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BASIC STUDIES

Rat CCl_4-induced cirrhosis plus total portal vein ligation: a new model for the study of hyperammonaemia and brain oedema

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Abstract

Introduction: Animal models used to study hyperammonaemic disorders related to chronic liver disease are unsatisfactory. These animals only develop hyperammonaemia and brain oedema when fed with diets supplemented with amonium acetate. *Aim:* To develop a novel experimental model of hyperammonaemia and brain oedema in CCl_4-induced cirrhosis in rats. *Methods:* Four groups were studied: rats with sham intervention (S), rats with total portal vein ligation (TPVL), cirrhotic rats (LC), and cirrhotic rats with TPVL (LC+TPVL). When ascites was diagnosed, oral glutamine challenge (OGC) test was performed. Blood, liver, lungs and brain samples were collected to quantify liver function parameters, plasmatic and cerebral ammonia, endotoxaemia, liver and brain histology, brain oedema and portosystemic shunting degree. *Results:* LC+TPVL rats showed a significant increase in portosystemic shunting when compared with LC group and a significant derangement in liver function when compared with TPVL group. These alterations resulted in a significant increase in plasmatic and brain ammonia concentrations and a higher plasmatic endotoxaemia as compared with others. Similarly, the area under OGC curve was significantly increased in LC+TPVL group as compared with the others, and correlates with portal shunting. Low-grade brain oedema was only observed in LC+TPVL group. All cirrhotic groups showed liver regeneration nodules and type-II Alzheimer astrocytes *Conclusion:* LC+TPVL reproduce the main alterations – portosystemic shunting, plasmatic and cerebral hyperammonaemia and low-grade brain oedema – observed in cirrhotic patients with hepatic encephalopathy.

Cirrhosis produces a wide spectrum of complications from portal hypertension, bleeding and bacterial infections to neurological disorders related with hyperammonaemia and brain oedema, which play a central role in the pathogenesis of hepatic encephalopathy (HE) (1, 2).

The most generally accepted hypothesis is that ammonia neurotoxicity is because of an alteration in neurotransmission mechanisms and a reduction in metabolic activity of central nervous system. Moreover, increased levels of brain ammonia induce histological changes such as Alzheimer type II astrocytes and low-grade brain oedema because of an increase in osmotic pressure (3).

Ammonia is produced by many tissues but its major external source results from the large intestine as a result of bacterial degradation of nitrogenous compounds because of deaminase and urease activities, as well as from the small intestine, mainly as a consequence of the metabolism of glutamine by the enzyme glutaminase (4). This ammonia reaches the liver carried by the portal vein where, in normal conditions, it is metabolized into urea.

The presence of porto-systemic shunts and the loss of parenchymal cells in the liver of cirrhotic patients lead, mainly, to an increase in plasma ammonia levels and are mainly responsible for the appearance of hepatic encephalopathy in these patients (5).

In this sense, oral glutamine load not only produces a marked increase of plasmatic ammonia, cerebral water

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and glutamine but also compromises neurological and psychometric clinical parameters. The relationship between the increase in ammonia levels and the risk to develop an HE episode makes oral glutamine challenge test a good predictor for HE (4, 6).

Whereas several animal models aiming to study acute liver failure-related hyperammonaemia (thioacetamine, azoxymethane, galactosamine) (7–12) or surgery procedures such as porto-cava anastomosis (13, 14) as well as the mere administration of ammonium acetate to healthy rats (15) are well established, there is a lack in the development of a suitable model for hyperammonaemia and brain oedema related to cirrhosis that is closest to that observed in cirrhotic patients who develop Type C HE (16).

In this sense, bile duct ligation or CCl_4 cirrhosis models, even though producing a wide spectra of alterations related to cirrhosis (liver function derangement, portal hypertension, presence of some porto-systemic shunts, ascites and bacterial infections) resulted in a mild increase of plasma ammonia. In spite of this, only when hyperammonaemic diet is added to those animals, plasmatic and brain ammonia and brain water content are significantly increased (17, 18).

Taking these data into account, we proposed to develop a new animal experimental model able to produce hyperammonaemia and brain oedema combining total portal vein ligation (TPVL) and chronic liver damage induced by CCl_4. The portal vein ligation model resulted in a high degree of shunting without parenchymal hepatic damage, while the CCl_4 model, which results in liver function alterations, did not always produce enough degree of shunting.

