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# Role of Manganese as Mediator of Central Nervous System: Alteration in Experimental Portal Hypertension

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## 1. Introduction

Portal hypertension (PH) is a major syndrome that frequently accompany chronic liver diseases such as cirrhosis. Prehepatic PH develops a splanchnic hyperdynamic circulation and hyperemia with increased splanchnic resistance and production of collateral vessels that drive splanchnic blood flow to systemic circulation (Chojkier & Groszmann, 1981). Several substances have been proposed as mediators of this hypodynamic circulatory state including prostacyclins, nitric oxide and endotoxins (Bosch et al., 1992; Reiner & Groszmann, 1999; Palma et al., 2005). PH is found in patients with cirrhosis, and in portal vein thrombosis. It is characterized by an increase in splanchnic blood flow and pressure, among others caused by abdominal blood flow resistance, secondary to important liver parenchyma alterations (fibrosis or cirrhosis).

Recent studies have demonstrated that experimental PH in rats is also a sub-clinic model of Minimal Hepatic Encephalopathy (MHE) (Butterworth et al., 2009), since rats with PH develop hyperammonemia, electrophysiology alterations, blood-brain barrier (BBB) breakdown, hippocampal mitochondrial dysfunction and changes in frontal cortex and hippocamus on glutamate uptake (Scorticati et al., 2004; Lores-Arnaiz et al., 2005; Eizayaga et al., 2006; Acosta et al., 2009; Bustamante et al., 2011).

Chronic hepatic encephalopathy (HE) is a complex neuropsychiatric syndrome associated with liver dysfunction, such as cirrhosis. The pathophysiology of HE is poorly understood and there are few high-quality diagnostic tests and markers. As a result, its treatment has

improved only slightly over the last several decades (Zafirova & O'Connor, 2010). The current classificaton of HE is: Type A HE associated with acute liver failure, Type B with portosystemic bypass without intrinsic liver disease and Type C with cirrhosis (Merino et al., 2011; Ferenci et al., 2002). In chronic liver dysfunction, such as cirrhosis, it occurs more insidiously causing a range of neuropsychiatric disturbances which include psychomotor dysfunction, impaired memory, increased reaction time, sensory abnormalities and poor concentration (Albrecht, 1998; Scorticati et al., 2005; Albrecht et al., 2007). In its severest forms, patients may develop confusion, stupor, coma and death (Ferenci et al., 2002).

Hyperammonemia is a well-known toxic substance for the central nervous system (CNS), especially when levels exceed the antitoxic capacity of the brain cells. Arterial blood ammonia concentrations are frequently elevated in patients with portal-systemic encephalopathy and studies in experimental animal models of chronic liver failure reveal blood and brain ammonia concentrations approaching the millimolar range (normal range 0.05-0.10 mM) (Butterworth, 1991; Therrien et al., 1991).

The CNS is an important target for manganese (Mn), an essential element that is normally excreted via the hepatobiliary route (Papavasiliou et al., 1966; Teeguarden et. al., 2007). Manganese has a key role in the normal functioning of several enzymes including mitochondrial superoxide dismutase, glutamine synthetase, and phosphoenolpyruvate carboxykinase (Bentle et al., 1976; Stallings et al., 1991). The metal was first considered to be neurotoxic more than 150 years ago, when workers employed in grinding black oxide of Mn developed an unsteady gait and muscle weakness (Couper, 1837). Since that time, many cases of Mn neurotoxicity (manganism), a neurologic disease characterized by psychological and neurologic abnormalities, have been reported, particularly in miners, smelters, welders, and workers involved in the alloy industry (Mena et al., 1967; Eamara et al., 1971).

As manganese acts as a cofactor for many enzymes and therefore, it plays important biological functions (Keen et al., 1984). Nevertheless, high concentration of Mn exerts toxic effects in the brain (Yamada et al., 1986) and the accumulation of Mn in the basal ganglia produces an irreversible neurological syndrome similar to Parkinson's disease. Typically, patients exhibit extrapyramidal changes that include hypokinesia, rigidity and tremor (Cotzias, 1958; Mena, 1974). High levels of this metal can cause alterations in development as well as reproductive dysfunction (Grey & Laskey, 1980; Laskey et al., 1982). Manganese deficiencies produce impairment of growth and reproduction in rats of both sexes (Boyer et al., 1942; Smith et al., 1944; Prestifilippo et al., 2008). Manganese exists as divalent and trivalent forms in the plasma (Nandedkav et al., 1973; Scheuhammer and Cherian, 1985) and both may be transported into the brain across the BBB and reach the blood–cerebral spinal fluid (CSF) and accumulates in the brain (Aschner 1992; 1999).

