World Journal of Gastroenterology

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DOI: 10.3748/wjg.v21.i30.9103

World J Gastroenterol 2015 August 14; 21(30): 9103-9110
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Case Control Study

Endozepine-4 levels are increased in hepatic coma

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Institutional review board statement: The study was reviewed and approved by the Hospital Cannizzaro of Catania Institutional Review Board.

Informed consent statement: All the legal guardian of study participants, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: No potential conflicts of interest relevant to this article were reported.

Data sharing statement: No additional data are available.

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Received: December 21, 2014
Peer-review started: December 22, 2014
First decision: February 10, 2015
Revised: March 11, 2015
Accepted: May 7, 2015
Article in press: May 7, 2015
Published online: August 14, 2015

Abstract

AIM: To evaluate the serum levels of endozepine-4, their relation with ammonia serum levels, the grading of coma and the severity of cirrhosis, in patients with hepatic coma.

METHODS: In this study we included 20 subjects with Hepatic coma, 20 subjects with minimal hepatic encephalopathy (MHE) and 20 subjects control. All subjects underwent blood analysis, Child Pugh and Model for End - stage liver disease (MELD) assessment, endozepine-4 analysis.

RESULTS: Subjects with hepatic coma showed significant difference in endozepine-4 ($P < 0.001$) and NH3 levels ($P < 0.001$) compared both to MHE and controls patients. Between NH_3 and endozepine-4 we observed a significant correlation ($P = 0.009$; Pearson correlation 0.570). There was a significant correlation between endozepine-4 and MELD ($P = 0.017$; Pearson

correlation = 0.529). In our study blood ammonia concentration was noted to be raised in patients with hepatic coma, with the highest ammonia levels being found in those who were comatose. We also found a high correlation between endozepine-4 and ammonia ($P < 0.001$). In patients with grade Ⅳ hepatic coma, endozepine levels were significantly higher compared to other groups.

CONCLUSION: This study suggests that an increased level of endozepine in subjects with higher levels of MELD was observed. In conclusion, data concerning involvement of the GABA-ergic system in HE coma could be explained by stage-specific alterations.

Key words: Endozepine-4; Hepatic encephalopathy; Hepatic coma; Cirrhosis, Benzodiazepine; Peripheral benzodiazepine receptor; Model for End - stage liver disease; Glutamate-related neurotoxicity which in turn may alter the γ-aminobutyric acid

Core tip: Endozepine-4 is an endogenous ligand for the benzodiazepine recognition site of the glutamate-related neurotoxicity which in turn may alter the γ-aminobutyric acid (GABA)$_A$ receptor. Endozepine-4, may play an important role in the pathogenesis of hepatic encephalopathy (HE). The pathogenesis of HE has been viewed as a multifactorial etiology, but ammonia has a pivotal role in the genesis of the disease. Ammonia exerts a GABA$_A$ receptor system Involvement of the GABA-ergic system in HE coma could be due to stage-specific alterations.

Malaguarnera G, Vacante M, Drago F, Bertino G, Motta M, Giordano M, Malaguarnera M. Endozepine-4 levels are increased in hepatic coma. *World J Gastroenterol* 2015; 21(30): 9103-9110 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i30/9103.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i30.9103

INTRODUCTION

Hepatic encephalopathy (HE) is a neuropsychiatry syndrome that covers a wide range of disturbances in which cerebral function deteriorates due to failure in liver function. Features of HE may include manifestations of extrapyramidal dysfunction, such as hypomimia, muscular rigidity, bradykinesia, hypokinesia, monotony of speech, and a Parkinson-like tremor[1]. The pathogenesis of HE has been viewed as a multifactorial etiology, but ammonia has a pivotal role in the genesis of the disease[2,3].

Brain oedema is observed in HE and recent reports showed the presence of cerebral swelling[4-6]. HE is a consequence of extracellular fluids of toxic products not metabolized by the failing liver and ammonia. Ammonia is not only related to a decreased general brain metabolism, but also to its ability to damage astrocytes. Moreover ammonia which rises in HE has been demonstrated to exert a glutamate-related neurotoxicity which in turn may alter the γ-aminobutyric acid (GABA)$_A$ receptor system[7]. Endogenous compounds acting at the benzodiazepine (BZD) receptor and found within the central nervous system (CNS) have been termed and are called here endozepines[8,9]. Endozepines are a class of non-benzodiazepine, non-protein molecules, recently found to act as positive allosteric modulators of GABA$_A$ receptors and mimicking the pharmacological activity of exogenous benzodiazepines. The endozepines are distributed in different parts of nervous system such as glial cells, cerebellum, amigdala, hippocampus, hypothalamus, and substantia nigra[10], but they are also found in peripheral organs, in particular liver, kidneys, adrenal glands and testis[11-13] and in some cells (platelets, lymphocytes, mononuclear cells, endothelium, vascular smooth muscle, mast cells)[14,15]. Consistent with their blood distribution endozepines appear to exert multiple biological activities such as modulation of melanotropin release and stimulation of steroid synthesis[16-18].