This combination would provide a model closer to the pathophysiological events occurring in hyperammonaemia associated to human cirrhosis. Parameters leading to the appearance of HE, such as portal-systemic shunting, derangement in liver function, brain and plasma hyperammonaemia, brain osmolytes concentration, low-grade cerebral oedema and cerebral histological changes (Alzheimer type II astrocytes) were evaluated. Moreover, the response to oral glutamine challenge, a test that induces an increase in blood ammonia in cirrhotic patients but not in healthy controls or in patients who have been successfully transplanted (19), was also assessed.

Materials and methods

Male Sprague–Dawley OFA rats weighing 100 g were included in this study. All animals were caged individually at a constant room temperature of 21 °C, exposed to a 7/17 h light/dark cycle, fed 20–25 g/day of standard rodent chow (A04, Harlan Ibérica S.A, Barcelona, Spain), and received 1.5 mmol/L of phenobarbital in drinking water. This study was conducted according to the guidelines set by the Guide for the Care and Use of Laboratory Animals, and was approved by the Ethical and Research Committee of our hospital.

Experimental design

Four groups of rats were studied: S group ($n = 10$): control rats with a sham intervention; TPVL group ($n = 16$): rats with TPVL; LC group ($n = 22$): rats with CCl_4-induced cirrhosis; and LC+TPVL group ($n = 30$): rats with CCl_4-induced cirrhosis plus TPVL.

When animals reached 200 g of body weight, cirrhosis was induced by weekly intragastric administrations of CCl_4 to 55 animals (LC and LC+TPVL groups). The initial dosage was 20 µl, and subsequent doses were adjusted to body weight changes according to Runyon *et al.* (20). After six CCl_4 dosages, animals were randomized into LC and LC+TPVL groups. In LC group, cirrhosis induction continued until ascites developed. Rats belonging to LC+TPVL group underwent a two-step portal vein ligation. In order to avoid fulminant hepatic failure allowing the collateral vessels formation, firstly partial portal vein ligation was assessed (tutorized with a 20 G needle to obtain a > 0.9 mm portal diameter). Forty-eight hours later, animals were relaparotomized and portal vein was occluded, and then cirrhosis induction continued until ascites developed. Animals were checked daily for ascites by visual examination. When this was suspected (abdominal distension, sudden weight increase), paracentesis was performed to confirm the presence of intraperitoneal fluid. Groups S and TPVL underwent a sham intervention or portal vein total ligation, respectively, in parallel with LC and LC+TPVL groups. All surgical procedures were performed under anaesthesia (ketamine, diazepam and atropine). When ascites was diagnosed, in LC and LC+TPVL, animals underwent several procedures and then were sacrificed with an anaesthetic's overdose to obtain peripheral and portal blood and solid tissue samples (brain, lung and liver). Rats from groups S and TPVL were sacrificed in parallel with LC and LC+TPVL groups respectively.

Routine blood biochemistry profile

Blood samples were obtained during sacrifice. Biochemical determinations [aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, glucose, urea, creatinine, sodium, triglycerides and cholesterol] were made using an auto analyzer (Dimension Clinical chemistry system, Dade Behring, Siemens, Madrid, Spain).

Endotoxin levels

Endotoxaemia was quantified by means of the Lymulus amoebocyte lysate kinetic test (Endosafe, Charles River, L'Abresle Cedex, France). Briefly, plasma samples were diluted in endotoxin-free water to 1:5 and then heated to 70 °C for 5 min. Afterwards, they were rediluted to a final dilution of 1:50 and assessed.

Portal-systemic shunting

To measure the presence of collaterals, 40 000 fluorescent microspheres (15 µm diameter) in 0.2 ml saline were

infused during 30 sec (0.4 ml/min) through the ileal vein (21). When sacrificed, liver (portal blood flow) and lung (systemic circulation) samples obtained were weighed and processed for microsphere recovery. Briefly, samples were placed in tubes for 2 weeks for autolysis, then ethanol KOH plus Tween-20 were added, and samples were incubated in a shaking bath at 50 °C for 48 h. Afterwards, samples were centrifuged (2000*g* 20 min), the supernatant was removed and 8 ml of 1% Triton X-100 were added, and after shaking samples was centrifuged and the supernatant was removed again. Seven millilitres of phosphate-buffered saline was added to each pellet, shaken and centrifuged again. After the supernatant was removed, 3 ml of ethoxyethyl acetate was added and samples were shaken until microsphere dissolution to release the fluorochrome. Finally, samples were centrifuged again and the supernatant was collected for fluorimetry analysis (Manual for Using Fluorescent Microspheres to Measure Regional Organ Perfusion, Fluorescent Microspheres Research Center, U. Washington, Seattle, WA, USA; October 1999). The percentage of portal-systemic shunting was calculated as lung fluorescence/total (lung+liver) fluorescence.