Importantly, these not only occurs in animal models but in human since the patients with chronic liver failure have been shown to exhibit increased serum and brain levels of Mn and display many of the clinical and pathological features associated with manganese toxicity (Krieger et al., 1995; Spahr et al., 1996; Hauser et al., 1994; 1996; Sassine et al., 2002). Excessive deposition of Mn in brain has also been demonstrated in a rat model of cirrhosis (Rose et al., 1999). This elevation is believed to be due to decrease elimination of manganese via biliary excretion (Papavasiliou et al., 1966; Teeguarden et al., 2007), and to increase systemic availability due to portal-systemic shunting associated with chronic liver disease (Spahr et al., 1996; Rose et al., 1999).

### 1.1 Study of the effect of manganese in plasma and hypothalamus levels in portal hypertensive rats

Different studies indicated that participation of manganese in HE (Hauser et al., 1994; Matsuda et al., 1994; Krieger et al., 1995; Pomier-Layrargues et al., 1995; Siger-Zajde et al., 2002). Therefore we determinate manganese concentration on plasma and the effects of this metal in hypothalamus in PH rats.

### 1.2 Investigate the action of manganese of manganese on amino acids and nitric oxide levels

Amino acids play an important role in the maintenance of homeostasis on the brain. Considering that manganese may also have a role in the pathogenesis of chronic HE (Hauser et al., 1994; Matsuda et al., 1994; Krieger et al., 1995; Poimier-Layrargues et al., 1995). The second point was to analyze the effects of manganese on amino acids levels in hypothalamus using the same animal model.

The third point to consider in this work was whether changes produced by manganese in PH may be due to the mechanism of nitric oxide pathway.

## 2. Materials and methods

### 2.1 Animals and surgical procedures

Adult male Wistar rats (240–260 g of body weight) were kept under controlled conditions of light (12 h light/dark cycle: 8 a.m. to 8 p.m.). They were housed under constant temperature and a 12-hour light-dark cycle and kept in an acclimatized animal room (21-23 $^{o}$C) with *ad libitum* access to dry food and tap water. Special care for perfect air renewal was taken.

All animal procedures were performed in accordance with our institutional guidelines after obtaining the permission of the Laboratory Animal Committee and with the U.S. National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication N*8* 80-23/96).

Prehepatic PH in rats was induced by a calibrated stenosis of the portal vein according to Chojkier & Groszmann (1981). Rats were lightly anesthetized with ether and then a midline abdominal incision was made. The portal vein was located and isolated from 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 alongside the portal vein. The needle was subsequently removed to yield a calibrated stenosis of the portal vein, after which the abdominal incision was sutured. Operations were performed at 2 p.m. to obey circadian rhythm. Fourteen days after portal vein ligation, animals exhibit an increase in portal pressure. 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. Rats were sacrificed by decapitation at two weeks after surgery.

All efforts were made to minimize suffering of animals and to reduce the number of animals used.

### 2.2 Portal pressure measurement

Fourteen days after the corresponding operation, the rats were anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg). 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, USA).

### 2.3 Determination of plasma ammonia

Blood samples were obtained by abdominal aortic artery puncture for the determination of biochemical parameters. Ammoniac Enzymatic UV kits (Biomerieux-France) were used to determine plasma ammonia concentration.

| | **Sham operated** | **PH** |
|---|---|---|
| **Portal pressure (mmHg)** | 7.3±1.4 | 13.5±1.3* |
| **Plasma Ammonia(µm/L)** | 26±4 | 82±17 ** |

Table 1. Determination of portal pressure and plasma ammonia levels

Portal pressure was 7.3 ± 1.4 mm Hg in the sham-operated group versus PH group vs 13.5 ± 1.3 mm Hg by an enhanced 184% (* $p<0.05$). In other hand, plasma ammonium levels was 26 ± 4 µm/L in the sham-operated group versus PH group was 82 ± 17 µm/L, by an increase of 315% (** $p <0.01$).