The involvement of the GABA$_A$ receptor system in HE, demonstrated in the 1980s, was considered likely when specific BZD receptor antagonists were shown to revert the symptoms of HE in animal models and in patients[19,20]. Endozepine-4, an endogenous ligand for the benzodiazepine recognition site of the GABA$_A$ receptor, may play an important role in the pathogenesis of HE and can be useful in the monitoring of patients with hepatic coma[21,22]. The purpose of this study was to evaluate the serum levels of endozepine-4, the relation with ammonia serum levels, the grading of coma and the severity of cirrhosis.

MATERIALS AND METHODS

Twenty patients (age, range 41-65, mean 48 ± 10.8) referred to our department with a history of severe HE at stage Ⅳ according of the West Haven criteria were enrolled. Twenty healthy subjects (10 men and 10 women, mean age 49 ± 10.2 years, range 35-64 years) were also included in the study. All of them were clinically examined to exclude both neurological and liver disorders[23,24].

Twenty patients with minimal hepatic encephalopathy (MHE) (19 men and 9 women, mean age 48.2 years, range 34-65 years) were included in the study. The diagnosis of MHE has been made with psychometric and neurophysiological measures. All patients were evaluated with psychometric hepatic encephalopathy score (PHES). This battery consist of five tests: the number connection test A and B, digit symbol test, serial dotting test and line tracing test[25-27]. The PHES was also administered to normal subjects to exclude cognitive dysfunction. All subjects

gave the informed consent.

The diagnosis of liver cirrhosis was based on biochemical tests on liver cirrhosis and on liver biopsy. This protocol was reviewed and accepted by the Medical Ethics Committee of our institution. Informed consent was obtained from the patients' relatives. Patients enrolled and Exclusion criteria were as follows: (1) intake of synthetic benzodiazepines or other sedative drugs in the previous 12 d; (2) alcohol abuse in the previous 10 d; (3) uncontrolled diabetes mellitus; (4) final-stage severe kidney failure, as defined by a BUN > 90 mg/dL and/or serum creatinine > 4 mg/dL; (5) severe respiratory failure, as defined by PO2 < 60 mmHg and/or PCO2 < 50 mmHg; (6) acidosis, with blood pH < 7.30; (7) pre-existing neurological disease; (8) heart failure; (9) hemodynamic instability; (10) intake of any drug for the specific treatment of HE in the previous 24 h (except lactulose); (11) endocrine diseases; and (12) refusal to sign informed consent by patients' relative. The neurological assessment was conducted taking into consideration the following parameters: verbal ability, eye opening, pupillary light reflex, corneal reflex, spontaneous eye movements, oculocephalix reflex, motor response, and pattern of respiration.

Clinical grading of coma

We used the Glasgow (23-24) coma scale (score range, 3-13) to evaluate the following parameters: (1) eye response: 1 = no response; 2 = open in response to pain; 3 = open under verbal command; 4 = open without a stimulus; (2) motor response: 1 = no response; 2 = extension in response to pain; 3 = flexion in response to pain; 4 = appropriate motor response to pain; 5 = execution of commands; and (3) verbal response: 1 = no response; 2 = grunting in response to pain; 3 = noncoherent speech; 4 = understandable speech. The minimum and the maximum score were respectively 3 and 13.

EEG

HE was graded with EEG as follows: Grade 0: HE was defined as the presence of background activity (alpha rhythm); Grade 1: An alpha rhythm with some scattered theta waves; Grade 2: Background activity of theta rhythm mixed with some delta and alpha waves; Grade 3: Background of polymorphic delta activity characterized by a high amplitude with spontaneous variability; Grade 4: Delta activity characterized by a low amplitude.

