Université de Montréal

Pathogenèse de l'œdème cérébral dans l'encéphalopathie hépatique minimale: rôles du stress oxydatif et du lactate

par

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Résumé

L’encéphalopathie hépatique (EH) est un syndrome neuropsychiatrique découlant des complications de l'insuffisance hépatique. Les patients souffrant d'une insuffisance hépatique chronique (IHC) présentent fréquemment une EH minimale (EHM) caractérisée par des dysfonctions cognitives subtiles qui affectent leur qualité de vie. L'insuffisance hépatique entraîne une hyperammoniémie, le facteur central dans la pathogenèse de l'EH. Pourtant, les taux d'ammoniaque sérique ne sont pas corrélés avec la sévérité de l'EH lors d'une IHC, suggérant que d'autres facteurs y contribuent. L'œdème cérébral est une caractéristique neuropathologique décrite chez les patients souffrant d'une EHM et plusieurs facteurs dont le stress oxydatif, les altérations du métabolisme énergétique et l'augmentation de la glutamine cérébrale pourraient contribuer à la pathogenèse de l'œdème cérébral lors d'une EHM induite par une IHC. Les mécanismes sous-jacents exacts ainsi que les relations entre ces facteurs et l'ammoniaque ne sont pas connus. Présentement, le seul traitement efficace de l'IHC est la transplantation hépatique, une option thérapeutique très limitée.

Le but de cette thèse est de contribuer à l'avancement des connaissances sur les mécanismes sous-jacents liés au rôle du stress oxydatif, de la glutamine et du lactate dans la pathogenèse de l'œdème cérébral lors d'une EHM induite par une IHC afin d'envisager de nouvelles options thérapeutiques. Les objectifs précis étaient: 1. Déterminer le rôle de l’ammoniaque dans la pathogenèse de l'œdème cérébral lors d'une EHM induite par une IHC. 2. Investiguer le rôle du stress oxydatif, en décrivant sa présence au niveau systémique et au niveau cérébral dans la pathogenèse de l'œdème cérébral lors d'une EHM induite par une IHC. 3. Déterminer la relation entre l’ammoniaque et le stress oxydatif dans la pathogenèse de l'œdème cérébral. 4. Établir le rôle du lactate et de la glutamine dans la pathogenèse de l'œdème cérébral et leur relation avec l’ammoniaque. Pour atteindre ces objectifs, 2 modèles animaux d'EHM obtenus par microchirurgie chez le rat ont été utilisés: 1) la ligature de voie biliaire, un modèle d'IHC et 2) l'anastomose porto-cave, un modèle d'hyperammoniémie induite par la dérivation portosystémique.

Nos résultats démontrent que l'ammoniaque et le stress oxydatif indépendamment n'induisent

pas l'œdème cérébral lors d'une EHM. Pourtant, lorsque les 2 facteurs agissent ensemble ils présentent un effet synergique qui entraîne le développement de l'œdème cérébral, le stress oxydatif étant une première insulte, qui est suivie par l'hyperammoniémie comme deuxième insulte. En plus, le stress oxydatif a été mis en évidence seulement au niveau systémique, et non au niveau central dans notre modèle d'IHC en association avec l'œdème cérébral, suggérant que le stress oxydatif systémique est une conséquence de la dysfonction hépatique et que l'hyperammoniémie n'induit pas le stress oxydatif ni systémique ni central.

Nous avons démontré qu'une augmentation du lactate cérébral est une conséquence directe de l'hyperammoniémie et joue un rôle important dans la pathogenèse de l'œdème cérébral lors d'une EHM induite par une IHC, tandis qu'une augmentation de la glutamine au niveau cérébral n'est pas un facteur clé.

La compréhension de ces mécanismes a entraîné la proposition de 3 nouvelles stratégies thérapeutiques potentielles pour l'EHM. Elles ciblent la diminution de l'ammoniaque sérique, la réduction du stress oxydatif et l'inhibition de la synthèse du lactate.

Mots-clés: encéphalopathie hépatique, hyperammoniémie, œdème cérébral, stress oxydatif, espèces réactives d'oxygène, ligature de la voie biliaire, anastomose portocave, AST-120, allopurinol, diéthyl maléate, résonance magnétique nucléaire, lactate, glutamine, dichloroacétate.

Abstract

Hepatic encephalopathy (HE) is a metabolic neuropsychiatric syndrome which occurs as a complication of liver failure/disease. Patients with chronic liver disease (CLD) present often with minimal HE (MHE) characterized by subtle cognitive dysfunction which impairs their quality of life. Impaired liver function leads to hyperammonemia which is a central factor in the pathogenesis of HE. However, ammonia alone is poorly correlated with the severity of HE during CLD, strongly suggesting other factors may contribute. Brain edema is a neuropathological feature described in MHE patients and several factors such as oxidative stress, energy metabolism alterations and an increase in glutamine may to contribute to the pathogenesis of brain edema during HE related to CLD. However the exact underlying mechanisms and the relationships between these factors and ammonia are poorly understood. To date, the only effective treatment of CLD remains liver transplantation, a limited therapeutic option.

The aim of this thesis is to advance the knowledge into the mechanisms underlying the role of oxidative stress, glutamine and lactate in the pathogenesis of brain edema during MHE associated with CLD in order to uncover new therapeutic options. The study objectives were: 1. Determine the role of ammonia in the pathogenesis of brain edema in chronic liver disease. 2. Investigate the role of oxidative stress, depicting between its presence systemically and centrally, in the pathogenesis of brain edema in chronic liver disease. 3. Determine the relationship of ammonia and oxidative stress in the pathogenesis of brain edema. 4. Define the roles of lactate and glutamine in the pathogenesis of brain edema and their relationship with ammonia. To achieve these objectives, we used 2 microsurgical rat models: 1) bile-duct ligation, a cirrhosis model and 2) portacaval anastomosis, a hyperammonemia model following portal-systemic shunting.

Our findings demonstrate that ammonia and systemic oxidative stress independently do not induce brain edema in MHE related to CLD. However, when both factors are present, they exert a synergistic effect leading to the development of brain edema with oxidative stress presenting as a “first hit”, followed by hyperammonemia as a “second hit”. Moreover, solely systemic and not central oxidative stress was observed in our CLD rat model in relation to brain edema implying that

systemic oxidative stress is a consequence of liver dysfunction and that central oxidative stress is not a direct effect of hyperammonemia in the setting of CLD. Moreover, we revealed that increased cerebral lactate is a direct consequence of hyperammonemia and also plays an important role in the pathogenesis of brain edema, while increased cerebral glutamine does not.

The understanding of these mechanisms led to the proposal of three different strategies as potential HE therapies. These are directed towards lowering ammonia, reducing oxidative stress and inhibiting lactate synthesis.

Keywords: hepatic encephalopathy, hyperammonemia, brain edema, oxidative stress, reactive oxygen species, bile-duct ligation, portacaval anastomosis, AST-120, allopurinol, diethyl maleate, nuclear magnetic resonance, lactate, glutamine, dichloroacetate

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Abbreviations:

HE:	hepatic encephalopathy
MHE:	minimal hepatic encephalopathy
ALF:	acute liver failure
CLD:	chronic liver disease
ICP:	intracranial pressure
TIPS:	transjugular intrahepatic portosystemic shunt
BBB:	blood-brain barrier
AQP:	aquaporin
CBF:	cerebral blood flow
NH_3, NH_4^+	ammonia, the gas, ion form respectively
GA:	phosphate-activated glutaminase
GS:	glutamine synthetase
AMPA:	α-amino-3-hydro-methyl-4-isoxasole-propionic acid
NMDA:	N-methyl-D-aspartate
GABA:	γ-aminobutyric acid
ROS:	reactive oxygen species
SOD:	superoxide dismutase
CAT:	catalase
GSH:	glutathione
XO:	xanthine oxidase
TCA cycle:	tricarboxylic acid cycle
LDH:	lactate dehydrogenase
MCT:	monocarboxylate transporter
MARS:	molecular adsorbent recirculating system
PCA:	portacaval anastomosis

BDL:	bile-duct ligation
GPB:	glycerol phenylbutyrate
BCAA:	branched-chain amino acids
LOLA:	L-ornithine–L-aspartate
OP:	L-ornithine phenylacetate
LT:	liver transplantation
CSF:	cerebrospinal fluid
AST:	aspartate aminotransferase
ALT:	alanine aminotransferase
SEM:	standard error of the mean
SSAO:	semicarbazide-sensitive amine oxidase
MAO-A:	monoamine oxidase A
MAO-B:	monoamine oxidase B
GR:	glutathione reductase
GP:	glutathione peroxidase
GSSG:	oxidized glutathione
TBARS:	thiobarbituric acid-reactive substances
Da:	dalton
NMR:	nuclear magnetic resonance
PDH:	pyruvate dehydrogenase
PC:	pyruvate carboxylase
DCA:	dichloroacetate
DEM:	diethyl maleate

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Chapter 1: Introduction

1.1 Hepatic encephalopathy

1.1.1 Definition and epidemiology

Hepatic encephalopathy (HE) is a metabolic neuropsychiatric syndrome manifesting by a wide spectrum of symptoms, ranging from mild cognition and attention deficits to coma and death. It represents a major complication of acute liver failure (ALF) as well as of chronic liver disease (CLD). HE is classified in 3 categories depending on the type of liver injury: type A results following ALF, type B is induced by portal-systemic bypass and type C is associated with CLD. The latter is further classified by the severity of symptoms in minimal and overt HE (Ferenci et al., 2002).

ALF is a rare condition, affecting around 2000 patients annually in the USA with a stable annual incidence and a mean survival rate of 45%, varying between 10- 65% depending on the cause (Lee, 2012). ALF is defined as a rapid and severe deterioration of a previously healthy liver leading to HE along with jaundice and coagulopathy (Hoofnagle et al., 1995). Close to 55% are a result of drug intoxication (acetaminophen occurring in 46% of cases), with the remaining cases caused by autoimmune hepatitis, acute viral hepatitis (A and B) and 15% unknown. HE induced by ALF, classified as type A HE, is characterized by a rapid, severe deterioration of mental, brain edema and an increase in intracranial pressure (ICP). Brain stem herniation, instigated through intracranial hypertension, is a frequent cause of death in these patients (Bernal et al., 2007; Bhatia et al., 2006; Jalan et al., 2004a).

CLD is defined as a progressive liver function deterioration occurring as a consequence of a persistent hepatic insult such as chronic hepatitis B and C, alcoholic hepatitis or non-alcoholic steatohepatitis (Rosen, 2011; Sanyal et al., 2010; Wang and Beydoun, 2007). The prevalence of these conditions is on the rise and is estimated that 3 million Canadians suffer from liver disease (Canadian Liver Foundation, 2013). In time, over a period of 10-20 years, under the aggression of the hepatic insult, normal liver architecture is replaced by fibrosis and regeneration nodules. This status is defined as cirrhosis which represents the fifth cause of death in Canada (Statistics Canada, 2010). Cirrhosis leads to numerous complications such as portal hypertension, impaired protein

synthesis, multi-organ dysfunction with 50-70% of patients presenting with HE (type C) (Bajaj, 2010). HE in this setting is the result of multiple pathogenic processes: decreased hepatic detoxification and synthetic capacity, formation of portal-systemic shunts (consequence of portal hypertension) and increased production and release of toxins by the ailing liver. These conditions are favored by numerous precipitating factors such as: infections, dehydration, renal failure, aggravation of the hepatic disease by a novel superimposed hepatic insult, the development of hepatocellular carcinoma, gastrointestinal bleeding, constipation or insertion of a transjugular intrahepatic portosystemic shunt (TIPS) (Munoz, 2008).These conditions lead to a variable clinical picture, classified into minimal and overt HE (figure 1).

The third type of HE is type B which results from portal-systemic shunting (bypass) without the presence of an underlying parenchymal liver disease, a rare condition. However, animal models, such as animals subjected to a portacaval anastomosis, are frequently used to study the effects of gut-derived toxic metabolites by-passing the liver on brain function (Butterworth et al., 2009).

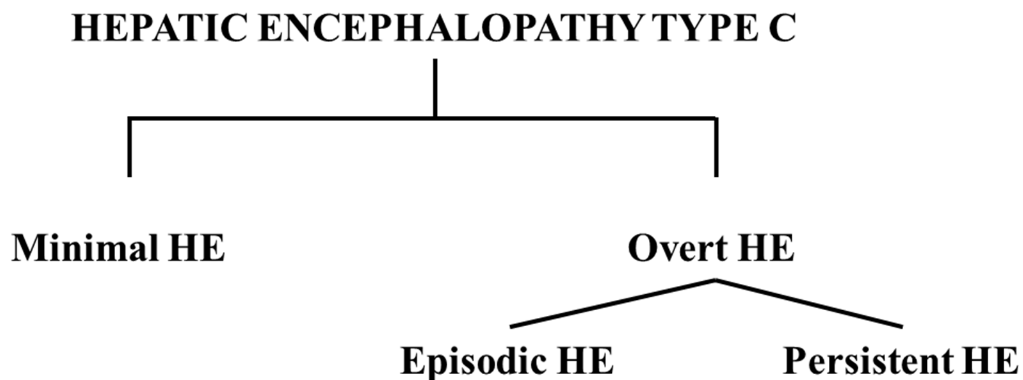

Figure 1. Classification of the subtypes of hepatic encephalopathy type C as described in the text. HE, hepatic encephalopathy.

1.1.1.1 Minimal HE

Minimal HE (MHE), the mildest form of HE, is characterized by neuropsychological and neuropsychiatric changes in the absence of any clinically evident symptoms. The symptoms consist in attention and cognition deficits and psychomotor performance impairments detectable only by neuropsychological and neurophysiological testing (tests such as the psychometric hepatic encephalopathy score, the repeatable battery for the assessment of neuropsychological status, the critical flicker frequency, the inhibitory control test or the EncephalApp, (Bajaj et al., 2013; Córdoba, 2011)). Most tests are time-consuming and not routinely performed during clinical examinations, therefore MHE remains widely underdiagnosed. In spite of the lack of obvious symptoms, MHE strongly impacts the quality of life of the patients, leading to impairments of operating heavy machinery and to an increased risk of having vehicle accidents (Amodio, 2009; Montgomery and Bajaj, 2011). Moreover, MHE patients have a 4-fold increased risk of developing overt HE (Hartmann et al., 2000) as well as a higher probability of leading to persisting neurological complications following liver transplant (Chavarria and Cordoba, 2013).

1.1.1.2 Overt HE

Overt HE is the clinically detectable form of HE. The highly variable and nonspecific symptomatology has been classified into 4 grades known as the West Haven Criteria (Conn et al., 1977) (table I). Grade 0 represents MHE. Grade I is characterized by sleep-wake rhythm alterations, shortened attention span, lack of awareness; grade II manifests as lethargy, apathy, overt personality changes and disorientation; grade III is defined by somnolence, stupor and severe confusion and grade IV represents the coma stage.

Symptoms may occur in surges (termed episodic HE) or be continuously present (persistent HE). The former is characterized by brief episodes, while the latter by continuous presence of overt HE symptoms.

Table I. West Haven criteria for the grading of hepatic encephalopathy. Modified after Bajaj, 2010 with the publisher's permission.

Grade	Consciousness	Intellect and behaviour	Neurological findings
0	Normal	Normal	Normal examination; impaired psychomotor testing
1	Mild lack of awareness	Shortened attention span; impaired addition or subtraction	Mild asterixis or tremor
2	Lethargic	Disoriented; inappropriate behaviour	Obvious asterixis; slurred speech
3	Somnolent but arousable	Gross disorientation; bizarre behaviour	Muscular rigidity and clonus; hyper-reflexia
4	Coma	Coma	Decerebrate posturing

1.2 Brain edema in liver disease

1.2.1 Brain edema

Brain edema is the result of water accumulation in the brain and may be a consequence of astrocyte or neuronal swelling, but also of an accumulation of water in the extracellular space. The maintenance of a constant volume is critical for cerebral cell homeostasis. Brain edema can occur as a result of either a disruption of the blood-brain barrier (BBB, vasogenic brain edema) or following metabolic alterations which lead to an accumulation of osmolytes within cells followed by the entry of water (cytotoxic brain edema). Brain edema can have a direct effect on cerebral function through physical stress (in addition brain edema can lead to an increase in intracranial pressure (ICP) as the brain lies within a non-compliant skull). Also, changes in cell water may affect signaling cascades, modify the intracellular and cell-to-cell communication or disturb the architecture of the cell or

intracellular compartments (Pasantes-Morales and Cruz-Rangel, 2010).

Vasogenic edema involves the breakdown of the BBB. It is induced following conditions such as traumatic brain injury or neoplasms, where the BBB is physically disrupted, or following inflammatory and infectious cerebral diseases, where the BBB opens following the agression of inflammatory factors. The BBB is composed of endothelial cells connected by tight junctions, resting on a basal lamina and connected with astrocytes and pericytes (figure 2), structures which, together with neurons, form the neurovascular unit (Hawkins and Davis, 2005). The BBB regulates the passage of molecules from the blood into brain, specifically allowing some through specific channels and transporters. The endothelial cells line the cerebral capillaries and their particularity compared to non-cerebral endothelial cells resides in the abundance of tight junctions proteins (Kniesel and Wolburg, 2000) which restrain the paracellular space (Abbott, 2000) not allowing macromolecules to pass. Pericytes contribute to the stability of the cerebral capillaries (von Tell et al., 2006). Astrocytes are the most abundant cell of the human brain with important roles as a metabolic support for neurons and endothelial cells. Therefore, alterations of the BBB's tight junctions, affecting BBB integrity, allows the free passage of macromolecules (such as circulating proteins) into the cerebral extracellular space thus pulling more water into the brain and causing vasogenic edema (figure 3). The alterations of the BBB are evidenced by two methods. The first one is the direct visualization of the BBB breakdown by electronic microscopy (Kato et al., 1992). The second one consists in infusing intravenously substances that do not normally pass through the BBB, but will pass if a BBB breakdown is present. These substances are detected in cerebral tissue by spectrophotometry, fluorescence, Western Blot or radioactivity measurement. Small molecules such as sodium fluorescein or [^{14}C]-γ-aminobutyric acid may detect small breakdowns, while big molecules such as Evans Blue or immunoglobulin G detect large ruptures of the BBB.

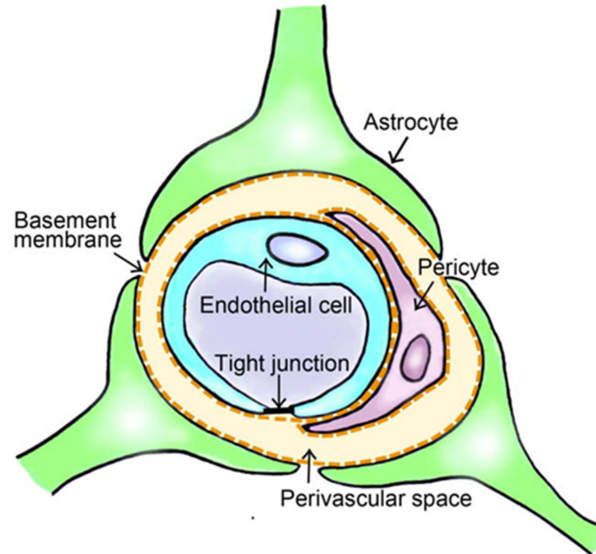

Figure 2. Structure of the BBB. Explanations are provided in the text. After Takeshita and Ransohoff, 2012 with the publisher's permission.

Numerous cerebral metabolic reactions produce osmotic molecules; in the normal brain they are rapidly cleared by enzymatic metabolism or excretion into the blood. However, numerous pathologies such as cerebral ischemia, infections or diabetic ketoacidosis (Donkin and Vink, 2010; Levin, 2008; Papadopoulos et al., 2000) induce an increase in osmolyte production or impaired clearance, leading to their accumulation into the brain. This hypertonicity will induce an increased water entry into the brain in order to restore the osmotic equilibrium (figure 3) thus inducing cytotoxic brain edema.

Different ion and water transporters located on the BBB contribute to the development of brain edema. One example is the family of bi-directional transmembrane water channels aquaporin (AQP). Of those, particulary AQP4 is highly expressed on the astrocytic end-feet (Amiry-Moghaddam et al., 2004) and plays an important role in the development of brain edema in traumatic brain injury, tumors and cerebrovascular disease (Badaut et al., 2011; Donkin and Vink, 2010; Nico and Ribatti, 2011). Other examples of transporters implicated in inducing brain edema are the NKCC, the Na^{+}-K^{+}-$2Cl^{-}$ co-transporter which maintains an inwardly-directed net ion flux, therefore inducing

brain edema by increasing cell volume (Kahle et al., 2009); the nonselective cation channel (NC_{Ca-ATP}), which is regulated by sulphonylurea receptor 1, a nonselective cation channel expressed in all cells of the neurovascular unit only in central nervous system injuries, including cerebral ischemia and traumatic brain injury (Simard et al., 2012).

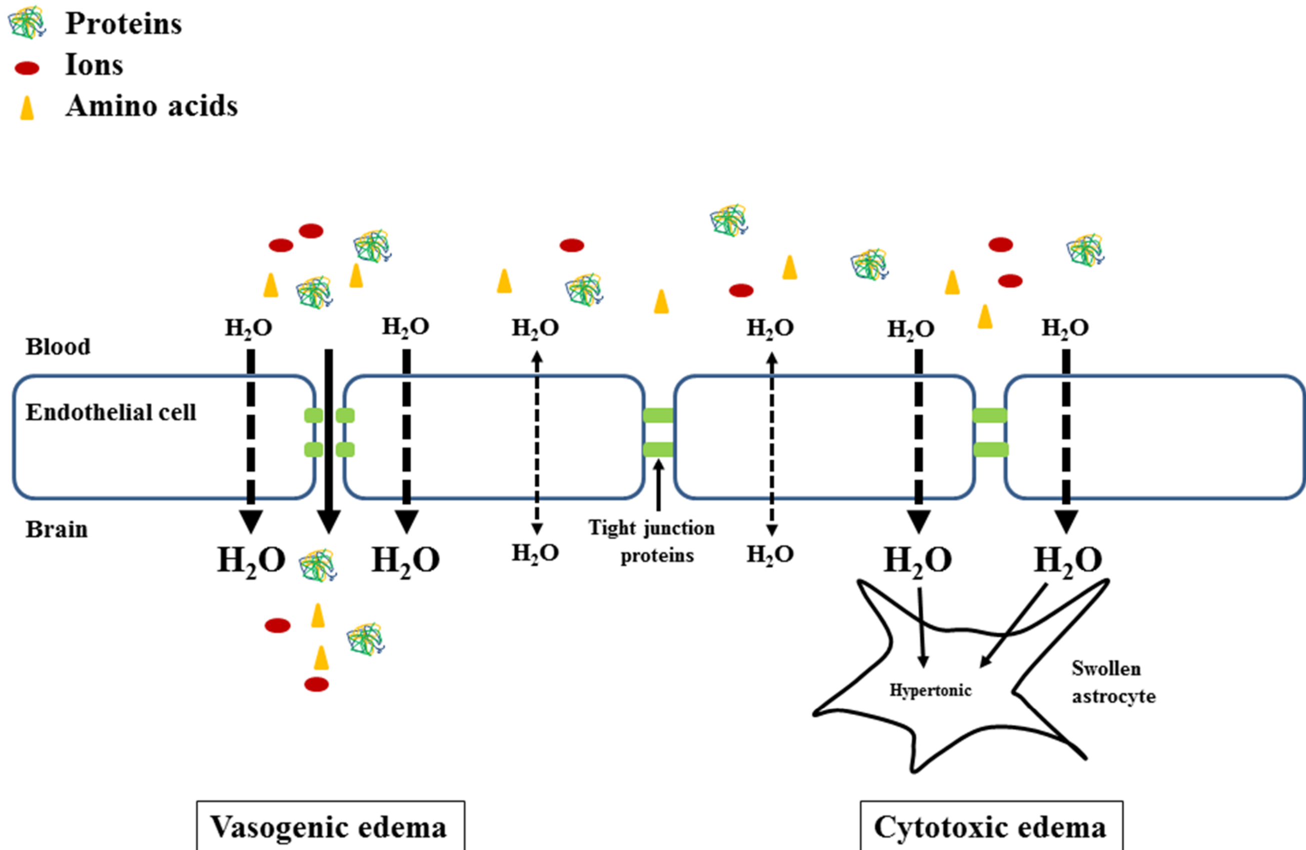

Figure 3. Vasogenic and cytotoxic brain edema. Left panel: vasogenic brain edema appears as a consequence of a physical breakdown of the BBB that allows plasma macromolecules (proteins) and other compounds to cross the BBB and accumulate in the extracellular space followed by an entry of water in attempt to re-establish the osmotic equilibrium and consequently results in brain edema; right panel: cytotoxic brain edema is the result of cellular metabolic alterations that cause an intracellular accumulation of osmotic molecules such as ions and amino acids, followed by an entry of water in order to re-establish the osmotic equilibrium. After Bosoi and Rose, 2013 with the publisher's permission.