Determination of ammonia

Ammonia was measured in plasma and cerebral cortex. Blood (150 µl) was taken from the femoral vein. Deproteinized brain samples were collected for ammonia measurement, which was performed enzymatically using a commercial kit (Sigma Chemical Co., St Louis, MO, USA).

Oral glutamine challenge test

A load of 100 mg/kg of glutamine (L-glutamine, SHS, Barcelona, Spain) was administered through an orogastric stainless steel tube (Popper and sons, New Hyde Park, NY, USA). Venous blood samples (150 µl) from the femoral vein were drawn preload (baseline), and every 30 min for 4 h for ammonia determination. Body temperature was monitorized and maintained between 36–38 °C using an infrared lamp. Samples were centrifuged *in situ* for 10 min at 2000 *g* and plasma was frozen − 80 °C until analysis. The area under the curve (AUC) of ammonaemia response was also calculated using the trapezoidal rule.

Brain oedema and organic brain osmolytes

A brain sample of each rat was excised, weighed and heated to 90 °C, for 48 h in a dryer oven in order to evaporate all water content. Then, dried samples were weighed again. The difference between the initial and final weight was considered the water content.

Another sample of brain was used to quantify myo-inositol and six other organic osmolytes (glutamine, glutamate, taurine, creatine, alanine, and *N*-acetylaspartate). Samples were collected and immediately frozen to –80 °C until their analysis. Briefly, they were weighed and homogenized. In order to deproteinize, five volumes of cold perchloric acid (6%) were added and then centrifuged to 12 000*g* for 20 min. After neutralization ($KHCO_3$, 25% wt/vol), samples were lyophilized. Measurement of organic osmolytes was performed using a Bruker Avance 400 9.4 T equipment (Bruker, Madrid, Spain) using nuclear magnetic resonance-spectroscopy method (22, 23).

Liver and brain histology

Liver and brain samples for histological examination were collected in 4% formaldehyde, subsequently embedded in paraffin wax, sliced in 5 µm sections and stained with haematoxylin and eosin. Liver samples were examined using the Scheuer's score system (24). Brain samples were examined by light microscopy and Alzheimer type II astrocytes were searched for.

Statistical analysis

Unless otherwise indicated, results are expressed as mean ± SD or proportions as required. Comparison of means among groups was performed using the one-way analysis of variance or its corresponding non-parametric test (Kruskal–Wallis). *Post hoc* comparisons, to identify pairs of groups significantly different at the 0.05 level, were made with the Duncan test or the Mann–Whitney *U*-test respectively. Comparisons of proportions among groups were made with the χ^2-test. Statistical analysis was performed with the SPSS (version 15.0) for Windows software (SPSS Inc., Chicago, IL, USA).

Results

Mortality, body weight and ascites development

As expected, the mortality in the cirrhotic groups was higher (55 and 67% in the LC group and in the LC+TPVL group respectively) as compared with the other two groups (0 and 37% in the S group and in the TPVL group respectively). Body weight at sacrifice was similar in both, LC and LC+TPVL group (336.8 ± 52.8 vs. 321.6 ± 58.9; P = NS). Ascites developed only in both cirrhotic groups without differences in the time elapsed from the first CCl_4 dose (range: 8–19 weeks). None of the ascitic rats showed any sign of infection or sepsis.

Serum biochemistry and endotoxaemia

Results are summarized in Table 1. As compared with their respective controls (S and TPVL groups), cirrhotic rats (LC and LC+TPVL groups) showed significantly higher levels of serum AST, ALT and bilirubin, as well as lower serum concentrations of glucose and albumin. Moreover, LC+TPVL group had significantly higher urea levels than the other cirrhotic and non-cirrhotic groups. Bilirubin, glucose and albumin alterations showed a trend to be more intense in the LC+TPVL group than in the LC group.

Endotoxaemia was increased in cirrhotic groups (LC and LC+TPVL) as compared with their controls (S and TPVL), but the differences only reached statistical significance for the LC+TPVL group.

Portal-systemic shunting

Portal vein occlusion resulted in a marked degree of portal-systemic shunting, being similar in TPVL and TPVL+LC groups (57.07 ± 32.59 and $58.76 \pm 30.31\%$, $P = $ NS) and significantly higher to that observed in LC group without TPVL ($33.45 \pm 35.19\%$; $P < 0.040$). No collateral circulation was detected in rats from the sham group S ($0.27 \pm 0.19\%$) (Fig. 1).

