Ammonia and Endogenous Benzodiazepine-like Compounds in the Pathogenesis of Hepatic Encephalopathy

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Background: Ammonia and endogenous benzodiazepines (BDZs) are two of the most important agents among those taken into consideration in the pathogenesis of hepatic encephalopathy (HE). Methods: Venous ammonia and endogenous BDZs sera levels were assayed in 58 liver cirrhosis patients (34 male, 24 female) free of commercial BDZs. Endogenous BDZs were measured by binding assay after high-performance liquid chromatography purification. Ammonia was assessed by colorimetric test. Results: Endogenous BDZs and ammonia were significantly higher in Child-Pugh class C than in class B and class A (P < 0.05), correlating to the severity of the liver dysfunction but not with the degree of HE. A significant difference, in fact, was noted between degree 0 (no HE) versus III–IV of HE (P < 0.05), but not between degrees I–II versus III–IV. Regression analysis performed to find a correlation between the ammonia and BDZ levels in HE resulted negative. Conclusion: Clinical evidence is provided in cirrhotic patients that ammonia and endogenous BDZ levels do not correlate with each other in the outcome of HE.

Key words: Ammonia; endogenous benzodiazepines; hepatic encephalopathy

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Hepatic encephalopathy (HE) is a complex neuropsychiatric disorder secondary to acute or chronic liver failure characterized by an impairment of the central nervous system. Two important factors of the syndrome are the increased tone of the inhibitory γ-aminobutyric acid (GABA) receptor system and the elevated levels of ammonia (1, 2). Endogenous benzodiazepine-like compounds (BDZs) that are positive allosteric modulators of GABA_A receptors, have been proposed as contributing factors (3). An increased presence of BDZs has, in fact, been found in the brain and in the blood of animals (4) and patients with HE (5, 6) owing to fulminant hepatic failure (FHF) or to liver cirrhosis (7) when compared with normal subjects. These compounds seem to be increased in 60% of cases of HE. However, the weak correlation between the levels of BDZs and the degree of HE due to FHF suggests a potential role of these compounds only in the neuronal dysfunction, which characterizes HE (8). Further evidence for the possible involvement of BDZs in the development of HE derives from clinical studies, where a selective BDZ antagonist has been shown to rapidly improve HE in responding patients (5, 9, 10), working as a symptomatic drug. Evidence in the mechanism that enhances GABAergic neurotransmission during HE, implicating BDZs as pathogenetic factors, has been provided (11). Indeed, a unifying concept relating to the pathogenesis of HE considered that ammonia contributes to this syndrome by directly potentiating inhibitory GABAergic neurotransmission. Endogenous BDZs may potentiate inhibitory GABAergic neurotransmission by acting synergistically with ammonia (12, 13).

To evaluate the possible correlation between ammonia and BDZs in the pathogenesis of HE, we assayed venous ammonia and BDZ plasma levels in patients with liver cirrhosis with different impairment of the liver function and with different degree of HE, free of commercial BDZ drugs.

Materials and Methods

We assayed venous ammonia and BDZ plasma levels in 58 liver cirrhosis (34 male, 24 female), free of commercial BDZ drugs. The patients, evaluated clinically and on the basis of electroencephalographic pattern, were divided into the following groups: 34 cases degree 0, 4 degree I, 7 degree II, 5 degree III and 8 degree IV. Regarding the liver function, the patients were classified according to the Child-Pugh classification: Child A 15, B 18, C 25 patients. The study was a

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retrospective analysis and cases with overt precipitating factors such as bleeding were excluded.

Ammonia was assayed in sera by standard colorimetric test (Ammonia test Kit II, Menarini diagnostics, Florence, Italy).

