Low myo-inositol and high glutamine levels in the brain are associated with neuropsychological deterioration following induced hyperammonemia

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Abbreviations in the text: HE: hepatic encephalopathy, mI: myo-inositol, Glx: glutamate/glutamine, aa: amino acid, $^1$H-MRS: proton magnetic resonance spectroscopy

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Six of the patients in this study have also been reported in our previous paper. (Balata S, Olde Damink SWM, Ferguson K, Marshall I, Hayes PC, Deutz NEP, Williams R, Wardlaw J, Jalan R. Induced hyperammonemia alters neuropsychology, brain MR spectroscopy and magnetization transfer in cirrhosis. Hepatology 2003; 37:931-939).

KEYWORDS

Neuropsychological function, hyperammonemia, amino acid solution, myo-inositol, MR spectroscopy
ABSTRACT

Background: The neuropsychological effect of hyperammonemia is variable. This study tests the hypothesis that the effect of ammonia on the neuropsychological function in patients with cirrhosis is determined by the ability of the brain to ‘buffer’ ammonia-induced increase in glutamine within the astrocyte by losing osmolytes like myo-inositol (mI) and not by the magnitude of the ‘induced hyperammonemia’.

Methods: Fourteen cirrhotic patients with no evidence of overt HE were given a 75 gram amino acid (aa) solution mimicking the hemoglobin molecule to induce hyperammonemia. Measurement of a battery of neuropsychological function tests including immediate memory, ammonia, aa and short-echo time proton magnetic resonance spectroscopy were performed prior to and 4 hours after administration of the aa solution.

Results: Eight patients showed deterioration in the Immediate Memory Test at 4 hours. Demographic factors, severity of liver disease, change in plasma ammonia and aa profiles following the aa solution were similar in those that showed a deterioration compared with those who did not. In the patients who showed deterioration in the memory test, the mI/creatine ratio was significantly lower at baseline than those that did not deteriorate. In contrast, the Glx/creatine ratio was significantly greater in the patients that deteriorated.

Conclusions: The observation that the deterioration in the memory test was greater in those with lower mI/creatine ratio supports the hypothesis that the neuropsychological effects of induced hyperammonemia is determined by the capacity of the brain to handle the ammonia-induced increase in glutamine.
INTRODUCTION

Ammonia has consistently been shown to be important in the pathogenesis of Hepatic Encephalopathy (HE). (7; 26) Current hypotheses support the ammonia-glutamine-brain swelling hypothesis of neuropsychological dysfunction in cirrhosis. Astrocytes are the site of ammonia detoxification in the brain and eliminate ammonia by the synthesis of glutamine through amidation of glutamate. Accordingly, accumulation of glutamine in astrocytes induced by hyperammonemia produces osmotic stress and astrocyte swelling. (18) In patients with HE, the observation that myoinositol (mI; a sugar involved in the synthesis of phosphoinositides) is reduced with increasing concentrations of glutamine suggests that mI may be an important osmotic regulator within the astrocyte. (13; 22; 34) Recent studies in patients with cirrhosis (11) have shown that the metabolic disturbances are associated with an increase in brain water, the severity of which correlates with worsening of the neuropsychological state.

In keeping with these observations, we have recently demonstrated that oral administration of a solution mimicking the amino acid (aa) composition of the hemoglobin molecule led to a significant alteration in neuropsychological function, an increase in brain glutamine/glutamate signal (Glx) and a reduction in mI. The increase in Glx was associated with a reduction in magnetization transfer ratio (suggesting an increase in brain water) supporting the ammonia-glutamine-brain swelling hypothesis of HE. (2) However, the correlation between the aa-induced increase in ammonia concentration was not directly related to the change in neuropsychological function suggesting that the neuropsychological effects of hyperammonemia may be determined, not by the degree of hyperammonemia, but by the ability of astrocytes to maintain the osmotic equilibrium by using mI as a compensatory mechanism for the
ammonia-induced increase in glutamine. According to this hypothesis a low mI level prior to administration of the aa solution would increase the susceptibility of the brain to the effects of induced hyperammonemia. Therefore, the aims of this study were to evaluate the neuropsychological changes following induction of hyperammonemia and correlate these changes with the brain mI and Glx measured with proton magnetic resonance spectroscopy (¹H-MRS).
METHODS