Blood and brain ammonia levels and oral glutamine challenge test

CCL_4-cirrhosis induction (LC group) and total portal vein ligation (TPVL group) resulted in a significant and similar increase in blood ammonia levels near twofolds when compared with control group (S) (148.6 ± 26.8 and 162.3 ± 13.7 vs. 89.1 ± 11.4 µmol/L, $P < 0.05$). When combined, (LC+TPVL group) blood ammonia increased more than threefolds (284.6 ± 44.1 µmol/L, $P < 0.05$ vs. all others) (Fig. 2). By contrast, brain ammonia levels were similar in LC, TPVL and S groups (0.27 ± 0.03, 0.29 ± 0.03 and 0.25 ± 0.02 mmol/kg). Only the combined group (LC+TPVL) showed significantly higher brain ammonia concentrations when compared with all other groups (0.42 ± 0.05 mmol/kg vs. all others, $P < 0.05$) (Fig. 3). Accordingly, the AUC after oral glutamine challenge test (OGC) was significantly higher in LC+TPVL group as compared with all others, as well as, in TPVL group with respect to the group S. LC group only showed a trend to be higher than the S group (Fig. 4). A significant direct correlation between shunting degree and AUC after OGC test was found among LC+TPVL, LC and C+TPVL ($r^2 = 0.65$; $P < 0.05$).

Table 1. Serum biochemistry and endotoxaemia

	Sham	TPVL	LC	LC+TPVL
Glucose (mmol/L)	22.0 ± 8.2	19.8 ± 7.0	18.6 ± 9.7	$10.8 \pm 5.4^{*,\dagger}$
Triglycerides (mmol/L)	0.32 ± 0.1	0.56 ± 0.2	$0.83 \pm 0.4^{*,\dagger}$	$0.76 \pm 0.3^{*}$
Urea (mmol/L)	12.6 ± 1.2	12.1 ± 2.1	11.3 ± 1.6	$18.8 \pm 2.4^{*,\dagger,\ddagger}$
Creatinine (µmol/L)	124 ± 39	95.9 ± 32	90.7 ± 29	$63 \pm 28^{*}$
Cholesterol (mmol/L)	2.6 ± 0.5	2.5 ± 0.7	$3.6 \pm 0.8^{*,\dagger}$	$3.5 \pm 0.7^{*,\dagger}$
Bilirubin (µmol/L)	0.9 ± 0.5	1.3 ± 0.4	2.1 ± 1.6	$4.6 \pm 2.3^{*,\dagger}$
ALT (UI/L)	109 ± 48	100 ± 41	$331 \pm 473^{*,\dagger}$	$260 \pm 281^{*,\dagger}$
AST (UI/L)	259 ± 92	184 ± 71	$622 \pm 812\dagger$	$427 \pm 295\dagger$
Albumin (g/L)	34.6 ± 4.7	32.0 ± 1.5	$29.8 \pm 3.2^{*}$	$25.5 \pm 3.2^{*,\dagger}$
Na^+ (mmol/L)	130 ± 2.1	133 ± 1.2	136 ± 1.4	134 ± 2.2
Endotoxin UE	0.034 ± 0.004	0.035 ± 0.005	0.09 ± 0.037	$0.14 \pm 0.067^{*,\dagger}$

$^{*}P < 0.05$ vs. sham group.

$\dagger P < 0.05$ vs. TPVL group.

$\ddagger P < 0.05$ vs. LC group.

Values are means ± SD.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; TPVL, total portal vein ligation.

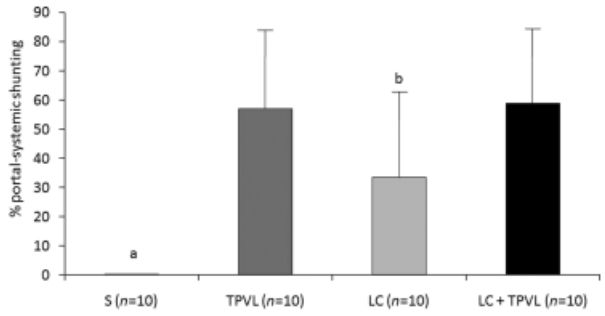

Fig. 1. Shunting degree. Both CCl_4-induced cirrhosis and total portal vein ligation produce portal-systemic shunting development assessed by fluorescent microspheres ($^{a}P < 0.05$ vs. S group). Total portal vein ligation (TPVL) induces a marked degree of portal-systemic shunting either in TPVL or in LC+TPVL groups, being significantly higher to that observed in the LC group without TPVL ($^{b}P = 0.040$).

