Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **30** (2): 412-6 (2011)

Short communication
Received: June 9, 2010
Revised version: July 4, 2010
Accepted: July 11, 2010

Effect of Monocrotaline on Blood-BrainBarrier Permeability in Rats

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***SUMMARY*. We studied if monocrotaline (MCT) portal hypertensive model modifies blood-brain barrier (BBB) condition. Male Wistar rats were used: Group MCT injected i.p. with MCT (60 mg/kg of body weight) and Group Sham (GS) with saline. Forty-four days after injection rats were sacrificed. Trypan blue and Evans blue tests were performed to evaluate BBB integrity in both groups. In cerebrospinal fluid (CF), protein and glucose were determined. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (AP) were measured in serum samples. Portal pressure rose after MCT injection. Trypan blue diffused into hippocampus, Evans blue increased concentrations in brain of Group MCT and CF showed an increase in protein and glucose content in Group MCT. Serum AST, ALT and AP activities were significantly increased in Group MCT rats. It is suggested that liver damage and vasoconstrictor substances could produce portal hypertension, associated to toxic effects on brain and modifying thereby the BBB permeability.**

INTRODUCTION

Monocrotaline (MCT) is a pyrrolizidine alkaloid, found in several plants, distributed around the world in different genera (Crotalaria, Symphitum, Heliotropium, and Senecio), that was involved in human intoxication after the consumption of herbal medicine, teas, and food grains of these plants [1,2].

The intoxication is associated to liver injury, showing in histopathological studies [3]: centrilobular necrosis, hepatic vein thrombosis, dilated and congested sinusoids, associated to sinusoidal endothelial cell (ECS) damage [4,5], portal hypertension (PH) [6] and cardiopulmonar and kidney damage were described as well [7,8]. Previous experiments induced us to study this model of PH, based on MCT toxicity [7] with liver involvement.

The partial portal vein stricture, as described by Chojkier & Groszmann [9], developed in the rat, fourteen days after the surgical procedure, prehepatic portal hypertension (PPH), accompanied by functional and morphological brain alterations, and an increase in blood-brain barrier (BBB) permeability as well [10].

BBB is the site of regulatory mechanisms, which controls the exchange of substances between the brain and the blood through the wall of brain capillaries with tight junctions between endothelial cells. In some pathological situations the permeability of the BBB is increased of a partial proteolytic degradation of some constituents of the capillary basement lamina. BBB complex integrity was quantitatively and qualitatively studied utilizing the Trypan blue and Evans blue dyes. When BBB integrity is altered its components may showed different changes like the detachment of ECS, distorted tight junctions, severe mitochondrial swelling and perivascular astroglial edema [11].

It can also be suggested that a vasculitis can be an earlier event that leads to the different parenchymal inflammation and to the development of pulmonary hypertension. Although,

KEY WORDS: Blood-brain barrier, Liver damage, Monocrotaline, Portal hypertension.

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ISSN 0326-2383

these events appear to depend upon the vasculitis, the development of pulmonary hypertension does not seem to depend upon parenchymal inflammation. Eventually a severe chronic inflammatory process involves the whole pulmonary parenchyma with many macrophages, erythrocytes, and plasma cells present in the reduced alveolar lumen, while the septa are markedly thickened with heavy deposition of collagen [12].

In the present work we put forward the hypothesis that this model of MCT intoxication generates derangement in CNS, including modifications in the BBB structure and PH, associated to liver injury.

MATERIALS AND METHODS

Animals and treatment

Male Wistar rats (240 - 250 g of body weight) were utilized. A 12h of light cycle (8 a.m. - 8 p.m.) were applied, and animal welfare was followed according to the guidelines of the Faculty of Pharmacy and Biochemistry of Buenos Aires and approved by Ethical Committee according to Helsinki's Declaration. Animals were placed in individual cages allowed to access to water and food ad libitum (Purina Chow).

Animals were divided in two groups: Group MCT was injected i.p., with a single dose of MCT (60 mg/Kg of body weight). Group Sham, control rats, was injected i.p., with saline solution. At the 44th day of injection, animals were sacrificed by decapitation. Previously, under light ether anaesthesia, blood samples were collected from the abdominal aorta and cerebrospinal fluid were obtained by cisternal puncture.