1.2.1.1. Intracranial hypertension in ALF and not CLD

Intracranial volume consists of 10% of cerebral tissue, 10% of blood and 80% cerebrospinal fluid (Rengachary and Ellenbogen, 2005). Cerebral water accumulation leads to increased intracranial volume. Since the skull is not compliant an increase in brain volume will lead to an increase in ICP which in turn may trigger brain stem herniation and death (a frequent cause of death in ALF). However intracranial hypertension rarely occurs in CLD. It is believed the degree of brain edema in CLD is "low-grade", not sufficient enough to cause an increase in ICP ((Häussinger et al., 2000), figure 4).

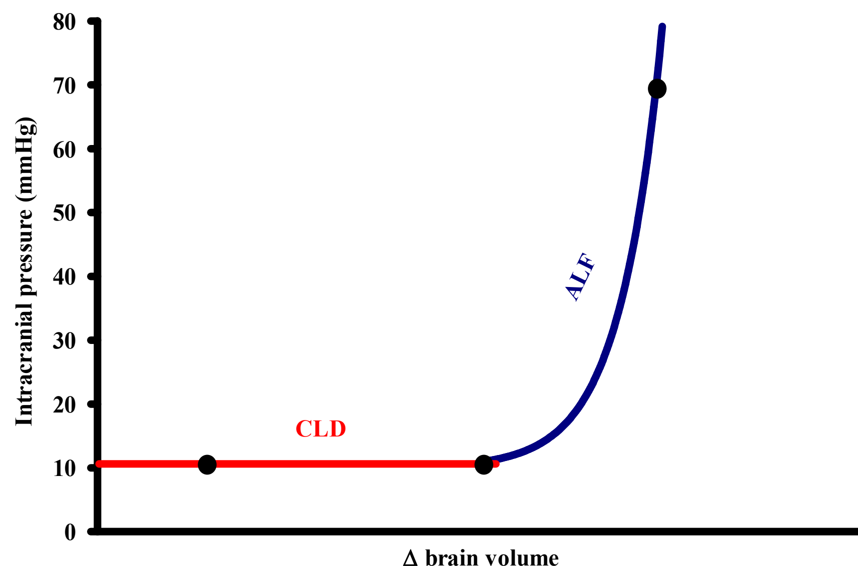

Figure 4. Relationship between brain volume and intracranial pressure (ICP): in chronic liver disease (CLD) a certain increase in brain volume does not lead to an increase in ICP; in acute liver failure (ALF) the water accumulation exceeds the brain volume capacity and leads to an increase in ICP.

Several other explanations for why an increase in ICP is found in ALF and not CLD exist. An increase in cerebral blood flow (CBF) will lead to an increase in brain volume. In ALF an increase in

CBF is observed (Jalan et al., 2004b; Larsen et al., 1996; Strauss et al., 1997; Wendon et al., 1994), while in CLD it decreases (Almdal et al., 1989; Burra et al., 2004; Dam et al., 1998; Iversen et al., 2009; Iwasa et al., 2000; Trzepacz et al., 1994). Second, since CLD develops over years of hepatic insults, cirrhotic patients are much older than ALF patients, with a median age of 56 years at diagnosis (Fleming et al., 2012) compared to a mean of 38 years in ALF (Ostapowicz et al., 2002). In the aging CLD population, the physiological aging atrophy of the brain, characterized by a steady volume loss, is present (Garcia-Martinez et al., 2011). A smaller brain volume in these patients grants more ‘‘space’’ for expansion in the eventuality of swelling. Therefore, a higher increase in brain volume constituents (compared to ALF) is needed in order for intracranial hypertension to develop in CLD.

1.2.2 Vasogenic versus cytotoxic brain edema in liver disease

Brain edema in liver disease has been widely described. It has been evidenced in HE induced by ALF (Jiang et al., 2009a; Rose et al., 2007) and CLD (Davies et al., 2009; Wright et al., 2007) in different animal models, as well as HE in patients with ALF (Bhatia et al., 2006; Gupta et al., 2010) and cirrhosis (Lodi et al., 2004). Brain edema has also been described in MHE patients, considered to have "low-grade" brain edema since they do not present an increase in ICP (Córdoba et al., 2001; Häussinger, 2006; Häussinger et al., 2000; Kale et al., 2006; Shah et al., 2008; Sugimoto et al., 2008).

Brain edema induced by BBB breakdown (vasogenic edema) has been reported in different animal models of ALF and CLD, but the conclusions are inconsistent. Toxin-induced ALF following galactosamine or azoxymethane administration leads to cerebral extravasation of plasmatic Evans Blue and [^{14}C] alpha-aminoisobutyric acid (Cauli et al., 2011; Dixit and Chang, 1990; Horowitz et al., 1983; Yamamoto and Nguyen, 2006). This effect is probably due to altered tight junctions as modifications of their composing proteins such as occludin, claudin-5, zonula occludens 1 and 2 were demonstrated (Chen et al., 2009; Lv et al., 2010; Sawara et al., 2009; Shimojima et al., 2008). However, other reports in the same models performed in other animal species demonstrated a lack of

extravasation of horseradish peroxidase (Traber et al., 1987) and observed a microscopically intact BBB (Alexander et al., 2000; Potvin et al., 1984; Wright et al., 2007). The use of different animal species, various HE models and diverse brain edema evaluation techniques may explain this discrepancy and therefore further investigations looking into BBB integrity in liver failure/disease are warranted.

On the other hand, cytotoxic evidences of brain edema are well described. Alterations in numerous metabolites, such as lactate and glutamine, are well known to play a role in inducing brain edema (Chavarria et al., 2010; Zwingmann et al., 2004). Moreover, these alterations have been proven both *in vitro* and in ALF and CLD animal models, but also in patients due to the development of non-invasive advanced magnetic resonance imaging techniques (for review see (McPhail et al., 2012)).

The neuropathology of HE involves swelling of the astrocytes, while neurons are resistant to the accumulation of water. Electron microscopy studies showed the cell most affected by swelling to be the astrocyte (Kato et al., 1992; Traber et al., 1987; Wright et al., 2007), while only one study demonstrated neuronal swelling (Kristiansen et al., 2010). In CLD, astrocytes present a characteristic morphology, named Alzheimer type II astrocytes: they are large, swollen and present enlarged swollen nuclei and cytoplasm, margination of chromatin, mitochondrial and rough endoplasmic reticulum proliferation and accumulation of glycogen (Norenberg, 1977; Wright et al., 2007). Given the metabolic support role of astrocytes for neurons, the accumulation of osmotic metabolites is more likely to occur in astrocytes than in neurons. Moreover, differences in mechanisms of volume regulation such as osmotic gradients and membrane channels ionic fluxes (Kelly and Rose, 2010; Olson and Li, 2000) between neurons and astrocytes explain why astrocytes are more prone to swelling (Pasantes-Morales and Cruz-Rangel, 2010). In regard to this, Nase et al. demonstrated that hypo-osmotic stress in normal mice induces an increased water entry only in astrocytes, not in neurons (Nase et al., 2008).

1.3. Pathogenesis of hepatic encephalopathy and brain edema

1.3.1 Ammonia

Genetic, environmental factors and type of liver disease may all impact the clinical manifestations of HE. However, ammonia has been long considered the main pathogenic factor involved in HE, since high ammonia concentrations lead to neurotoxicity (Felipo and Butterworth, 2002). Ammonia is produced in large quantities by the gastrointestinal system and efficiently removed by the hepatic urea cycle.

1.3.1.1 Ammonia chemistry and metabolism

Ammonia exists either as a gaseous weak base (NH_3), or as an ion (NH_4^+), forms which are in equilibrium depending on the pH accordingly to the Henderson-Hasselbach equation:

$$\log_{10}[NH_3 / NH_4^+] = pH - pKa$$, where pKa is the acid dissociation constant.

At the physiological pH, 98% of ammonia is in its ionic form and 2% is gaseous. Both ammonia forms can cross any membranes, NH_3 by freely diffusing through the phospholipid layer of cells. NH_4^+ however, has similar ionic properties with the K^+ ion, therefore it crosses membranes using K^+ channels and transporters, such as inward rectifying and voltage-gated K^+ channels or Na^+/K^+ and H^+/K^+ ATP-ase (Moser, 1987) and $Na^+/K^+/Cl^-$ cotransporters (Aickin et al., 1982; Kelly et al., 2009). In addition, ammonia can also be transported through membranes by specific ammonia transporters, the nonerythroid Rhesus (Rh) glycoprotein B (RhBG) and C (RhCG) (for review see (Bakouh et al., 2006)), as well as by the aquaporin-8 channel (Saparov et al., 2007). Through these chemical properties, ammonia has a direct effect on the cell pH, membrane potential and cellular metabolism (Bosoi and Rose, 2009).

NH_4^+ is a substrate as well as a product of numerous metabolic reactions. The primary source of ammonia in the body is the gut either by enterocytes where glutamine is deaminated to glutamate by the phosphate-activated glutaminase (GA) or by intestinal bacteria through urealysis and protein

deamination. Further, ammonia is absorbed into the portal system and is filtered through the liver, which represents the main ammonia removing organ due to the presence of the urea cycle which maintains circulating concentrations of ammonia within 35-65 μM.

Other organs important in maintaining the homeostasis of ammonia are the muscle, the kidney and the brain. The muscle has a high protein and amino-acid turnover, therefore it contains numerous enzymes that metabolize ammonia. The most important is glutamine synthetase (GS) which incorporates ammonia into glutamate to form glutamine. The kidney contains both GA and GS, however its main role in ammonia metabolism is NH_4^+ excretion in order to maintain the urinary pH, as well elimination of liver-derived urea.

Ammonia metabolism in the brain has some particularities. GS is found exclusively in astrocytes (Martinez-Hernandez et al., 1977), while GA is primarily found in the neurons, forming the glutamate–glutamine metabolic cycle between astrocytes and neurons. This cycle is important in recycling the glutamate released from neurons in the synaptic cleft, thus preventing neuronal excitotoxicity (Cooper, 2001). Astrocytes take up the glutamate by excitatory amino acid transporters (EAAT1 and EAAT2, (Gegelashvili et al., 2007; Rothstein et al., 1994)), and then converted it to glutamine through GS. Then, the non-neuroactive glutamine is released into the extracellular space through glutamine transporters (N and ASC-system transporter SN-1, SN-2 and ASCT2 (Chaudhry et al., 1999; Cubelos et al., 2005; Dolińska et al., 2004)), from where is uptaken by neurons (sodium-coupled amino acid transporter, SAT/ATA (Varoqui et al., 2000)), and transformed to glutamate by GA.

1.3.1.2 Ammonia metabolism during liver failure

During ALF or CLD, several mechanisms lead to an increase in circulating ammonia which may reach up to 1.5 mM in ALF (Jiang et al., 2009a; Rose et al., 2007), and presents around a 2-3-fold increase in CLD, up to 150-200 μM (Montoliu et al., 2011; Ong et al., 2003). First, cirrhotic patients present a switch of normal gut flora to ammoniagenic bacteria, therefore leading to an

increase in intestinal ammonia production (Romero-Gómez et al., 2009). Moreover, intestinal GA increases (Jover-Cobos et al., 2014; Romero-Gómez et al., 2004). Secondly, hepatic fibrosis induces portal hypertension and formation of portal-systemic shunts, therefore the portal blood bypasses the liver and its ammonia content fails to be detoxified by the liver. Thirdly, due to hepatocyte necrosis urea production is impaired and the ammonia contained by the portal blood reaching the liver is released into the hepatic veins and into the systemic circulation.

As a consequence of hyperammonemia, an increase of ammonia is seen in every organ and compensatory mechanisms are developed. The muscle presents an elevation of ammonia uptake (Bessman and Bessman, 1955) as well as an increase in GS activity (Desjardins et al., 1999; Jover-Cobos et al., 2014), leading to excess glutamine formation (Chatauret et al., 2006). These mechanisms however, do not suffice in order to obtain an efficient whole-body ammonia removal; in addition glutamine can be converted back to ammonia and glutamate by GA (Wright et al., 2011). On the other hand, the kidney does not excrete more ammonia during hyperammonemia (Tyor et al., 1960), instead it becomes an ammonia producer through increased GA activity (Dejong et al., 1993; Olde Damink et al., 2003).

1.3.1.3 Cerebral metabolism and neurotoxicity of hyperammonemia

During hyperammonemia, ammonia crosses the BBB (both as an ion and as a gas) and increased cerebral ammonia exerts numerous toxic effects by affecting membrane potential, calcium signaling, cellular pH, metabolism as well as mRNA and protein expression (Bosoi and Rose, 2009). Inflammation, oxidative/nitrosative stress, energy metabolism alterations will ensue leading to impairment of neurotransmitter systems, cerebral blood flow, astrocyte morphology, mitochondrial function and other ((Bosoi and Rose, 2009), figure 5).

High ammonia concentrations have been shown to be toxic to other organs, including the liver, the skeletal muscle, the respiratory and gastrointestinal tracts (Jia et al., 2014; Qiu et al., 2013; Seo et al., 2011; Wise et al., 2013). However, ammonia's neurotoxicity remains particularly dangerous due

to underlying mechanisms that remain incompletely understood. A possible explanation for the brain being so sensitive to ammonia might be the fact that it is a huge energy consumer (20% of the body's energy for an organ representing 2% of the body weight). Ammonia has been shown to trigger direct alterations to the TCA cycle, by inhibiting enzyme α-ketoglutarate dehydrogenase (Lai and Cooper, 1986). At the same time, ammonia increases the glycolysis flux, by stimulating phosphofructokinase activity (Lowry and Passonneau, 1966). In spite of these alterations, ATP levels remain unchanged during ammonia intoxication and ALF (Fitzpatrick et al., 1988; Mans et al., 1994).

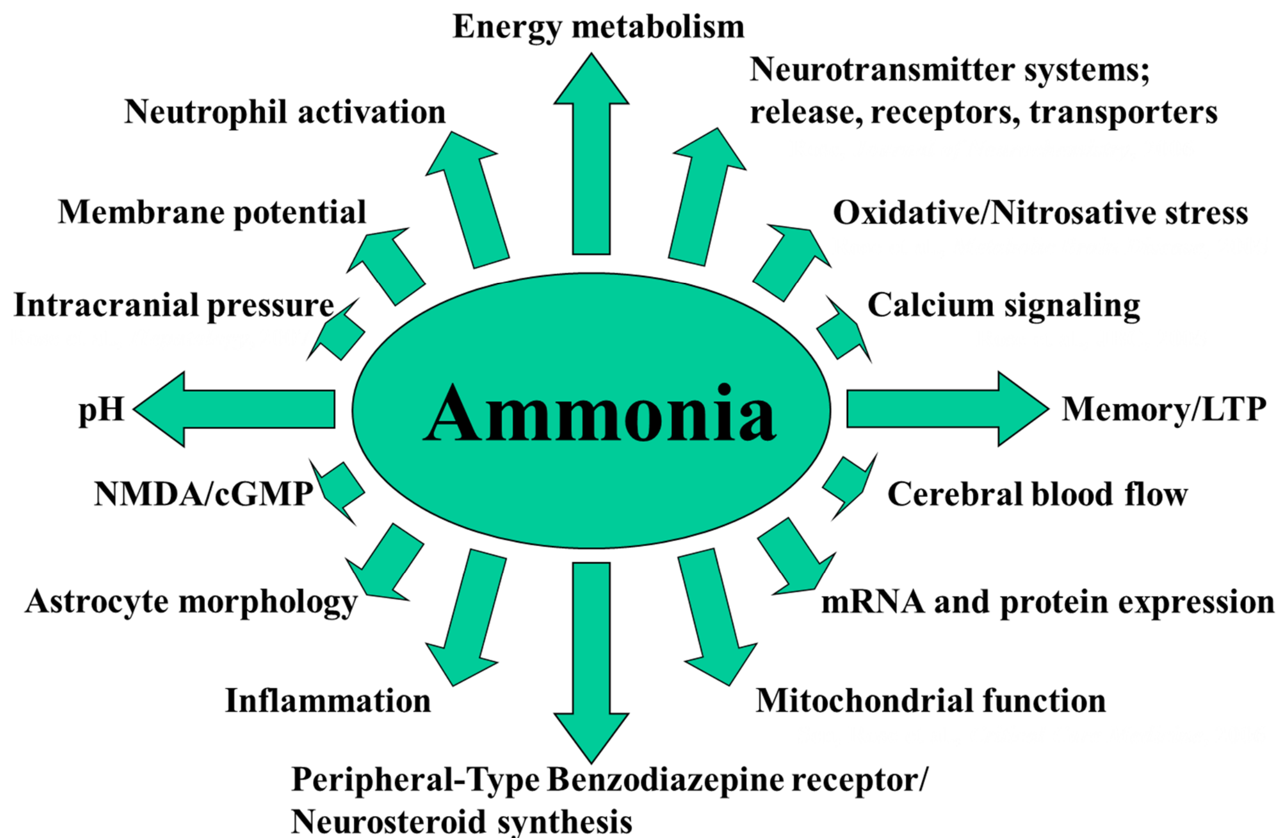

Figure 5. Neurotoxic effects of ammonia. After Bosoi and Rose, 2009 with the publisher's permission.

In the brain, exclusively in the astrocytes, ammonia is metabolized to glutamine by GS. This

reaction leads to an excess of glutamine, considered an osmotic molecule important in the development of brain edema (astrocytic swelling, (Brusilow and Traystman, 1986)). Intracellular accumulation of glutamine will lead to a decrease in glutamate and other osmolytes such as taurine and myo-inositol and to disturbances of other amino acids such as aspartate and alanine (Heins and Zwingmann, 2010; Zwingmann, 2007; Zwingmann et al., 2003).

In ALF, high arterial ammonia concentrations are correlated with increased ICP and cerebral herniation (Clemmesen et al., 1999). However, a correlation between ammonia levels and severity of HE in CLD is weak (He et al., 2011; Kundra et al., 2005; Nicolao et al., 2003; Ong et al., 2003; Weissenborn et al., 2007; Wilkinson et al., 2011) therefore suggesting pathogenic factors other than ammonia are involved in the pathogenesis of brain edema and HE in CLD.

1.3.1.4 Impairment of neurotransmitter systems induced by ammonia

Whether neurotransmitter disturbances play a causal or causative role in the pathogenesis of HE is still debated. However, neurotransmitter disturbances are important cerebral dysfunctions seen in HE that explain the clinical manifestations characterizing the syndrome. Neurotransmitter metabolism is strongly related to cerebral ammonia levels; therefore neurotransmitter disturbances are an important chapter in ammonia neurotoxicity. Moreover, differential regulation in different brain regions of neurotransmitters and their receptors may explain the heterogenous symptoms seen in different HE patients.

Glutamate

Glutamate is the main excitatory neurotransmitter in the brain. As explained above, glutamate is directly linked to the metabolism of ammonia through the enzyme GS. As a consequence cerebral glutamate levels decrease during HE, as it is consumed in order to detoxify ammonia into glutamine. In spite of that, synthesis of glutamate and its synaptic release are both increased (Palomero-Gallagher and Zilles, 2013). Glutamate acts on three different ionotropic receptors: α-amino-3-hydro-methyl-4-isoxasole-propionic acid (AMPA), kainate and *N*-methyl-D-aspartate (NMDA).

These receptors play an important role in synaptic plasticity and long-term potentiation, processes related to learning and memory. HE animal models and human autopsy studies determined AMPA and kainate receptors to have a differential response, as they are either up- or down-regulated in different brain areas (as reviewed by (Palomero-Gallagher and Zilles, 2013)). Therefore, their role remains unknown. On the other hand, NMDA receptors are known to be excessively activated by the extracellular glutamate during acute hyperammonemic conditions leading to activation of the glutamate – nitric oxide – cyclic guanosine monophosphate pathway and thus to coma and death in cerebellum and cortex of hyperammonemic rats (as reviewed by (Montoliu et al., 2010)). Altered glutamate neurotransmission may explain the learning and memory problems observed in HE patients.

γ-aminobutyric acid

γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian brain. In HE, an increased "GABAergic tone" is present, which is induced by an increase in cerebral GABA, increased expression of GABA receptors, increased concentration of endogenous benzodiazepine-like compounds known to activate GABA receptors and by ammonia activation of GABA receptors (Cauli et al., 2009a). The GABAA receptor is particulary of interest because, aside being a binding site for GABA, different subunits present different allosteric binding sites for benzodiazepines or neurosteroids. Cerebral endogenous benzodiazepines and neurosteroids are increased during liver disease (Ahboucha and Butterworth, 2005). Their action on the GABAA receptor potentiates the inhibitory effect of GABA contributing to the increased "GABAergic tone", characterized by sedative and anxiolytic effects.

Acetylcholine

Acetylcholine is a neurotransmitter with functions in the peripheral nervous system (as main muscle activator), in the autonomic nervous system and in the central nervous system where it modulates plasticity, arousal and reward. Moreover, acetylcholine suppresses the inhibition of GABA. In cirrhotic patients and rats, cerebral acetylcholine levels are reduced as a consequence of an increase in acetylcholinesterase, the enzyme responsible for metabolizing acetylcholine (García-

Ayllón et al., 2008). This decrease results in a reduced GABA inhibition, thus contributing to the increase in the “GABAergic tone” seen in HE.

Histamine and serotonin

These two monoamines are neurotransmitters important in the regulation of sleep and circadian rhythm. They interact with each other: serotonin stimulates histamine release (Laitinen et al., 1995) and histamine inhibits serotonin release through H_3 receptors (Schlicker et al., 1988). Increased cerebral histamine levels have been found both in vivo and post mortem in patients with HE, along with an increase in extracellular serotonin levels in several rat models of HE and a decrease in histamine H_3 receptors (as reviewed by (Palomero-Gallagher and Zilles, 2013)). The increase in histamine is related indirectly to hyperammonemia by an increase in cerebral neutral aminoacid transport through the BBB determined by increased glutamine (Cascino et al., 1982). Serotonin levels are correlated with the degree of portacaval shunting and to ammonia levels in a rat model of HE (Lozeva et al., 2004). These changes in histamine and serotonin lead to the sleep-wake rhythm alterations and somnolence seen in HE patients.

1.3.2 Oxidative stress

Oxidative stress (OS) is defined as an imbalance between the production and detoxification of free radicals leading to an increase in reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), superoxide ion ($O_2^{\cdot-}$) and peroxynitrite ($ONOO^-$). ROS play an important role in cell signaling (Valko et al., 2007). They are constantly produced during oxygen metabolism by oxidant enzymes, such as NADPH-, xanthine-, monoamine-, and aldehyde-oxidase or nitric oxide synthetase and rapidly metabolized by ubiquitary antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), or ROS neutralizing molecules, such as glutathione and albumin.

ROS are highly reactive due to the presence of unpaired valence shell electrons. In excess, they react with cellular molecules such as proteins, lipids and nucleic acids. This leads to functional

alterations of enzymes, transporters and receptors (Stadtman and Levine, 2000), alterations in membrane permeability (Poon et al., 2004) and abnormal gene translation and protein synthesis. Since they are highly reactive, ROS do not cross the BBB, instead they might contribute to increased BBB permeability by modifying transporters located at this level (as detailed in chapter 2.1), by modifying membrane lipids or activating different signaling pathways (Pun et al., 2009).

Since systemic OS is present in liver disease and ammonia toxicity has been shown to induce ROS in the brain, a thorough investigation in relation to HE is warranted.

1.3.2.1 Systemic oxidative stress and liver disease

OS in liver disease represents a systemic phenomenon not only in the stage of cirrhosis, but long before, since the stage of hepatitis, whether the etiology is viral, alcoholic or autoimmune (Ikegami et al., 2014; Kaffe et al., 2015; Wang et al., 2012). OS is known to be induced directly by the hepatitis C virus or by alcohol (Koike, 2014; Wang et al., 2012). However, the systemical persistence of OS in these patients is associated with the progression to liver fibrosis, cirrhosis and development of hepatocellular carcinoma (Choi et al., 2014).

