® version 14.8.1 and the statistical tests employed were as follows: ANOVA for testing the difference between the means of continuous variables in the three lots, Pearson's correlation test to verify correlation between continuous variables, and Receiver Operating Characteristic (ROC) for evaluating the diagnostic performance of MRS and ammonia as diagnostic tests. The results were considered statistically significant when p values were lower than 0.05.

Results and discussions

The values of the glutamate-glutamine-gamma-aminobutyric acid peaks were noted in the table 1, along with the ratios to creatine and the statistical significance of the difference between lots.

The value of Glx in predicting minimal hepatic encephalopathy is described by an area under ROC curve of 0.955 with a standard error of 0.0397, 95% confidence interval between 0.765 and 0.999 and a P value of < 0.0001 (area = 0.5). The ROC curve is presented in figure 2a, and demonstrates a specificity of 90.9% and sensitivity of 90% for diagnosing MHE with a cut-off value of 150.15 in reference to PHES testing. However, using the Glx/Cr ratio as a predictive factor, increases the specificity to 100% while maintaining a 90% sensitivity, with a cutoff value of 0.693 and an AUC of 0.973 (fig. 2b).

Venous blood ammonia levels obtained in the three lots were, as follows: for the MHE+ lot 69.8 ± 5.793 μg/100 mL, in the MHE- lot 59.545 ± 5.598 μg/100 mL and for the control lot, 54.727 ± 4.750μg/100 mL. There are statistically significant differences between the three lots (p < 0.001).

The venous blood ammonia values correlate with the PHES score with a Pearson's correlation coefficient r of -0.591, and a statistical power p = 0.0050. The strength of ammonia predicting MHE is defined by an AUC of 0.886 with a sensitivity of 80% and a specificity of 72.7 for a cut-off value of 64μg/100 mL.

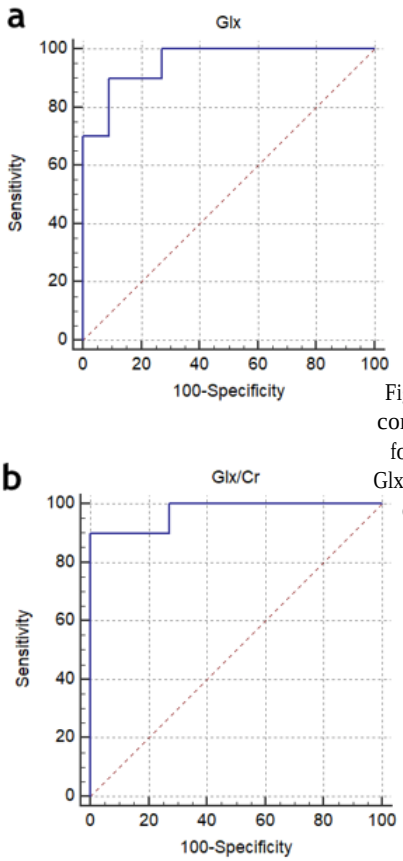

Fig. 2. a) ROC curve of Glx complex as a diagnostic test for MHE. b) ROC curve of Glx to creatine (Cr) ratio as a diagnostic test for MHE

There is no correlation between the venous blood ammonia and Glx values measured by MRS in the study lot.

Glutamate is the major mediator of excitatory signals as well as of nervous system plasticity, including cell elimination, and is continuously being released to the extracellular fluid [10]. Neurons cannot synthesize glutamate, however, astrocytes can accordingly synthesize glutamate and transport it, together with accumulated neuronal-released glutamate and GABA, after conversion to glutamine to neurons in a very active pathway: the glutamine-glutamate cycle [11]. Glutamate is converted to glutamine in the astrocytes by glutamine synthetase, an enzyme specific to astrocytes and absent in the neurons [12]. Once inside the neuron cytosol, glutamine is then converted back to glutamate in neurons by glutaminase, and then loaded into synaptic vesicles for presynaptic release [13]. After release by the neurons, glutamate is removed by transporters in order to limit the activation, and is transported back to the astrocytes.

$$Glutamate + ATP + NH_3 \xrightarrow{Glutamine\ synthetase} Glutamine + ADP + Pi \quad (1)$$

$$Glutamine + H_2O \xrightarrow{Glutaminase} Glutamate + NH_3 \quad (2)$$

Hepatic encephalopathy is a neuropsychiatric disorder in patients with liver insufficiency or after porto-systemic shunting with pathophysiological and structural alterations, manifesting by a wide spectrum of neuropsychiatric and cognitive impairments from subclinical disturbance to

Table 1

THE ABSOLUTE AND RELATIVE AVERAGE VALUES OF THE GLUTAMATE-GLUTAMINE-GAMMA-AMINOBUTYRIC ACID PEAKS IN THE THREE STUDY LOTS AND THE STATISTICAL SIGNIFICANCE OF THE DIFFERENCE BETWEEN THEM.