Ethical Considerations

Studies were undertaken with the approval of the Hospital Research Ethics Committee and the written informed consent from each patient in accordance with the Declaration of Helsinki (1989) of the World Medical Association. The safety of administering 75 grams of an aa solution mimicking the composition of the hemoglobin molecule to patients with cirrhosis has been extensively studied (2; 21; 27) without any complication such as development of overt HE. Similar studies have used other formulations of a combination of aa (15) or glutamine challenge (24; 28; 31), which were shown to produce ammoniagenesis without any significant adverse effects.

Patients

Sixteen haemodynamically stable patients with biopsy-proven cirrhosis enrolled from the outpatient clinic were studied. Patients were excluded if they had clinical evidence of overt HE (10), diabetes, cardiovascular disease, renal dysfunction (serum creatinine >150 µmol/l), serum sodium <130 mmol/L, serum potassium <3.2 mmol/L or >5 mmol/L, concomitant neurological disease, recent gastrointestinal bleeding (within the previous 4 weeks), malignancy or pregnancy. Patients had to be abstinent from alcohol and benzodiazepines for at least 1 month prior to the study. Patients were studied after an overnight fast. Patient details are summarised in Table 1.

Oral amino acid solution

Simulation of the upper gastrointestinal bleed was by administration of an oral bolus of 75 grams of a specially prepared solution (Nutricia Cuijk, The Netherlands, Product Number: 24143) that mimics the aa composition of the hemoglobin molecule [leucine 99.8, isoleucine 0, valine 85.9, glycine 55.4, tryptophan 8.3, threonine 44.4, lysine 61,
glutamate 33.3, asparagine 27.7, glutamine 11.1, methionine 8.3, arginine 16.6, tyrosine 16.6, proline 38.8, aspartate 41.6, alanine 99.8, cysteine 8.3, serine 44.4, phenylalanine 41.6 and histidine 52.7 mmol per gram of aa solution]. (19) The solution was freshly made in 200 mls of water and xanthum gum was added to prevent sedimentation.

**Measurement of neuropsychological function**

A construct-driven neuropsychological test battery was used to test concentration, memory, visuospatial-construction skills and motor function. This consisted of: Trails B Test (14); the Digit Symbol Substitution test (DSST) (20); the Immediate Story Recall subtest of the Randt test battery (30); and Choice reaction time (16). The test battery was performed immediately before the administration of the aa solution and 4 hours afterwards. The total time taken to perform this battery was less than 20 minutes. The same investigator performed the neuropsychological tests. All patients had one practice session of each test. All of the tests have been well validated, and parallel forms were used. The Trails B Test (14) is a derivative of the Trail Making Test and measures visual conceptual and visuomotor tracking. The test has to be completed in 420 seconds. The Digit symbol substitution test (DSST) (20) is part of the Wechsler Adult Intelligence Schedule and is used to assess visuomotor coordination and vigilance. The test score was the number of symbols correctly substituted in 90 seconds. The Immediate Story Recall subtest of the Randt test battery (30) measures immediate memory function. The subject is asked to recall 20 words from a paragraph read to him or her that has an emotionally charged substance and includes fire and disaster. One point was awarded for each word that was recalled immediately after presentation (acquisition). The Choice reaction time (16) is part of the Continuous Performance Task and measures motor function, sustained
concentration, and the ability to suppress inappropriate responses. The task was to press the space bar of the computer as quickly as possible every time the letter E appeared, except when it was immediately preceded by the letter X. The mean reaction time and the number of observations were recorded.