During liver disease, both a decrease in antioxidants and an increase in systemic oxidants are observed. The liver is responsible for synthetizing the major antioxidants glutathione and albumin. Glutathione (GSH) represents the main intracellular antioxidant and although it is produced by every cell, the liver synthetizes it in large quantities. During CLD, GSH synthesis significantly decreases due to hepatocyte necrosis which leads to a decrease in glutamate-cysteine ligase and glutathione synthetase, enzymes in the GSH synthesis pathway (Yang et al., 2009). Another important antioxidant produced by the liver is albumin, the most abundant plasmatic protein which possesses multiple cysteine and methionine residues (Roche et al., 2008). Liver disease results in decreased protein synthesis and a significant reduction in albumin levels (Chen et al., 1997).

Xanthine oxidase (XO) is an oxidant enzyme highly expressed in the liver. Following hepatocyte necrosis, XO is released into the systemic circulation where it oxidizes hypoxanthine and xanthine to uric acid, releasing H_2O_2. XO is increased in plasma of cirrhotic patients (Battelli et al.,

2001). Moreover, XO inhibition with allopurinol has been previously demonstrated to reduce systemic OS in stable cirrhotic patients (Spahr et al., 2007). The increase in XO, along with the decrease in antioxidants leads to increased systemic ROS during CLD (Chen et al., 1997; Ljubuncic et al., 2000).

1.3.2.2 Cerebral oxidative stress and hepatic encephalopathy

Cerebral OS in HE is considered to be the result of ammonia neurotoxicity (Häussinger and Görg, 2010; Norenberg et al., 2004), but whether it may also be triggered by systemic OS remains to be determined.

One of the toxic effects of ammonia in the brain is induction of OS. *In vitro*, high ammonia concentrations (> 5mM) lead to the generation of ROS in astrocyte cultures (Görg et al., 2008; Jayakumar et al., 2006; Mehrotra and Trigun, 2012; Murthy et al., 2001). *In vivo*, acute ammonia intoxication (intraperitoneal administration of ammonium acetate to naïve rats at a high concentration of 12 mmol/kg; sacrificed after 11 minutes) leads to an increase in cerebral ROS due to increased xanthine-, monoamine-, and aldehyde-oxidase accompanied by decreased activities of SOD, CAT and GP (Kosenko et al., 2003). In ALF rats following hepatic devascularisation, high hyperammonemia and brain ammonia are accompanied by the development of brain edema and severe HE (coma stage); systemic and central OS are associated features (Jiang et al., 2009a).

These evidences suggest a direct relationship of ammonia and cerebral OS in the pathogenesis of HE related to ALF, however such a relationship and its underlying mechanisms in CLD and MHE remains undefined. Moreover, the relationship between systemic ROS and ammonia also remains unknown.

1.3.3 Lactate

Lactic acid is a carboxylic acid which, in solution, donates a proton from its carboxyl group,

forming the lactate ion. It is produced in every cell from pyruvate via a reversible reaction catalyzed by the enzyme lactate dehydrogenase (LDH), which involves the oxidation of NADH to NAD^+.

1.3.3.1 Lactate metabolism

Glucose-derived pyruvate (via glycolysis) is a substrate of the tricarboxylic acid (TCA) cycle, the metabolic hub of the cell and final common pathway for the aerobic oxidation of fuel molecules. The conversion of pyruvate to lactate during anaerobic situations (such as exercise or hypoxia) is needed in order to regenerate NAD^+ which allows the glycolytic pathway to function (figure 6). Muscle-derived lactate is metabolized in the liver via gluconeogenesis which will return to fuel other organs, including the muscle, completing the Cori cycle ((Woll and Record, 1979), figure7). Imbalances between lactate production and removal lead to lactic acidosis, a serious condition characterized by a low plasmatic pH which may impair all systems leading to severe respiratory, cardiovascular and neurological symptoms.

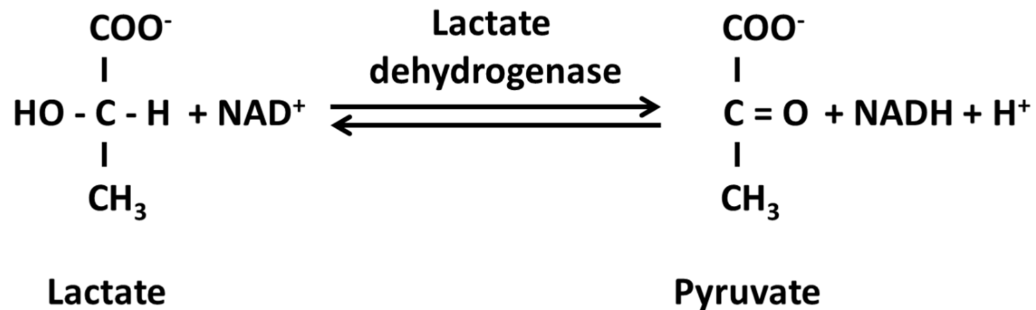

Figure 6. The reversible reaction from lactate to pyruvate catalyzed by lactate dehydrogenase.

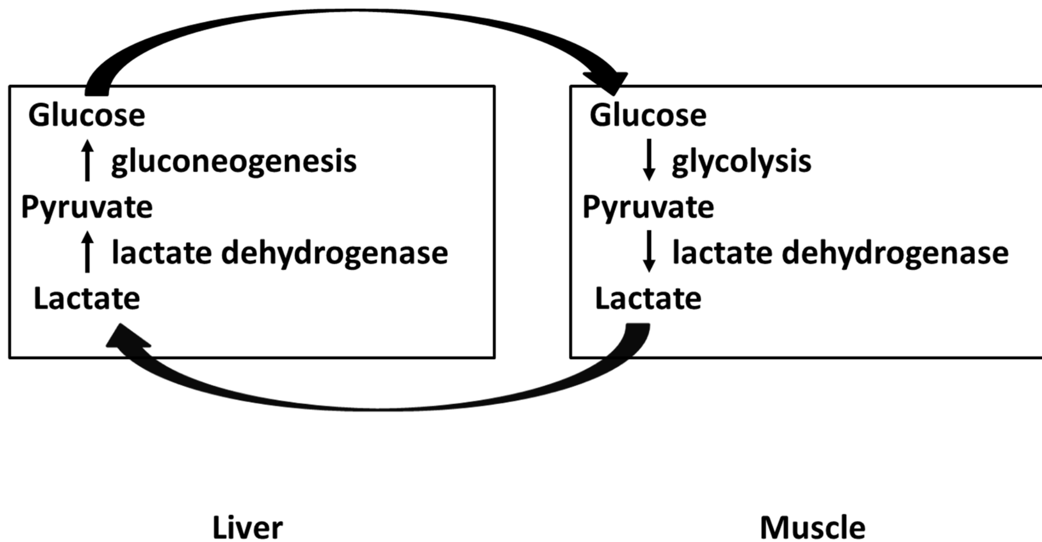

Figure 7. The Cori cycle. Explanations are given in the text.

In the brain, glucose is traditionally considered the only fuel molecule; however, new evidence demonstrates that lactate is a preferred oxidative energy substrate over glucose by neurons (as reviewed by (Pellerin and Magistretti, 2012)). This theory is known as the “astrocyte-neuron lactate shuttle” (ANLS) and it states that lactate is primary produced by astrocytes, released extracellularly and taken up by neurons where it is used a fuel for the TCA cycle (Pellerin et al., 2007; Schurr, 2006).

The ANLS is supported by the different localizations of specific transporter and enzyme isoforms involved in lactate metabolism of astrocytes and neurons. As astrocytes are part of BBB, they come in close contact with the cerebral capillaries and therefore are the first “brain” cells to take up blood-derived glucose. Astrocytes play a metabolic supportive role for neurons as they produce and release molecules required by neurons. Different isoforms of LDH enzymes and lactate transporters have different isoforms in astrocytes and neurons: astrocytes express LDH5 and monocarboxylate transporters MCT1 and MCT4, while neurons LDH 1 and MCT2 with a higher affinity for lactate than the astrocytic isoforms. This suggests astrocytes are better equipped to produce and release lactate, while neurons to take up and metabolize lactate in back into pyruvate to fuel the TCA cycle (for review see (Pellerin and Magistretti, 2012)).

1.3.3.2 Lactate and liver failure

Increased plasmatic lactate is present in animal models of ALF (Chatauret et al., 2003; Rose et al., 2007) and is considered a prognostic marker in patients with ALF (Bernal et al., 2002). In CLD, hyperlactatemia correlates with the severity of cirrhosis (Jeppesen et al., 2013) and is associated with mortality (Tas et al., 2012; Zauner et al., 2000). The role of systemic lactate in MHE has not been evaluated.

Several factors contribute to hyperlactatemia during CLD: (i) impaired Cori cycle consequent to hepatic necrosis (Levraut et al., 1998; Woll and Record, 1979), (ii) increased lactate release from necrotic hepatocytes (Clemmesen et al., 1999); and (iii) increased extra-hepatic lactate production following multi-organ dysfunction (Bernal et al., 2002).

1.3.3.3 Lactate and hepatic encephalopathy

In ALF patients, increased cerebral lactate is correlated to severe HE (brain edema, intracranial hypertension and coma) (Tofteng et al., 2002) or rats (Chatauret et al., 2002; Zwingmann et al., 2003). Ammonia-lowering therapies such as hypothermia, ornithine phenylacetate and the albumin hepatic dialysis system (MARS, Molecular Adsorbent Recirculating System) have shown to reduce cerebral lactate along with brain edema and the development of severe HE in ALF (Chatauret et al., 2002; Rose et al., 2007; Sen et al., 2006).

Contrary to ALF, the role of lactate in HE and CLD is poorly known. A 1.37-fold increase in cerebrospinal fluid lactate has been described in patients with end-stage liver disease and overt/severe HE (grades 3 and 4) (Yao et al., 1987). However the role of cerebral lactate in the pathogenesis of brain edema and MHE is unknown. Moreover, it still remains unclear whether lactate is a cause or a consequence of HE (Rose, 2012).

1.3.4 Glutamine

Glutamine is one of the 20 amino acids encoded by the standard genetic code. Also, glutamine is the most abundant plasmatic amino acid (Brosnan, 2003). Its metabolism is strongly related to NH_4^+ since it is formed from glutamate and ammonia by the enzyme GS (present in the brain, the liver and the muscle) and degraded by the enzyme GA back to glutamate and ammonia (present in the brain, the intestine, the kidney and the liver), as explained above (chapter 3.1.2). Along with glutamate, N-acetylaspartate, creatine and myo-inositol, glutamine is one of the major cerebral osmolytes, accounting for approximatively 10-15% of the cerebral osmolar pool (Pasantes-Morales and Cruz-Rangel, 2010) (table II)

Table II. Rat and human brain organic osmolyte content (mM). Modified after Pasantes-Morales and Cruz-Rangel, 2010 with the publisher's permission.

Osmolyte	Rat brain	Human brain
Glutamate	10–15.4	7.5–9.5
N-Acetylaspartate	7.5–10	8.8–8.9
Creatine/P-Creatine	6.6–9.7	7.4–8.4
Myo-inositol	3.3–5.8	6.5–9.0
Glutamine	3.4–5.1	5.0–5.9

1.3.4.1 Glutamine metabolism during hyperammonemia

Glutamine represents the end-product of ammonia detoxification during CLD and hyperammonemia. Therefore, an increase in both plasmatic and cerebral glutamine is observed in animal models as well as ALF patients (Chatauret et al., 2002; McConnell et al., 1995; Zwingmann et al., 2003), but also in animal models and CLD patients (Fries et al., 2014; Hourani et al., 1971; Laubenberger et al., 1997; Lavoie et al., 1987), including patients with MHE (Singhal et al., 2010;

Taylor-Robinson et al., 1999).

ALF following liver devascularisation in the rat induces a significant increase of expression and activity of GS in the muscle and a decrease in the cortex, whereas glutamine levels remain increased in both organs (Chatauret et al., 2006). Hyperammonemia induced by portacaval shunt in the rat shows the same pattern: GS is reduced in the brain (cortex and cerebellum) and increased in the muscle (Desjardins et al., 1999). Moreover, an increase in GA activity both in the muscle and in the brain is described in cirrhotic rats (Jover-Cobos et al., 2014). This means that, during hyperammonemia, the muscle becomes an important organ in ammonia detoxification into glutamine; however glutamine metabolism through GA leads to the production of ammonia (the reverse reaction). Meanwhile in the brain in spite of the compensatory reduction in GS glutamine levels remain high (Cordoba et al., 1996). This effect is the result of trying to balance the neurotoxic effects of ammonia and those of glutamine which will be detailed below.

1.3.4.2 Glutamine neurotoxicity

Glutamine is harmful to the brain as it is an osmolyte considered important in the development of brain edema but also by direct neurotoxic effects.

Firstly, glutamine is considered an osmolyte important in the development of brain edema (Brusilow and Traystman, 1986). Several studies demonstrated a correlation of glutamine with severity of HE (Hourani et al., 1971; Laubenberger et al., 1997). Moreover, pre-treatment of cultured astrocytes with the irreversible GS inhibitor, methionine sulfoximine (MSO), results in an attenuation of the increase in glutamine and also of swelling (Norenberg and Bender, 1994). Similar results have been observed *in vivo* following ammonia-infusion in portacaval shunted rats pre-treated with MSO (Master et al., 1999; Willard-Mack et al., 1996). Although this evidence supports an important role of glutamine in the development of brain edema, ammonia-lowering treatments, which significantly attenuate brain edema in animal models of ALF, do not induce a decrease of cerebral glutamine levels (Ytrebø et al., 2009; Zwingmann et al., 2004). Normally, the increase in

glutamine is followed by a decrease in other cerebral osmolytes such as myo-inositol and taurine (Cordoba et al., 1996). However, this mechanism is not able to fully rebalance the osmotic equilibrium during severe HE related to ALF (Zwingmann, 2007).

Secondly, glutamine has been shown to accumulate in mitochondria, impairing its function by mechanisms involving OS. The mechanism by which glutamine exerts its toxic effects in astrocytes was proposed as the “Trojan horse” hypothesis by Dr. Albrecht and Dr. Norenberg. They state that mitochondrial metabolization of glutamine to ammonia and glutamate by GA facilitates the glutamine transport in excess from the cytoplasm to mitochondria (as the Trojan horse) serving as a carrier of ammonia (Albrecht and Norenberg, 2006). Although this hypothesis remains highly disputed, some evidences link glutamine to OS and swelling. *In vitro*, glutamine induces the mitochondrial transition pore, resulting in a sudden increase in permeability of the mitochondrial membrane to osmolar molecules less than 1500 Da followed by swelling of cultured astrocytes (Pichili et al., 2007; Ziemińska et al., 2000). Moreover, MSO treatment reduces ROS formation in ammonia-treated astrocytes suggesting a direct role of glutamine in inducing OS (Murthy et al., 2001). The role of these mechanisms *in vivo* remains to be determined in the future.

1.4. Models of hepatic encephalopathy

1.4.1 *In vivo* models

HE animal models are classified corresponding to the classification of HE into types A, B and C ((Ferenci et al., 2002), detailed in chapter 1.1).

Several species are used to induce similar manifestations as those seen in humans. Large animals such as pigs have the advantages of obtaining high quantities of samples, providing repetitive sampling and using human compatible therapeutic strategies such as hepatic dialysis devices. Small animals such as mice or rats are largely used because of the availability of information (atlases, literature, specific antibodies or transgenic models) and of the low cost

(Butterworth et al., 2009).

1.4.1.1 Chronic hepatic encephalopathy models (types B and C)

Different models available for the study of type B and C HE are discussed below.

Type B hepatic encephalopathy models

Portal-systemic shunt (or anastomosis, PCA, figure 8) allows gut-derived toxins such as ammonia to bypass the liver, to enter the systemic circulation and affect the brain. This model permits the study of hyperammonemia alone, without factors released by an ailing liver. PCA rats survive long term and present with normal liver function markers and behavior alterations (Butterworth et al., 2009).

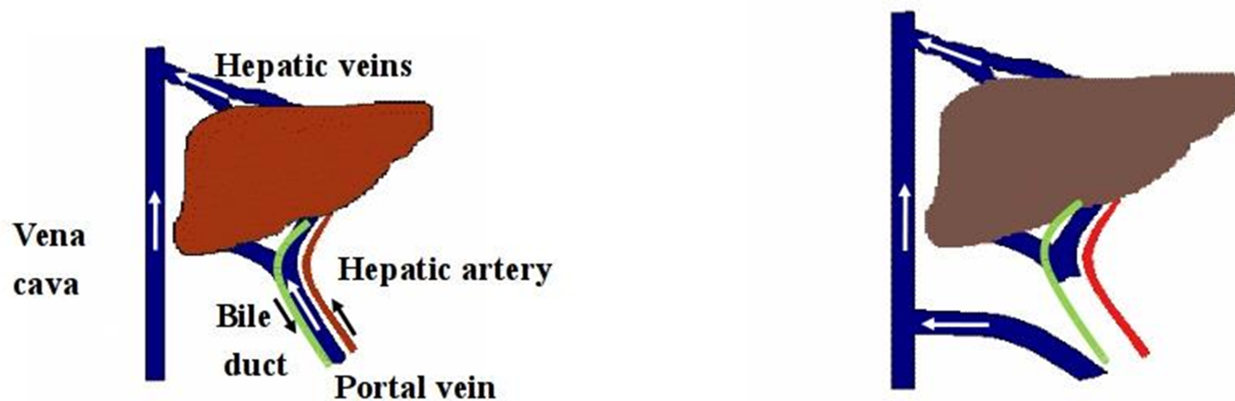

Figure 8. Portacaval anastomosis (PCA).

Type C hepatic encephalopathy models

The ideal animal model of type C HE should present all features of cirrhosis: liver function and histopathology alterations, jaundice, portal hypertension, portal-systemic shunting. The two most

frequent etiologies of cirrhosis are alcoholic and viral, however it is difficult to induce liver disease by administering these factors to animals. Nevertheless, manifestations and complications of cirrhosis arise independent of the etiology (Anand, 1999) and several toxin-induced and bile duct ligation (secondary biliary cirrhosis) are well characterized animal models.

Hepatic toxins are used to induce CLD in animal models. They act through several mechanisms but have a poor reproducibility as a major disadvantage. The most frequent used toxins are:

- *Carbon tetrachloride* is a toxin inducing hepatic necrosis through mechanisms which involve OS. Administered daily to naïve rats until the development of ascites (between 8 and 19 weeks), it reproduces the features of cirrhosis and increased ammonia levels (Miquel et al., 2010). However the difficulty of daily dose adjustment to body weight, the long duration of the model and a wide variability limit the utilization of this model (Butterworth et al., 2009);
- *Thioacetamide* is a thioamide used as a source of sulfide ion in chemistry. Administered for a period of 20 weeks in rats, it increases circulating liver function markers and it alters behavioral tests along with elevation of ammonia blood levels (Kawai et al., 2012);

Bile duct ligation (BDL, figure 9) in the rat or mouse also reproduces the features of cirrhosis. Moreover, the animals develop MHE: hyperammonemia, inflammation, OS, behavior alterations and edema are present (Jover et al., 2006). This model becomes an overt HE model following administration of different pathogenic factors such as ammonia or LPS (lipopolysaccharide, a bacterial toxin which induces inflammation) (Jover et al., 2006; Wright et al., 2007).

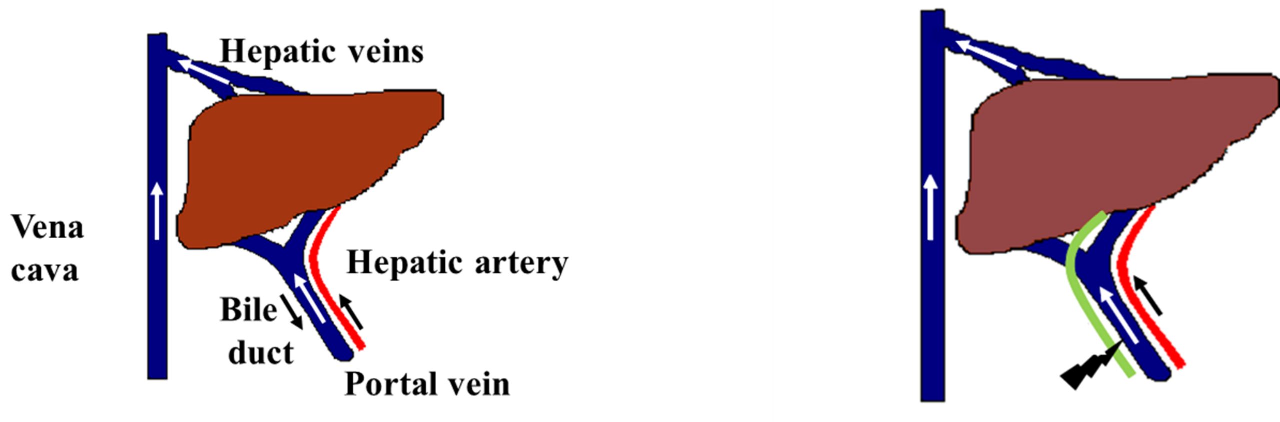

Figure 9. Bile-duct ligation (BDL).

At this moment, no experimental animal reproduces perfectly the HE pathology seen in humans. However, available models help understand the pathogenic mechanisms of HE, studying the relation between different factors and testing new therapeutic options.

1.4.1.2 Acute hepatic encephalopathy models (type A)

ALF is not the focus of this thesis. However, some of the mechanisms we investigated have been previously described in different ALF animal models. Therefore a short description of these models merits to be mentioned. Two main types of models are available for the study of HE during ALF: anhepatic and toxic models. Since several organs are affected by ALF, several parameters including temperature and glycaemia must be monitored and corrected because they may affect the time course of the model (Vaquero et al., 2005).

Anhepatic models

This category excludes the liver from circulation through surgery. The hepatic devascularization implies the derivation of the portal blood flow into the systemic circulation (portacaval anastomosis,

PCA) along with a ligation of the hepatic artery. For the hepatectomy model, the liver is completely removed. Those two models lead to cerebral edema followed by increased ICP (Potvin et al., 1984; Rose et al., 2000; Ytrebø et al., 2009). The hepatic devascularization model is characterized by ammonia levels similar as those present in humans with ALF (Kundra et al., 2005). Also, other factors such as inflammation or OS are present (Jiang et al., 2009a, 2009b).

Toxic models

Several hepato-toxins are used to induce ALF in animals similar to those used to induce CLD but at a much higher dose. As with CLD model, the poor reproducibility remains a major disadvantage. The most frequent toxins are:

- *Galactosamine* is a hexosamine which induces hepatic necrosis by directly altering the hepatocyte metabolism. In the rat, galactosamine induces the apparition of brain edema as well as a breakdown of the BBB (Dixit and Chang, 1990);
- *Acetaminophen* is toxin responsible for 30-50% of ALF cases. Acetaminophen is metabolized by the liver through glucuronidation (45-55%), sulfation (20–30%) or N-hydroxylation and dehydration, followed by GSH conjugation (less than 15%). In excess, these mechanisms are overcome and the hepatic cytochrome P450 enzyme system metabolizes it, forming NAPQI (N-acetyl-p-benzoquinone imine), compound which irreversibly inactivates glutathione (Moyer et al., 2011) leading to hepatic necrosis. In the rat, acetaminophen induces hepatic necrosis and cerebral edema, however, the model is rarely used due to a large variability of hepatic toxicity (Newsome et al., 2010);
- *Thioacetamide* in higher doses than those used to induce CLD, administered once, induces hepatic necrosis and brain edema in the rat (A. R. Jayakumar et al., 2014; Zimmermann et al., 1989);
- *Azoxymethane*`s mechanism of action is not fully understood, however it induces hepatic necrosis, hyperammonemia and cerebral edema (Bélanger et al., 2006; Chastre et al., 2012). This model has the best reproducibility among toxic models (Bélanger et al., 2006).

1.4.2 *In vitro* models

In vitro studies have multiple advantages: reproducibility, excellent control on the evaluated factors and low cost. Moreover, cell cultures allow the study of individual cerebral cells (astrocyte, neuron, microglia, endothelial cell) but also the interactions between cells by co-cultures. The effect of individual pathogenic factors or of their combinations may also be evaluated. For example, ammonia administration induces OS, cell swelling and energy metabolism alterations (Atanassov et al., 1995; Görg et al., 2008; Norenberg et al., 2005). A study on neuron-astrocyte co-cultures showed that the latter protects the first against ammonia's neurotoxic effects (Rao et al., 2005). Lactate administered to cultured astrocytes induces morphological changes and inflammation (Andersson et al., 2009, 2005). Inhibition of GS with its irreversible inhibitor MSO, results in attenuation of the increase in ammonia-induced glutamine and swelling in pre-treated cultured astrocytes (Norenberg and Bender, 1994).