Laboratory tests

All subjects included in the study were evaluate for blood test (serum hemoglobin, hematocrit, complete blood cell count) and liver function tests (serum alanine amino transferase, aspartate alanine transferase, γ-glutamyl transpeptidase, cholinesterase activity, serum bilirubin concentration, serum albumin concentration, prothrombin time and partial tromboplastin time).

Child pugh and model for end-stage liver disease scores

The Child-Pugh score was determined to assess the severity of cirrhosis, including 3 biochemical variables (serum albumin, bilirubin, and prothrombin time) and 2 clinical characteristics (presence or absence of ascites and clinical HE). A patient had Child-Pugh score A cirrhosis if the score was < 6 points, Child-Pugh B cirrhosis if the score was 7-9 points, and Child-Pugh C cirrhosis if the score was > 9 points. Patients without signs of ascites were scored as 2 points for ascites[28]. The Model for End - stage Liver Disease (MELD) scale is a reliable measure of mortality risk in patients with end-stage liver disease. The score was calculated by the formula 9.6 × log (creatinine mg/dL) + 3.8 × [log (bilirubin mg/dL) + 11.2 × log (INR) + 6.4 × cause of cirrhosis (0 if alcoholic or cholestatic liver disease, 1 if otherwise)[29].

Venous ammonia concentration

Serum levels of ammonia were evaluated by an enzymatic method employing glutamate dehydrogenase in a rapid and interference-free photometric determination (340 nm) of NH_4+ in blood plasma according to the De Fonseca - Wollheim method[30]. Within 15 min from withdrawal, the blood sample was frozen (-20 ℃) for later determination of NH_4+.

Endozepine-4 assessment

Serum sample was collected and stored at -70 ℃ until analysis. One millilitre of serum was extracted with 1 mL of chloroform, the lower organic phase was then collected, dried by vacuum centrifugation, then reconstituted with 500 mL of distilled deionized water containing 0.1% trifluoroacetic acid[31]. Samples were then injected onto a high-pressure liquid chromatography (HPLC) 25034 mm reversed phase column (BIO-SIL ODS 10, Bio-Rad) equilibrated with 0.1% trifluoroacetic acid in H_2O[32]. Samples were chromatographed by reverse phase HPLC as described previously[31,32] to elute and separate the endozepines. Effluent was monitored by UV219 nm and 1 min fractions were collected. Fractions were vacuum dried and reconstituted with distilled deionized water (250 mL) for endozepine-4 quantification. In some cases, fractions containing endozepine-4 activity were liophilysed, then reconstituted with a small volume (1-2 mL) of methanol: ethylacetate (1:1) and chromatographed by normal phase HPLC as described previously[31,32]. Using these HPLC methods, the only synthetic benzodiazepine that elutes within 4 min of endozepine-4 activity is lorazepam. Endozepine-4 was quantified in HPLC fractions by competitive displacement studies[32]. Aliquots of the reconstituted HPLC fractions (5-100 mL) from either serum or CSF extracts were tested for their ability to inhibit 1 nmol/L [3H] flunitrazepam

Table 1 Subjects characteristics at enrollment

	Patients (n = 20)	Controls health (n = 20)	Patients *vs* control healthy	MHE controls (n = 20)	Patients *vs* MHE controls (P)	Control healthy *vs* MHE controls (P)
Age (yr)	48 ± 10.8	49 ± 10.2	0.765	48.2 ± 10.7	0.953	0.81
Males/females	12/8	10/10	-	11/9	-	-
Alcoholic cirrhosis	5/20	-	-	4/20	-	-
Post hepatitis B cirrhosis	4/20	-	-	4/20	-	-
Post hepatitis C cirrhosis	8/20	-	-	9/20	-	-
Unknown cirrhosis	3/20	-	-	3/20	-	-
SBP (mmHg)	137.2 ± 12.8	134.1 ± 10.4	0.406	138.4 ± 11.7	0.759	0.227
DBP (mmHg)	82.4 ± 10.7	80.6 ± 10.3	0.591	82.7 ± 10.6	0.929	0.529
Heart rate (bpm)	78.1 ± 11.2	80.2 ± 11.8	0.567	84.1 ± 10.7	0.091	0.280
Smokers/no smokers	11/9	11/9	-	10/10	-	-

SBP: Systolic blood pressure; DBP: Diastolic blood pressure; Bpm: Beats per minute.