### 2.4 Determination of manganese levels and in Hypothalamus

For the determination of manganese levels in tissue, brains were rapidly dissected and the hypothalamus was removed. Tissue blocks were snap frozen in liquid nitrogen and saved at -80 $^{o}$C and blood was digested by digestion in oxidizing acid, both were analysis by inductively coupled plasma mass spectrometry as described (Melnyk et al., 2003). The method was considered in Sham operated when the duplicates were ± 15% of the expected value and blank values were < 0.001 ppb.

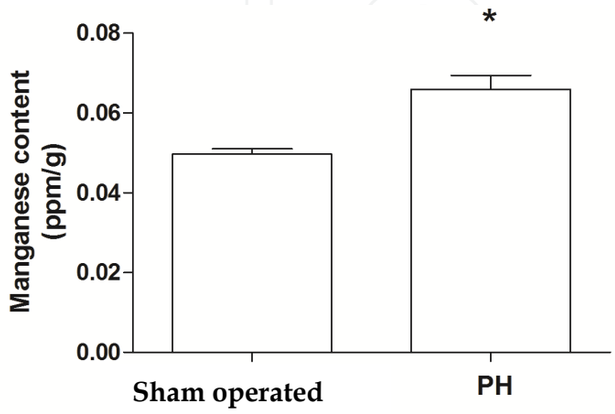


Fig. 1. Manganese analyses. A significant increase of manganese (*$p < 0.05$) was observed in plasma levels in PH groups compared with the respective Sham operated.

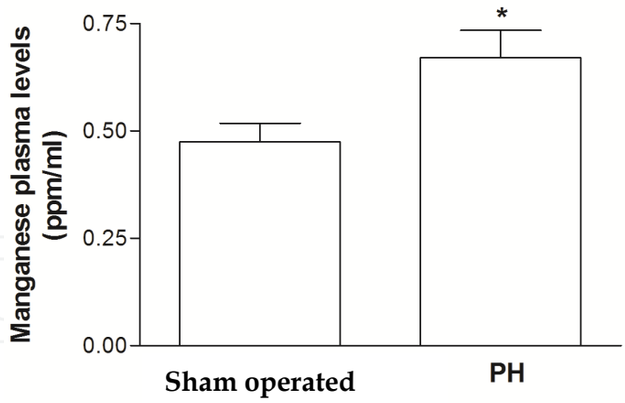


Fig. 2. Effects of Manganese on hypothalamus. The stenosis of the portal vein produced an accumulation of Manganese in the brain by 14 days after surgery versus sham operated resulting an increase in Manganese levels in hypothalamus *($p < 0.05$).

### 2.5 In vivo studies

The rats were anesthetized (ketamian/xilasiana) and implanted a cannula into the lateral cerebral ventricle, using a stereotaxic instrument and coordinates from the atlas. The correct localization of the cannula in the ventricle was confirmed at the end of the experiment. The experiments were performed a week after the implantation of the cannula. The day of experiment, conscious, freely moving rats were divided into two groups of 10 animals each. The rats were microinjected intracerebroventricularly (i.c.v.) during 1 min with 5 µl of sterile saline (control group) or 10 µg of MnCl2/5 µl sterile saline. After decapitation, the brains were rapidly dissected and the hypothalamus was removed. All incubations were carried out in a Dubnoff shaker (50 cycles per min; 95% $O_2$/5% $CO_2$) at 37°C. The hypothalami (seven to eight for each group) were preincubated individually in glass tubes in 500µl of Krebs-Ringer bicarbonate-buffered medium (NaCl 124.40 mM, KCl 4.98 mM, NaHCO3 24.88 mM, CaCl2 1.50 mM, MgCl2 1.42 mM, KH2PO4 1.25mM containing 0.1% glucose, pH: 7.4). After this preincubation (15 min) the medium was discarded and replaced with fresh medium alone or containing the substances to be tested. The incubation continued for 30 min. At the end of the incubation period the media were removed and the tissues were homogenized and submitted to appropriate extraction procedure and stored at −20 °C until the respective assays were conducted.