Quantification of BZDs was performed as previously described (7, 14). Aliquots of all the serum samples (1 ml) were acidified with acetic acid (1 M) and centrifuged at 3000 x g for 10 min. The supernatant was passed through previously washed Sep-Pak C18 cartridges (Millipore, Medford, MA, U.S.A.). The material was eluted from Sep-Pak with CH3CN/0.1% trifluoroacetic acid (TFA) and then lyophilized. The lyophilized samples were reconstituted with 1 ml H2O and aliquots (200 μl) were chromatographed in duplicate at 0.8 ml/min on a LiChrospher 100 RP-18 column (250 x 4.0 mm; 5 μm) equilibrated with 80% water/0.1% trifluoroacetic acid and 20% acetonitrile. Absorbance was monitored at 230 nm. Samples were chromatographed using a water/0.1% TFA and acetonitrile gradient at 0.5%/min from 20% to 58% acetonitrile. Seventy-five fractions (1/min) from each sample were collected, lyophilized and reconstituted with water before radioreceptor assay. Known concentrations of diazepam, N-desmethyl diazepam, oxazepam, lorazepam, delorazepam and 2’-chlordiazepam were run in parallel with the plasma samples.

Unless otherwise indicated, all reagents were obtained from Sigma Chemical Co. and were all HPLC grade. All the fractions were then tested for their ability to inhibit [3H]flunitrazepam (1 nM, specific activity 87 Ci/mmol, NEN, Boston, MA, U.S.A.) binding to rat cerebellar membrane preparations (180–200 μg protein/100 μl measured by Bradford’s method). Dua were expressed as diazepam equivalents (DE) based on extrapolation from standard displacement curves generated using diazepam. The total concentrations of BDZ-like compounds present in each serum were calculated by determining the DE derived from the displacement activity of any single peak and then summing the amount of values of all peaks. Since the chemical identity of all components of the BDZ-like material are unknown, their extraction efficiencies could not be determined. The limit of detection of diazepam by [3H]flunitrazepam binding was 2 nmol DE/L with a coefficient variation of 0.52. The assays were done in triplicate and variations from the mean were less than 15%.

Values of BDZs and ammonia were analysed using the Kolmogorov-Smirnov test. Comparisons of BDZs and ammonia between the Child-Pugh classes and HE degrees were performed by ANOVA. With α set to 0.05, the employed sample size ensured a power of 0.99 and 0.90 for BDZ comparisons in the HE and Child-Pugh groups, respectively. Corresponding values for ammonia were 0.88 and 0.71. Linear regression was used to test the hypothesis that BDZ levels have the tendency to increase with increasing levels of ammonia (residuals were normally distributed). Statistical analysis was performed on a MacOS computer using the Statview 5.0 software package.

**Results**

The values of BZDs and ammonia in cirrhotic patients classified according to Child-Pugh score are, respectively: geometric mean (minimum value–maximum value) Child A 17 (2–701) and 38 (12–154); Child B 111 (2–4019) and 67 (4–235); Child C 238 (2–3508) and 73 (14–410) (BZDs and ammonia in Child A versus B and C: P < 0.05). The values of BZDs and ammonia in cirrhotic patients classified according to the degree of HE (0, I and II; III and IV) are, respectively: geometric mean (minimum value–maximum value) degree 0 35 (2–820) and 47 (12–235); degrees I and II 183 (2–4019) and 72 (14–145); degrees III and IV 770 (2–3508) and 100 (21–410) (BZDs and ammonia in degree 0 versus degree III and IV: P < 0.05). On the pooled samples, there is no correlation between BZDs and ammonia levels (R = 0.275), indicating that the increase in BDZS is independent of the increase in ammonia levels (see Fig. 1). No statistical differences between age and sex were found.

**Conclusions**

We have shown in this study, which includes fully characterized liver cirrhotic patients, that BDZ levels rise in the serum of cirrhotic patients in proportion to the worsening of the liver function, but not to the degrees of HE and levels of ammonia.

The finding that encephalopathy may occur in liver cirrhotic patients with low levels of circulating BDZ-like compounds is in line with the results of previous studies of patients with HE due to fulminant hepatic failure (15), and support the concept that BDZs rather than pathogenetic agents

![Fig. 1. Regression analysis between benzodiazepine (BZD) and ammonia (NH₃) levels in serum of patients with liver cirrhosis.](image-url)
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