Metabolite	MHE+	MHE-	Control	Relevance
Glx	176.55 ± 21.43	116.26 ± 35.43	150.53 ± 32.81	P = 0.0007
Cr	215.82 ± 18.59	243.20 ± 17.20	224.15 ± 12.20	P = 0.0027
Glx/Cr	0.82 ± 0.08	0.48 ± 0.16	0.67 ± 0.14	P < 0.0001

stupor and coma [14]. Liver failure secondary to cirrhosis impairs the body's capacity to transform ammonia into urea, the metabolite that disposes of waste nitrogen molecules in normal conditions. In patients with cirrhosis, plasma ammonia concentration increases, and this increase together with high levels of inflammatory mediators can be toxic to the brain tissue and may lead to the development of neurologic manifestations [15]. The ammonia accumulation in the plasma together with increased permeability of the blood-brain barrier induce an increase of ammonia in the astrocytes, which will quickly be incorporated into glutamine that will increase in concentration. The accumulated glutamine leads to astrocyte swelling that can trigger a downward spiral leading to increase in production of reactive oxygen and nitrogen species, which can downstream target gene transcription and translation [16]. Glutamine accumulation may be also related to a decreased capacity of astrocytes to take up glutamate, thus leading to glutamate excitotoxicity [15].

Magnetic Resonance Spectroscopy is a non-invasive technique capable of providing information on brain metabolites such as choline (Cho), creatine (Cr), N-acetyl aspartate (NAA), glutamine, glutamate and GABA (Glx), as well as osmoles such as myo-inositol (mIns) and taurine [14, 17]. The conventional findings of minimal hepatic encephalopathy consist of decreased Cho and mIns peaks and increased glutamate-glutamine-ã aminobutyric acid complex peak [18]. Unfortunately, most available magnetic resonance machines destined for patient use have magnetic field strengths of 1.5 or 3T, that do not allow a robust separation of the glutamate and glutamine peaks, which resonate at adjacent frequencies; moreover, GABA is usually found in very low concentrations, obscured to the conventional MRS capabilities in these conditions, and is indistinguishable from Glu and Gln [19]. Therefore, the three substances are overlapped in a complex peak, usually labeled Glx.

In our study, the Glx complex peak was significantly higher in patients with minimal hepatic encephalopathy than the controls (fig. 3). The Glx complex peak was also demonstrated to yield a strong predictive power for diagnosing MHE. This predictive value is further increased by referring it to corresponding creatine values in each patient. It is most likely that the increased complex peak is due primarily to the glutamine concentration rise, even if at 1.5 Tesla it is not possible to separately distinguish the signal of glutamine from glutamate or GABA due to their overlap in resonating frequencies. In vivo and in vitro studies on 7 and 9.4 Tesla resolved the issue of separation, but currently there are no available solutions for clinical investigation [20].

Despite the many pathophysiological processes incriminated in the development of minimal hepatic encephalopathy, the glutamate-glutamine cycle seems to play a central role due to ammonia excess in liver insufficiency. Astrocytes are not able to induce an increase in glutamine synthetase activity and fail at transforming the ammonia excess into glutamate [21]. Nevertheless, both glutamate and glutamine are increased in patients with hepatic encephalopathy, even subclinical forms, as it is also suggested by our experimental study. Accumulation of glutamate and glutamine leads to astrocyte swelling, which induces oxidative stress and amplifies the edema [22]. The toxic effect on the mitochondria has been traditionally attributed to glutamine, even though some studies suggest that astrocyte swelling may occur in normal venous blood ammonia levels . However, our study