The main disadvantage of *in* vitro models is the difficulty of reproducing the multitude of alterations seen during liver failure. Also, sometimes concentrations much higher than those seen *in vivo* are needed to induce a similar effect: often, the ammonia concentrations used *in vitro* reach 5 mM (see above studies), compared to a max of 1.5 mM seen in ALF models with severe HE.

1.5. Treatments of hepatic encephalopathy

Several therapeutic strategies are used for the treatment of HE, however all have shown inconclusive results, adverse effects or inconsistent beneficial reports. Moreover, all the up-to-date available treatments focus on ammonia: either decreasing its production or on increasing its removal (Rose, 2012). Today, lactulose, a nonabsorbable disaccharide, remains the primary treatment for HE. Liver transplantation is considered the only curative treatment of HE, however increasing number of cases demonstrating persisting neurological complications occurring after liver transplantation are

being reported (Chavarria and Cordoba, 2013). The poor neurological outcome following liver transplantation is believed to a result of a history of HE pre-liver transplantation. Therefore, HE treatment remains an unmet clinical need. The most frequent used therapies or those tested in animal models with a great potential in humans will be discussed below.

1.5.1 Protein restriction

Protein restriction used to be commonly advised to cirrhotic patients in order to prevent a rise in gut-derived blood ammonia. This is no longer recommended since cirrhotic patients already have a poor nutritional status and restricting proteins is more harmful than a normal protein diet (1.2–1.5 g protein/kg/day) (Bémeur et al., 2010). Malnutrition affects between 25 and 80% of cirrhotic patients and is associated with an increased prevalence of HE and poor prognosis.

1.5.2 Nonabsorbable disaccharides (lactulose)

Lactulose represents the first-line therapy for HE patients. It is a synthetic disaccharide composed of the monosaccharides fructose and galactose. It acts within the colon, where it is metabolized by colonic bacteria into acetic and lactic acid. Therefore, the intraluminal pH becomes acidic leading to the replacement of ammoniagenic with nonammoniagenic flora. Lactulose also decreases the absorption of ammonia through a cathartic effect, clearing it from the gut before it is systemically absorbed, resulting in increased fecal nitrogen excretion. In 2004, a Cochrane review on 22 clinical trials concluded that there was not enough convincing evidence of nonabsorbable disaccharides in the treatment of HE (Als-Nielsen et al., 2004a). However, since the Cochrane review, 2 large studies compared lactulose to placebo in cirrhotic patients and demonstrated that lactulose reduces circulating ammonia (Sharma, 2012) and improves cognitive function and quality of life in patients with cirrhosis and MHE (Prasad et al., 2007). Poor adherence to treatment and side effects such as abdominal cramping, bloating, nausea, vomiting, flatulence, abdominal distension and impairment of intestinal absorption, limit the use of lactulose in the treatment of HE.

1.5.3 Antibiotics

Oral antibiotics inhibit the ammoniagenic colonic bacteria, thus decreasing intestinal ammonia production. Traditionally, neomycin, metronidazole, and vancomycin were successfully used to lower blood ammonia in patients (Conn et al., 1977; Morgan et al., 1982; Tarao et al., 1990). However, their use was limited due to adverse effects secondary to their systemic absorption. Rifaximin is a recently approved antibiotic for the treatment of HE. It reduces the risk of recurrence of overt HE in patients with end-stage liver disease (Bass et al., 2010) and efficiently lowers ammonia (Mas et al., 2003). Rifaximin has the advantage of a poor systemic absorption (<0.4%) and has a broad spectrum of antibacterial activity, therefore has fewer adverse effects and a greater adherence to treatment. The only negative point of rifaximin remains the high cost.

1.5.4 Probiotics

Cirrhosis is characterized by changes in the gut microbiome resulting in a switch from normal flora (*Lachnospiraceae, Ruminococcaceae* and *Clostridiales*) to ammoniagenic bacteria (*Enterobacteriaceae* and *Streptococcaceae*) (Bajaj, 2014). Moreover, bacterial translocation occurs, process through bacteria and/or bacterial products (lipopolysaccharides, bacterial DNA, etc.) pass from the gut to mesenteric lymph nodes, ascites or into the hepato-splanchnic as well as systemic circulation.These changes are related to the severity of cirrhosis (Bajaj et al., 2014).

Probiotics are a mixture of live microorganisms used to replace the ammoniagenic with nonammoniagenic colonic flora. This leads to a decrease in ammonia, but also in endotoxins, which are harmful products for the liver. Although the exact underlying mecanisms are poorly explained, probiotics are beneficial in the treatment of cirrhosis as demonstrated by two studies where an improvement of cirrhosis (evaluated by the Child-Pugh score) was observed (Lata et al., 2007; Liu et al., 2004). Probiotics decrease ammonia (Malaguarnera et al., 2007), reduce circulating endotoxins (Bajaj et al., 2014), and are effective in preventing HE when administered long-term in cirrhotic patients (Lunia et al., 2014). In spite of these benefic effects, the mechanism of action of probiotics remains poorly understood.

Probiotics are well-tolerated, with no harmuful effects yet demonstrated and excellent patient adherence; however, their efficacy in altering clinically relevant outcomes remains inconclusive (McGee et al., 2011). Moreover, another recent study showed no difference between probiotics and lactulose in the secondary prophylaxis of HE in patients with cirrhosis (Agrawal et al., 2012). Concerns regarding variations of the live microorganisms and a lack of standardization among probiotics manufacturers, as well as establishing optimal doses and treatment duration remain issues requiring additional research.

1.5.5 Sodium benzoate and sodium phenylacetate/phenylbutyrate

These two substances have been used to treat hyperammonemia in children born with urea cycle disorders. Sodium benzoate conjugates with the amino acid glycine, inhibiting the metabolism of the latter which leads to generation of ammonia. Sodium benzoate has been shown to reduce ammonia in cirrhotic patients (Sushma et al., 1992). However, sodium benzoate can inhibit the production of urea, inducing hyperammonemia, therefore its efficacy increases when urea cycle is impaired (Maswoswe and Tremblay, 1989).

Sodium phenylbutyrate is oxidized into phenylacetate which conjugates with glutamine to form phenylacetylglutamine, which is excreted in the urine. Since cirrhotic patients often present with water retention and renal impairment, administration of sodium may contribute to their clinical deterioration. Therefore, sodium phenylbutyrate was replaced with glycerol phenylbutyrate (GPB), which showed similar safety and tolerability (McGuire et al., 2010). A recent study showed GPB to efficiently reduce HE episodes and ammonia levels in cirrhotic patients with previous HE episodes (Rockey et al., 2013). The effectiveness of sodium benzoate and sodium/glycerol phenylbutyrate/phenylacetate in the context of liver disease require further evaluation.

1.5.6 Benzodiazepine-like antagonists

Since cerebral endogenous benzodiazepines potentiate the inhibitory effect of GABA and contribute to the increased "GABAergic tone", their inhibition may have a beneficial effect in the treatment of HE. Flumazenil is a GABAA receptor antagonist, which reverses the effects of

endogenous benzodiazepines by competitive inhibition. In a Cochrane meta-analysis of thirteen trials including 805 cirrhotic patients, flumazenil had a beneficial effect in half of the patients on short-term improvement of HE, with no significant effect on recovery or survival (Als-Nielsen et al., 2004b). It has a short half-life and requires multiple doses and careful patient monitoring to prevent recurrence of overdose symptoms. Therefore, it remains the drug of choice in selected patients such as those where HE was triggered by the use of exogenous benzodiazepines or in patients with HE grade III – IV who do not respond to standard therapeutic measures (Romero-Gómez, 2010).

1.5.7 Branched-chain amino acids

Branched-chain amino acids (BCAA, valine, leucine, and isoleucine) are amino acids with an aliphatic side-chains branch. They have been reported to be decreased during CLD (Holecek, 2013). They may be involved in ammonia detoxification by forming glutamate through the enzyme branched-chain aminotransferase and then forming glutamine through GS. BCAA treatment has been shown to decrease ammonia levels in cirrhotic patients (Marchesini et al., 1990). Moreover, they help maintain the muscle mass by stimulating protein synthesis in the liver and attenuating the degradation of muscle protein. However, a study showed that BCAA treatment in patients with cirrhosis resulted in an increase of blood ammonia levels (Dam et al., 2011) most probably due to degradation of glutamine by GA which results in ammonia production. Although BCAA treatment is regarded as safe, noncompliance with long-term treatment has been reported due to its poor palatability and solubility, requiring consumption of large quantities of water. The efficacy of BCAA treatment remains therefore questionable.

1.5.8 L-ornithine L-aspartate

L-ornithine–L-aspartate (LOLA) are two amino acids that are substrates of the urea cycle, so they were first believed to lower blood ammonia by stimulating ureagenesis in the residual hepatocytes. However, LOLA has also been shown to stimulate GS in the muscle in PCA rats and form glutamine (Rose et al., 1998). The possibility of glutamine degradation back to ammonia questions the efficacy of this drug and explains why studies have demonstrated contradictory effects

on ammonia levels and clinical outcomes (Acharya et al., 2009a; Jiang et al., 2009c; Kircheis et al., 1997). Although it has a good tolerability and few side-effects, LOLA is currently not available for clinical use in North America.

1.5.9 L-ornithine phenylacetate

L-ornithine phenylacetate (OP) is a combination strategy. L-ornithine contributes to increasing glutamate in the muscle (by transamination of ornithine to glutamate) and increasing glutamine production through GS. The glutamine resulted from this reactions will be conjugated with phenylacetate and excreted by the kidneys instead of being reconverted to glutamate and ammonia. This product showed promising results in liver-devascularized pigs and BDL rats (Davies et al., 2009; Ytrebø et al., 2009). As a consequence, ongoing clinical trials are evaluating the safety and tolerability of OP treatment.

1.5.10 Liver transplantation

Liver transplantation (LT) remains the only curative treatment option for HE due to CLD. However, the limited number of organs and the difficulty of timing this intervention, restrain its benefit. Historically, HE has always been considered to be a reversible metabolic disorder and has therefore been expected to completely resolve following LT. However, even following a LT, persisting neurological complications remain a common problem affecting as many as 47% (8-47%) of liver transplant recipients (Amodio et al., 2007; Atluri et al., 2010; Mechtcheriakov et al., 2004; Sotil et al., 2009). Although these studies show an important role of pretransplant HE in persisting neurological symtpoms after LT, numerous other factors must be considered. First, an advanced age of the patient and the presence of comorbidities at time of transplantation may slow post LT recovery. A patient with multiple episodes of HE before LT or severe HE at the time of LT is more likely to have concomitant multiple complications such as renal failure at the time of LT, therefore a slower or poorer recovery after. These conditions present at time of LT may get aggravated during the surgery, which differs for each patient in terms of duration, degree of hypotension due to extracorporeal circulation or anesthesia effects. Moreover, after LT all patients have to take

immunosupressants, also known to have neurological side-effects including sleep troubles, confusion, weakness, depression, seizures and neuropathy. Depicting between the roles of each of these factors in persisting neurological complications after LT is challenging but merits to be thoroughly investigated. Consequently, these enduring neurological complications following LT (that are not defined as HE since the diseased liver has been replaced with a healthy liver) continue to weigh severely on the patients' quality of life, and lead to longer stays in the hospital, thus causing further financial burden on the health care system (Bajaj et al., 2011).

To resume, HE is common in clinical practice and the routinely administered treatment consists of lactulose, which is beneficial in the majority of patients. Patients with refractory or recurrent HE despite optimal lactulose treatment or those unable to tolerate it may benefit from rifaximin; however the expensive cost limits its use. LT is also a limited treatment option, by the number of available organs, moreover neurological symptoms seem to persist in a certain percentage of cases. Promising new molecules such as probiotics, OP or glycerol phenylbutyrate are still under investigation. In this context, treatment for HE associated to CLD remains an unmet clinical need and new therapeutic strategies targeting other factors than ammonia are worth to be investigated.

1.6. Hypothesis and objectives

HE is a metabolic neuropsychiatric syndrome which occurs as a complication of liver failure/disease. Patients with CLD present often with MHE characterized by subtle cognitive dysfunction leading to a decreased quality of life and impairment in daily activities. To date, the only effective treatment of CLD remains liver transplantation, a limited therapeutic option. The precise prevalence of MHE in CLD patients remains unknown, however a proper treatment would allow them to be functional and increase their quality of life. Brain edema is a feature often described in these patients.

Ammonia is known as an important factor the pathogenesis of the syndrome. Several evidences suggest a relationship of ammonia and oxidative stress as well as an important role of lactate and

inconsistent results regarding the role of glutamine in the pathogenesis of severe encephalopathy (intracranial hypertension) related to acute liver failure. In CLD, brain edema is a feature often described; however, the implications and the relationships between ammonia, oxidative stress, lactate and glutamine in CLD and MHE remain poorly unknown.

The main aim of this project is to advance the knowledge into understanding the mechanisms underlying the role of oxidative stress, lactate and glutamine in the pathogenesis of brain edema during minimal hepatic encephalopathy associated with chronic liver disease in order to undercover new therapeutic options. To address the roles of the mentioned factors, we assessed two different rat models: 1) bile-duct ligation (BDL), a hepatic encephalopathy model induced by secondary biliary cirrhosis; and 2) portacaval anastomosis (PCA), a hepatic encephalopathy model induced following a surgical portacaval shunt.

The specific objectives followed within this project were:

1. Determine the role of ammonia in the pathogenesis of brain edema in chronic liver disease.

2. Investigate the role of oxidative stress, depicting between its presence systemically and centrally, in the pathogenesis of brain edema in chronic liver disease.

3. Determine the relationship of ammonia and oxidative stress in the pathogenesis of brain edema.

4. Define the roles of lactate and glutamine in the pathogenesis of brain edema and their relationship with ammonia.

Chapter 2: Paper presentation

First paper: AST-120 (spherical carbon adsorbent) lowers ammonia levels and attenuates brain edema in bile duct–ligated rats

Aim of first publication

This paper aims to define the role of ammonia and its relationship with oxidative stress in the pathogenesis of brain edema in CLD. To attain this objective, BDL rats were treated with AST-120 (spherical carbon adsorbent), an oral adsorbent of engineered activated carbon microspheres with surface areas exceeding 1600m^2/g working as a sink for neuro- and hepato–toxins generated in the gut. The capacity of AST-120 to adsorb ammonia *in vitro* and to lower blood ammonia, OS and brain edema was evaluated. This treatment paradigm is also an excellent model to study the relationship between ammonia and OS in blood and in the brain.

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AST-120 (Spherical Carbon Adsorbent) Lowers Ammonia Levels and Attenuates Brain Edema in Bile Duct–Ligated Rats

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Authors contributions to the present publication

CRB contributed to the study design, performed the animal experiments, treatments and locomotor activity, the brain water content measurement and the spectrophotometric assays (ammonia and ROS), analyzed the data, and wrote the manuscript. CPR assisted to the animal experiments. KA provided the AST-120 and conceived the study. MT participated to the study design, data analysis and corrected the manuscript. CFR conceived the study, supervised the research group and approved the final manuscript.

Abstract

The pathogenesis of hepatic encephalopathy (HE) is multifactorial involving gut-derived toxins such as ammonia, which has demonstrated to induce oxidative stress. Therefore a primary HE treatment target is reducing ammonia production in the gastrointestinal tract. AST-120, an oral adsorbent of engineered activated carbon microspheres with surface areas exceeding 1600 m^2/g, acts as a sink for neuro- and hepato–toxins present in the gut. We evaluated the capacity of AST-120 to adsorb ammonia *in vitro* and to lower blood ammonia, oxidative stress and brain edema in cirrhotic rats. Cirrhosis was induced in rats by bile-duct ligation (BDL) for 6 weeks. AST-120 was administered by gavage preventively for 6 weeks (0.1, 1 and 4 g/kg/day). In addition, AST-120 was evaluated as a short-term treatment for 2 weeks and 3 days (1 g/kg/day) and as a sink to adsorb intravenously infused ammonium acetate. *In vitro*, AST-120 efficiently adsorbed ammonia. Ammonia levels significantly decreased in a dose-dependent manner for all AST-120 treated BDL rats (non-treated: 177.3 ± 30.8 µM; AST-120, 0.1 g/kg/day: 121.9 ± 13.8 µM; AST-120, 1 g/kg/day: 80.9 ± 30.0 µM; AST-120, 4 g/kg/day: 48.8 ± 19.6 µM) and significantly correlated with doses of AST-120 (r=-0.6603). Brain water content and locomotor activity normalized following AST-120 treatments, while arterial ROS levels remained unchanged. Furthermore, AST-120 significantly attenuated an increase in arterial ammonia following ammonium acetate administration (iv). **Conclusions**: AST-120 treatment decreased arterial ammonia levels, normalized brain water content and locomotor activity but did not demonstrate an effect on systemic oxidative stress. Also, AST-120 acts as an ammonia sink, efficiently removing blood-derived ammonia. Additional studies are warranted to evaluate the effects of AST-120 on HE in patients with advanced liver disease.

Introduction

Hepatic encephalopathy (HE) is a neuropsychiatric metabolic syndrome, a major complication of both acute and chronic liver disease. HE is characterized by cognitive, psychiatric and motor dysfunctions and can rapidly progress to hepatic coma and death. The prevalence of hepatic diseases is estimated at 5.5 million cases in US and as much as 80% of these patients will develop minimal HE (1). The pathogenesis of HE is multifactorial involving gut-derived toxins, the most important one being ammonia. During liver failure, gut-derived ammonia is not metabolized by the liver leading to hyperammonemia and consequently to neurotoxic ammonia levels in the brain (2,3). Ammonia neurotoxicity has been associated with a number of pathophysiological, biochemical and molecular changes in the brain, which consequentially lead to cerebral dysfunction (4). One important pathological change related to ammonia is represented by astrocyte swelling which leads to brain edema (5). Brain edema, a common finding in patients with acute liver failure (6) is also observed in patients with cirrhosis (7-9), as well as in animal models of chronic liver failure (10).

The correlation between ammonia and severity of HE in chronic liver failure remains however controversial (11-14) and other factors are believed to act synergistically with ammonia to induce brain edema (15). Recently it has been demonstrated that acute, high ammonia concentrations induce oxidative stress both *in vitro* (16) and *in vivo* (17), suggesting an important role for oxidative stress in the pathogenesis of HE (18). Systemic oxidative stress occurs during cirrhosis and represents a systemic phenomenon (19) however its relationship to HE or brain edema is unresolved. Reactive oxygen species (ROS) are believed to be produced at the intestinal level in relation to bacterial translocation and increased intestinal permeability (20), but also in the liver in relation to gut-derived toxins (21). ROS are known to be involved in blood-brain barrier dysfunction (22) which could enhance the neuropathological effects of ammonia. One of these effects could be brain edema since *in vitro*, astrocyte swelling is related to ammonia-induced oxidative stress (23).

Reducing ammonia levels remains a primary treatment strategy in patients with HE and gut-derived ammonia represents an obvious treatment target. AST-120 consists of engineered activated carbon microspheres (0.2 - 0.4 mm in diameter) with high non-specific adsorptive surface area

(>1600 m^2/g). It is not adsorbed or degraded in the gastrointestinal tract and provides sustained binding surface for low molecular weight compounds (<10 kDa) present in the bowel (24). AST-120 has proven to lower plasma ammonia levels in portacaval-shunted dogs (25) and attenuate oxidative stress in uremic rats (26). Also, preliminary studies in humans have showed neurocognitive improvements in patients with low-grade HE following AST-120 treatment (27).

The aim of this study was to evaluate the capacity of AST-120 to lower arterial ammonia (gut- and blood-derived) and oxidative stress and to investigate its effect on brain edema and locomotor activity in rats with cirrhosis induced by bile-duct ligation.

Materials and methods

In vitro adsorption of ammonia by AST-120

To determine the *in vitro* capacity of AST-120 to adsorb ammonia, 50 ml of two different ammonium chloride solutions (100 µM and 1 mM, pH = 5.5) were incubated at room temperature with 2 g of AST-120 for 1, 3 and 6 hours. Ammonia chloride solutions were incubated without AST-120 as controls. Samples were collected at each time point and ammonia concentration was measured. Results were expressed as percent of ammonia recovered in the solution after AST-120 adsorption.

Animal model

Secondary biliary cirrhosis was induced in male Sprague-Dawley rats (250 g) (Charles River, St. Constant, QC) following 6 weeks of bile-duct ligation (BDL). Prior to the double ligation and resection, formalin (1 µl/mg) was injected intracholedochal in order to prevent the dilatation of the ligated bile ducts (28). Control rats underwent a SHAM operation in which the bile duct was isolated without formalin injection, ligation or resection.

AST-120 treatment

AST-120 (Ocera Therapeutics, San Diego, CA) was administered by gavage every 12 hours, for a period of 6 weeks, beginning day 1 after surgery. SHAM and BDL rats (n=6/group) received AST-120 dissolved in methylcellulose (Sigma, St. Louis, MO) (10 ml/kg/gavage) at a dose of 0.1, 1 and 4 g/kg/day. Control SHAM and BDL rats (n=6/group) received equivalent volumes of methylcellulose. Animals were sacrificed after 6 weeks. In the second part of the study the effect of AST-120 administered as short-term treatment was evaluated. 1 g/kg/day of AST-120 was administered for 2 weeks (starting 4 weeks after surgery) and 3 days (starting day 39 after surgery) in both SHAM and BDL rats.

To determine the capacity of AST-120 to adsorb ammonia from the periphery (blood), non-treated and AST-120 treated BDL rats (1 g/kg/day for 2 weeks) were infused intravenously with ammonium acetate (55 µmol/kg/min) for 3 hours (29). Under isoflurane anesthesia, 22G catheters (Smiths Medical, United Kingdom) were placed in the aorta and vena cava and connected by PE60 tubing (Solomon Scientific, Plymouth Meeting, PA) to an infusion pump (CMA 100, Sweden). Rats were allowed to recover 30 min before starting the venous infusions. Aortic blood samples were collected at baseline and hourly to assess ammonia levels. Rats were sacrificed after 3 hours or at precoma stage (loss of righting reflex), if this occurred before 3 hours. All experiments were conducted following the Guidelines of Canadian Council on Animal Care and were approved by the Animal Protection Committee of CHUM Research Center.

Daily food/protein intake was monitored in BDL and SHAM-operated rats both non-treated and treated with AST-120 (1 g/kg/day) for 2 weeks; one week before starting the treatment and during the 2 weeks treatment. Mean daily food/protein intake/100 g body weight was calculated and expressed as % compared to non-treated SHAM-operated controls.

Plasma liver function markers

Plasmatic aspartate and alanine aminotransferase (AST; ALT), bilirubin, alkaline phosphatase (AP), γ-glutamyl transpeptidase (GGT) and albumin were measured using routine biochemistry techniques. Liver pathology was assessed as described in the supplemental information.

Ammonia

Ammonia levels were measured in arterial plasma using a commercial kit (Sigma, St. Louis, MO). Ammonia levels were assessed based on the reaction with α-ketoglutarate and reduced nicotinamide adenine dinucleotide phosphate (NADPH) in presence of L-glutamate dehydrogenase. Oxidation rate of NADPH was recorded by the absorbance decrease at 340 nm. Ammonia

concentration was calculated according to the manufacturer's protocol.

Reactive oxygen species (ROS)

ROS were assessed using the oxidation reaction of dichlorofluorescein diacetate (DCFDA; Invitrogen, Carlsbad, CA) to dichlorofluorescein (DCF) (30). 100 µM DCFDA was incubated 30 min in dark with hydroxylamine hydrochloride (40 mM) and hydrolysed to non-fluorescent DCF. DCF was incubated with arterial plasma and oxidation rate was recorded by changes in fluorescence over a 10 min period with a spectrofluorometer (BioTek, Winooski, VT) at 485 nm excitation and 520 nm emission wavelengths.

Brain water content

Brain water content was measured using the sensitive densitometry technique, as previously described (31). Briefly, after the animal was sacrificed frontal cortex was freshly dissected at 4°C and cut into 2 mm^3 pieces. Tissue pieces were placed in density gradient columns and equilibrium point was recorded after 2 min. Columns were made with different kerosene and bromobenzene mixtures and precalibrated with K_2SO_4 solutions of known densities. 8 samples measurements were averaged in each rat. Water content was calculated based on tissue density, according to the formula described by Marmarou *et al*.