### 2.6 NOS enzimatic activity determination

Determination of NOS activity was performed by a modification (Canteros et al., 1995) of the $^{14}$C-arginine method of Bredt & Snyder (1989). After the incubation period (30 min) the hypothalamus were immediately homogenized in 0.5 ml of N-(2-hydroxyethyl)-piperazine-N-2-ethanesulfonic acid (HEPES) (20mM, pH: 7.4) with addition of $CaCl_2$ (1.25mM) and DL-dithiothreitol (DTT, 1mM). The reaction was started by adding NADPH (nicotinamide adenine dinucleotide phosphate, reduced) (120µM) and 200.000 dpm of $^{14}$C-arginine (360 mCi/mmol) to the homogenates. The tubes were incubated for 15 min at

37ºC in a Dubnoff metabolic shaker (50 cycles per min and 95%$O_2$,5%$CO_2$ atmosphere). At the end of this incubation period, the tubes were immediately centrifuged at 10.000 g for 10 min at 4ºC. The supernatants were immediately applied to individual columns containing 1 ml of Dowex AG 50 W-X8 200 mesh sodium form, and washed with 2.0 ml of double distilled water. All collected fluid from each column was counted for $^{14}$C-citrulline activity in a scintillation counter. NOS converts arginine into equimolar quantities of citrulline and NO, the data were expressed as pmol of NO produced per hypothalamus per min.

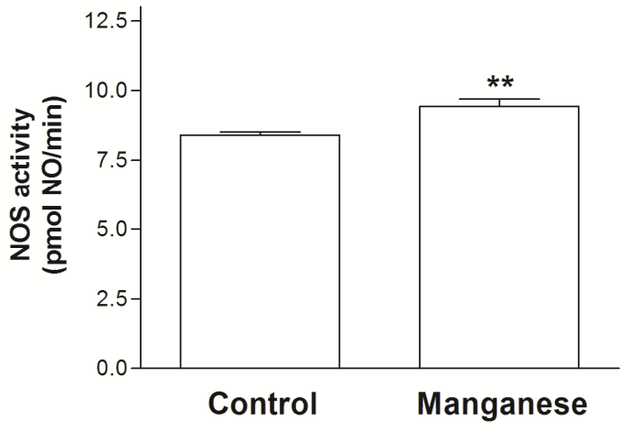


Fig. 3. Determination of NOS activity. Results show that Manganese increased NOS activity (**p<0.01) evaluated by the conversion of $^{14}$C-arginine into $^{14}$C-citrulline compared with the control group.

### 2.7 Quantification of GABA, aspartate and glutamate

The method described by Durkin et al. (1988) allowed the isolation of γ-aminobutiric acid (GABA), aspartate and glutamate. Aliquots of 50 µl of homogenates were mixed with 400 µl of O-phthalaldehyde, 50 µl of 2-mercaptoethanol in 50 µl of ethanol, and 400 µl of 0.5 M sodium borate, pH 9 (reaction mixture). After 30 min at room temperature, 50 µl of the reaction mixture were injected into the HPLC column. The O-phthalaldehyde derivates were then separated on a reverse-phase column and eluted with a buffer of acetonitrile/sodium acetate 1:9 (v/v), pH 4 at a flow rate of 1.6 ml/min. The concentrations of amino acids were extrapolated from curves made with known amounts of standard amino acids.

Different functions of the CNS are mediated by the action of diverse amino acids neurotransmitters such as aspartate, glutamate and GABA. Therefore, with the purpose of determining whether manganese could affect their secretions, evaluating the release and the content of aspartate, glutamate and GABA from the hypothalamus obtained after i.c.v. injection of manganese determined by high-performance liquid chromatography (HPLC). GABA release by 2 folds (* p< 0.05) compared with the respective control group. The others neurotransmitters not shown significative differences.

| | Aspartate | | Glutamate | | GABA | |
|---|---|---|---|---|---|---|
| | **Release** | **Content** | **Release** | **Content** | **Release** | **Content** |
| **Control** | 192 ± 11 | 1505± 171 | 157±10 | 309± 39 | 78 ± 7 | 84 ± 24 |
| **Manganese** | 206± 11 | 1679± 63 | 163± 9 | 271± 47 | 106± 7 * | 115 ±21 |

Table 2. Release and concentration of different amino acids such as aspartate, glutamate and GABA following the injection of manganese.