**Proton MR spectroscopy (\(^1\)H-MRS)**

The patients underwent \(^1\)H-MRS immediately before and 4 hours after administration of the aa solution to determine changes in the brain osmolytes Glx and mI. This was performed using an Elscint Prestige scanner (GE Medical Systems, Haifa, Israel) in the SHEFC Brain Imaging Research Centre for Scotland, operating at 1.9 Tesla. Using T2-weighted axial images for positioning, PRESS-localized spectra were acquired from 15mm cubical volumes of interest including both grey and white matter in the left basal ganglia and the left temporal cortex. We chose to study the dominant hemisphere, and in the case of the patients reported, the left hemisphere was dominant in all. The sub-thalamic structures were chosen to be studied as they are critical to memory and learning, (9) which are important in executive attention. The repetition time was 1500 milliseconds and the echo time was 36 milliseconds. Following localised shimming and water-suppression calibration for each volume of interest, 200 acquisitions with water suppression were collected. Eight acquisitions without water suppression were also collected to serve as a phase reference. The volume of interest positions within the head coil were noted, as was the scanner radiofrequency calibration figure to adjust for the effects of head coil loading. Spectroscopy data were transferred to a Sun workstation for analysis. Analysis consisted of phase correction using the water reference data (29) and removal of the residual water signal using Hankel-Lanczos singular value decomposition.(8) Spectral peak areas were quantified using the AMARES method within the Magnetic Resonance User Interface software
A model consisting of 12 Gaussian peaks was developed with reference to in vitro measurements on metabolites, in vivo measurements on healthy volunteers on the same scanner with the same sequences, and literature values for peak assignments. Three observers studied the fitted results independently. The observers discussed any peak assignments on which they did not all agree. In this way, a consensus was reached for all of the spectra in the study. The following cerebral metabolites were quantified: N-acetyl aspartate (NAA), choline (Cho), creatine (Cr), mI and Glx. Cho resonance reflects changes in phospholipid metabolism and osmotic regulation in glial cells (6) and NAA is a normal neuronal marker. (4) Finally, peak areas for each subject were corrected for head coil loading and volume of interest position within the head coil. The resulting ‘institutional units’ enabled intersubject comparisons.

**Blood Sampling and Analysis**

A peripheral venous blood sample was taken for analysis of ammonia and plasma aa profile at the beginning of each study immediately prior to the administration of the aa solution and was repeated 4 hours following administration.

**Ammonia.** Plasma was obtained by centrifugation, deproteinised with trichloroacetic acid and stored at -80 °C for spectrophotometric determination of ammonia (CobasMiraS, Hoffman-LaRoche, Switzerland) at a later date.

**Amino Acids.** Plasma was obtained by centrifugation and deproteinised with sulphosalicylic acid for determination of aa’s by high-performance liquid chromatography (Pharmacia, Woerden, The Netherlands).

**Statistics**

All the data are expressed as median and range. The neuropsychological data were compared before and after the administration of the aa solution using a Wilcoxon’s
Sign Rank test. In the post-hoc analysis, the neuropsychological deteriorators and non-deteriorators were compared using the Mann Whitney test. (Prism software version 3.0, GraphPad, San Diego USA). A p-value of <0.05 was considered significant.

Prism software was also used to calculate linear regression of correlation between 2 variables (r value) with 95% confidence intervals. A p-value of <0.05 was considered significant.
RESULTS

Patients
Sixteen patients were recruited; of these 14 completed the study and are the subject of this report. One patient was unable to tolerate the ¹H-MRS due to claustrophobia and we were unable to gather sufficient data for one other due to excessive movement inside the magnet. None of the patients entered into the study showed any evidence of altered mental state or overt HE following the administration of the aa solution.

Neuropsychological Function
There were no significant differences in the scores for the Trails B Test (p=0.06), DSST (p=0.06), and Reaction Time (p=0.72) prior to and 4 hours after receiving the aa solution. Significant differences were however apparent in the Immediate Story Recall subtest of the Randt memory test (p=0.001). In view of this apparent difference in the continuous data of the Immediate Story Recall subtest of the Randt memory test, we decided to perform a post-hoc analysis to identify if there were any differences between the 14 patients which might determine whether they would deteriorate in the memory test in response to hyperammonemia. When the total deterioration in the score (out of 20) was calculated, 8 of the 14 subjects demonstrated deterioration in the score of 1 standard deviation (2.74) (mean 7) or more, irrespective of the degree of induced hyperammonemia. We therefore went on to compare the group which deteriorated (n=8) with the group which did not (n=6).