Locomotor activity

Locomotor activity was assessed using an infrared beam computerized auto-track system (Columbus Instruments, Columbus, OH) (32). SHAM-operated controls, non-treated BDL and BDL treated with AST-120 (1 g/kg/day for 2 weeks) were individually placed in plexiglas cages (29 x 22 x 22 cm) for 6 h before beginning to record activity. Cumulative distance travelled during the night (active) and day (inactive) period was recorded for 24 h and expressed as night/day ratio.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Significance of difference was tested with Student t-test and ANOVA followed by Newman-Keuls post-test; correlation was calculated with Spearman test using GraphPad Prism 4 (La Jolla, CA). Probability values $p<0.05$ were considered to be statistically significant.

Results

***In vitro* adsorption of ammonia by AST-120**

AST-120 significantly adsorbed ammonia at a pH similar to the physiological pH of the human gastrointestinal tract (33). The adsorption of ammonia by AST-120 is rapid, ~ 60% occurring during the first incubation hour, for both 100 µM and 1 mM solutions. After 6 h of incubation, 2 g of AST-120 removed 93.5% (93.5 µM) and 77.2% (772.3 µM) of 100 µM and 1 mM ammonia solutions respectively (fig. 1).

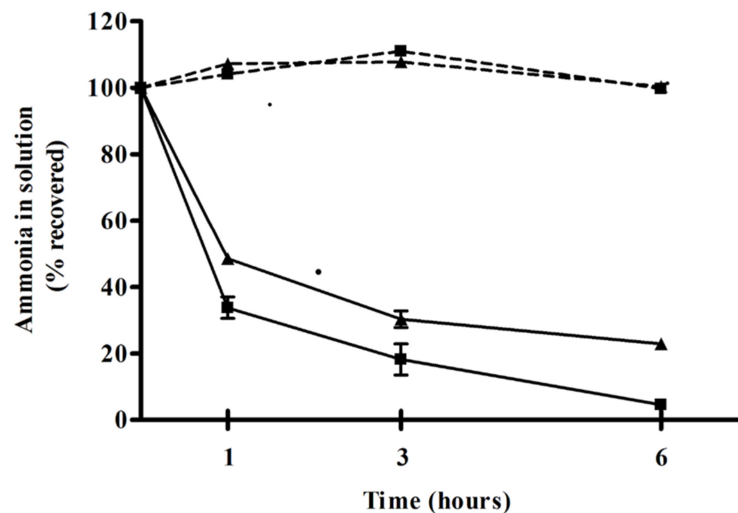


Figure 1. Ammonia recovered (%) in a volume of 50 ml of two different ammonium chloride solutions (100 µM and 1 mM) after 1, 3 and 6 hours of incubation *in vitro* with 2 g of AST-120 (—■— = ammonium chloride [100 µM] incubated with 2 g of AST-120 ; —▲— = ammonium chloride [1 mM] incubated with 2 g of AST-120; - -■- - = ammonium chloride [100 µM]; - -▲- - = ammonium chloride [1 mM])

***In vivo* effect of AST-120 on body weight and protein intake**

BDL rats gained significantly less weight than their respective SHAM-operated controls during the 6 week course of the model. No significant difference was found between all four groups of SHAM-operated rats or between all 4 groups of BDL rats (non-treated and AST-120 treated) (fig. 2A). As no significant differences were found between the 3 groups of AST-120 treated SHAM-operated rats, the data was pooled and presented as mean values in order to simplify the figures.

The amount of protein ingested was not significantly different between BDL and SHAM-operated rats before starting the treatment and neither group was affected by AST-120 (fig. 2B).

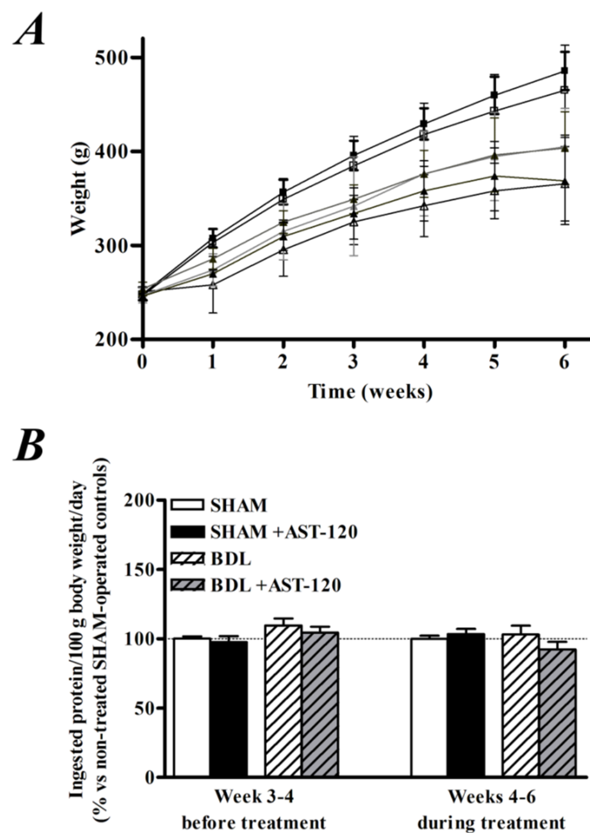


Figure 2. A) Weight curves of bile-duct ligated (BDL) and SHAM-operated rats under treatment with AST-120 0.1, 1 and 4 g/kg/day for 6 weeks. As no significant differences were found between the 3

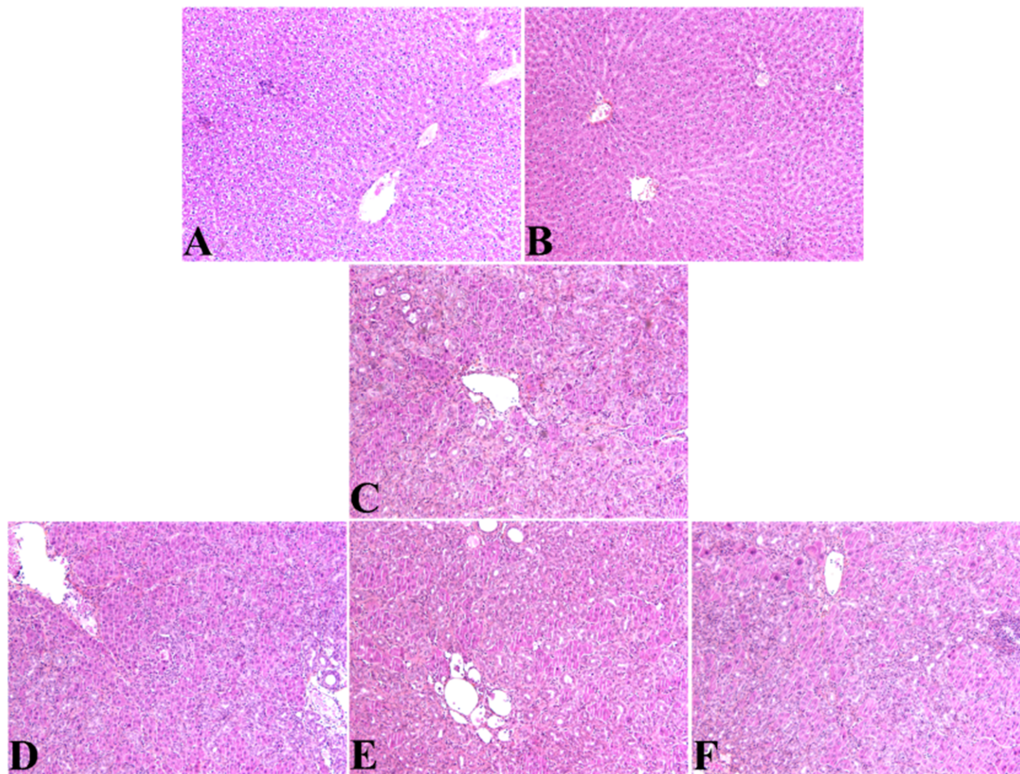
groups of AST-120 treated SHAM-operated rats, only the mean value for these groups is presented in order to simplify the figure (□ = SHAM; ■ = treated SHAM; Δ = non-treated BDL; ▲ = AST-120, 0.1 g/kg/day treated BDL; ▲ = AST-120, 1 g/kg/day treated BDL; ▲ = AST-120, 4 g/kg/day treated BDL). B) Daily protein intake/ 100 g body weight in bile-duct ligated (BDL) and SHAM-operated rats under treatment with AST-120 1 g/kg/day for 2 weeks (starting 4 weeks after surgery). Data are expressed as percent change relative to SHAM-operated controls one week before starting the treatment (week 3-4) and during the treatment period (weeks 4-6).

Liver biochemistry

At time of sacrifice, all BDL rats (non-treated and AST-120 treated) presented clinical signs of cirrhosis: jaundice, ascites and enlarged, nodular, discolored liver. Levels of plasma AST, ALT, bilirubin, AP and GGT were significantly increased in BDL groups compared to their corresponding SHAM-operated controls. In addition, albumin levels were significantly lower in all BDL rats compared to SHAM-operated controls (table I). Histopathological examination of liver sections revealed enlarged portal spaces with cholangiolar proliferation and hepatic necrosis in all BDL groups (suppl. fig. 1). AST-120 treatment did not significantly improve liver function markers or histopathological changes.

Table I. Liver biochemistry markers in bile-duct ligated and SHAM-operated rats under treatment with AST-120 0.1, 1 and 4 g/kg/day for 6 weeks. Data are expressed as mean ± SEM. BDL, bile-duct ligation; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase. *** $p<0.001$, significantly different from non-treated SHAM-operated controls. † $p<0.05$, ††† $p<0.001$, significantly different from corresponding treated SHAM-operated controls group. $ $p<0.05$, significantly different from non-treated BDL.

	AST (U/l)	ALT (U/l)	Bilirubin (μmol/dl)	AP (U/l)	GGT (U/l)	Albumin (g/l)
SHAM	72 ± 5	46 ± 3	6.8 ± 1.0	196 ± 12	2.1 ± 0.7	20.2 ± 0.5
treated SHAM	67 ± 3	48 ± 2	6.0 ± 0.4	251 ± 16	3.2 ± 0.4	20.4 ± 0.2
BDL	401 ± 35***	125 ± 30***	148.0 ± 13.7***	520 ± 65***	47.3 ± 8.2***	<10***
BDL 0.1 g/kg/day	417 ± 39†††	135 ± 24†††	138.3 ± 9.2†††	622 ± 57†††	61.8 ± 9.1†††	<10†††
BDL 1 g/kg/day	366 ± 46†††	80 ± 7$	155.3 ± 13.8†††	771 ± 69†††$	68.2 ± 5.8†††$	<10†††
BDL 4 g/kg/day	486 ± 67†††	100 ± 13†	183.4 ± 18.2†††$	730 ± 79†††$	58.9 ± 7.7†††	<10†††



Supplemental figure 1. Liver histopathology following treatment with AST-120, 0.1, 1 and 4 g/kg/day for 6 weeks in bile-duct ligated (BDL) and SHAM-operated rats. Representative liver sections of: A) non-treated SHAM operated controls and B) AST-120 treated SHAM-operated controls show normal liver architecture. Liver sections from: C) non-treated BDL rats and AST-120 D) 0.1, E) 1 and F) 4 g/kg/day show typical morphology of biliary cirrhosis: cholangiolar proliferation, hepatocyte loss, enlargement of portal spaces. No differences were noted between non-treated and treated BDL rats groups.

Ammonia

Arterial ammonia significantly increased in non-treated BDL rats (177.3 ± 30.8 μM vs SHAM: 66.5 ± 18.2 μM, $p < 0.01$). All doses of AST-120 significantly decreased ammonia in BDL rats to similar levels found in respective SHAM-operated controls: 0.1 g/kg/day: 121.9 ± 13.8 μM vs 81.1 ± 8.9 μM, $p > 0.05$; 1 g/kg/day: 80.9 ± 30.0 μM vs 72.2 ± 6.3 μM, $p > 0.05$; 4 g/kg/day: 48.8 ± 19.6 μM vs 53.8 ± 16.8 μM, $p > 0.05$. Furthermore, ammonia levels were significantly lower in treated BDL compared to non-treated BDL rats (fig. 3A). Ammonia levels significantly correlated

with dose of AST-120 (Spearman r = -0.6603; p = 0.0006) (fig. 3B).

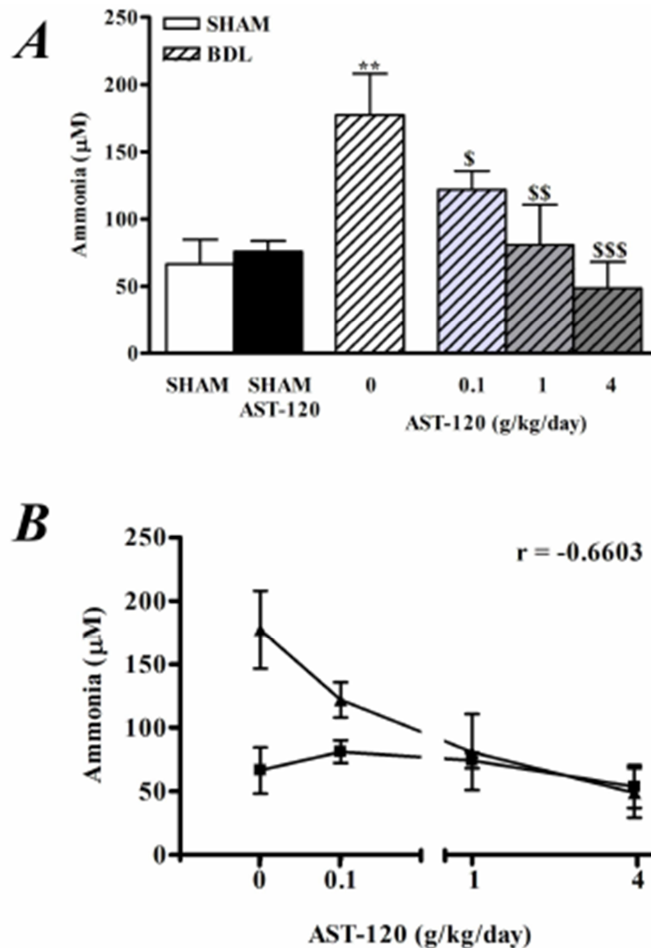


Figure 3. A) Arterial ammonia concentrations following treatment with AST-120, 0.1, 1 and 4 g/kg/day for 6 weeks in bile-duct ligated (BDL) and SHAM-operated rats. As no significant differences were found between the 3 groups of AST-120 treated SHAM-operated rats, only the mean value for these groups is presented in order to simplify the figure. Data are expressed as mean ± SEM. ** $p<0.01$, significantly different from non-treated SHAM-operated controls; $^{\$}$ $p<0.05$, $^{\$\$}$ $p<0.01$, $^{\$\$\$}$ $p<0.001$, significantly different from non-treated BDL. B) Correlation between ammonia levels and AST-120 dose (Spearman r = -0.6603; p = 0.0006, ■ = SHAM, ▲ = BDL).

ROS

Arterial ROS levels significantly increased in non-treated BDL compared to SHAM-operated rats ($p<0.01$). Following AST-120 treatment, ROS levels remained significantly higher compared to respective SHAM-operated controls and no significant change was observed between non-treated and AST-120 treated BDL rats (fig. 4).

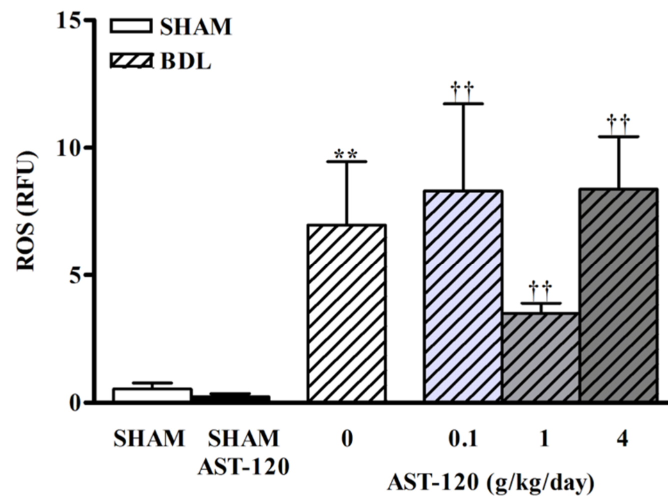


Figure 4. Arterial ROS concentrations following treatment with AST-120, 0.1, 1 and 4 g/kg/day for 6 weeks in bile-duct ligated (BDL) and SHAM-operated rats. As no significant differences were found between the 3 groups of AST-120 treated SHAM-operated rats, only the mean value for these groups is presented in order to simplify the figure. Data are expressed as mean ± SEM. ** $p<0.01$, significantly different from non-treated SHAM-operated controls; †† $p<0.01$, significantly different from corresponding treated SHAM-operated controls group.

Brain edema

Brain water content in frontal cortex was found to be significantly increased in non-treated BDL rats (79.39 ± 0.22 % vs non-treated SHAM: 78.60 ± 0.19 %, $p<0.05$) and in 0.1 g/kg/day AST-120 treated BDL rats (79.53 ± 0.30 % vs respectively treated SHAM: 78.76 ± 0.15 %, $p<0.05$). AST-120, at 1 and 4 g/kg/day, lowered brain water content in the frontal cortex to values similar to their respective SHAM-operated controls, although no difference was observed when compared to non-treated BDL (fig. 5A).

Brain water content significantly increased in non-treated BDL rats in cerebellum (78.89 ± 0.16 % vs non-treated SHAM: 77.96 ± 0.16 %, $p<0.01$) and brain stem (69.50 ± 0.25 % vs non-treated SHAM: 68.78 ± 0.41 %, $p<0.01$). All doses of AST-120 lowered brain water content in cerebellum and brain stem to values similar to their respective SHAM-operated controls. In cerebellum, no difference was found between AST-120 at 0.1 g/kg/day treated BDL rats vs non-treated BDL rats, while AST-120, at 1 and 4 g/kg/day, presented a significant reduction vs non-treated BDL ($p<0.05$). In brain stem, no difference was observed between AST-120 treated and non-treated BDL rats (fig. 5B and C).

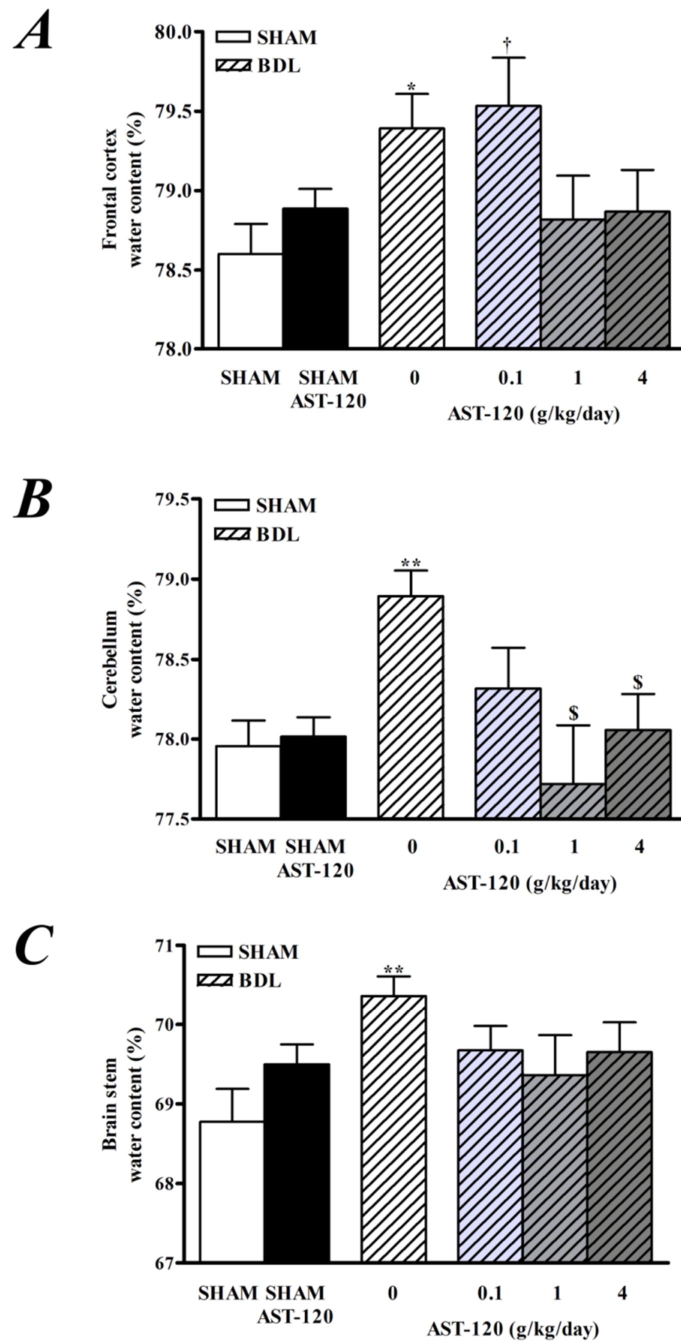


Figure 5. A) Frontal cortex B) cerebellum and C) brain stem water content following treatment with AST-120, 0.1, 1 and 4 g/kg/day for 6 weeks in bile-duct ligated (BDL) and SHAM-operated rats. As no significant differences were found between the 3 groups of AST-120 treated SHAM-operated rats, only the mean value for these groups is presented in order to simplify the figure. Data are expressed as

mean ± SEM. * $p<0.05$, ** $p<0.01$, significantly different from non-treated SHAM-operated controls; † $p<0.05$, significantly different from corresponding treated SHAM-operated controls group; $ $p<0.05$, significantly different from non-treated BDL.

Effect of AST-120 administered as short-term treatment

Since AST-120 administered preventively for 6 weeks lowered arterial ammonia levels and normalized brain water content, we investigated the effect of AST-120 when administered as short-term treatment. 1 g/kg/day of AST-120 was investigated as the treatment dose, since it was the lowest dosage which reduced both ammonia and brain edema following 6 weeks of treatment.

AST-120 (1 g/kg/day) administered for 2 weeks (week 4-6) and 3 days (days 39-42) significantly reduced arterial ammonia levels compared to non-treated BDL rats (non-treated BDL: 267% vs. AST-120 treated BDL: 108% (2 weeks) and 105% (3 days) relative to respective sham-operated controls). Brain water content decreased in all 3 regions (frontal cortex, cerebellum and brain stem) to similar levels as SHAM-operated controls (fig. 6A).

Locomotor activity

Locomotor activity in BDL rats was reduced compared to SHAM-operated controls but normalized following AST-120 treatment (1 g/kg/day for 2 weeks) (fig. 6B).

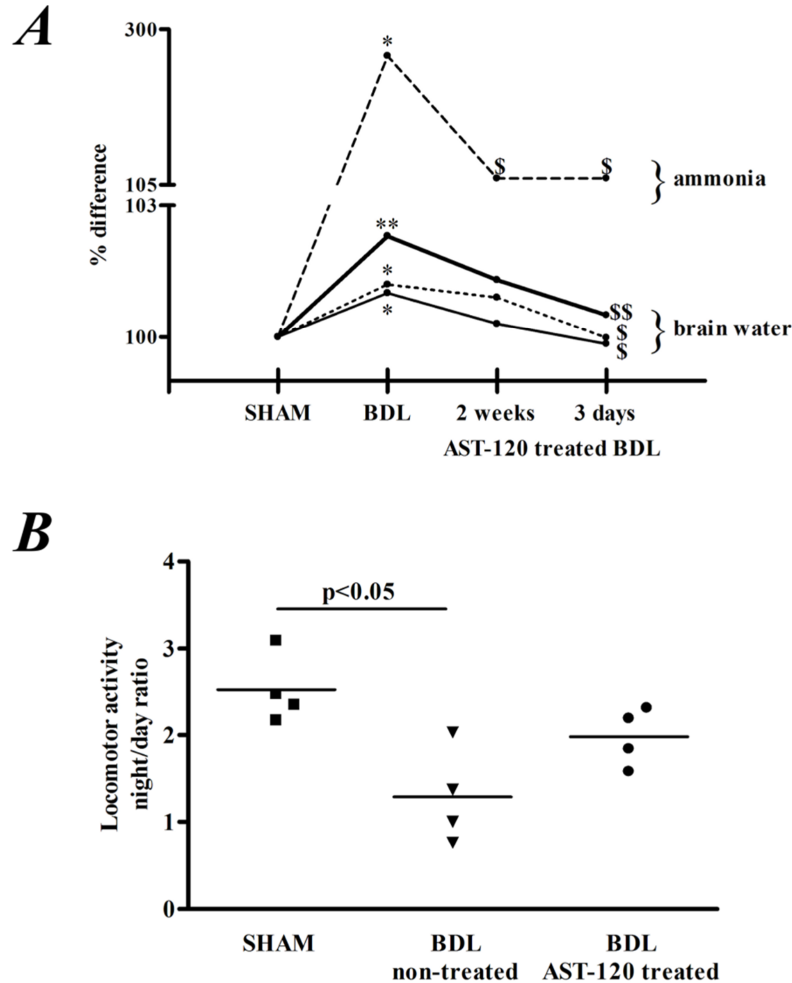


Figure 6. A) Arterial ammonia and frontal cortex, cerebellum and brain stem water content percent change following treatment with AST-120, 1 g/kg/day for 2 weeks and 3 days in bile-duct ligated (BDL) and SHAM-operated rats. Data are expressed as percent change relative to SHAM-operated controls. * $p<0.05$, ** $p<0.01$, significantly different from non-treated SHAM-operated controls; $\$$ $p<0.05$, significantly different from non-treated BDL; $\$\$$ $p<0.01$, significantly different from non-treated BDL (**- - -** Ammonia; —— Frontal cortex water content; ······ Cerebellum water content; **━━** Brain stem water content). B) Locomotor activity following treatment with AST-120, 1 g/kg/day for 2 weeks in bile-duct ligated (BDL) and SHAM-operated rats. Data are expressed as night/day ratio of cumulative distance travelled recorded over 12 hours active period (night) and 12 hours inactive period (day).