### 2.8 Drugs, chemicals and radiolabeled compounds

*Manganese chloride (MnCl2) was purchased from Anedra (San Fernando, Buenos Aires, Argentina). HEPES, DTT, NADPH, Glutamate, Aspartate and GABA were purchased from Sigma Aldrich (St Louis, MO , USA). Dowex AG 50 W-X8 200-400 mesh sodium form was obtained from Bio-Rad (Hercules, CA), and the $^{14}$C-arginine-monohydrochloride 360 mCi/mmol was from Amersham Pharmacia (Buckinghamshire, HP, UK). All other chemical materials used in this work were from analytical grade.*

### 2.9 Statistical analysis

Experiments were repeated at least twice employing seven to eight animals per group in each experiment. All data are expressed as the mean ± SEM. Comparisons between groups were performed by using a one-way ANOVA followed by the Student-Newman-Keuls multiple comparison tests for unequal replicates. Student's t-test was used when comparing two groups. Differences with p values $< 0.05$ were considered significant.

## 3. Conclusions

Experimental prehepatic PH produces a hyperdynamic redistribution of splanchnic circulation and minimal liver damage. Ammonia was considerate the major responsible of the alterations in CNS included cytotoxic brain edema characterized by swelling of astrocyte. However the ammonia is not the only toxic and as Shawcross & Jalan (2005) demonstrated the participation of other relevant metabolic molecules such as manganese.

In the present work we showed for the first time that rats with experimental prehepatic PH presented increase of manganese level in plasma and hyphotalamus. The manganese is transported to the liver after absorption from the gut and the liver may be important as a deposit for manganese, with hepatic manganese later delivered to the brain (Takeda, 1998). Rats with PH show a redistribution of splanchnic circulation and increase the different toxic in blood including ammonia and manganese as shown in this work. Even more, patients with abnormal deposit of manganese in the basal ganglia has been estimated by magnetic resonance imaging was associated with the elevated levels of manganese in the blood (Krieger et al., 1995; Siger-Zajdel et al., 2002).

This metal is able to enter the brain through the cerebral vasculature and the spinal fluid. The mechanism by which Mn crosses the BBB is not yet well understood, but involves binding of the metal to transport systems such as transferrin (Aschner & Aschner, 1992; 1999). Also, as Mn levels rise in blood, the influx into the spinal fluid rises and entry across the choroid plexus becomes more important (Murphy et al., 1991). Importantly, Mn

accumulates in the hypothalamus (Deskin et al., 1980; Pine et al., 2005) and is known to be taken up by both neurons and glial cells (Tholey et al., 1990) and, hence, suggesting a potential role in neuronal/glial communications within the developing hypothalamus.

We investigated the participation of hypothalamus NO production and we found that the rats with administer this metal increased the activity of NOS. So we can deduce that nitric oxide has been involved in this pathophysiological brain processes

This metal is able to enter the brain through the cerebral vasculature and the spinal fluid. The mechanism by which manganese crosses the BBB is not yet well understood, but involves binding of the metal to transport systems such as transferrin (Aschner & Aschner, 1992; 1999). On the other hand, has been observed a decrease of GABA concentration opposite to the chronic exhibition to manganese in certain regions of the CNS as the globo pallidum, but not in substance nigra or hippocampus (Bonilla et al., 1994; Zwingmann et al., 2003). This effect on GABA levels produces to itself across the direct action of the manganese on the expression of glutamic decarboxylase, enzyme that regulates GABA synthesis (Tomas-Camardiel et al., 2002).

When the Mn is accumulated in the synapsis it produces a consistent neuropathy with an excitocitotoxic effect, suggesting that the mechanism of glutamate is involved in the development of the pathology described by the manganese. These findings suggest that the manganese induce an increase in nitric oxide synthase production probably correlated to GABAergic and glutamatergic hypothalamic neurons that form a part of a network neuronal autoregulation.

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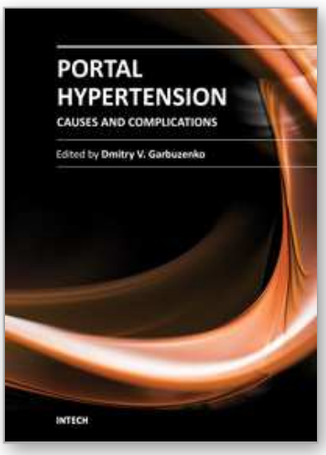


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Portal hypertension is a clinical syndrome defined by a portal venous pressure gradient, exceeding 5 mm Hg. In this book the causes of its development and complications are described. Authors have presented personal experiences on conducting patients with various displays of portal hypertension. Moreover, the book presents modern data about molecular mechanisms of pathogenesis of portal hypertension in liver cirrhosis, the information about the original predictor of risk of bleeding from gastro-esophageal varices and new methods for their conservative treatment.

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