Systemic Inflammatory Response Exacerbates the Neuropsychological Effects Of induced Hyperammonemia in Cirrhosis

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Abbreviations in the text. HE: hepatic encephalopathy, SIRS: Systemic Inflammatory Response Syndrome, aa: amino acid, NO: nitric oxide, IL-6: interleukin 6, IL-1ß: interleukin 1 beta, TNF-α: tumour necrosis factor alpha, UGIB: upper gastrointestinal bleed, BBB: blood brain barrier, CNS: central nervous system.

KEYWORDS

Minimal Hepatic Encephalopathy, Neuropsychological Function, Proinflammatory Cytokines, Amino Acid Solution

ABSTRACT

Background: Studies in acute liver failure show correlation between evidence of a systemic inflammatory response syndrome (SIRS) and progression of Hepatic Encephalopathy (HE). We tested the hypothesis that SIRS mediators, such as nitric oxide and proinflammatory cytokines, may exacerbate the neuropsychological effects of hyperammonemia in cirrhosis. **Methods**: Ten patients with cirrhosis were studied, 24-36 hours after admission with clinical evidence of infection, and following its resolution. Hyperammonemia was induced by oral administration of an amino acid (aa) solution mimicking hemoglobin composition. Inflammatory mediators, nitrate/nitrite, ammonia, aa profiles, and a battery of neuropsychological tests were measured. **Results:** The hyperammonemia generated in response to the aa solution was similar prior to and after resolution of the inflammation (p=0.82). With treatment of the infection there were significant reduction in WBC, CRP, nitrate/nitrite, Interleukin-6, Interleukin-1ß and Tumour Necrosis Factor α. Induced hyperammonemia resulted in significant worsening of the neuropsychological scores when patients showed evidence of SIRS but not after its resolution. **Conclusion:** The significant deterioration of neuropsychological test scores following induced hyperammonemia during the inflammatory state, but not after its resolution, suggests that the inflammation and its mediators may be important in modulating the cerebral effect of ammonia in liver disease.

BACKGROUND

Hepatic Encephalopathy (HE) is a neuropsychiatric syndrome, which incorporates a spectrum of manifestations, which include psychomotor dysfunction, impaired memory, increased reaction time, sensory abnormalities and poor concentration. This occurs on the background of severe liver dysfunction and has the potential for full reversibility.[1] Ammonia is thought to be central in the pathogenesis of HE.[2] It is detoxified in the brain by the synthesis of glutamine in astrocytes from amidation of glutamate by glutamine synthetase. Current hypotheses suggest that accumulation of glutamine in the astrocytes induced by hyperammonemia produces osmotic stress and causes the astrocytes to swell.[3] However, a direct correlation between plasma ammonia concentrations and severity of HE is not found and additional factors may be important. In patients with acute liver failure (ALF), the presence of a systemic inflammatory response syndrome (SIRS) was recently shown to confer a poorer neurological outcome.[4] In cirrhosis, infection is a common precipitant of HE suggesting that inflammation may be important in it's pathogenesis. Astrocytes belong to the macrophage lineage and therefore have an associated range of cytokine responses. Increased signalling factors such as nitric oxide (NO) and proinflammatory cytokines may be important in modulating the neuropsychological response to hyperammonemia.[5]

One of the most common precipitating events of HE in cirrhosis is upper gastrointestinal bleeding (UGIB) which produces hyperammonemia.[6] Simulating the metabolic effects of an UGIB by the oral administration of erythrocytes has been shown to induce hyperammonemia and behavioural disturbance in portacaval shunted rats.[7] In a recently published placebo-controlled study, we showed that simulating an UGIB by the oral administration of a specifically prepared amino acid (aa) solution

identical to the aa profile of hemoglobin to patients with well-compensated cirrhosis, resulted in hyperammonemia but was not associated with significant deterioration in neuropsychological tests. However, the neuropsychological function in the group administered the placebo improved significantly suggesting that induced hyperammonemia produced a learning difficulty in the population studied.[8]

The present study was designed to test the hypothesis that inflammatory mediators, such as NO and proinflammatory cytokines exacerbate the neuropsychological effects of hyperammonemia in patients with cirrhosis. We therefore evaluated the changes in neuropsychological function following induction of hyperammonemia by oral administration of a specifically prepared solution mimicking the aa composition of hemoglobin in patients with cirrhosis with evidence of SIRS, [9, 10] and after its resolution.