Table 2 Precipitant factors in patients with hepatic coma

	Patients endozepine-4 levels > 221	Patients endozepine-4 levels < 221
Hemorrage	10	2
Sepsis	-	3
Dehydration	-	2
Surgery	-	-
Unknown	-	3

(specific activity 87 Ci/mmol, New England Nuclear, Boston, Mass., United States) binding to rat cerebellar membranes. The aliquots were incubated in 0.25 mL of 50 mmol/L $NaPO_4$ (pH 7.4) which contained 5 mmol/L GABA, 1 nmol/L [3H] flunitrazepam, and rat cerebellar membranes (80 mg/protein per milliliter) for 1 h at 4 ℃. The reaction was terminated by vacuum filtration through glass fibre filters using a cell harvester (Cambridge Technology, Watertown, Mass., United States). The filters were transferred to vials (Wheaton Omnivials, Millville, NJ, United States) with scintillation cocktail (Safety Solve, Research Products International, Ill., United States) and the radioactivity measured. The amount of competition for the radioligand was compared with standard competition curves generated with known amounts of diazepam. Data were expressed as diazepam equivalents, defined as the number of pmoles of diazepam required to inhibit [3H] flunitrazepam binding to rat cerebellar membranes to the same extent as endozepines. All samples were analysed in triplicate; intra-assay variability was < 5%. To rule out the presence of contaminating synthetic benzodiazepine in patients' sera, extracts of sera from four patients with elevated endozepine-4 activity were analysed by gas chromatography-mass spectrometry. In parallel, normal serum doped with 5 nmol lorazepam was extracted and analyzed to determine instrumental sensitivity and response. Lorazepam was chosen because it is the only synthetic benzodiazepine that elutes from HPLC in the vicinity of endozepine-4. Chloroform extracts (2 mL) from 1 mL of serum were dried, re-suspended in 1 mL of chloroform and the clear supernatants transferred carefully by pipette to small vials and evaporated under a stream of nitrogen. Dried residues were dissolved in 8 mL methanol and 3 mL aliquots of each sample analyzed by gas-chromatography-electron ionization mass spectrometry. Mass spectra were recorded from 50 to 350 Da, and recorded spectra of lorazepam extracted from serum that matched from standards with a chemical signal to noise ratio of > 100.

Statistical analysis

The results are expressed as mean ± SD. Statistical significance in contingency tables was evaluated using the χ^2 and Fisher exact tests. Student's test for unpaired data, one-way ANOVA, and Mann-Whitney rank sum test were used for comparisons of continuous variables. Statistical analysis was performed using tests for repeated measures as well as by controls for multiple comparisons with correction by Duncan procedure. The statistical methods of this study were reviewed by Dr. Marco Vacante from Department of Clinical and Experimental Medicine, University of Catania, Italy.

RESULTS

The population characteristic at enrollment are showed in Table 1, based on Child Pugh score: subjects with grade A were 4/20 (20%), grade B were 7/20 (35%), and grade C were 9/20 (45%). Based on the grading of coma, subjects with grade 0 were 2/20 (10%), grade 1 were 3/20 (15%), grade 2 were 6/20 (30%), grade 3 were 6/20 (30%), and grade 4 were 3/20 (15%). And EEG grading 8/20 were grade 4, 4/20 grade 3, 4/20 grade 2, 4/20 grade 1. Precipitant factors in patients with hepatic coma in relation to the concentration of Endozepine-4 are showed in Table 2. Subjects with hepatic coma showed significant difference in endozepine-4 (P < 0.001) and NH3 levels (P < 0.001) compared to controls and MHE (Table 3). In the MHE patients endozepine-4 is not significantly elevated respect controls. In the HE patients with coma compared to controls and to MHE both NH3 and endozepine-4 were significantly increased. Between NH_3 and endozepine-4 we also observed a statistically significant correlation (P = 0.009; Pearson correlation