Ammonia
Overall, the median fasting basal venous ammonia concentration and the degree of induced hyperammonemia were similar within the whole group. When the group who had a deterioration in memory score was compared with the group who did not, there was no significant difference in the fasting basal venous ammonia concentration [50.5
Furthermore, the ammonia levels generated in response to the aa solution did not differ between the deteriorator and non-deteriorator groups at 4 hours [90 (73-140) vs 95 (46-221) µmol/L] (p=0.84).

**Amino Acids**

There were no significant differences in plasma aa profiles within the group as a whole. When the deteriorator and non-deteriorator groups were compared, the reduction in the concentration of isoleucine at 4 hours following the aa solution was greater in the deteriorator group [26 (16.7-31) vs 31 (28-41) µmol/L] (p=0.03). The remaining aa profiles were otherwise the same in both groups. (Table 2) The Fischer ratio (33) (branched chain aa’s/aromatic aa’s) was not significantly different between the deteriorator and non-deteriorator groups.

**Proton MR spectroscopy (1H-MRS)**

Table 3 gives the 1H-MRS data for the basal ganglia and temporal lobe obtained prior to, and 4 hours following, the administration of aa solution for both the group which showed a deterioration in memory score and the group which did not.

**mI/Cr**

The mI/Cr ratio prior to the administration of the aa solution was significantly lower in the patients that showed deterioration in the immediate memory test compared with those that did not (basal ganglia p=0.0007; temporal lobes p=0.001). The degree of deterioration in memory score correlated with the basal (t=0) mI/Cr, such that the deterioration in memory score was greater when the basal (t=0) mI/Cr was lower (basal ganglia r = -0.58; p=0.03; temporal lobe r = -0.59; p=0.03). (Figure 1) Four hours following administration of the aa solution, the mI/Cr ratio was significantly reduced in the basal ganglia of patients that showed no deterioration but not in the patients that showed deterioration in the immediate memory test (p=0.001).
**Glx/Cr**

The Glx/Cr ratio prior to the administration of the aa solution was significantly higher in the patients that showed deterioration in the immediate memory test compared with those that did not (basal ganglia p=0.007; temporal lobes p=0.001). The degree of deterioration in memory score correlated with the basal (t=0) Glx/Cr, such that the deterioration in memory score was greater when the basal (t=0) Glx/Cr was higher (basal ganglia r = 0.57; p=0.04; temporal lobe r = 0.54; p=0.05) (Figure 2). Four hours following administration of the aa solution, the Glx/Cr ratio was significantly increased in the patients that showed no deterioration but not in the patients that showed deterioration in the immediate memory test (basal ganglia p=0.003; temporal lobes p=0.01). The correlation between the degree of deterioration in memory score and Glx/Cr ratio in the basal ganglia was even greater at 4 hours following administration of the aa solution (r= 0.69; p=0.007) than prior to its administration.

**Cho/Cr & NAA/Cr**

No significant changes were seen between the groups at t=0 and 4 hours following the administration of the aa solution. (Table 3)
DISCUSSION

The results of this study show that the deterioration in the immediate memory test with induction of hyperammonemia was more likely to occur in the patients that had low levels of ml/Cr measured using $^1$H-MRS. This observation suggests that the effect of hyperammonemia is likely to be determined by the ability of the astrocytes to maintain osmotic equilibrium by losing osmolytes like ml in response to the ammonia-induced increase in glutamine.

The neuropsychological response to induced hyperammonemia is variable (24). Unlike the study, by Riggio et al. (32), who found no significant change in mental state following an aa load, the investigators from the Newcastle group (28; 31) have demonstrated significant changes in the mental state following administration of glutamine raising questions about neuropsychological factors that may determine the response to hyperammonemia. As we have recently shown, other factors such as systemic inflammatory response may exacerbate the neuropsychological effect of induced hyperammonemia. (35) In the present study we recruited a fairly homogenous group of patients with cirrhosis, who were metabolically stable without any evidence of infection/inflammation. It is therefore not surprising that there were no significant differences in the clinical and biochemical profile of the patients in whom a deterioration in the memory test was observed compared with those in whom no such deterioration was observed. In addition, the magnitude of change in ammonia and aa in the two groups following administration of the aa solution were similar.