METHODS

Ethical Considerations

Studies were undertaken with full approval of the local 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 an aa solution to patients with cirrhosis has been extensively studied [8,11,12] without any complication such as development of overt HE. Similar studies have used aa [13] and glutamine challenge [14-16] which produces ammoniagenesis and again no adverse effects were reported using this strategy.

Patients

Inclusion criteria

Patients were included if they had clinical and histological evidence of cirrhosis and had a SIRS score of 2 or more. The components of SIRS include the following: temperature >38 ^{o}C or <36 ^{o}C; heart rate >90 beats per minute; tachypnoea >20 breaths per minute or $PaCO_2$ <4.3 kPa; white blood cell count (WBC) >12 x 10^9/L or the presence of >10% immature neutrophils.

Exclusion criteria

Patients were excluded if they had clinical evidence of overt HE, [17] diabetes, cardiovascular disease, renal dysfunction (serum creatinine >150 μμmol/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.

Study protocol

Patients were studied within 24 - 36 hours after correction of volume and electrolyte

disturbances and again after resolution of the inflammatory response (defined as normalisation of clinical indices of infection, WBC and CRP). All patients were studied following an overnight fast. Antibiotics were commenced on the day of admission and the first study was performed on the following day. No patient received more than 3 doses of antibiotics prior to the first study being completed and all patients were given a 7-day course of a suitable antibiotic. The second study was performed on the day the patient was thought to be well enough for discharge [mean 9; range 7 - 12 days]. Each study involved assessment of the neuropsychological function and blood sampling prior to and after administration of the aa solution (Figure 1).

Oral amino acid solution

Simulation of the UGIB was by administration of an oral bolus of 75 grams of a specifically prepared solution (Nutricia Cuijk, The Netherlands, Product Number: 24143) that mimics the aa composition of the hemoglobin molecule.[8, 18] 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 designed. The following cognitive domains were tested: concentration, memory, visuospatial-construction skills and motor function. The neuropsychological test battery consisted of: Trails B Test;[19] the Digit symbol substitution test (DSST);[20] the immediate story recall subtest of the Randt test battery; [21] and choice reaction time.[22] A full description of the test battery used has been previously described. [11] The total time required to perform this battery was less than 20 minutes. The test battery was performed immediately before the administration of the aa solution**,** and at 2 and 4 hours afterwards (except Trails B

test which was only performed at 0 and 4 hours). The same investigator performed the neuropsychological tests and the patients had one practice session of each test. All the tests have been well validated and parallel forms were used.

Blood Sampling and Analysis

A peripheral venous blood sample was taken for analysis of WBC, neutrophil count, CRP, ammonia, aa's, nitrate/nitrite, IL-6, IL-1ß and TNF-α at the beginning of each study immediately prior to the administration of the aa solution. This was repeated after 2 and 4 hours for analysis of ammonia and aa's.

WBC, neutrophil count and CRP. Measured by standard automated laboratory techniques in the haematology and chemical pathology laboratories.

Ammonia. Plasma was obtained by centrifugation, deproteinised with trichloroacetic acid and stored at -80 ^{o}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 [23] (Pharmacia, Woerden, The Netherlands).

Nitrate/nitrite. Plasma was obtained by centrifugation and stored at -80 ^{o}C. Plasma nitrate and nitrite were determined by a Modified Griess Test. [24]

IL-6, IL-1ß and TNF-α. Plasma was obtained by centrifugation and measured by a commercially available ELISA (R&D Systems, Minneapolis, USA). The detection limit for the assays was 5 pg/ml. (Intra-assay coefficient of variation for the IL-6, IL-1β and TNFα assays was 3.7%, 3.2% and 4.8% respectively; inter-assay coefficient of variation for the IL-6, IL-1β and TNFα assays was 4.9%, 5.7% and 5.8%).

