Altered blood-brain barrier permeability in rats with prehepatic portal hypertension turns to normal when portal pressure is lowered

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CONCLUSION: The impairment of blood-brain barrier and subsequent normalization could be a mechanism involved in hepatic encephalopathy reversibility. Hemodynamic changes and ammonia could trigger blood-brain barrier alterations and its reestablishment.

Key words: Blood-brain barrier; Rats; Prehepatic portal hypertension

INTRODUCTION

Portal hypertension (PH) and hepatic encephalopathy are major complications in human cirrhosis or portal vein thrombosis. Hyperdynamic splanchnic circulation and hyperammonia associated to systemic circulatory alterations are usually present[1,2].

Our laboratory found in the last years, the presence of derangement in the CNS in a prehepatic portal hypertensive rat model. Increases in the uptake and release of norepinephrine in diencephalic and telencephalic discrete brain regions and significant increments in tyrosine hydroxylase activity in these nuclei were described in previous publications[3-5].

Finally, morphologic alterations in astrocytes (AS) and endothelial cells (EC) located in the hippocampal region (CA1 and CA4) were also found in this experimental model[6].

The morphological cell components of BBB are basically AS, EC, and pericytes. Two of this, AS processes and EC, were found altered in this model and in acute acetaminophen intoxication fourteen days after portal vein ligation[7,16,17].

Beside this, forty days after portal vein stricture, portal pressure spontaneously turns to normality. The aim of the present experiment was to investigate if normalization of PP was accompanied by the reestablishment of BBB properties and relate these findings with plasma ammonia concentration.
MATERIALS AND METHODS

Animals and surgical procedures
Male Wistar rats, with an average weight of 240 g were utilized and animal welfare was in accordance with the guidelines of the Faculty of Pharmacy and Biochemistry and approved by the Ethical Committee of the Faculty accordingly with Helsinki’s Declaration. The animals were placed in individual cages, with free access to food (standard laboratory rat chow) and water, and 12 h light cycle: 8 a.m. - 8 P.M. Special care for perfect air renewal was taken.

Four groups of rats were used: Sham14d- sham operated rats; PH14d - Portal vein stenosis; both groups were used 14 d post-surgery. Sham40- sham operated; PH40d- Portal vein stenosis; used 40 d after surgery.

Each group had separated subgroups for portal pressure determination, serum and CSF fluid determinations, Trypan blue and Evans blue injection respectively.

Portal hypertension was obtained by calibrated stenosis of the portal vein (PH) according to Chojkier et al.[1]. Rats were lightly anesthetized with ether and then a midline abdominal incision was made. The portal vein was located and isolated from the surrounding tissues. A ligature of 3.0 silk sutures was placed around the vein, and snugly tied to a 20-gauge blunt-end needle placed along side the portal vein. The needle was subsequently removed to yield a calibrated stenosis of the portal vein. Operations were performed at 2 PM to obey circadian rhythm. Fourteen days after portal vein ligation, animals exhibit an increase in portal pressure. After 20 d portal pressure begins to fall down to normal values approximately after 30 d.

Sham operated rats underwent the same experimental procedure except that the portal vein was isolated but not stenosed. Animals were placed in individual cages and allowed to recover from surgery. Portal pressure was measured the 14th d and the 40th d after surgery in the corresponding group, by puncture of the splenic pulp. Animals were sacrificed by decapitation between 2 and 4 PM to avoid circadian variations.

Experimental procedures
Portal pressure measurement
Fourteen and forty days after the corresponding operation, the rats were anesthetized with sodium pentobarbital (40 mg/kg), intraperitonally (ip). Portal pressure was measured through a needle placed in the splenic pulp, and maintained in place by cyanoacrylate gel. The needle was cannulated to a polyethylene catheter (50) filled with a heparinized saline solution (25 U/mL) and connected to a Statham Gould P23ID pressure transducer (Statham, Hato Rey, Puerto Rico) coupled to a Grass 79D polygraph (Grass Instruments, Quincy, MA).

Biochemical determinations
Plasma was obtained from blood drained from aorta artery puncture. Under anesthesia, samples of cerebrospinal fluid (CSF) were obtained by cisternal puncture for pro-tein determination according to Bradford[2]. Plasma ammonia concentrations were determined using Ammoniac Enzymatic UV Kits (Biomerieux, France). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined in plasma using a commercial standardized and optimized Boehringer-Mannheim (Germany) kits.

Brain water content
Cortical brain zones were utilized for the determination of water content in order to quantify possible brain edema. Gravimetric method was employed according to Marmorou et al.[3].