Adsorption of blood ammonia by AST-120

In order to determine AST-120's capability to remove ammonia from the blood, both non-treated and AST-120 treated BDL rats were infused (i.v) with ammonium acetate. In non-treated BDL rats, ammonium acetate infusions (55µmol/kg/min) lead to rapid increase in blood ammonia and consequently a fast deterioration in neurological status (80% progressed to precoma during the 3 hour observation period). Whereas AST-120 significantly attenuated an increase in blood ammonia and furthermore, only 14% of AST-120 treated BDL rats progressed to precoma (fig. 7).

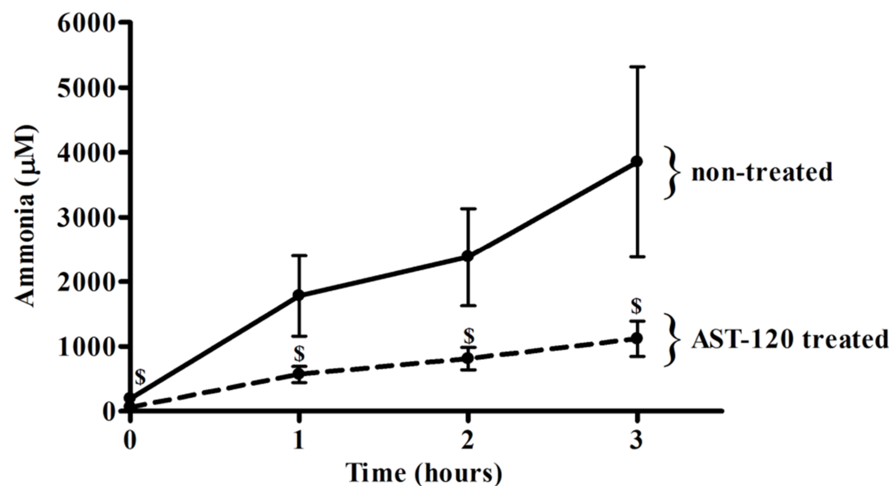


Figure 7. Time course of arterial ammonia concentrations following ammonium acetate intravenous infusions in non-treated and AST-120, 1 g/kg/day for 2 weeks treated bile-duct ligated (BDL) rats. Data are expressed as mean ± SEM. $^{\$}$ $p<0.05$, significantly different from non-treated BDL. (── non-treated BDL rats; **- - -** AST-120 treated BDL rats).

Discussion

Results of the present study demonstrate that AST-120 treatment (both as a preventive and short-term therapy) lowers arterial ammonia levels, attenuates brain edema and improves locomotor activity. No significant effect on circulating ROS was noted. These results demonstrate the important role of ammonia in the pathogenesis of brain edema. Furthermore, it suggests systemic oxidative stress independently does not lead to brain edema and is not induced by hyperammonemia. We also demonstrate that AST-120 has the capability of lowering ammonia by adsorbing both gut- and blood-derived ammonia.

Prior to testing AST-120 *in vivo*, we tested its capacity to adsorb ammonia *in vitro*. AST-120 significantly adsorbed ammonia from of 100 µM and 1 mM solutions, which represent the range reported in humans with HE (11), as well as in animal models of HE (28). *In vitro*, ammonia adsorption occurred rapidly and efficiently, providing a good rationale to test AST-120 as an ammonia-lowering treatment *in vivo*.

The different treatment periods were chosen accordingly to different pathological aspects in the time course of HE. In cirrhotic patients a preventive treatment would be useful for reducing the onset of minimal HE or the number of episodes of overt HE, while short-term treatment would be useful in treating episodes of HE (see nomenclature of HE (34)). In BDL rats, 4 weeks after surgery, blood ammonia levels are increased, but brain edema is not present (35); brain edema is observed only 5 weeks after BDL (10). We began AST-120 treatment: 1) immediately after surgery (for 6 weeks) to investigate the preventive effect of a long-term treatment on ammonia and brain edema; 2) 4 weeks after surgery (2 weeks treatment) to investigate the capacity of lowering ammonia levels before the apparition of brain edema; and 3) 39 days after surgery (3 days treatment) to investigate the short-term treatment capacity to lower ammonia and normalize brain water. Our data suggests AST-120 can be used to prevent an increase in blood ammonia as well treat hyperammonemia.

In BDL rats, AST-120 treatment was well tolerated; no adverse effects were observed; weight curves and daily protein intake were not altered. AST-120 treatment had no effect on liver necrosis and cholestasis markers, however ammonia levels significantly decreased in a dose-response manner.

Ammonia, as a gas (NH_3) and ion (NH_4^+), can easily diffuse and be transported across plasma membranes (4). To better understand the mechanism of action of AST-120 we investigated its capability to remove blood-derived ammonia and found AST-120 significantly prevented an increase in blood ammonia following intravenous ammonium acetate infusion (i.v). This suggests that: 1) ammonia diffuses not only from the gut to the portal system, but also from systemic circulation to the gut and 2) AST-120 in the gut acts as an ammonia sink, significantly clearing blood-derived ammonia.

To date, only acute, high ammonia concentrations have been proven to induce oxidative stress both *in vitro* (5 mM applied to the astrocytes in culture (16)) and *in vivo* (12 mmol/kg administered intraperitoneally in rat (17)). Interestingly, in the current study, lowering ammonia levels did not lead to a reduction in ROS, suggesting oxidative stress persists in the absence of hyperammonemia.

Previous studies demonstrated that AST-120 attenuated oxidative stress in rats with chronic kidney disease, by adsorbing uremic intestinal toxins (26). The current data suggest systemic oxidative stress in BDL rats is not directly related to gut-derived toxins such as ammonia and is likely related to a result of the primary liver injury (36).

Reduction of ammonia levels following AST-120 treatment leads to a normalization of brain water content. This data sustains the important role of ammonia in the pathogenesis of brain edema in chronic liver failure but does not exclude that oxidative stress could exacerbate ammonia effect on HE (18). The fact that AST-120 at 0.1 g/kg/day decreased water content only in cerebellum and brain stem and not in frontal cortex, despite of a significant decrease in ammonia levels sustains previous findings that white matter is more amenable to therapy than grey matter (37).

The protective effect of AST-120 on brain edema is the result of ammonia adsorption. This study confirms ammonia remains an important factor in the pathogenesis of HE, however the relation between ammonia, oxidative stress and brain edema in chronic liver failure requires further investigation.

Traditional HE treatments such as non-absorbable disaccharides and protein restriction are

limited due to the high frequency of adverse reactions (38). New treatments are required and gut-targeted ammonia therapies are warranted. The main mechanisms for eradicating ammonia include sterilizing the gut (antibiotic therapy), acidifying and flushing the gut (lactulose/lactilol), or selectively binding local toxins (AST-120). Thus far, some promising new therapies are being advanced in the clinic: OCR-002 (ornithine-phenylacetate) reduces systemic ammonia levels and brain edema in cirrhotic rats (10) and rifaximin (non-absorbable antibiotic) maintains remission and reduces risk of hospitalisation from HE (39). In the current study, AST-120 significantly lowered ammonia and protected against the development of brain edema in rats with chronic liver failure. In this context, further studies in humans are warranted to evaluate utility of AST-120 as a gut ammonia sequestering agent and its potential benefit in the management of HE.

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Second paper: Systemic oxidative stress is implicated in the pathogenesis of brain edema in rats with chronic liver failure

Aim of second publication

The previous paper demonstrated that lowering arterial ammonia levels following AST-120 treatment attenuates brain edema and improves locomotor activity without having a significant effect on systemic OS. This suggests systemic OS independently does not lead to brain edema and is not induced by hyperammonemia.

As a continuation, we aimed to specifically define the role of oxidative stress in the pathogenesis of brain edema, its relationship with ammonia as well as the effect of antioxidant treatment. To attain this objective, PCA and BDL rats were used to assess ammonia, OS markers both systemically and centrally as well as brain edema. Allopurinol (xanthine oxidase inhibitor) was administered in BDL rats in order to evaluate the impact of systemic OS on the development of brain edema.

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Systemic oxidative stress is implicated in the pathogenesis of brain edema in rats with chronic liver failure

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Authors contributions to the present publication

CRB contributed to the study design, performed the animal experiments and treatments, the brain water content measurement and the oxidative stress markers assays, analyzed the data, and wrote the manuscript. XY performed part of the oxidative stress markers assays. JH performed the BBB extravasation experiments. CPR and WJ assisted to the animal experiments. MT participated to the study design, data analysis and corrected the manuscript. CFR conceived the study, supervised the research group and approved the final manuscript.

Abstract

Background & aims: Chronic liver failure leads to hyperammonemia, a central component in the pathogenesis of hepatic encephalopathy (HE), however a correlation between blood ammonia levels and HE severity remains controversial. It is believed oxidative stress plays a role in modulating the effects of hyperammonemia. This study aimed to determine the relationship between chronic hyperammonemia, oxidative stress and brain edema (BE) in two rat models of HE: portacaval anastomosis (PCA) and bile-duct ligation (BDL). **Methods:** Ammonia and reactive oxygen species (ROS) levels, BE, oxidant and antioxidant enzyme activities as well as lipid peroxidation were assessed both systemically and centrally in these 2 different animal models. Then, effect of allopurinol (xanthine oxidase inhibitor, 100 mg/kg for 10 days) on ROS and BE and temporal resolution of ammonia, ROS and BE were evaluated only in BDL rats. **Results:** Similar arterial and cerebrospinal fluid ammonia levels were found in PCA and BDL rats, both significantly higher compared to their respective SHAM-operated controls ($p<0.05$). BE was detected in BDL rats ($p<0.05$) but not in PCA rats. Evidence of oxidative stress was found systemically but not centrally in BDL rats: increased levels of ROS, increased activity of xanthine oxidase (oxidant enzyme), enhanced oxidative modifications on lipids and proteins as well as decreased antioxidant defence. In PCA rats, a preserved oxidant/antioxidant balance was demonstrated. Treatment with allopurinol in BDL rats attenuated both ROS and BE suggesting systemic oxidative stress is implicated in the pathogenesis of BE. Analysis of ROS and ammonia temporal resolution in plasma of BDL rats suggests systemic oxidative stress might be an important “first hit”, which followed by increases in ammonia, leads to BE in chronic liver failure. In **conclusion**, chronic hyperammonemia and oxidative stress in combination lead to the onset of BE in rats with chronic liver failure.

Key Words: hepatic encephalopathy, hyperammonemia, bile-duct ligation, portacaval anastomosis, allopurinol

Introduction

Hepatic encephalopathy (HE) is a metabolic neuropsychiatric syndrome, a major complication of liver disease. Minimal hepatic encephalopathy (mHE), the mildest form of HE, is present in up to 80% of patients with chronic liver failure (CLF) [1]. Patients with mHE have no recognizable clinical symptoms of HE but show mild cognitive and psychomotor dysfunctions diagnosed with sensitive neuropsychological and neurophysiological tests [2–4]. mHE negatively impacts on the patients' quality of life and places these patients at a higher (4 fold) risk of developing severe HE [1,2,5]. Newly advanced highly sensitive imaging techniques have demonstrated brain edema is a major pathological feature present in patients with CLF and mHE [6–8]. Although these studies established a link between brain edema and alterations in cognitive function, the relationship between brain edema and the pathogenic factors of HE is still poorly described.

Ammonia is considered the most important factor in the pathogenesis of HE given that hyperammonemia consequently leads to toxic levels of ammonia in the brain [9,10]. Nevertheless whether a correlation exists between hyperammonemia and HE severity in CLF still remains controversial [11–15]. A recent study demonstrated ammonia administrated intravenously to healthy volunteers did not cause any significant neuropsychological impairment [16], supporting the premise that other pathogenic factors, besides ammonia, are involved in the pathogenesis of brain edema and HE in CLF.

Oxidative stress is believed to play a role in the pathogenesis of HE since acute doses of ammonia lead to the induction of oxidative stress [17]. For example, cultured astrocytes acutely exposed to 5 mM ammonia, show an increase in reactive oxygen species (ROS) and cell swelling, which are both prevented following antioxidant treatments [18,19]. To date, the only *in vivo* evidence of ammonia-induced oxidative stress in the brain has been reported in

animal models of acute ammonia intoxication [20–22], however the link between chronic hyperammonemia, oxidative stress (central and systemic) and brain edema in CLF remains undefined.

The aim of the present study was to determine the relationship between blood and brain ammonia and oxidative stress in the pathogenesis of brain edema associated with CLF. In order to clearly understand these relationships, two well characterized rat models of chronic hyperammonemia and mHE [23] were used: 1. portacaval anastomosis (PCA) – a HE type B model and 2. bile-duct ligation (BDL) – a type C HE model. The fact that brain edema is present in BDL rats [24] and not in PCA rats [25] sets up an excellent experimental paradigm to study the pathogenesis of brain edema in the context of chronic hyperammonemia.

Materials and methods

Animal models

Four groups of male Sprague-Dawley rats (250-275 g, n=6/group) (Charles River, St-Constant, QC) were included in the first part of this study. Group 1: Type B model of HE: rats with end-to-side portacaval anastomosis (PCA) [26]. Group 2: SHAM-operated control rats (PCA-SHAM). Groups 1 and 2 were sacrificed 4 weeks after surgery. Group 3: Type C model of HE: rats with secondary billiary cirrhosis induced by bile-duct ligation (BDL) [24]. Group 4: SHAM-operated control rats (BDL-SHAM). Groups 3 and 4 were studied 6 weeks after the intervention. For the second part of the study, separate groups of BDL-SHAM and BDL rats either: 1) received allopurinol (Cayman chemical company, Ann Arbor, MI) (100 mg/kg intraperitoneally for 10 days, starting 32 days after the intervention [27]); or 2) were studied at earlier time points (2 and 4 weeks after intervention). Locomotor activity was assessed in SHAM-operated controls, non-treated and allopurinol treated BDL rats using an infrared beam computerized auto-track system (Columbus Instruments, Columbus, OH) [24]. Rats were individually placed in plexiglass cages (29 x 22 x 22 cm) for 6 h before beginning to record activity to accommodate to the environment. Cumulative distance travelled was recorded for 12 h during the night (active period).

Experiments were conducted following the Guidelines of Canadian Council on Animal Care and were approved by the Animal Protection Committee of CHUM Research Center.

Cerebrospinal fluid sampling

Cerebrospinal fluid (CSF) was collected from cisterna magna. The rats were anesthetised with isoflurane and the skull was immobilized in a stereotaxic apparatus. An incision was made on the back of the head and the occipital bone was exposed. A dental burr was used to drill a hole on the sagittal midline rostral to the interparietal-occipital bone suture. A PE-10

tubing catheter was inserted into the cisterna magna through the dura mater. CSF was collected and immediately frozen in deeply cooled isopentane. Immediately following this procedure, arterial plasma and brain samples were collected and instantly frozen at -80°C.

Liver biochemistry

Plasma aspartate and alanine aminotransferase, bilirubin, alkaline phosphatase, γ-glutamyl transpeptidase and albumin were measured using routine biochemistry techniques.

Ammonia

Ammonia levels were measured in plasma and CSF using a commercial kit (Sigma, St-Louis, MO) according to the manufacturer's protocol.

Brain water content

Brain water content was measured using the sensitive densitometry technique. Frontal cortex was freshly dissected at 4 °C and cut into 2 mm^3 pieces. Tissue pieces were placed in density gradient columns and equilibrium point was recorded after 2 min. Columns were made with different kerosene and bromobenzene mixtures and precalibrated with K_2SO_4 solutions of known densities. At least 8 samples were measured in each rat. Water content was calculated based on tissue density, according to the formula described by Marmarou *et al*. [28].

Reactive oxygen species

ROS were quantified as previously described [24] following the oxidation of dichlorofluorescein diacetate (DCFDA; Invitrogen, Carlsbad, CA) to dichlorofluorescein

(DCF). 100 µM DCFDA was incubated 30 min in the dark with hydroxylamine hydrochloride (40 mM) and hydrolysed to non-fluorescent DCF. DCF was incubated with plasma and CSF samples and oxidation rate was recorded by changes in fluorescence over a 10 min period with a spectrofluorometer (BioTek, Winooski, VT) at 485 nm excitation and 520 nm emission wavelengths.

Hydrogen peroxide (H_2O_2): AmplexRed (10-acetyl-3,7-dihidroxyphenoxazine) reacts with H_2O_2 and releases resorufin, a fluorescent oxidation product. Plasma samples were incubated with a mixture of 50 µM Amplex Red (Invitrogen) and 0.1 U/ml horseradish peroxidase (HRP, Sigma) in 50 mM sodium phosphate buffer pH 7.4 for 30 min in dark. Fluorescence was read at 530 nm excitation and 590 nm emission wavelengths. H_2O_2 levels were calculated based on a standard curve of known H_2O_2 concentrations.

Tissue preparation

Frontal cortex was dissected and homogenized in lysis buffer (50 mM Tris pH 7.5, 1 mM EDTA, 1/500 cold Protease Inhibitor Cocktail (Roche, Indianapolis, IN). Homogenates were centrifuged at 30,000 g for 40 min at 4°C. The supernatant was used as the brain cytosolic fraction and the precipitate was resuspended in lysis buffer and used as the brain membrane fraction. Protein content was determined according to the method of Lowry et al. [29].

Oxidants and antioxidants

Xanthine oxidase (XO), amine oxidase (semicarbazide-sensitive amine oxidase (SSAO), brain monoamine oxidase (MAO)) and catalase (CAT) activities were determined by measuring produced or consumed H_2O_2, which reacts with Amplex Red and produces fluorescent resorufin, as described below.

XO: Plasma and brain samples were incubated with a mixture of 50 µM Amplex Red, 0.2 U/ml HRP and 25 µM xanthine in 100 mM Tris pH 7.5 for 45 min in the dark. Following xanthine oxidation H_2O_2 is produced, which reacts with Amplex Red and produces fluorescent resorufin. A control for each sample was performed without adding xanthine to the reaction mix to correct for background H_2O_2. XO activity was calculated based on a standard curve of known XO concentrations.

SSAO and MAO: To determine activity of SSAO, plasma samples were incubated with a mixture of 200 µM Amplex Red, 1 U/ml HRP and 2 mM benzylamine in 100 mM Tris pH 7.5 for 30 min in dark. To determine activity of MAO, brain membrane fractions were incubated with a mixture of 200 µM Amplex Red, 1 U/ml HRP and 2 mM benzylamine (MAO-B) or tyramine (MAO-A and MAO-B) in 100 mM Tris pH 7.5 for 30 min in dark. Oxidation of benzylamine and tyramine leads to H_2O_2 production, which further reacts with Amplex Red and forms fluorescent resorufin. A control for each sample was performed without adding benzylamine, respectively tyramine to the reaction mix to correct for background H_2O_2. 1 µU of SSAO/MAO was calculated as the quantity of enzyme that produced 1 µM of resorufin based on a resorufin standard curve.

CAT: Samples were incubated with 40 µM H_2O_2 for 30 min in the dark. Then, a mixture of 50 µM Amplex Red and 0.2 U/ml HRP in 100 mM Tris pH 7.5 was added and incubated for 30 min in the dark. CAT activity was determined by measuring the non-consumed H_2O_2 based on the reaction with Amplex Red. A control for each sample was performed by incubating samples with buffer instead of H_2O_2 to correct for background H_2O_2. CAT activity was calculated based on a standard curve of known CAT concentrations.

Superoxide dismutase (SOD) activity was assessed using a commercial kit (Biovision, Mountain View, CA), according to the manufacturer’s protocol.

Glutathione reductase (GR) activity was assessed as previously described [30], based on the reduction of oxidized glutathione (GSSG) to glutathione (GSH). Samples were added to a

mixture of 100 mM phosphate buffer pH 7.5 and 1 mM GSSG. Reaction was started by addition of 0.1 mM nicotinamide adenine dinucleotide phosphate (NADPH). The oxidation rate of NADPH was followed at 340 nm for 5 min. Controls were performed to correct for non-specific NADPH oxidation. 1 mU of GR was calculated as the quantity of enzyme that reduced 1 mM of GSSG per minute, reaction which induced the oxidation of 1 mM of NADPH.

Glutathione peroxidase (GP) activity was determined based on the reduction of GSH to GSSG followed by the recycling of GSSG back to GSH [31]. Samples were added to a mixture of 100 mM phosphate buffer pH 7.5, 0.2 U/ml GR, 2.1 mM GSH and 0.25 mM NADPH. Reaction was started by adding 0.3 mM tert-butyl hydroxyperoxide and NADPH oxidation rate was followed at 340 nm for 5 min. 1 mU of GP was calculated as the quantity of enzyme that oxidized 2 mM of GSH per minute, reaction followed by the reduction of 1 mM of GSSG accompanied by the oxidation of 1 mM of NADPH.

Reduced glutathione/oxidized glutathione ratio (GSH/GSSG): GSH and GSSG were measured by a commercial kit (Oxford Biomedical Research, Rochester Hills, MI), according to the manufacturer's protocol and expressed as % vs SHAM.

Oxidative stress effects on lipids

Lipid peroxidation was assessed by measuring TBARS, such as malondialdehyde (MDA), which are end-products of cell membrane lipid peroxidation and are considered reliable markers of oxidative stress. They were determined by the measurement of chromogen obtained from the reaction of TBARS with 2-thiobarbituric acid using TBARS assay kit (Cayman Chemical Company) in plasma samples. Lipid peroxidation in brain tissue was assessed using Bioxytech LPO-586 assay kit (Oxis International, Foster City, CA) according to the manufacturer's protocol. In both kits, MDA was used as a standard and therefore calculated as μM of MDA.

Blood-brain barrier extravasation

Evans Blue and sodium fluorescein (NaF) were used to assess blood-brain barrier (BBB) extravasation according to Kaya et al. [32]. PCA and PCA-SHAM as well as BDL and BDL-SHAM were anesthetised with isoflurane and injected with 2% Evans Blue (4 ml/kg) and 10% NaF (1 ml/kg) in the caudal vein [33]. 30 min after, rats were perfused with saline, the brain was removed and left frontal cortex was dissected on ice. Tissues were homogenized in phosphate buffered saline (PBS, 13.7 mM NaCl, 0.27 mM KCl, 1 mM Na_2HPO_4, 0.2 mM KH_2PO_4) and then mixed 1:1 with 60% trichloroacetic acid. Samples were vortexed, cooled on ice for 30 min and centrifuged at 10,000 g for 5 min. Evans Blue extravasation in frontal cortex was determined spectrophotometrically (610 nm) and expressed in ng/mg tissue. NaF extravasation was determined fluorometrically (at 485 nm excitation and 528 nm emission wavelenghts). In order to validate the technique, a toxic dose of mannitol was used to open the BBB (positive control, [34]): naïve rats were injected within 5 min with 25% mannitol (5 ml/kg) in the left carotid artery. 5 min after, Evans Blue and NaF were injected over 5 min (5 ml/kg) and measured as described above.