Statistics

All the data are expressed as median and range. Results were compared using the Wilcoxon Sign Rank test. (Prism software version 3.0 ,GraphPad, San Diego USA). A p-value of <0.05 was considered significant.

RESULTS

Patients

Twelve patients with cirrhosis were recruited, of these ten completed the study and are the subject of this report. Two patients were excluded because of worsening in clinical condition with progression to renal dysfunction in one and development of 'overt' HE in the other. Each patient had end-stage biopsy-proven cirrhosis, was on the waiting list for an orthotopic liver transplant and had been admitted to the hospital with clinical evidence of mild SIRS.[9,10,25] The SIRS was due to confirmed infections. All patients were clinically stable and had no overt evidence of HE. Six of the patients were male (mean age 52; range 43 to 61). Patient details are summarised in Table 1.

Clinical and Biochemical Indices:

The median CRP [on admission: 55mg/L (34 - 112) and post-antibiotic therapy: 20.5 mg/L (18 -31) {p=0.002}], WBC [admission: 14.2 x 10^9 /L (9 -23) and post: 9 x 10^9 /L (5.6 - 12.2) {p=0.002}] and neutrophil count [admission: 82 x 10^9 /L (78 - 82) and post: 69.5 x 10^9 /L (65 - 78) {p=0.002}] were reduced significantly with resolution of the inflammatory state.

No change was observed in Child Pugh Score[26] and the grade of HE remained unchanged at zero throughout the study. There was no significant change in liver function tests, albumin or prothrombin time between the 2 studies. The median bilirubin on admission was 78μμmol/l (56 - 132) and 81.5 (71 - 143) {p=0.106}in the post-inflammatory state. Table 2 outlines the clinical and biochemical indices of each patient.

Ammonia and Amino acids:

The median basal venous ammonia concentration was similar in the inflammatory state and following antibiotic therapy [83 (56 - 112) and 73.5 (59 - 132) μmol/L

respectively). The ammonia generated in response to the simulated bleed did not differ between the 2 studies (p=0.77) (Figure 2).

The concentrations of leucine (p<0.01), valine and phenylalanine (p<0.001) increased following administration of the aa solution and the concentration of isoleucine decreased (p<0.001), but there was no difference between the inflammatory and post-inflammatory states (Table 3).

Inflammatory Mediators:

Measured inflammatory markers, nitrate/nitrite, IL-6, IL-1ß and TNF-α were significantly reduced with resolution of the infection (nitrate/nitrite p=0.002, IL-6 p= 0.002, IL-1ß p=0.031 and TNF-α p=0.031) (Figure 3). IL-1ß and TNF-α levels were detectable in only 6 (patients 1,2,4,6,7 and 8). Patients 3 and 5 had undetectable levels of IL-1ß and TNF-α in both the inflammatory and post-inflammatory states. IL-1ß and TNF-α assays were not available in patients 9 and 10.

Neuropsychological Function:

None of the patients showed any evidence of an altered mental state or overt HE following the administration of the aa solution.

Trails B Test: Data analysis could only be completed in 6 of the 10 patients. This is because the test has to be completed in 420 seconds and if the patient took any longer, the test was abandoned and a time of 420 seconds recorded. 4 of the patients took > 420 seconds to complete the test at 0 and 4 hours following administration of the aa solution in the inflammatory state and 1 patient took >420 seconds to complete the test in both the studies. There were no significant differences in the time taken to complete the test between the inflammatory and post-inflammatory state both at baseline (p=1) and at 4 hours (p=0.63). There were however marked changes at baseline between the inflammatory and post-inflammatory state in individual patients,

with 8 patients improving their performance time and 2 patients showing a marked deterioration (Figure 4a).

The digit symbol substitution test: There was a significant overall improvement in the baseline scores between the inflammatory and post-inflammatory states (p=0.002). Induced hyperammonemia resulted in significant worsening (p=0.004) of the test scores at 4 hours when patients showed evidence of inflammation (Figure 4b). After resolution of inflammation, there was no significant change in the score from baseline following induced hyperammonemia (p=0.8).