The results of the spectroscopy provide support for the hypothesis that the ‘buffering capacity’ of the brain measured by the relative concentrations of ml and Glx may be important in determining the effect of hyperammonemia. Accordingly, a state of cellular osmotic equilibrium may be maintained even with high brain glutamine
levels, which are compensated for by very low levels of mI (or other unmeasured organic solutes). Therefore, in such a patient, a small increase in ammonia may cause a marked increase in osmotic stress by further increasing the accumulation of glutamine and therefore deterioration in the neuropsychological state. On the other hand, in the very well compensated patient, the proportion of Glx to mI may be low, making this patient resistant to the effects of induced-hyperammonemia. One way to gauge this potential ‘buffering capacity’ is to calculate the ratio of mI to mI + Glx, which accounts for the total measured osmolar capacity. The rationale for having mI as the denominator was to try and express the role of mI in relation to the total measurable osmolality (using $^1$H-MRS). Therefore conceptually, buffering capacity would be the buffer (mI in this case) divided by the total osmolytes. However, this is hypothetical and will have to be looked at prospectively in future studies. This ratio was significantly lower in the group that had deterioration in their immediate memory test at t=0 (p=0.0007) following administration of aa solution in the Basal Ganglia. This was less pronounced in the Temporal Lobe but still highly significant (p=0.001). Furthermore, the degree of deterioration in memory score correlated with the mI /mI +Glx ratio, such that the deterioration in memory score was greater when the basal (t=0) mI /mI +Glx ratio was lower (Basal Ganglia r = -0.77; p=0.003; Temporal Lobe r = -0.66; p=0.01) (Figure 3).

A decrease in the concentration of brain organic osmolytes such as mI indicates the activation of the process of regulatory volume decrease. (37) Therefore, the release of mI from the astrocytes may be indicative of an adaptive change. (40) In keeping with this, Cordoba et al. (12) have shown that brain swelling induced by hyperammonemina is made significantly worse by hyponatremia, which is a condition known to reduce brain mI levels. (5) The mechanism of why the mI levels were
different in the two groups of patients (those that deteriorated and those that showed no deterioration) has not been answered in the present study given that both the groups of patients had similar severities of liver disease and ammonia concentrations at baseline. Although plasma sodium concentration was similar (within the normal range) at the time of the study, variations in its levels over the preceding weeks may contribute to lower mI levels. Furthermore, we have no data about other factors such as the duration of hyperammonemia, cerebral blood flow or the degree of activation of neurohormones which may be important as they are known to alter the signalling pathways in the brain and therefore mI levels. (3; 23; 36)

The simulated upper gastrointestinal bleed by the oral administration of an aa solution is a safe and well-validated. Over 80 patients have been studied so far using this protocol where we administer 75 grams of the aa solution that is not significantly different in protein content from average daily protein intake of these patients. We were very careful in selecting patients for the study and excluded all those with any clinically detectable encephalopathy. Furthermore, the safety of administering aa solution to the cirrhotic patient has been extensively studied by our group (2; 21; 27) and now 4 other published studies (15; 24; 28; 31) that have also evaluated both glutamine challenge and aa challenge as safe methods to induce hyperammonemia in cirrhotic patients.

The observed deterioration in the memory function with induced hyperammonemia confirms the result of our previous study in which we administered an oral aa solution mimicking the haemoglobin molecule to examine neuropsychological changes. We showed that patients in the placebo group had a significant improvement in all of the neuropsychological function tests except the Immediate Story Recall subtest of the Randt memory test. In contrast, the patients
who were administered the aa solution showed significant deterioration in the memory
test but the results of the other 3 tests (Trails B test, DSST and Choice reaction time)
did not change significantly. (2) Changes in memory tests have been suggested as an
important component of minimal HE by other investigators (17; 38) and is commonly
used as part of a standard test battery when characterizing minimal HE. (1) The
pathophysiological basis of the memory deficit in minimal HE, is the likely result of
the effect of hyperammonemia on neuronal pathways controlling attention, which is
crucial for learning. (39) Looking at this from a different pathophysiological
perspective, the concentration of ml which is located mainly in glial cells may vary in
different parts of the brain, which may also explain why there was a more significant
difference between the deteriorators and non-deteriorators when looking at the ml /ml
+ Glx ratio in the basal ganglia than in the temporal lobe.