Spleen pulp pressure was determined to evaluate splanchnic pressure. A needle was connected to a Gould Statham P23ID transducer and punctured to splenic pulp, and pressure was obtained using a Grass polygraph model 790.

Trypan blue transcardial perfusion

Rats were perfused transcardially with Trypan blue (TB) solution and fixed in paraformaldehyde. TB solution (5 g/L) was made by dissolving 1g of TB in 200 ml of PBS buffer (pH 7.4) with gentle heat. The solution was allowed to cool room temperature and then added to the filtrate. Heparin (500 units) was added to the filtrate, and then the solution was placed on ice and used immediately. The temperature of TB solution was 10-12 °C at the time of perfusion. Rats were anaesthetized with ethyl urethane (1 mg/Kg of body weight). The transcardiac perfusion was made with 200 ml of TB solution, and followed by 300 ml of ice-cold paraformal-dehyde (20 g/L) and dissolved in PBS adjusted to pH 7.4. The low rate of perfusate was maintained at 25 ml/min. Brains were dissected and post fixed over night in 0.3 Kg/L sucrose solution for 2 days. Subsequently, brains were frozen in powdered dry ice and stored at –80 °C, until processed for microscopic studies. Slices of brain tissue were obtained with cryostat in sections of 300 microns according to Paxinos & Watson 15. Brain tissue 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.* [11].

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 body weigh in rats under light ether anaesthesia. One hour after the injection, animals were sacrificed by decapitation. Brains were weighed, clipped and individually placed within formamide p.a. (2 ml/brain). These tubes were kept at 37 °C for 48 h. The content of dye extracted from each brain was determined by spectrophotometer (Metrolab 1600) 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 [14,15].

Biochemical determination

After 44 days of MCT injection the animals were sacrificed by decapitation. Previously, under light ether anaesthesia, samples of cerebrospinal fluid (CSF) were obtained by cisternal puncture for protein and glucose determination. Blood samples were collected by abdominal aortic puncture for the determination of biochemical determinations. The activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP) and albumin was determined. Biochemical parameters were determined using standardised and optimised commercial Roche kits (Germany).

Statistical analysis

Results are presented as the mean ± SEM. Comparisons among group means were made using the Student's t test. The criterion for significance was $p < 0.05$ for all studies.

RESULTS

Portal pressure

A significant increase in portal pressure was observed in Group MCT (12.1 ± 1.0 mmHg) as compared to Group Sham (7.6 ± 0.2 mmHg) ($p < 0.01$). (Fig. 1).

Evans Blue test

As can seen in Fig. 2, Group MCT rats (n = 8) showed a significant increase of Evans blue in brain tissue homogenates (8.34 ± 0.25 µg /g) when compared to Group Sham (n = 8) (6.12 ± 0.27 µg/g), $p < 0{,}001$). These results confirmed us an increased BBB permeability.

Trypan Blue test

The intracardial injection of TB in Group MCT, indicated the presence of the dye in the hippocampus region, limited to the vascular area and diffused to perivascular zone, suggesting an increased permeability of the BBB (Fig. 3B). In Group Sham no diffusion was observed (Fig. 3A).

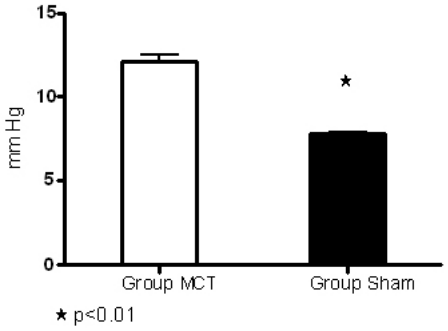

Figure 1. Measurement of portal pressure. There are significant differences between Group MCT (monocrotaline, n = 8) and Group Sham (n =8). * $p < 0.01$; SEM, standard error of the mean.