Statistical analysis

Data are expressed as mean±standard error of the mean (SEM). Significance of difference was tested with ANOVA followed by Newman-Keuls post-test using GraphPad Prism4 (La Jolla, CA). Probability values $p<0.05$ were considered to be statistically significant.

Results

Liver biochemistry

As expected, at time of sacrifice, BDL rats presented clinical signs of cirrhosis: jaundice, ascites, and enlarged, nodular, discoloured liver. These signs were absent in PCA rats, which only developed liver atrophy (decreased liver/body weight). Plasma levels of AST, ALT, bilirubin, AP and GGT levels were significantly increased in BDL rats compared to BDL-SHAM. Albumin levels were significantly lower in BDL rats compared to BDL-SHAM. Liver markers remained unchanged in PCA vs PCA-SHAM (table I).

Table I. Liver/body weight and biochemistry markers .PCA, portacaval anastomosis; BDL, bile-duct ligation; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase; n.d., not detected, below the detection limit of the method. Data are expressed as mean±SEM. $^{**}p<0.01$, $^{***}p<0.001$, significantly different from SHAM. $^{\#}p<0.05$, $^{\#\#\#}p<0.001$, significantly different from PCA.

	PCA-SHAM	PCA	BDL-SHAM	BDL
Liver/body weight (%)	3.84 ± 0.06	$2.59 \pm 0.09^{***}$	4.25 ± 0.39	$6.43 \pm 0.24^{***\#\#\#}$
AST (U/l)	68 ± 5	84 ± 3	65 ± 4	$355 \pm 76^{***\#\#\#}$
ALT (U/l)	46 ± 2	53 ± 3	44 ± 4	$72 \pm 8^{**\#}$
Bilirubin (µmol/dl)	7.6 ± 0.8	6.8 ± 0.4	7.1 ± 1.1	$156.0 \pm 17.7^{***\#\#\#}$
AP (U/l)	310 ± 30	360 ± 53	257 ± 22	493 ± 101
GGT (U/l)	2.3 ± 0.9	3.5 ± 0.7	3.2 ± 0.8	$54.5 \pm 10.1^{***\#\#\#}$
Albumin (g/l)	22.7 ± 0.8	21.8 ± 0.5	22.0 ± 0.8	n.d.

Ammonia

Arterial ammonia (fig. 1A) significantly increased in both PCA (164.0±19.0 µM vs PCA-SHAM: 68.7±16.5 µM, $p<0.001$) and BDL rats (119.7±15.2 µM vs BDL-SHAM: 41.0±8.3 µM, $p<0.01$). Consequently, CSF ammonia levels (fig. 1B) also increased in PCA (106.4±16.9 µM vs PCA-SHAM: 45.1±11.9 µM, $p<0.05$) and BDL rats (128.4±36.7 µM vs BDL-SHAM: 23.3±6.1 µM, $p<0.05$). No significant difference was found in either plasma or CSF ammonia levels between PCA and BDL rats.

Brain edema

Brain water content in frontal cortex was found to be significantly increased in BDL rats (79.46±0.28 % vs BDL-SHAM: 78.35±0.17 %, $p<0.05$) and not in PCA rats vs PCA-SHAM (fig. 1C).

Reactive oxygen species

Arterial ROS levels significantly increased in BDL compared to BDL-SHAM rats ($p<0.001$). No significant difference in plasma ROS levels was found between PCA and PCA-SHAM rats or in CSF ROS levels of the two experimental groups compared to their respective SHAM-operated controls (table II). Supporting these results, arterial H2O2 displayed a similar pattern as ROS levels with a 2.4 fold increase in BDL rats compared to SHAM-operated controls ($p<0.001$) and no change between PCA rats and PCA-SHAM (table II). CSF H2O2 was not detected in either BDL or PCA rats along with their respective controls.

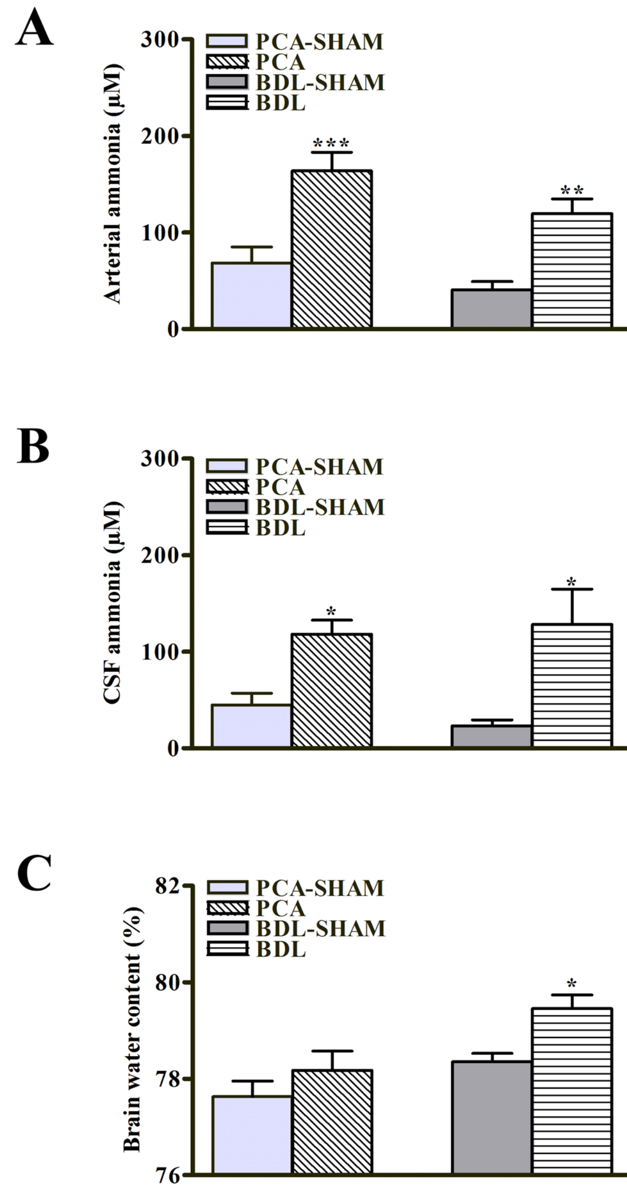

Figure 1: A) Arterial ammonia, B) cerebrospinal fluid (CSF) ammonia, C) frontal cortex brain water in rats with portacaval anastomosis (PCA) and bile-duct ligation (BDL) compared to respective SHAM-operated controls. Data are expressed as mean±SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significantly different from SHAM.

Oxidants

Plasma XO activity significantly increased in BDL rats whereas there was no significant difference found in plasma in PCA rats or in the frontal cortex in both BDL and PCA groups compared to their respective SHAM-operated controls (table II). Plasma SSAO as well as brain MAO-A and MAO-B did not show any significant difference in either of the 2 experimental models compared to their respective SHAM-operated controls (table II).

Antioxidants

Plasma CAT activity significantly decreased by 40% in BDL compared to BDL-SHAM rats. There was no significant difference in plasma CAT between PCA and PCA-SHAM rats. In the frontal cortex no significant difference was found in both BDL and PCA groups compared to their respective SHAM-operated controls. SOD activity, in both plasma and frontal cortex, showed no significant difference in either of the experimental groups compared to their respective SHAM-operated controls. GR activity significantly increased in plasma of BDL rats (4.5 fold vs BDL-SHAM, $p<0.001$) and in brains of both PCA (2.6 fold vs PCA-SHAM, $p<0.001$) and BDL rats (2 fold vs BDL-SHAM, $p<0.001$). No significant changes were observed in plasma of PCA vs PCA-SHAM rats. There were no changes in GP activity between the two experimental groups and their respective SHAM-operated controls in both plasma and frontal cortex. Total GSH significantly decreased by 44 % ($p<0.001$) in plasma in BDL rats, whereas GSH/GSSG ratio decreased 2.3 fold ($p<0.05$) compared to BDL-SHAM rats. No significant difference was demonstrated in plasma between PCA rats and their SHAM-operated controls. In the frontal cortex, no significant difference was found in both BDL and PCA groups compared to their respective SHAM-operated controls. All antioxidants activities are listed in table II.

Table II. Reactive oxygen species, oxidant and antioxidant enzyme activities in plasma and frontal cortex of rats with portacaval anastomosis (PCA) and bile-duct ligation (BDL) compared to respective SHAM-operated controls. ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; XO, xanthine oxidase; SSAO, semicarbazide-sensitive amine oxidase; CAT, catalase; SOD, superoxide dismutase; GR, glutathione reductase; GP, glutathione peroxidase; GSH/GSSG, reduced glutathione to oxidized glutathione ratio; MAO-A, monoamine oxidase A; MAO-B, monoamine oxidase B; n.d., not detected, below the detection limit of the method. Data are expressed as mean±SEM. *p<0.05, **p<0.01, *** p<0.001, significantly different from SHAM.

PLASMA

	PCA-SHAM	PCA	BDL-SHAM	BDL
REACTIVE OXYGEN SPECIES				
ROS (RFU)	0.33 ± 0.11	0.25 ± 0.12	0.15 ± 0.04	5.49 ± 1.93***
H_2O_2 (µM)	2.27 ± 0.59	1.42 ± 0.61	3.35 ± 0.53	8.02 ± 1.20***
OXIDANTS				
XO (mU/ml)	4.93 ± 0.14	9.45 ± 0.64	6.46 ± 0.75	30.47 ± 5.04***
SSAO (µU/ml)	162.1 ± 11.7	143.2 ± 15.2	178.4 ± 15.4	209.5 ± 35.4
ANTIOXIDANTS				
CAT (U/ml)	249.3 ± 28.2	196.8 ± 32.2	323.4 ± 56.2	126.5 ± 19.7**
SOD (fold change)	0.99 ± 0.15	1.12 ± 0.07	1.00 ± 0.12	1.07 ± 0.05
GR (mU/ml)	12.45 ± 1.47	16.07 ± 1.42	9.23 ± 1.02	42.20 ± 8.94***
GP (mU/ml)	69.73 ± 1.96	76.56 ± 5.08	47.42 ± 3.97	57.27 ± 2.66
total GSH (mM)	1.18 ± 0.04	1.15 ± 0.05	1.37 ± 0.04	0.77 ± 0.09***
GSH/GSSG (% vs SHAM)	100.00 ± 13.18	168.90 ± 30.96	100.00 ± 8.97	43.68 ± 16.88*

BRAIN

	PCA-SHAM	PCA	BDL-SHAM	BDL
REACTIVE OXYGEN SPECIES				
ROS (RFU)	15.03 ± 6.91	34.03 ± 8.86	38.89 ± 12.67	25.97 ± 8.21
H_2O_2 (µM)	n.d.	n.d.	n.d.	n.d.
OXIDANTS				
XO (mU/100 µg protein)	18.44 ± 3.18	26.68 ± 2.65	17.16 ± 1.12	17.54 ± 2.86
MAO-A (µU/100 µg protein)	10.02 ± 0.63	8.25 ± 1.35	11.71 ± 0.59	10.10 ± 0.55
MAO-B (µU/100 µg protein)	2.52 ± 0.91	3.93 ± 0.92	5.27 ± 0.41	5.75 ± 0.62
ANTIOXIDANTS				
CAT (U/100 µg protein)	23.41 ± 6.79	13.20 ± 2.10	20.75 ± 2.77	18.20 ± 2.20
SOD (fold change)	1.00 ± 0.13	1.01 ± 0.08	1.00 ± 0.17	1.23 ± 0.10
GR (mU/100 µg protein)	0.33 ± 0.02	0.88 ± 0.08***	0.39 ± 0.03	0.79 ± 0.03***
GP (mU/100 µg protein)	3.24 ± 0.08	3.70 ± 0.35	3.71 ± 0.18	3.16 ± 0.55
total GSH (mM)	0.42 ± 0.01	0.34 ± 0.04	0.37 ± 0.07	0.25 ± 0.05
GSH/GSSG (% vs SHAM)	100.00 ± 43.81	106.10 ± 36.25	100.00 ± 24.85	207.20 ± 77.73

Oxidative stress effects on lipids and proteins

Plasma levels of TBARS significantly increased in BDL rats compared to BDL-SHAM (9.87±0.62 µM vs BDL-SHAM: 41.91±7.40 µM, $p<0.001$). No significant difference in levels of TBARS were observed in plasma of PCA rats or in the frontal cortex of either

experimental group compared to their respective SHAM-operated controls (fig. 2A, B).

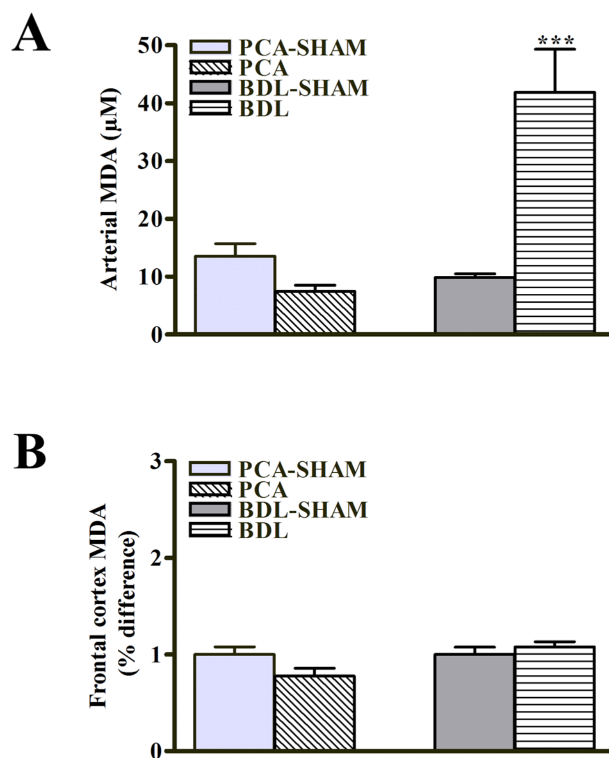

Figure 2. Oxidative stress effects on lipids in rats with portacaval anastomosis (PCA) and bile-duct ligation (BDL) compared to respective SHAM-operated controls: A) Arterial, B) frontal cortex malondialdehyde (MDA). Data are expressed as mean±SEM. ***$p<0.001$, significantly different from SHAM.

BBB extravasation

Evans Blue and NaF were measured in the frontal cortex in order to evaluate BBB extravasation. Evans Blue (961 Da) binds to albumin in plasma, forming a macromolecule, which, present in brain tissue denotes a large rupture of the BBB. The presence of NaF (376 Da) in brain tissue points to damages reflecting paracellular diffusion of small size molecules. No trace of Evans Blue or NaF extravasation was found in neither BDL nor PCA rats (fig. 3).

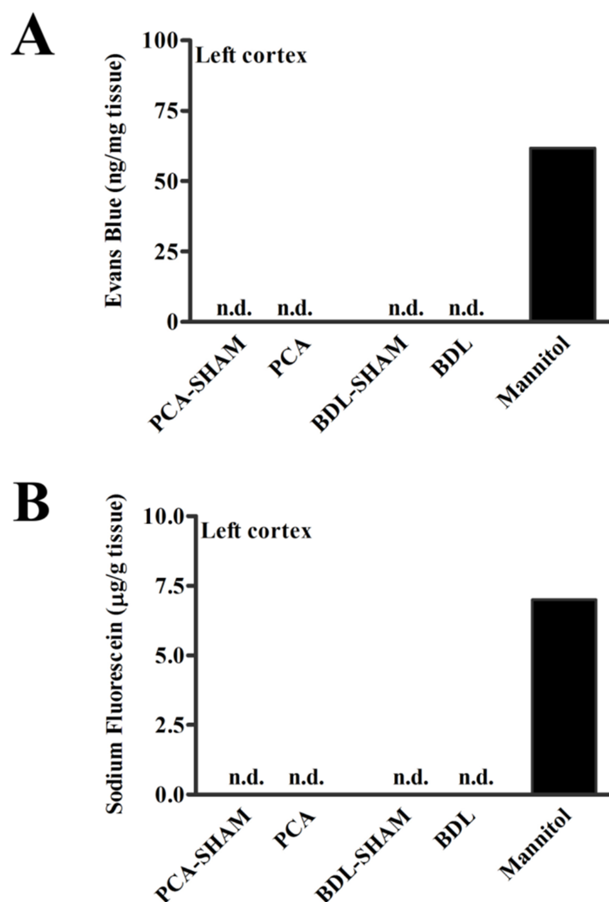

Figure. 3. Blood-brain barrier permeability to A) Evans Blue and B) sodium fluorescein in frontal cortex of rats with portacaval anastomosis (PCA) and bile-duct ligation (BDL) compared to respective SHAM-operated controls. Mannitol injected naïve rats were used as positive controls in order to validate the technique. n.d., not detected.

Effect of allopurinol in BDL rats

In order to delineate the effect of systemic oxidative stress on brain edema, BDL rats were treated with allopurinol (xanthine oxidase inhibitor). Following allopurinol treatment in BDL rats, both arterial ROS and brain edema decreased to levels similar to those seen in BDL-SHAM rats (fig. 4A, B). Allopurinol treatment did not improve liver function, as no changes were found between levels of AST, ALT, bilirubin, AP and GGT in allopurinol-treated versus non-treated BDL rats (fig. 4C). Locomotor activity (total distance travelled by BDL rats) was

reduced compared to SHAM-operated controls and ameliorated following allopurinol (fig. 4D).

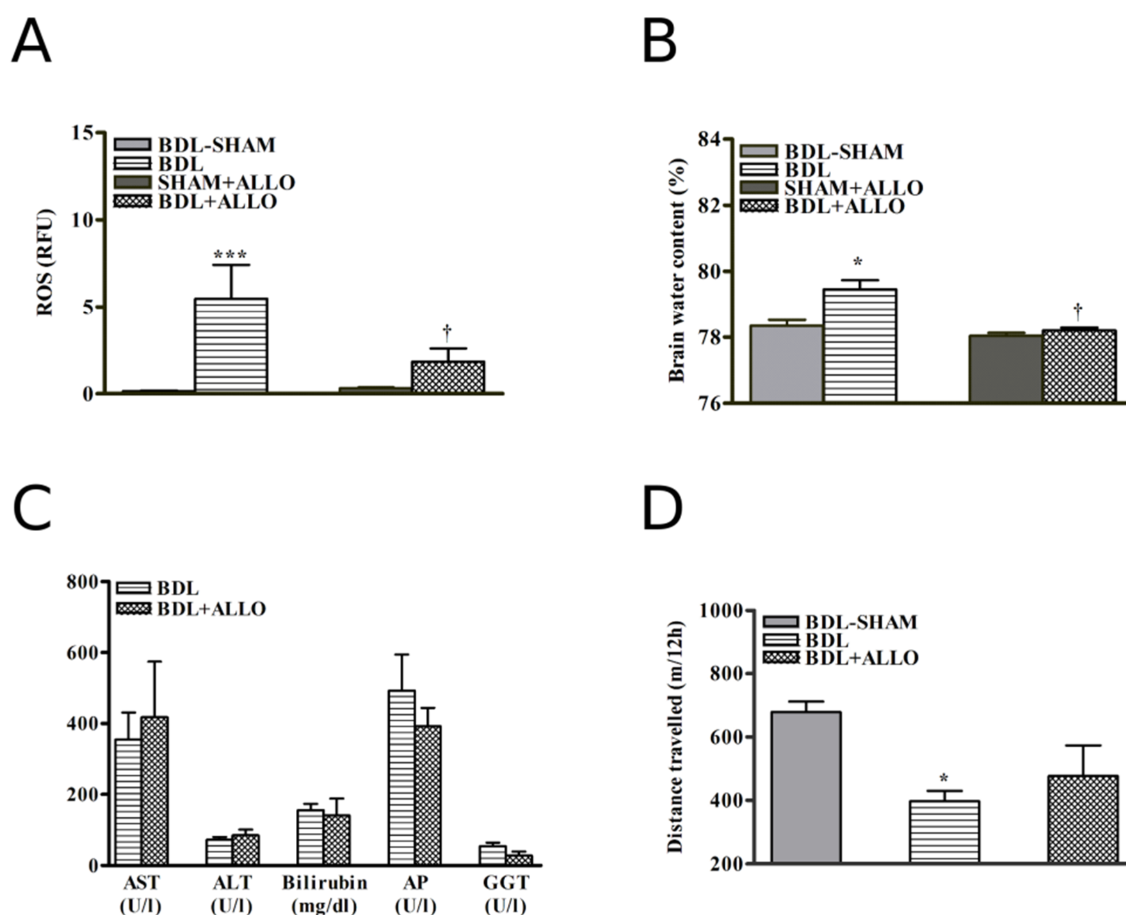

Figure 4. A) Arterial reactive oxygen species (ROS), B) frontal cortex brain water, C) liver biochemistry markers (AST, aspartate aminotransferase; ALT, alanine aminotransferase; AP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase), D) distance travelled over a period of 12 h during the night in bile-duct ligation (BDL) and allopurinol-treated bile-duct ligation (BDL+ALLO) rats compared to respective SHAM-operated controls. Data are expressed as mean±SEM. *$p<0.01$, ***$p<0.001$, significantly different from SHAM; †$p<0.05$, significantly different from non-treated BDL.

Temporal resolution of ammonia, oxidative stress and brain edema in BDL rats

Following 2 weeks of BDL, an increase in plasma oxidative stress is observed with no appearance of hyperammonemia, increased brain ammonia or brain edema. Following 4 weeks of BDL, in addition to an increased systemic oxidative stress an increase in plasma and brain ammonia is demonstrated but still no evidence of brain edema. Brain edema appears at 6 weeks, along with a further increase in plasma and brain ammonia compared to 4 weeks but with similar levels of systemic oxidative stress (fig. 5). Furthermore, another oxidative stress marker, TBARS, demonstrated significant increase in plasma of BDL rats vs SHAM-operated controls at 2 (1.19 fold, $p<0.05$), 4 (1.49 fold, $p<0.05$) and 6 (4.23 fold, $p<0.001$) weeks respectively. No increase in ROS levels in the brain (fig 5) or levels of TBARS in CSF (data not shown) were found in BDL rats.

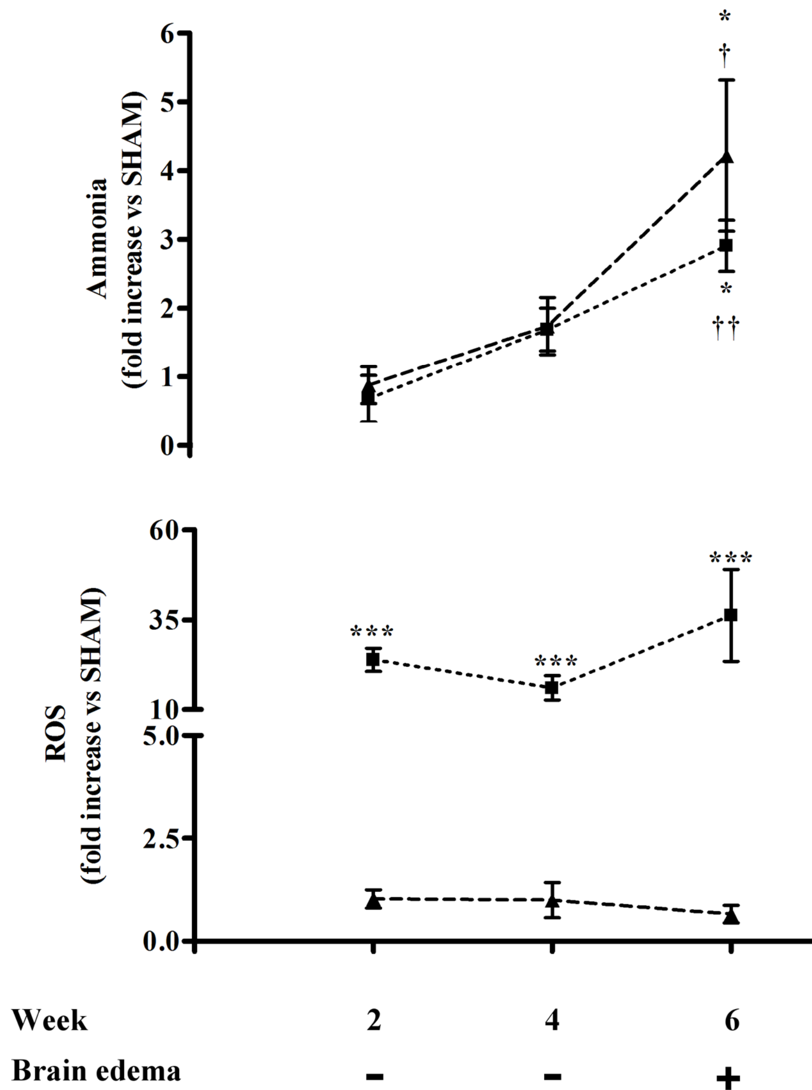

Figure 5: Temporal resolution of ammonia, reactive oxygen species (ROS) and brain edema in rats with bile-duct ligation (BDL) compared to respective SHAM-operated controls. The timepoint 0 represents the mean value for the 3 groups of respective SHAM-operated controls. Data are expressed as mean±SEM. *p<0.05, ***p<0.001, significantly different from SHAM and BDL 2 weeks; †p<0.05, ††p<0.01, significantly different from BDL 4 weeks (¨■¨ plasma ammonia and ROS temporal resolution; - ▲ - cerebrospinal fluid (CSF) ammonia and ROS temporal resolution).