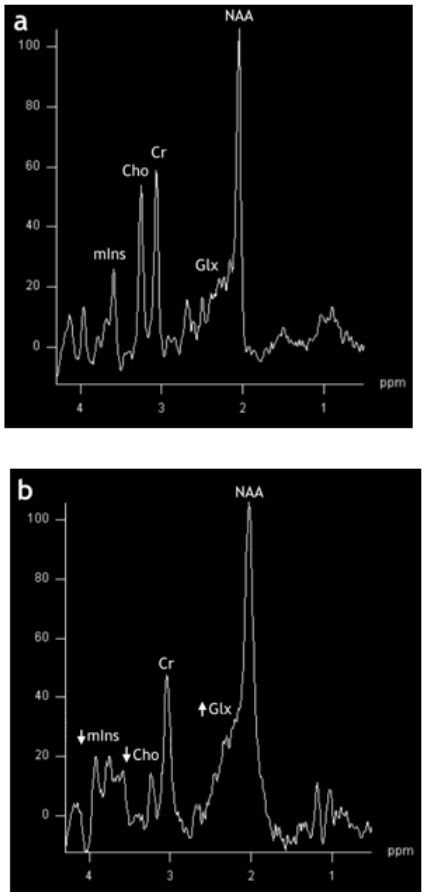

Fig. 3. MR spectrum in a patient without MHE (a) and with MHE (b). Note the increased values of the Glx peak and decreased mIns and Cho peaks, with relatively constant NAA and Cr peaks

demonstrated invariably increased values of ammonia in patients with MHE.

Other structures of the central nervous system such as neurons, capillaries or other membranes are not affected by the edema process due to low water permeability [23]. In the case of astrocytes, edema seems to be determined by the water translation from the extracellular towards the intracellular space, which alters the volume and geometry of the interstitial space, leading to a stimulation of the compensatory mechanisms that regulate cell volume by release of ions and aminoacids [24, 25]. This mechanism may account for the MRS changes of choline and myo-inositol, which may act as osmotic buffers.

Our study demonstrates that referring the Glx complex values to creatine yields a stronger statistical relevance of the difference between the study groups. Creatine is considered a marker for energy, and is commonly used as a reference metabolite due to its consistency in intra and interindividual observations [26]. However, some studies suggest an ATP depletion may occur in hepatic encephalopathy which may somewhat decrease creatine levels by activating the NMDA receptor and the Na^+/K^+ ATP pump [27].

Remarkably, the highest values of Glx metabolites were found in the MHE+ lot, seconded by the normal controls, while the lowest values were identified in the MHE- lot. Other authors cited similar results [28]. Patients in the MHE- lot, which suffer from chronic liver disease, may present an enabling of compensating mechanisms which keep the pathophysiological processes under control, and the onset of MHE occurs when these mechanisms are overrun [29]. The precise pathways of this process are still a subject for scientific speculation.

There is no correlation between the Glx changes explored by MRS and venous blood ammonia values, even though the latter ones correlate with the PHES score. This finding was expected, as ammonia plays a key role in the pathophysiology of hepatic encephalopathy, even though

not all similar studies demonstrated a statistically relevant correlation [30, 31].

The limitations of this study include the small number of patients, the possible artifacts in the acquisition of the MRS sequence, and the possible contamination of the voxel with structures adjacent to the area of interest. The intrinsic deficiencies of the PHES test may also influence the study results as they are subjected to errors due to patients misunderstanding the tasks, or their visual or motor impairment.

Future studies using multivoxel or repeated multiple locations of single voxel acquisitions may lead to more accurate results. Also, high-field MRS could provide good separation of the metabolites in the Glx complex and further explore the pathophysiological processes involved in MHE. Studies testing the usage of 14.1 Tesla MRI machines and molecular labeling techniques are becoming available and encourage the progress of understanding the glutamate and glutamine definitive roles in the neuro-astrocyte metabolism in normal and pathological conditions [32].

Conclusions

The glutamate-glutamine cycle plays a central role in the pathophysiological processes of minimal hepatic encephalopathy. The accumulation of these metabolites may be demonstrated by magnetic resonance spectroscopy, and this method may be used to predict the onset of minimal hepatic encephalopathy as they correlate with the psychometric testing in these patients. The use of Glx to creatine ratio demonstrates a higher predictive strength for demonstrating minimal hepatic encephalopathy.

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