Reaction time: There was a significant overall improvement in the baseline scores between the inflammatory and post-inflammatory states (p=0.01). The induced hyperammonemia resulted in a significant deterioration (p=0.002) in the reaction time at 4 hours when patients showed evidence of inflammation (Figure 4c). After resolution of inflammation, there was no significant change in the score from baseline following induced hyperammonemia (p=0.4).

Immediate Story recall subtest of the Randt memory test: There was no significant overall improvement in the baseline scores between the inflammatory and post-inflammatory states (p=0.58). The induced hyperammonemia resulted in a significant deterioration (p=0.002) in the number scored at 4 hours when patients showed evidence of inflammation (Figure 4d). After resolution of inflammation, there was no significant change in the score from baseline following induced hyperammonemia (p= 0.6).

DISCUSSION

The results of this study show that hyperammonemia induced by administration of an aa solution to cirrhotic patients, results in a deterioration in neuropsychological function in the presence of a systemic inflammatory response. Following resolution of SIRS, there was no deterioration in neuropsychological function despite equivalent severity of induced hyperammonemia. We believe that these data provide direct evidence suggesting that inflammation plays a crucial role in the neuropsychological effects of hyperammonemia in patients with cirrhosis.

The most important observation of this study is that the induction of a similar degree of hyperammonemia produced significant deterioration in all the neuropsychological function tests only in the inflammatory state. This suggests that inflammatory mediators may modulate the effects of hyperammonemia. SIRS is the clinical manifestation of inflammation and therefore the end product of the activation of a normally quiescent system comprising many components, including leucocytes, endothelial cells and cytokine networks. Patients performed significantly better at baseline in the post-inflammatory state in 2 of the 4 neuropsychological tests (DSST and Choice Reaction time). In the Trials B tests there were marked changes at baseline between the inflammatory and post-inflammatory state in individual patients, with 8 patients improving their performance time. The difference in the direction of the changes and the exclusion in the analysis of anyone taking longer than 420 seconds to complete the test (4 of the 8 patients who had an improvement in their performance time) is likely to explain the lack of statistically significant differences in the group when tested as a whole. The lack of significant improvement in overall baseline scores for the Immediate Recall Memory test is also likely to be for similar reasons. 4 patients showed a dramatic improvement, 3 remained the same and 3 marginally

deteriorated.

The association between infection and impaired brain function is well recognised. [27] Septic encephalopathy can arise from the action of inflammatory mediators on the brain or a cytotoxic response by brain cells to these mediators. Septic encephalopathy is usually a sequelae of severe sepsis complicated by multiple organ failure and hypotension. [28] The patients in this study however, although fulfilling criteria for SIRS, had relatively mild infections and no evidence of 'overt' HE. We showed that resolution of inflammation was associated with improvement in the mental state despite similar liver function and ammonia concentration further highlighting the importance of inflammation in modulating derangement in neuropsychological dysfunction. It is not possible from the present study to define the neuropsychological effects of mild sepsis, which need to be further explored. Severe sepsis causes plasma and brain aa's to become deranged with a decrease in branched chain aa's (eg isoleucine) and an increase in neutral aa's in the brain similar to the findings in portosystemic encephalopathy. [29,30] In patients with sepsis it was shown that aromatic aa's (eg phenylalanine) correlated with APACHE II scores and mortality. Scores were greater in shock patients with higher levels of ammonia and sulphur containing aa's with a higher mortality rate. [31] However, aa derangements such as these could not be demonstrated in this study, making it unlikely that septic encephalopathy could explain our findings.