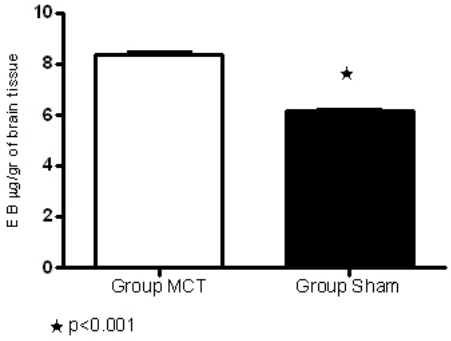

Figure 2. Evans Blue Test. There are significant differences between Group MCT (monocrotaline, n = 8) and Group Sham (n = 8). * $p < 0.001$; SEM, standard error of the mean.

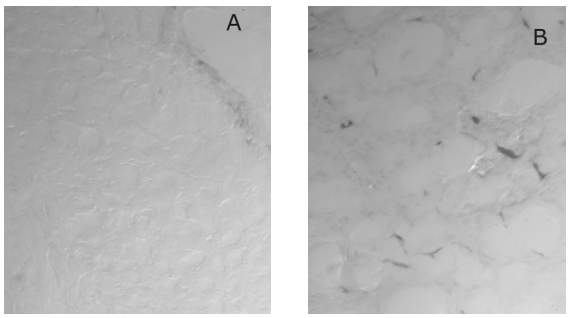

Figure 3. Light microscopy, magnification 400X, showed a marked diffusion of intravascular Trypan Blue dye injected in brain sections belongs to Group MCT (**B**), and in Group Sham the image was negative (**A**).

Cerebrospinal fluid parameters

Protein and glucose content in CSF were 0.41 ± 0.07 g/L and 1.12 ± 0.08 g/L in Group MCT and 0.19 ± 0.04 g/L and 0.61 ± 0.14 g/L in Sham rats respectively ($p < 0.001$) , indicating an augment in both parameters in rats treated with MCT (Table 1).

Serum biochemical parameters

Table 1 shows that a significant increase in the hepatic enzymes (p.<.0.05) was detected in serum of Group MCT against Group Sham, and a significant decrease in albumin and protein concentrations were also found.

DISCUSSION

Human and animal exposure to plant alkaloid MCT produces damage to several tissues, fundamentally liver and lung. In liver parenchyma it was found centrilobular parenchymal necrosis, endothelial cell damage and fibrin deposition [6]. Pulmonary hypertension and portal hypertension were also described [7].

MCT is a no reactive compound, which requires activation by cytochrome P450 to produce MCT-pyrrole and other metabolites and adducts, that are suggested to be responsible for the toxic effects observed by Yee *et al.* [5] in ECS, showing the presence of activating enzymes in these cells.

Moreover, MCT metabolized by cytochrome 3A (CYP3A) produce some metabolites as dehydromonocrotaline, by hydrolysis, retronectine, DHP-derived DNA, adducts and monocrotaline-N-oxide, that by themselves produce toxic actions.

In previous experiments with MCT, we documented hepatocytes edema, congestion, steato-

Plasma	Group MCT	Group Sham
ALT (UI/L)	41 ± 4	68 ± 9
AST (UI/L)	149 ± 14	239 ± 25
AP (UI/L)	396 ± 16	698 ± 44
Albumin (g/dl)	4.13 ± 0.11	3,22 ± 0.23
CSF	**Group MCT**	**Group Sham**
Protein (g/dl)	0.41 ± 0.07	0.19 ± 0.04
Glucose (g/L)	1.12 ± 0.08	0.61 ± 0.14

Table 1. Biochemical parameters in plasma and cerebrospinal fluid. ALT: alanine amino transferase, AST: aspartate amino transferase, AP: alkaline phosphatase, CSF: cerebrospinal fluid. The samples were obtained in Group MCT (monocrotaline, n = 8) and in Group Sham (n = 8) as described in the text. Results are means ± SEM (standard error of the mean).

sis, portal vein thrombosis and necrosis, similar to that found in the present study [16]. Liver electron microscopy showed also marked mitochondria swelling, disruption and nuclear alterations, among others findings.

Lemberg *et al.* [16] documented marked and progressive alteration in prostanoids production, with a significant decrease in vasodilator prostanoids, and the presence of vasoconstrictor prostanoids, in isolated Kupffer cells (KCS) and ECS from MCT injected rat livers, sacrificed 25 and 45 days after injection. Similar findings were also described by Bizzi *et al.* [17]. Also, this toxin stimulates the production of eicosanoids and cytokines by KCS, as was found by Brower *et al.* [18].