In conclusion, the results of our study have shown that the
neuropsychological response to induced hyperammonemia is determined by the
underlying brain biochemistry and the ability of the brain to buffer the ammonia-
induced increase in glutamine rather than the degree of induced hyperammonemia.
Our data support the hypothesis that low brain ml levels may be used as a marker in
patients with cirrhosis to predict those that are at risk of developing HE and should be
tested prospectively in suitably designed studies.
FIGURE LEGEND

Figure 1
Graphs showing the correlation between the degree of deterioration in Immediate Story Recall subtest of the Randt memory test score and the ml/Cr in the Basal Ganglia and Temporal Lobes at baseline (t=0). An improvement in memory score is represented by a negative value and deterioration in memory score is represented by a positive value.

Figure 2
Graphs showing the correlation between the degree of deterioration in Immediate Story Recall subtest of the Randt memory test score and the Glx/Cr in the Basal Ganglia and Temporal Lobes at baseline (t=0). An improvement in memory score is represented by a negative value and deterioration in memory score is represented by a positive value.

Figure 3
Graphs showing the correlation between the degree of deterioration in Immediate Story Recall subtest of the Randt memory test score and the ml / ml + Glx in the Basal Ganglia and Temporal Lobes at baseline (t=0). An improvement in memory score is represented by a negative value and deterioration in memory score is represented by a positive value.

Table 1
## Table 2

<table>
<thead>
<tr>
<th>Ammonia/Amino Acid (µmol/L)</th>
<th>Pre-amino acid solution (t=4 hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>53 (41 - 112)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>71.75 (42 - 94)</td>
</tr>
<tr>
<td>Leucine</td>
<td>77.5 (36 - 140)</td>
</tr>
<tr>
<td>Valine</td>
<td>129 (60 - 220.6)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>614.5 (461 - 890)</td>
</tr>
<tr>
<td>Total essential amino acids (TEAA’s)</td>
<td>80.5 (4 - 246.9)</td>
</tr>
<tr>
<td>Total non-essential amino acids (TNEAA’s)</td>
<td>99.5 (15 - 400)</td>
</tr>
</tbody>
</table>

* Patients who had a significant deterioration in Immediate memory score of 1 standard deviation or more (2.74) of the population mean (7).
<table>
<thead>
<tr>
<th>Brain Area</th>
<th>Basal Ganglia</th>
<th>Temporal Lobe</th>
<th>Basal</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours</td>
<td>T=0</td>
<td>T=4</td>
<td>T=0</td>
<td>T=4</td>
</tr>
<tr>
<td>T=0</td>
<td>T=4</td>
<td>T=0</td>
<td>T=4</td>
<td></td>
</tr>
<tr>
<td>mI/Cr</td>
<td>0.42 (0.19-0.58)</td>
<td>0.3 (0.1-0.43)</td>
<td>0.5 (0.3-0.67)</td>
<td>0.89 (0.76-1.47)</td>
</tr>
<tr>
<td>Glx/Cr</td>
<td>0.99 (0.74-1.27)</td>
<td>1.26 * (0.92-1.5)</td>
<td>1.03 (0.56-1.46)</td>
<td>1.29 (0.7-1.65)</td>
</tr>
<tr>
<td>Cho/Cr</td>
<td>0.71 (0.57-1.3)</td>
<td>1.3 (0.4-2.1)</td>
<td>1.5 (1.37-2.34)</td>
<td>1.8 (1.2-2.3)</td>
</tr>
<tr>
<td>NAA/Cr</td>
<td>1.84 (1.47-4.58)</td>
<td>1.85 (1.18-4.44)</td>
<td>1.53 (0.82-4.27)</td>
<td>1.57 (0.92-3.78)</td>
</tr>
<tr>
<td>mI = myoinositol, Cr = creatine, Glx = glutamate/glutamine, Cho = choline, NAA = N-acetylaspartate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significant differences between t=0 and 4 hrs are represented as *p&lt;0.05, ** p&lt;0.01</td>
<td></td>
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</tbody>
</table>
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