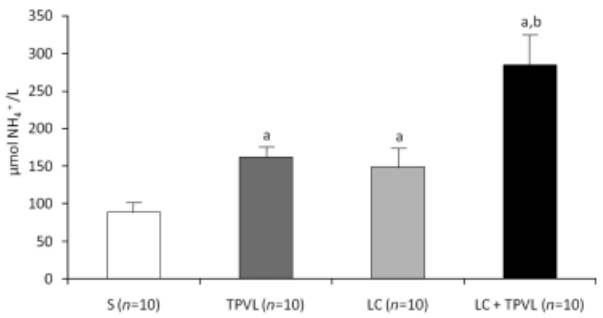

Fig. 2. Plasma ammonia levels. Plasma ammonia levels were significantly higher in cirrhotic rats (LC), total portal vein ligation (TPVL) and LC+TPVL groups when compared with S group ($^{a}P < 0.05$). Moreover, this increase was more exacerbated in the LC+TPVL group ($^{b}P < 0.05$ vs. TPVL and LC groups).

Brain water content and brain osmolytes

LC+TPVL group showed a mild but significant increase in brain water content as compared with the other groups (LC+TPVL: 79.07% ± 0.66 vs. LC: 78.56% ± 0.25, TPVL: 78.63% ± 0.24, and S: 78.24% ± 0.19; $P < 0.05$) as shown in Fig. 5.

Brain myo-inositol, taurine and creatine levels were significantly lower in LC+TPVL when compared with controls (LC+TPVL vs. S). In addition, we found a trend of increased glutamine levels in LC+TPVL group. Moreover, glutamine/myo-inositol and glutamine/creatine ratios showed a significantly increase in LC+TPVL group when compared with the others (Table 2). A typical NMR spectrum of brain extracts is shown in Fig. 6.

Histology

All rats treated with CCl_4 developed cirrhosis with regeneration nodules, necrosis and steatosis Fig. 7. All CCl_4-treated animals were scored F4 with the Scheuer system (25).

Brain histological changes consisting of swollen astrocytes with enlarged and vesicular nuclei (Alzheimer type II cell) were observed in animals of groups LC and LC+TPVL, while they were not found in animals from groups S and TPVL (Fig. 7).

Discussion

In the present study, we proposed to combine two different animal models of portal hypertension that separately are unable to reproduce the entire picture of hyperammonaemia-related alterations usually observed in HE: extrahepatic portal hypertension achieved by TPVL, and intrahepatic hypertension obtained by CCl_4-induced cirrhosis with ascites. Only when combining both models, a closest approach to the findings in well-established human cirrhosis (high degree of shunting+ profound liver function alterations) were achieved.

It must be acknowledged that the mortality rate of the combined model is high (67%). This is mainly because of the well-known high mortality rate of CCl_4-induced cirrhosis. However, as our aim was to produce a model of hyperammonaemia in the setting of chronic

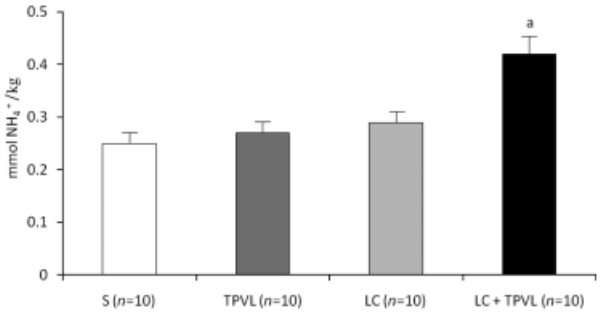

Fig. 3. Brain ammonia levels. In contrast to than observed in plasma ammonia levels, only LC+TPVL group showed a significant increase in brain ammonia levels ($^aP < 0.05$ vs. all other groups).

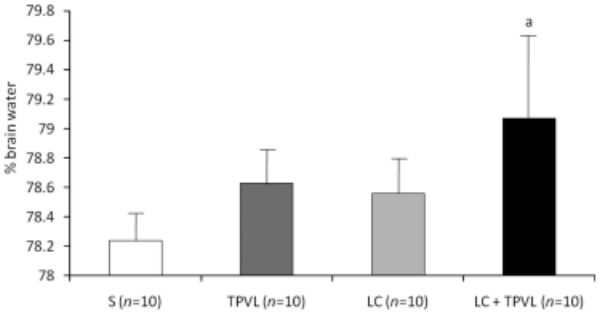

Fig. 5. LC+TPVL rats had a significant increase in brain water content (%) that indicates low-grade brain oedema ($^aP < 0.05$).