Table 3 Laboratory parameters of subjects included in the study

	Hepatic coma patients (n = 20)	Controls (n = 20)	P1 patients *vs* Control	MHE patients (n = 20)	P2 patients *vs* MHE controls (P)	P3 control *vs* MHE controls (P)
Endozepine-4 (pmol/mL)	252.55 ± 25.06[b]	2.8 ± 0.87[b]	< 0.001	2.96 ± 1.28[b]	< 0.001	0.646
NH_3 (mg/dL)	139.30 ± 87.46[b]	36.0 ± 8.40[b]	< 0.001	45.8 ± 10.2[b]	< 0.001	0.002
Serum albumin (g/dL)	2.78 ± 0.6[b]	4.1 ± 0.31[b]	< 0.001	3.9 ± 0.61[a,b]	< 0.001	0.199
Serum BUN (mg/dL)	71.2 ± 7.2[b]	48.4 ± 4.70[b]	< 0.001	56.4 ± 5.40	< 0.001	< 0.001
Serum creatinine (mg/dL)	1.56 ± 0.44	0.96 ± 0.32	< 0.001	1.01 ± 0.30	< 0.001	0.613
Serum bilirubin (mg/dL)	2.96 ± 1.56	0.84 ± 0.21	< 0.001	1.22 ± 0.36	< 0.001	< 0.001
Serum AST (IU/L)	60.2 ± 10.8	22.8 ± 8.20	< 0.001	47.5 ± 10.2	< 0.001	< 0.001
Serum ALT (IU/L)	58.8 ± 11.9	36.9 ± 11.2	< 0.001	45.2 ± 10.8	< 0.001	0.022

[a]P < 0.05, [b]P < 0.001 *vs* control. MHE: Minimal hepatic encephalopathy.

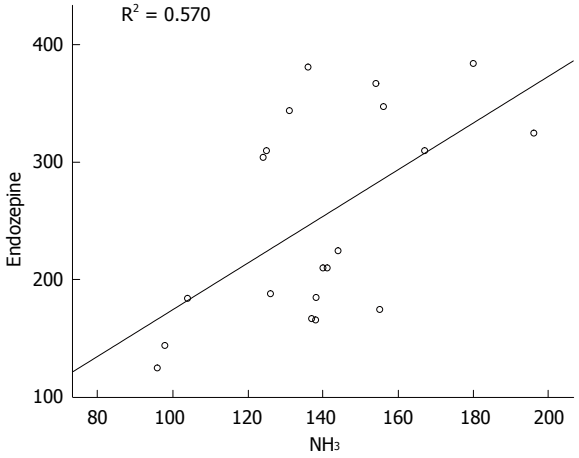

Figure 1 Correlation between endozepine levels and NH_3.

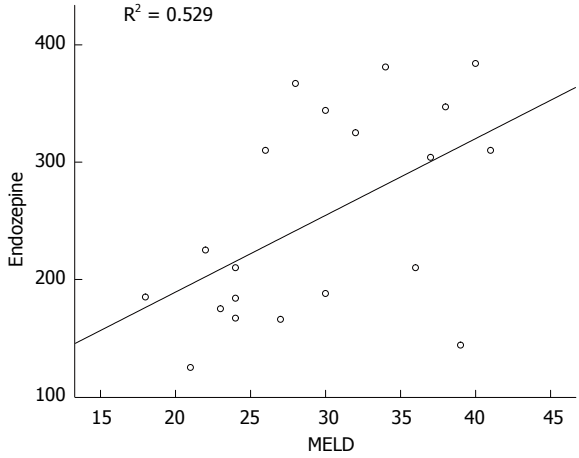

Figure 2 Correlation between endozepine levels and model for end - stage liver disease (Model for End - stage Liver Disease). MELD: Model for End - stage liver disease.

0.570) (Figure 1). We did not observed a significant correlation between MELD and NH_3 levels (P = 0.208; Pearson correlation = 0.294), while there was a significant correlation between endozepine-4 and MELD (P = 0.017; Pearson correlation = 0.529) (Figure 2).

DISCUSSION

Results of the present study reveal a markedly increase in serum endozepine-4 in patients with hepatic coma. The present study has also demonstrated that endozepine are highly correlated with the degree of hepatic encephalopathy and with ammonic concentrations**.** Blood ammonia concentration was noted to be raised in patients with hepatic coma, with the highest ammonia levels being found in those who were comatose.

The role of increased ammonia in the development of HE has been a puzzle[33]. Ammonia is the best-characterized neurotoxin that precipitates HE. It is directly toxic to the brain in high concentrations, through modulation of inhibitory and excitatory neurotransmission. Itzhak *et al*[34] have hypothesized that increased ammonia induces peripheral benzodiazepine receptors in brain glia cells. Hyperammonemia is associated with an increased GABA synthesis *via* the neuronal tricarboxylic acid cycle (indirect pathway)[35]. Comparable ammonia concentrations also enhanced synergistically the binding of a GABA agonist and a benzodiazepine. However, glutamic acid decarboxylase activity (direct GABA synthesis) was described to be unaltered or down-regulated as a consequence of HE. Endozepines are present in significant amounts in the brain and can be released by neurons *in vitro* and *in vivo*[32]. It has been suggested that the high affinity of endozepine-4 for the GABA A receptor might explain its ability to induce coma[36]. Similarly, its affinity for the hippocampal GABA receptors might explain the disturbance of memory that has been observed in patients with recurrent stupor in the prodromal and recovery phases[32,37-40].