The decrease in vasodilators and the persistence of vasoconstrictors prostanoids, probably participates, in the complex mechanism of the splanchnic and systemic circulation derangements found in this intoxication, including PH production.

The described liver damage and portal vein trombosis may explain, in part, the increase in portal pressure, usually associated to pulmonary hypertension, as described in cirrhotic patients and the intoxication of MCT.

Plestina & Stomer [8] and Copple *et al.* [4] described ECS injury produced by MCT intoxication in liver sinusoids, a few hours after MCT injection, suggesting that the functional and structural damage take place in a progressive manner, in a later period of the intoxication.

As was described earlier [16], the toxic effect on ECS and KCS in MCT treated rats, progresses at least up to 45 days, time of duration of the experiment. The increased activity of liver enzymes in serum may correspond to the toxic liver injury.

We described that in PH rats, portal pressure was normalized at the 40th day, when portal vein stricture disappears, and is followed by a normal BBB condition of permeability, associated to functional and morphological reconstitution [11]. Brain alteration, and BBB impaired permeability documented by the presence of TB and EB in brain, and the ultramicroscopic alterations in hippocampus [19] found in the PH rat model allowed us to put forward the hypothesis that perhaps these MCT intoxicated rats with PH and brain alterations follow a related pathophysiological mechanism. This observation suggests that the presence of PH could play an important role in the BBB alterations in both rat models, in the present case perhaps associated to some toxic local effect in MCT rats. Furthermore, the increase in protein content found in CSF of MCT rats, add another sign of BBB impaired permeability, suggesting a toxic action at this level.

When BBB is damage, by MCT, swelling and edema of nervous tissue can develop, associated perhaps, to the penetration of some other substances, as neurotransmitters, drug metabolites to the brain, producing alterations of its microvascular ECS, including its tight junctions, damaging the neighbour astrocyte processes, consequently modifying its function, and possibly including an injury at the mitochondrial level, as occurred in PPH [10]. It seems that as in liver, brain ECS is a special MCT target.

Considering that endothelial cells are targets of MCT and adducts toxicity, it must be recalled that at any tissue, and brain tissue included ECS, are joint one to another by tight junctions, constituted by a set of continuous membrane unions [20]. The union is constituted by fibrils and adherents junctions, forming complexes (cadherin-catenins) associated to proteins. It may be suggested that MCT and derivates, act on ECS and it tight junctions [21].

This complex formation described between brain ECS, in some pathological conditions, may disturb normal BBB behaviour, as may occur in stroke, inflammatory or toxic processes [22,23].

The alterations of liver morphology, as we observed by microscopy examination, confirms MCT toxic action on liver parenchyma, logically associated to ALT, AST, and ALP serum increased activities, that represent a sign of hepatic aggression, and associated to a decrease in albumin and proteins. The serum glucose increas-

es is originated from liver glycogenolysis, probably attributed to the same toxic activity.

The increase in vasoconstrictor prostanoids in this model may also have some action on the brain microcirculation, including the BBB region, but more experiments are needed to demonstrate this probable event.

Astrocytes have a significant influence on the morphogenesis and organization of the vessel wall. Furthermore, in BBB, pericytes are supposed to play an important role in angiogenesis and the formation of tight junctions. Therefore, ECS, astrocytes and pericytes are key cells in BBB constitution. It can be suggested that these can be involved in BBB permeability induced by MCT damage.

Consequently, MCT and metabolites, in addition to liver, lung and kidney damage, are capable to induce BBB alteration in its permeability as demonstrated by the permeation of BE and TB, systemically injected, and also by the alteration observed in CSF chemical constitution.

CONCLUSION

In the present study, it was shown that MCT intoxication produce, besides of the important liver damage, with fibrosis and portal vein thrombosis, an increase in portal pressure and an alteration in BBB permeability, also characterized by a diffusion of both dyes (EB and TB) through the BBB in hippocampal region. This was associated with a significant increase in protein content in CSF. We can conclude that our results suggest clearly that brain and specifically its BBB region participate in MCT intoxication.

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