Studies in patients with ALF have shown rapid progression to severe HE in those patients that have evidence of SIRS suggesting a possible link between inflammation and HE. [4] The effect of inflammation on neuropsychological function in patients with cirrhosis has not been systematically studied. The patient population that we chose to study had evidence of SIRS fulfilling 2 or more criteria. Furthermore, all

the patients had raised CRP, nitrate/nitrite and IL-6 values on admission, which significantly decreased following antibiotic therapy. It is unclear why 2 of the patients had undetectable levels (<5 pg/ml) of IL-1ß and TNF-α in the inflammatory state despite other markers of inflammation being raised. This however, is not an unusual finding in our experience of other, ongoing studies, and may reflect the fact that the cytokines are measured in peripheral blood rather than at sites of inflammation. Inflammation is associated with increased NO possibly by induction of nitric oxide synthase in response to proinflammatory cytokines. [32,33] We have shown in this study a significant reduction in the levels of nitrate/nitrite with resolution of inflammation.

The specific mechanism by which an inflammatory response modulates the neuropsychological response to hyperammonemia remains to be elucidated. There are several possibilities. First, recent work on cytokine effects on glutamate uptake by human astrocytes has shown that proinflammatory cytokines inhibit astrocyte glutamate uptake by a mechanism involving NO resulting in altered glutaminergic neurotransmission. [34] Second, it has been shown that IL-1ß and TNF-α increase the expression of peripheral-type benzodiazepine binding sites in cultured astrocytes which may alter cellular osmotic homeostasis.[35] Third, it is possible that the difference in neuropsychological deterioration following the aa solution is related to changes in cerebral blood flow. Recent work by Moller et al.[36] has shown that high circulating levels of TNF-α following an intravenous bolus of endotoxin resulted in reduced cerebral blood flow. Fourth, it is possible that cytokines may modulate ammonia diffusion within the central nervous system (CNS). It has been shown that TNF-α and IL-6 increase fluid phase permeability and ammonia diffusion in CNS-derived endothelial cells. [37]

There has previously been doubt that the peripheral immune system could

signal the brain. Cytokines (15 - 20 kD) cannot directly cross the blood brain barrier (BBB) and are unable to have a direct affect. [38] Recent studies have however suggested that peripheral cytokines can effect the brain by three routes.[5] Firstly, peripheral tissues, innervated by the peripheral and autonomic nervous systems, can send direct signals to the brain via the vagus nerve.[39] Secondly, the brain vasculature can convey signals through secondary messengers, such as NO and prostaglandins by induction of their synthesising enzymes [40] produced in response to cytokines binding to their receptors expressed in cerebral blood vessels. For example, in the brain, TNF-α and IL-1ß are potent stimuli for inducible nitric oxide synthase production.[41] Thirdly, cytokines can directly act at the level of the brain parenchyma after crossing the BBB by active transport or after entering brain areas that lack a BBB.

In summary, the results of this study provide the first direct evidence that mediators of the systemic inflammatory response may modulate the neuropsychological effects of hyperammonemia in patients with cirrhosis. Further research elucidating the mechanisms involved may lead to the development of newer therapeutic approaches.

LEGENDS TO FIGURES

Figure 1.

Summary of study design.

Figure 2.

Change in venous ammonia concentration (μμmol/L) in each of the ten patients administered the aa solution at 0, 2 and 4 hours in the study performed in the inflammatory state and then in the repeat study following antibiotic therapy (p=0.77).

Figures 3 a-d.

Measured concentrations of IL-6 (pg/ml), IL-1ß (pg/ml), TNF-α (pg/ml) and nitrate/nitrite (μmols/L) in each of patients in the inflammatory and the post-inflammatory state (TNF-α and IL-1ß were measured in only 8 patients and values were undetectable in 2 patients).

Figure 4.

Change in neuropsychological function (Trails B test, Digital symbol substitution test, Choice reaction time (secs) and Immediate story recall) in patients administered the amino acid solution at 0, 2 and 4 hours performed in inflammatory state (pre) and in the post-inflammatory state. [Trails B test: data from 4 of the patients are not shown as they took more than 420 seconds to complete the test at 0 and 4 hours following administration of the aa solution in the inflammatory state and 1 of them took more than 420 seconds to complete the test in both the studies]. The difference between groups was tested using the Wilcoxon Signed Rank test.

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