They could be physiological neurotransmitters or neuromodulators[21]. Endozepines, peripheral BZ receptors, are located in the mitochondrial membrane of cells in the periphery and in astrocytes in the central nervous system and not allosterically coupled to $GABA_A$ receptors are consistently up-regulated in several experimental models of HE[38-40]. Human studies revealed that peripheral BZ receptor densities are not altered during the early stages of the disease (HE grades Ⅰ-Ⅲ) but were up-regulated in autopsy

material obtained from patients who had deceased in hepatic coma (HE grade Ⅳ)[41,42]. In our patients with grade Ⅳ hepatic coma, endozepine levels were significantly higher compared both to controls subjects and MHE patients. Meanwhile, no significative difference were found between MHE patients and control subjects. It may be correlated to inflammatory status present during HE. Infact, in a prospective observational study, Clavier *et al*[43] showed increased plasma levels of endozepines during systemic inflammation. This results show that increased levels of Endozepine-4 concentration occur in the advanced and critical phases of HE. We also observed an increased level of endozepine in subjects with higher levels of MELD. Plasma levels of GABA - like activity tend to be increased several fold in patients with hepatic encephalopathy[44,45]. The correlation between plasma levels of GABA - like activity and the stage of hepatic encephalopathy is not close. However this negative finding does not necessary detract from the GABA hypothesis since data on the prevailing permeability of the blood barrier in the patients studied are not available. Plasma levels of GABA - Like activity have been shown to increase several fold in cirrhotic patients following hemorrages into the gastrointestinal tract, an observation that suggests that blood in the gut acts as a substrate for GABA synthesis by intestinal bacteria. The liver probably plays an important role in extracting gut - derived GABA from portal venus blood. Accordingly, reduced hepatic catabolism of gut - derived GABA may contribute to elevated plasma levels of GABA - like activity found in liver failure[44-47]. Besides HE, it remains unknown whether endozepines are increased in other human disorders characterized by decreased vigilance and resembling endozepine stupor. Some limitations of our analysis deserve comment. First we selected biomarkers based on the knowledge about HE pathogenesis and results from previous clinical studies. We acknowledge that other not sufficiently tested biomarkers might have provided additional information. Second inter-subject biological variability needs further validation not only in a different setting, but also in different population. In addition, even though we included in the study a sample of patients, with a well characterized subjects, it would be important to validate these results in large scale longitudinal studies increasing the sample size. In conclusion there is a growing body of evidence concerning involvement of the GABAergic system in the pathophysiology of HE coma. Excessive central GABAergic tone and increase in benzodiazepine receptor ligands contribute to hepatic encephalopathy[48].

COMMENTS

Background

The pathogenesis of hepatic encephalopathy (HE) has been viewed as a multifactorial etiology, but ammonia has a pivotal role in the genesis of the disease.

Research frontiers

The involvement of the glutamate-related neurotoxicity which in turn may alter the γ-aminobutyric acid $(GABA)_A$ receptor system in HE, demonstrated in the 1980s, was considered likely when specific benzodiazepine (BZ) receptor antagonists were shown to revert the symptoms of HE in animal models and in patients.

Innovations and breakthroughs

The purpose of this study was to evaluate the serum levels of endozepine-4, the relation with ammonia serum levels, the grading of coma and the severity of cirrhosis.

Applications

The present study has also demonstrated that endozepine are highly correlated with the degree of hepatic encephalopathy and with ammonic concentrations. Blood ammonia concentration was noted to be raised in patients with hepatic coma, with the highest ammonia levels being found in those who were comatose.

Peer-review

Endozepine-4, an endogenous ligand for the BZ recognition site on the $GABA_A$, may play an important role in the pathogenesis of HE. In this article, the authors evaluated the serum endozepine-4 levels to explore its correlation with clinical scales and plasma concentration of ammonia. They reported that endozepine-4 was markedly elevated in patients with grade Ⅳ hepatic coma and/or high scores of the Model for End-Stage Liver Disease. They also identified the significant correlation between serum endozepine-4 levels and the ammonia concentration.

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P- Reviewer: Cao GW, De Ponti F **S- Editor**: Qi Y **L- Editor**: A
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ISSN 1007-9327