Trypan blue transcardial perfusion
Rats were perfused with Trypan blue (TB, Sigma Chemical Co. St. Louis MO. USA.) solution and then fixed with paraformaldehyde. TB solution (0.5%) was prepared by dissolving 1 g of TB in 200 mL of PBS, with gentle heat. The solution was allowed to cool at room temperature, added to the filtrate and placed on ice for immediate use. The temperature of TB solution was 10-12 °C at the time of perfusion. Rats were anesthetized with ethyl urethane (1mg/kg) and perfused transcardially with 200 ml of TB solution; followed by 300 mL of ice-cooled paraformaldehyde (2% in PBS). The flow rate of perfusate was maintained at 25 mL/min. Brains were dissected and post-fixed overnight in 30 % sucrose for 2 d. Subsequently, the brains were placed in powdered dry ice and stored at -80 °C until processed for microscopic studies. Slices of brain tissue were obtained with cryostat in section of 300 microns according to Paxinos and Watson[4]. Hippocampal slices were evaluated under light microscope and expressed as positive (+) or negative (-) for TB staining. Medial eminence and choroids plexus staining were used as control of TB adequate perfusion. This method was adapted from Ikeda et al[5].

Evans blue test
Evans blue (EB, Sigma Chemical Co. St. Louis MO. USA.) dye (25% in 0.9% NaCl solution) was intravenously injected at dose of 25 mg/kg in rats under ether anesthesia. One hour after the injection, animals were sacrificed by decapitation. Brains were weighed, clipped and individually placed within formamide p.a. (2ml./brain). These tubes were kept at 37°C for 48 h. The content of dye extracted from each brain was determined by spectrophotometer (Photometer 4010, Boehringer) at 620 nm and compared to standard graph created through the recording of optical densities from serial dilutions of EB in 0.9% NaCl solution[11, 12].

Statistical analysis
Results were expressed as mean ± SE. For multiple comparisons ANOVA, followed by Newman-Keuls or Student Newman Keuls tests were used. A P<0.05 was considered significant.

RESULTS
Portal pressure
The portal pressure (Figure 1) in Group Sh14d was 7.6 ± 1.90 (n = 6), in Group PH14d 14±1.80 (n = 6), in Group PH40d was 2.52 ± 1.35 (n = 3), Sh40d 7.7 ± 1.98 (n = 5). Differences between the following groups were significant: Sh14d vs PH14d (P<0.001); PH14d vs. PH40d (P<0.001); Sh14d vs PH40d (P<0.01). When sham groups (Sh14d vs Sh40d) were compared differences were not significant.

Plasma protein concentration
Group PH14d presented 87.40 ± 8.00 mg/mL (n = 4); Group PH40d 107.60 ± 2.10 mg/mL (n = 5); Group Sh14d, 108.80 ± 7.60
Ammonia plasma levels (Figure 3)

Portal hypertensive rats showed that ammonia plasma level was 79.00 ± 15.00 µm/L in Group PH14d (n = 8), 44.76 ± 4.51 µm/L in Group PH40d (n = 5), 19.52 ± 0.80 µm/L in Group Sh14d, and 21.70 ± 2.27 µm/L in Group Sh40d. Statistical analysis indicated that there were significant differences between Sham14d and Group PH14d (P < 0.05) and between Group PH14d and Group Sh40d (P < 0.01) (Figure 1). According to these results PH14d rats had increased protein permeability through the BBB. This increment was reverted when CSF protein was measured 40 days after portal vein stricture (Figure 2). These changes were not related to the above-cited variations in plasma protein values.

Plasma transferases activity (Figure 4)

Plasma ALT activity was 63.00 ± 9.00 IU/L in Group PH14d (n = 6), 44.00 ± 4.50 IU/L in Group PH40d (n = 7), 39.00 ± 4.00 IU/L in Group Sh14d (n = 6) and 39.00 ± 3.50 IU/L in Group Sh40d (n = 6). Statistical analysis shows an increase of PH14d levels when compared to groups Sh14d and Sh40d (P < 0.05).

The activity of plasma AST was 316.0 ± 23.0 IU/L in Group PH14d (n = 6), 167.0 ± 14.0 IU/L in Group PH40d (n = 7), 155.0 ± 25.0 IU/L in Group Sh14d (n = 6) and 155.0 ± 32.0 IU/L in Group Sh40d (n = 6). Statistical calculations outline
The presence, Sham

Evans blue test. The systemic injection of Evans blue showed a statistically significant increase of the dye presence in the hippocampal region in PH14d, presence in the indicate p<0.01 when compared with the other 3 groups.

![Figure 5 Evans blue test](image)

**Figure 5** Evans blue test. The systemic injection of Evans blue showed a statistically significant increase of the dye presence in the hippocampal region in PH14d presence in the indicate p<0.01 when compared with the other 3 groups.

an increment of the enzyme in PH14d group, when compared with groups of SH14d (P<0.001), PH14d (P<0.01), and SH14d (P<0.001). The elevation of their activity usually indicates some level of rat liver alteration, which is observed in this model of prehepatic portal hypertension.