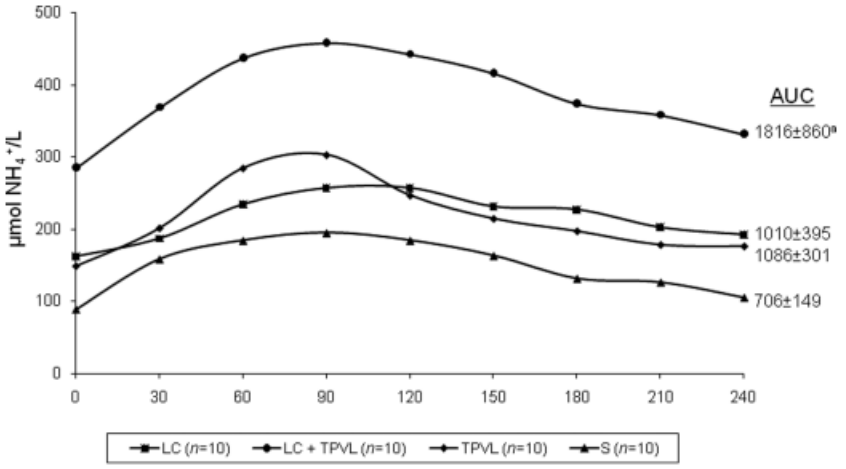

Fig. 4. Area under curve (AUC) after oral glutamine challenge (OGC) test. The AUC after 100 mg/kg of oral glutamine administration was significantly higher in LC+TPVL group as compared with all others ($^aP < 0.05$).

parenchymal liver damage, the high mortality rate of the combined model should be considered as unavoidable. In this study, we found that either LC or TPVL alone increased twofold plasma ammonia levels but in the combined model (LC+TPVL) this increase reached threefold. In spite of this, and taking into account that %PSS was similar in TPVL and LC+TPVL groups, other factors, in addition to %PSS are involved in hyperammonaemia. There are several sources of ammonia production: (i) microbiota from large (and also small) intestine and (ii) glutaminase activity from kidney and small bowel. In this sense, it is well known that cirrhosis is associated to bacterial overgrowth and to an alteration of gut pH. This results in an increase of ammonia from bacteria and in a glutaminase activity enhancement (26). Moreover, several tissues and organs are involved in ammonia detoxification: (i) liver (urea synthesis), and (ii) muscle and brain (glutamine synthetase activity). In this sense, liver function is compromised in cirrhosis and malnutrition resulting in muscle catabolism, becomes in this tissue an extra source of ammonia instead of an ammonia scavenger. These alterations could explain why only LC+TPVL rats showed a significant delay in ammonia plasmatic clearance after an oral glutamine challenge test, similarly to that found in patients where a pathological OGC test is a prognostic factor for the development of overt HE (27).

On the other hand, brain ammonia levels were only increased in the combined model. Interestingly, in this model, brain alterations (ammonia, presence of oedema, osmolites and the presence of Alzheimer Type II astrocytes) occur without the administration of hyperammonaemic diet. In fact, only brain ammonia levels are well correlated with HE intensity. It seems necessary not only high levels of plasma ammonia but also haematoencephalic brain barrier alterations (28–30). Probably, there is a synergistic effect because of liver impairment related to CCl_4-induced cirrhosis, an increased shunting degree related to a higher portal pressure because of TPVL and

Table 2. Brain osmolytes

Osmolites (μmol/g)	Sham	LC	LC+TPVL
Myo-inositol	3.82 ± 1.28	3.27 ± 1.9	2.22 ± 0.96*
Taurine	1.59 ± 0.45	1.37 ± 0.98	1.08 ± 0.38*
Glutamate	3.80 ± 1.51	2.91 ± 2.32	3.27 ± 0.95
Glutamine	3.50 ± 0.69	3.26 ± 1.42	4.78 ± 1.99
Creatine	5.01 ± 1.52	4.32 ± 2.59	3.44 ± 0.69*
N-acetylaspartate	3.44 ± 1.07	2.96 ± 1.6	2.54 ± 0.69
Alanine	0.29 ± 0.13	0.29 ± 0.23	0.28 ± 0.17
Glutamine/myo-inositol	0.91 ± 0.49	0.94 ± 0.23	2.78 ± 2.01*,‡
Glutamine/creatine	0.70 ± 0.16	0.76 ± 0.40	1.42 ± 0.64*,‡

*$P < 0.05$ vs. sham.
‡$P < 0.05$ vs. LC group.
Values are means ± SD.
TPVL, total portal vein ligation.

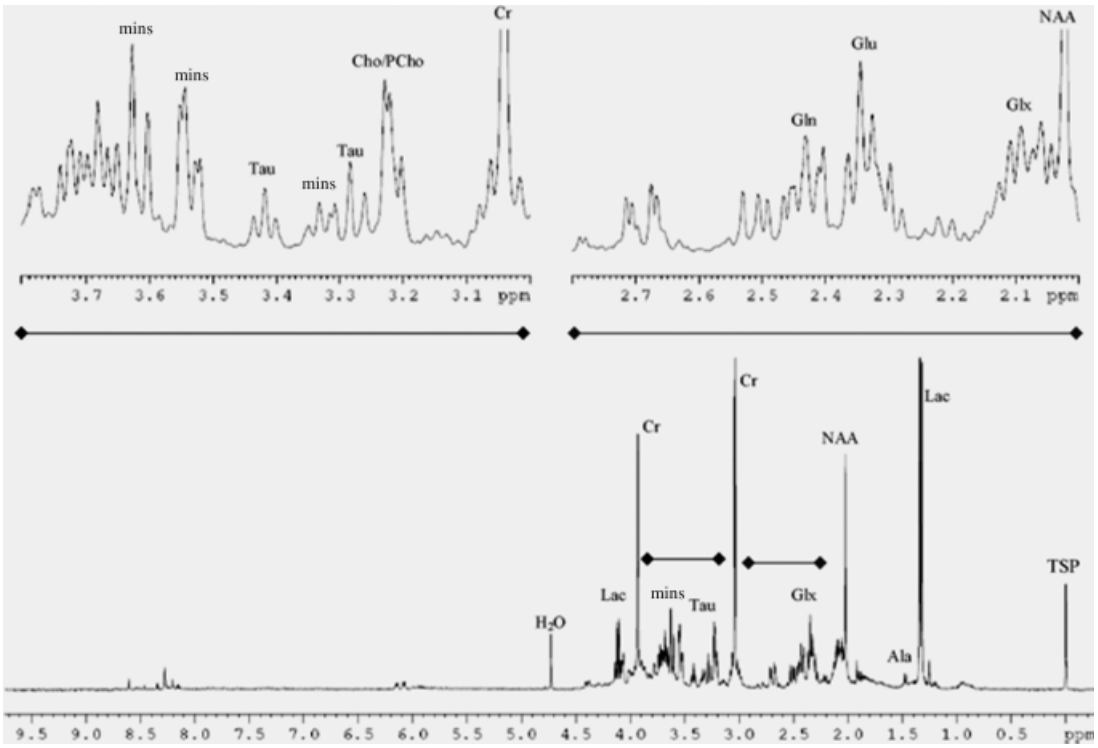

Fig. 6. A typical nuclear magnetic resonance spectrum of one sample from LC+TPVL group is represented. Several osmolytes were analszed: myo-inositol (mins), choline (Cho), fosfocholine (Pcho), creatine (Cr), glutamine (Gln), glutamate (Glu), glutamate+glutamine (Glx), *N*-acetylaspartate (NAA), alanine (Ala), lactate (Lac) and sodium 3-trimethylsilyl-2, 2, 3, 3-d4-propionate (TSP).