**Water content in cortical brain areas**

Water content in cortical brain areas (H₂O/ gr %) was 79.21 ±0.17 in Group PH14d (n = 6), 79.21 ±0.17 in Group PH14d (n = 7), 78.95 ±0.18 in Group SH14d (n = 6) and 77.26 ±0.29 in Group SH14d (n = 6). No significant differences were found between the groups.

**Trypan blue and Evans blue tests**

Trypan blue test showed the presence of the dye limited to the vascular area, not involving other areas in Groups SH14d and SH40d. Meanwhile, EB appeared positively in vascular area and diffusely in perivascular area in Group PH14d. In Group PH40d both were detected in vascular area and very slightly in perivascular areas. These findings demonstrate the existence of an increased BBB permeability to the dye in PH14d rats, which turns to normal when pressure levels are normalized 40 d later (Figures 6 A and B).

The systemic injection of EB confirmed quantitatively the results found with TB. The results, expressed in µg/g of brain tissue, were 6.170±0.380 in Group SH14d (n = 12), 8.680 ±0.700 in Group PH14d (n = 9), 6.300 ±0.590 in Group PH40d (n = 6) and 6.250 ±0.450 in Group SH40d (n = 6). Statistical comparison showed that there was significant difference between Group PH14d and Group PH40d (P<0.01) and between Group SH14d and Group PH14d (P<0.01). There was no significant difference between Group SH14d and Group PH40d; Group SH14d and Group SH40d shared almost identical values (Figure 5).

**DISCUSSION**

The BBB is formed by complex tight junctions of the brain capillary endothelial cells in close relation with the astrocyte processes. These morphological and functional structures make possible a selective transport across BBB and a non-selective brain distribution of drugs. Three cellular components characterize BBB: Endothelial cells, astrocyte end feet and pericyte. Tight junctions present between cerebral endothelial cells form a diffusion barrier that restricts the influx of most blood-borne substances from entering the central nervous system (CNS). Dysfunction of BBB complicates a number of neurological diseases.

Alterations in astrocytes architecture as well as changes in its metabolism due to ammonia detoxification, osmotic balance and cell homeostasis are a well-known feature of hepatic or porto-systemic encephalopathy.

In previous publications, we described the alteration of BBB in this model of prehepatic portal hypertension, with or without acute acetaminophen intoxication. In this paper, the addition of Evans blue technique confirms previous results and adds a quantitative measurement to morphologic findings.

No difference in brain water content was found in this model of partial portal vein ligation, opposed to changes in brain water content found in rats with acetaminophen intoxication as previously described. The presence, however, of EEG modifications and rota rod test modifications, led us to classify it as a probable sub-clinic encephalopathy. The lack of a rise in brain water content observed in this model is probably due to the fact that the chronic nature of the portal pressure rise and porto-systemic circulation probably allows some kind of compensation mechanism.

Alterations of BBB permeability have been studied in different models of cirrhosis and porto-caval shunting, observing different kind of BBB changes like for some aminoacids but not others, Evans blue, but not for 14C mannitol or 3H-glutamate.
We confirm the spontaneous tendency of this model to faster recovery of hepatic encephalopathy in patients. It is interesting to stress out that in PH group, protein concentration in CSF is elevated despite a tendency to fall in plasma protein when compared to control (Sh). There were no differences in plasma or CSF protein content between Sh and PH or Sh, nor in the same groups when comparing Evans Blue concentration.

The fact that BBB altered permeability is resolved but plasma ammonia concentration is not completely normal remains unexplained. Further studies should be conducted to clarify the responsibility of vasoactive substances released in portal hypertension in BBB alterations in this rat model.

The elevated AST and ALT may indicate some kind of liver alteration present in this model with an otherwise almost normal liver function. Astrocyte changes and ammonia detoxification in the brain are probably some of the mechanisms implicated in BBB alteration and posterior normalization. Other factors have been implicated like cytokines and carbon monoxide [1-2, 20].

The normalization of BBB permeability could be a necessary part of hepatic encephalopathy reversibility. This could be a simple model for further studies in BBB function and reversibility of permeability mechanisms useful for a faster recovery of hepatic encephalopathy in patients.

We confirm the spontaneous tendency of this model to normalize portal pressure. No changes in cortical brain water content were found. Following the spontaneous decrease of portal pressure at 40 d, the BBB altered permeability reestablished its properties. Despite BBB permeability normalization, ammonia concentration remained moderately high, but significantly lower than the portal hypertension group 14 d after surgery. This could be a useful model to study BBB alterations and posterior normalization in portal hypertension with a low grade of encephalopathy.

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