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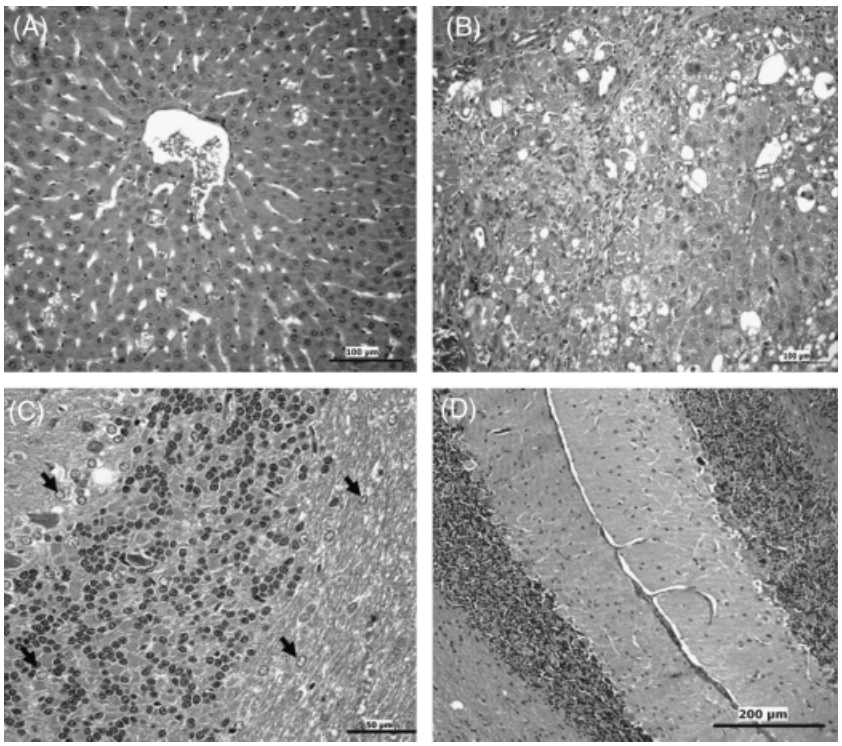

Fig. 7. Liver (A, B; × 20) and left cerebral cortex (C; × 40, D; × 10) section photomicrographs. S and TPVL groups (A) showed normal cellular architecture, whereas rats treated with CCl_4 developed cirrhosis with regeneration nodules, necrosis and steatosis (B). The presence of Alzheimer type II astrocytes (black arrows) were observed in brains from animals of groups LC and LC+TPVL (C), while they were not found in brains from groups S and TPVL (D).

increased plasma endotoxin levels. In this sense, it has been reported that endotoxins, in a synergistic way, increase cerebral blood flow and intracranial pressure related to blood brain barrier alterations in hyperammonaemic rats (15). Similarly, an increase in portal vein pressure enhances blood brain barrier permeability quantitatively and qualitatively (10), probably because of vasoactive substances and pro-inflammatory cytokines that, in turn, are overexpressed in response to endotoxins. Because the highest levels of plasma ammonia and LPS were detected in the combined group (LC+TPVL), one can speculate that some blood brain barrier alteration could be in the basis of this significant increase in brain ammonia levels in this group.

Neuropathologically, HE in cirrhosis is mainly characterized by astrocyte changes and not by neurological changes. We observed a trend of increased brain glutamine and a significant decrease of myo-inositol only in the combination model LC+TPVL, a pattern of change in brain osmolytes that is characteristic of human HE in cirrhosis (24, 31, 32). This scenario stimulates a mild but significant increase in brain water as we have observed in LC+TPVL rats. This increase, around 1%, is comparable with experimental HE models such as shunt porto-cava anastomosis plus hyperammonaemic diet (31) or bile duct ligation plus hyperammonaemic diet (17). Also, it is noteworthy that the method used in our study to determine low-grade brain oedema by desiccation in a drying oven is comparable with the gravimetric method. Moreover, we have observed histological alterations in the cerebral cortex such as the presence of Alzheimer type II astrocytes, similar to those found in human HE not only in LC+TPVL but also in LC animals. The fact that these features are also present in the LC group (mild hyperammonaemia without brain oedema) probably means that this is a previous and necessary condition but not enough by itself. In fact, this pathogenesis is the same of HE observed in human cirrhosis: encephalopathy progresses, the balance of glutamine to myo-inositol changes and finally when overt HE is established, the osmoregulatory dysfunction develops as the level of myo-inositol decreases and the levels of brain glutamine increase (33).

Finally, and taking into account that most of the alterations present in this combined model are in accordance with alterations observed in hepatic encephalopathy models described previously such as bile-duct ligation+hyperammonaemic diet, a set of behavioural, conductual and memory tests as well as neurophysiological assessment of possible functional abnormalities in central motor tract should be performed in order to characterize this new model of hyperammonaemia related to advanced cirrhosis as an hepatic encephalopathy model as well.

In conclusion, we propose a new model that reproduces not only hyperammonaemia and increased cerebral ammonia levels and brain oedema, but also the main pathophysiological and histological alterations observed in HE of cirrhotic patients. Of note, these changes occur in the setting of chronic parenchymal liver damage, without the need of exogenous ammonia supply. This model may allow the search of new targets and the development of new pathophysiological approaches and novel strategies in the treatment of hyperammonaemia related to advanced cirrhosis. Further work is necessary to demonstrate whether or not these biochemical and morphological changes result in those behavioural alterations occurring in hepatic encephalopathy.

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