Discussion

Results of the present study reveal that chronic hyperammonemia, which consequently leads to an increase in brain ammonia, independently does not induce oxidative stress (neither centrally nor systemically) or provoke brain edema. However, in combination, ammonia and systemic oxidative stress stimulate an increase in brain water. This conclusion was drawn from our results since brain edema is solely found in BDL rats and not in PCA rats even with similar levels of ammonia (arterial and CSF) observed in both animal models. The role of systemic (not central) oxidative stress in the pathogenesis of brain edema was confirmed when brain water normalized along with attenuation of systemic oxidative stress following treatment with allopurinol (inhibitor of the oxidant enzyme XO). These results depict ammonia and systemic oxidative stress act together in the pathogenesis of brain edema in CLF.

It has previously been demonstrated ammonia neurotoxicity leads to oxidative stress and subsequently brain edema (astrocyte swelling) [17]. However it must be noted that these studies involved acute ammonia intoxication models (*in vitro* and *in vivo*). First, cultured astrocytes acutely exposed to ammonia (1, 5 and 10 mM) showed an increase in ROS levels [18] and cell swelling [19]. Moreover, naïve rats injected with an acute dose of ammonia (12 mmol/kg injected intraperitoneally) [20] as well as rats with acute liver failure [21,22] displayed severe HE along with increased oxidative stress in brain. In our study, chronic hyperammonemia and CSF ammonia levels present in PCA rats (125-250 μM; lower than brain ammonia concentrations found in animal models of acute liver failure: 1-5 mM [22,35]) does not stimulate severe HE or oxidative stress in brain (or in circulation). Interestingly, a recent human study demonstrated oxidative stress markers in post mortem brain (cortical) tissue of cirrhotic patients that died with severe HE (grade 4) [36]. All in all, this suggests the degree and/or the acuteness of the onset of hyperammonemia is important for the induction of oxidative stress and that oxidative stress brain is associated with severe HE.

Only BDL rats (not PCA rats) demonstrated an increase in systemic oxidative stress. One obvious difference between the two animal models is liver function. BDL rats displayed with an increase in liver markers in comparison to PCA rats and their respective SHAM-operated controls. Accordingly to the Vienna consensus on classification of HE [37], PCA rats present type B HE (portosystemic shunting associated with hepatic atrophy and no significant intrinsic hepatocellular disease) while BDL rats present type C HE (associated with liver cirrhosis). In BDL rats, an increase in ROS and H_2O_2 blood levels was demonstrated along with an increase in XO activity and a decrease in antioxidant defense (decreased CAT activity and total GSH and GSH/GSSG ratio along with an increase in GR activity). A marker of oxidative stress TBARS, was also observed to be increased in blood of BDL rats. Moreover, plasma albumin levels, protein considered an important antioxidant [38], were decreased in BDL rats below the detection limit of our method. In PCA rats, a preserved oxidant/antioxidant balance was demonstrated with no changes in oxidant and antioxidant enzymes activities. Our results suggest systemic oxidative stress is a result of primary liver injury; a common finding observed in different types of liver disease such as non-alcoholic fatty liver disease, alcoholic liver disease and viral hepatitis [39–41], as well as cirrhosis [42–44].

In our study no signs of central oxidative stress were observed in BDL rats implying that systemic oxidative stress does not lead to oxidative stress in the brain. Systemic ROS could have an effect on the brain by acting directly on the luminal side of the BBB and causing BBB breakdown [45]; however Wright et al. [46] demonstrated the anatomical integrity of the BBB is intact in BDL rats. This was also confirmed by our group where no brain extravasation of Evans Blue and sodium fluorescein in BDL rats was observed. Nevertheless, systemic oxidative stress may induce post-translational modifications of proteins implicated in the BBB, leading to changes in signal transduction pathways across the BBB and

hyperpermeability. These aspects remain to be explored.

Furthermore, it is believed oxidative stress is closely associated with inflammation. It has been demonstrated proinflammatory cytokines are increased in plasma of cirrhotic patients with mHE as opposed to cirrhotic patients without HE [47,48]. Moreover, brain edema arises in endotoxemic BDL rats [46]. However the relationship and dependency between ROS and inflammation during cirrhosis as well as their effects on BBB permeability remain undefined.

XO released from the ailing liver is a major source of ROS [49]. Since an increase in XO activity was found in BDL rats, allopurinol, a XO inhibitor, was investigated as an antioxidant (oxidant inhibitor) treatment. Allopurinol, (a structural isomer of the XO substrates hypoxanthine and xanthine), is oxidized by XO to a more active metabolite, oxypurinol, which acts by irreversibly binding to the enzyme's active site [50]. Allopurinol has been previously demonstrated to reduce serum oxidative stress in stable cirrhotic patients [51]. In the present study, allopurinol-treated BDL rats displayed attenuated oxidative stress and brain edema. This protective effect was not due to an improvement in liver function since no change in hepatic function markers compared to non-treated BDL rats was found. Allopurinol treatment also resulted in an amelioration but not normalization of locomotor activity, suggesting other factors in addition to brain edema may contribute to neurological dysfunction. Our results sustain that XO plays an important role in oxidative stress production during liver disease and therefore we propose that an antioxidant treatment directed towards inhibiting sources of ROS may be more beneficial in HE treatment than one directed towards improving antioxidant defence, including ROS scavengers.

Overall, the relationship between ammonia, systemic oxidative stress and brain edema is

a complex one: in BDL rats hyperammonemia and oxidative stress lead to brain edema, while in PCA rats hyperammonemia alone does not lead to brain edema. Moreover, brain edema in BDL rats is attenuated following a reduction in systemic oxidative stress but also by lowering arterial levels of ammonia following AST-120 treatment as we previously demonstrated [24]. Moreover, in patients with CLF, serum 3-nitrotyrosine (an oxidative stress marker) was found to be related to mHE, but did not correlate with concentrations of blood ammonia [43]. Together, these studies strongly suggest a synergistic role between ammonia and oxidative stress in the pathogenesis of brain edema in BDL rats.

To further understand the relationship between blood ammonia and oxidative stress in the development of brain edema, we measured the pathogenetic factors along with brain water content at 2, 4 and 6 weeks following BDL. At 2 weeks, a significant increase in systemic ROS was observed without any signs of hyperammonemia or brain edema. At 4 weeks, both hyperammonemia and oxidative stress were present but no significant change in brain water content was observed. At 6 weeks, brain edema appeared and was associated with a significant further increase in ammonia levels compared to those seen at 4 weeks, and oxidative stress. Therefore the apparition of brain edema at 6 weeks is preceded by increased systemic oxidative stress (from week 2 to week 6) combined with a gradual chronic increase in ammonia (significantly increased at week 4 and significantly increased further at week 6). We propose systemic oxidative stress may be an important “first hit” followed by hyperammonemia as a “second hit” in the development of brain edema. Whether a certain threshold concentration of ammonia ($\approx 120\ \mu M$) or a degree of chronic hyperammonemia is necessary for the development of brain edema remains to be investigated.

We conclude that chronic hyperammonemia and systemic (not central) oxidative stress independently do not lead to brain edema, however when both factors are present brain edema

ensues. Our findings support the multifactorial pathogenesis of brain edema in HE and suggest systemic oxidative stress might be an important “first hit”, acting synergistically with ammonia to induce brain edema in chronic liver failure.

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Third paper: Induction of systemic oxidative stress leads to brain edema in portacaval shunted rats

Aim of third publication

The previous two papers suggested a synergistic effect between ammonia and systemic oxidative stress leads to the development of brain edema in MHE during CLD.

The aim of the third paper was to confirm this synergistic effect using a different approach. Hyperammonemic PCA rats were treated with the glutathione inhibitor DEM in order to induce oxidative stress. The ability of DEM to induce systemic OS as well as its effect on brain water content was evaluated.

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Induction of systemic oxidative stress leads to brain edema in portacaval shunted rats

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Authors contributions to the present publication

CRB contributed to the study design, performed the animal model and treatments, the brain water content measurement and the oxidative markers assays, analyzed the data, and wrote the manuscript. MT participated to the study design, data analysis and corrected the manuscript. CFR conceived the study, supervised the research group and approved the final manuscript.

Abstract

Background: The pathogenesis of hepatic encephalopathy (HE) is multifactorial and often associated with the development of brain edema. In addition to ammonia playing a central role, systemic oxidative stress is believed to aggravate the neuropsychological effects of ammonia in patients with chronic liver disease (CLD). The aim of this study was to i) induce systemic oxidative stress in hyperammonemic portacaval anastomosed (PCA) rats by inhibiting the antioxidant glutathione using diethyl maleate (DEM), and ii) investigate whether a synergistic relationship between ammonia and oxidative stress contributes to the pathogenesis of brain edema in CLD. Methods**:** 4-week PCA and sham-operated rats received DEM (0.4-4 mg/kg/day) for the last 10 days before sacrifice when oxidative stress markers (reactive oxygen species (ROS) and malondialdehyde (MDA)) were assessed in blood and frontal cortex. Brain water content was measured using a specific gravimetric technique. **Results:** DEM induced an increase in ROS and MDA in the blood, but not in the brain, of the PCA rats, compared to non-treated PCA rats. This was accompanied with an increase in brain water content (PCA+DEM: 78.45±0.13% vs PCA: 77.38±0.11%, $p < 0.001$). Higher doses of DEM induced systemic oxidative stress in sham-operated controls, but brain edema didn't develop. **Conclusions:** DEM provoked systemic, not central, oxidative stress in PCA rats, resulting in the development of brain edema. Independently, hyperammonemia and systemic oxidative stress do not precipitate brain edema; therefore, our findings sustain that a synergistic effect between hyperammonemia and systemic oxidative stress is responsible for the development of brain edema in HE.

Key Words: hepatic encephalopathy, oxidative stress, brain edema, portacaval shunt, diethyl maleate

Introduction

Hepatic encephalopathy (HE) is a debilitating condition affecting close to 30-84% of patients with chronic liver disease (CLD). This neuropsychiatric disorder is characterized by cognitive, psychiatric and motor disturbances and is primarily divided into overt (confusion, disorientation, ataxia and coma) and covert (reduced psychomotor speed, increased reaction time, sensory abnormalities, poor concentration) (1). Contrary to overt HE, the diagnosis of covert HE is not clinically evident and requires sensitive psychometric and neurophysiological testing (2). The burden of covert HE is multidimensional, affecting the patient's quality of life (ability to work and drive a car) and economically draining on health care systems (3). More importantly, covert HE places patients at a higher risk of developing overt HE with increased mortality (4).

The neuropathology of HE in CLD reveals morphological changes in astrocytes, including cell swelling, which consequently leads to brain edema (5). This feature is commonly observed in both patients and rats with cirrhosis suffering from covert HE (6–11), however HE also exists in the absence of brain edema (12–14). Brain edema has demonstrated to correlate with the severity of the disease (15–17), and has shown to improve following liver transplantation (6).

Liver failure induces hyperammonemia, which consequently leads to neurotoxic levels of ammonia, a principle factor long considered to be implicated in the pathogenesis of HE. However, a correlation between levels of blood ammonia and severity of HE in CLD remains controversial (18–22), suggesting other pathogenic factors are involved. Recently, it has been postulated that systemic oxidative stress can exacerbate the neurological effects of ammonia and play an important role in the pathogenesis of HE in CLD (23,24). Oxidative stress, a systemic phenomenon correlated with severity of liver disease (25,26), is an important condition present in patients with cirrhosis, and occurs due to an imbalance between the activities of pro-oxidants and antioxidants, leading to a surplus in reactive oxygen species

(ROS). Pro-oxidants constitutively generate ROS, which are important for proper cell signalling and function whereas antioxidants are responsible for regulating the levels of ROS and thus maintain a healthy equilibrium. Disturbance in pro-oxidant/antioxidant harmony has been demonstrated in the setting of liver disease. Xanthine oxidase, a liver derived pro-oxidant enzyme which produces ROS following oxidation of hypoxanthine to xanthine and to uric acid, has been found to be increased in circulation in both patients with CLD and cirrhotic rats (27,28). In addition , we recently demonstrated that by inhibiting xanthine oxidase with allopurinol, circulating levels of ROS were attenuated, brain edema was prevented, and HE was improved (28). Furthermore, glutathione (GSH), an important antioxidant synthesized by the liver, has been found to be decreased in cirrhosis (28–30).

Contrary to cirrhotic rats, rats with portacaval anastomosis (PCA) develop hyperammonemia in the absence of intrinsic hepatocellular disease (31) and neither brain edema nor any evidence of oxidative stress has been observed (28,32). The aim of this study was to induce systemic oxidative stress in PCA rats and investigate whether a synergistic relationship between ammonia and oxidative stress is key in the pathogenesis of brain edema in CLD. In order to induce oxidative stress, PCA rats were treated with dimethyl maleate (DEM), an irreversible GSH inhibitor (33).

Materials and methods

Animal model and experimental protocol

Adult male Sprague-Dawley rats weighing 200-225 g were anesthetised with isoflurane, and an end-to-side PCA was performed (28). Starting at day 18 after surgery, both PCA and sham-operated controls were administered DEM once daily (4, 3, 2, 1 or 0.4 mg/kg diluted in 1ml/kg saline; intraperitoneally) until day 28 when they were sacrificed. Vehicle-treated sham and PCA rats received equivalent volumes of saline. Experiments were conducted following the Guidelines of Canadian Council on Animal Care and were approved by the Animal Protection Committee of CRCHUM.

Markers of oxidative stress

Circulating ROS were evaluated following the oxidation of dichlorofluorescein diacetate (DCFDA; Invitrogen, Carlsbad, CA) to dichlorofluorescein (DCF) as previously described (28). Plasmatic and/or frontal cortex lipid peroxidation was estimated by measuring the levels of malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) formation. MDA levels were measured using TBARS Assay Kit (Cayman Chemical Company, Ann Arbor, MI), according to the manufacturer's protocol.

Total GSH was measured in the plasma and frontal cortex tissue, based on the sulfhydryl group oxidation by DTNB (5,5'-dithiobis-2-nitrobenzoic acid), which forms a yellow product (5-thio-2-nitrobenzoic acid, TNB) that is measured spectrophotometrically. Oxidized glutathione was previously reduced to GSH by glutathione reductase. Plasma and brain samples were extracted in 5 volumes of sulphosalicylic acid 5%, then homogenized and centrifuged 10 minutes at 13000g. 10 µl of the supernatant was incubated for 5 min with 150 µl sodium phosphate-buffer 95 mM containing EDTA 0.95 mM, glutathione reductase 0.115 U/ml and DTNB 0.031 mg/ml. The reaction was started by adding 50 µl reduced nicotinamide

adenine dinucleotide phosphate (NADPH) 0.038 mg/ml, the oxidation of which was followed at 412 nm for 32 minutes. Total GSH concentration was calculated based on a standard curve with known concentrations of GSH.

For HNE assessment, frontal cortex was homogenized in lysis buffer (50 mM Tris pH 7.5, 1 mM EDTA, 1/500 cold protease inhibitor cocktail). Homogenates were centrifuged 40 min at 13,000 g at 4°C. The supernatant was taken and protein content was determined according to the method of Lowry et al. (34). Samples containing 75 µg of cortex proteins were separated by 8 % sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE), and then transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% skim milk in TBS-T buffer (1 mM Tris pH=7.5, 10 mM NaCl and 0.5% Tween 20) for 1 h at room temperature and followed by 1 h incubation with rabbit polyclonal anti-HNE antibody (Calbiochem, Darmstadt Germany) at a dilution of 1:1000. Membranes were washed 3 times in TBS-T buffer for 5 min and then incubated for 1 h at room temperature, with the corresponding secondary antibody labelled with horseradish peroxidase (Perkin-Elmer, Waltham, USA) at a dilution of 1:10000 and washed 3 times in TBS-T buffer for 5 min. Immunoreactivity was detected with chemiluminescence reagent and probed on X-ray film. A monoclonal antibody to β-actin (Sigma, St- Louis, MO) was used at a dilution of 1:200000 as a control of protein loading.

Brain water content

Brain water content was measured using the sensitive densitometry technique. Frontal cortex was freshly dissected at 4°C and cut into $2mm^3$ pieces. Tissue pieces were placed in density gradient columns and equilibrium point was recorded after 2 min. Columns were made with different kerosene and bromobenzene mixtures and precalibrated with K_2SO_4 solutions of known densities. At least 8 samples were measured in each rat. Water content was calculated based on tissue density, according to the formula described by Marmarou *et al*. (35).

Liver biochemistry

Liver necrosis markers aspartate and alanine aminotransferase were measured using routine clinical biochemistry techniques.

Ammonia

Plasmatic ammonia levels were measured using a commercial kit (Sigma, St- Louis, MO) according to the manufacturer's protocol. The kit is based on the reaction of ammonia with α-ketoglutarate and reduced nicotinamide adenine dinucleotidephosphate in the presence of L-glutamate dehydrogenase. Oxidation rate of reduced nicotinamide adenine dinucleotide phosphate was recorded by the absorbance decrease at 340 nm. Ammonia concentration was calculated according to the manufacturer's protocol.

Statistical analysis

Data are expressed as mean±standard error of the mean (SEM). Significance of difference was tested with ANOVA, followed by Newman-Keuls post-test using GraphPad Prism4 (La Jolla, CA). Probability values $p<0.05$ were considered to be statistically significant.

Results

DEM treatment

It has previously been demonstrated that a single injection of DEM at doses up to 4 mg/kg administered to naïve rats was non-toxic and did not lead to mortality (33). In our study, since oxidative stress is a chronic feature observed throughout the time course of cirrhosis (28), we aimed to induce long-term oxidative stress in PCA rats by injecting DEM daily for 10 days at different doses (4, 3, 2, 1 or 0.4 mg/kg). DEM at doses 4 and 3 mg/kg lead to 100% mortality in PCA rats before the end of the 10-day treatment (4 mg/kg resulted in death after 1-2 days of treatment, and 3 mg/kg resulted in death following 7-9 days of treatment), whereas DEM at doses 2, 1 and 0.4 mg/kg did not lead to mortality in PCA rats. No mortality occurred in sham-operated controls treated with DEM at doses 4, 3, 2, 1 and 0.4 mg/kg for the duration of the 10-day treatment.

DEM induces systemic oxidative stress in PCA rats

The efficacy of DEM treatment in inducing systemic ROS was assessed by measuring various markers of oxidative stress. A significant increase in plasma ROS was observed in PCA rats vs. sham-operated controls following DEM treatment at doses 2 and 1 mg/kg, but not at 0.4 mg/kg (fig. 1A). Continuing with the dose 1 mg/kg, DEM treatment led to a significant decrease in total GSH in PCA rats, but not in sham-operated controls compared to respective non-treated controls (fig. 1B). MDA, a reliable marker of oxidative stress resulting from lipid peroxidation, was found to be significantly increased in DEM-treated PCA rats compared to sham-operated controls (fig. 1C).

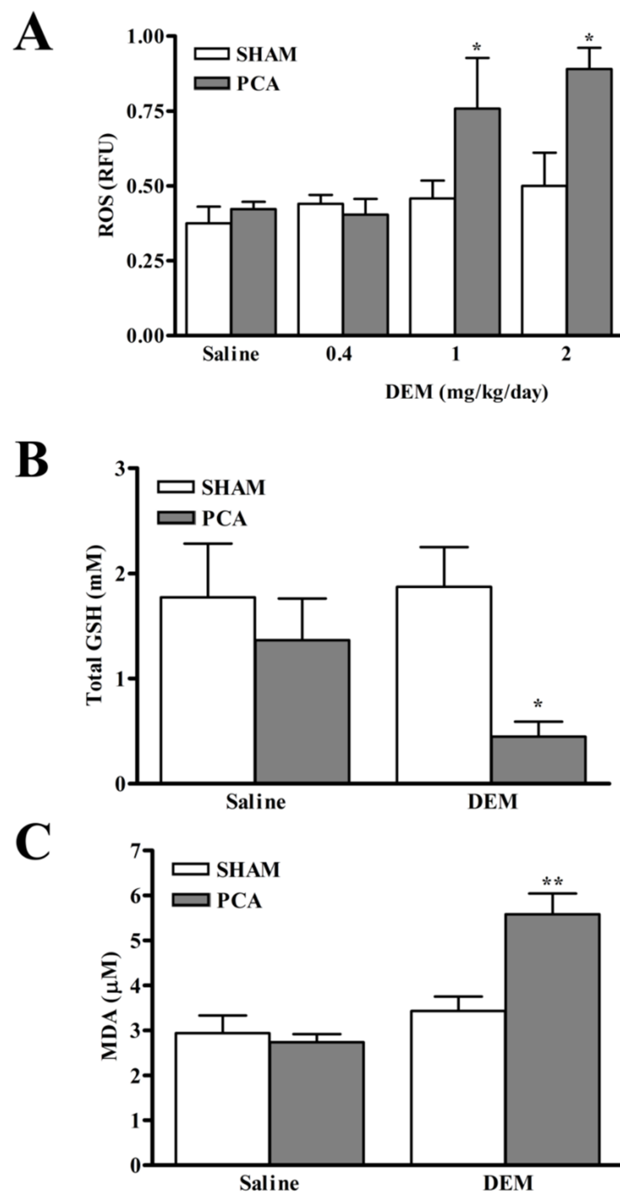

Figure 1. A) Circulating reactive oxygen species (ROS) in rats with portacaval anastomosis (PCA) and sham-operated controls treated and not treated with diethyl maleate (DEM) at a dose of 2, 1 or 0.4 mg/kg/day for 10 days.; B) plasmatic glutathione (GSH) and C) malondialdehyde (MDA) in rats with portacaval anastomosis (PCA) and sham-operated controls treated and not treated with diethyl maleate (DEM) at a dose of 1 mg/kg/day for 10 days.). Data are expressed as mean±SEM. *p<0.05, **p<0.01, significantly different from all other groups.

DEM induces brain edema in PCA rats

DEM treatment to PCA rats lead to a significant increase in brain water content in frontal cortex compared to non-treated PCA rats (PCA+DEM: 78.06±0.09% vs. non-treated PCA: 77.53±0.12%, p<0.05) and compared to sham-operated controls (77.54±0.11%, p<0.05) (fig. 2).

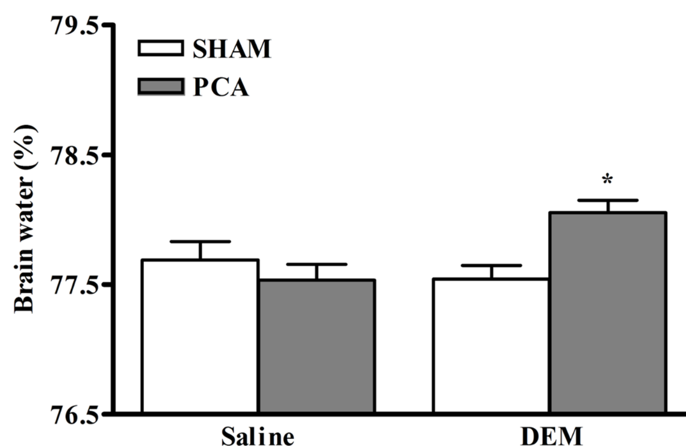

Figure 2. Frontal cortex water content in rats with portacaval anastomosis (PCA) and sham-operated controls treated and not treated with diethyl maleate (DEM) at a dose of 1 mg/kg/day for 10 days. Data are expressed as mean±SEM. *p<0.05, significantly different from all other groups.

DEM does not induce cerebral oxidative stress in PCA rats

DEM treatment did not lead to a significant increase in oxidative stress markers (total GSH, MDA and HNE) in either the frontal cortex of PCA nor sham-operated controls (fig. 3A, B, C).

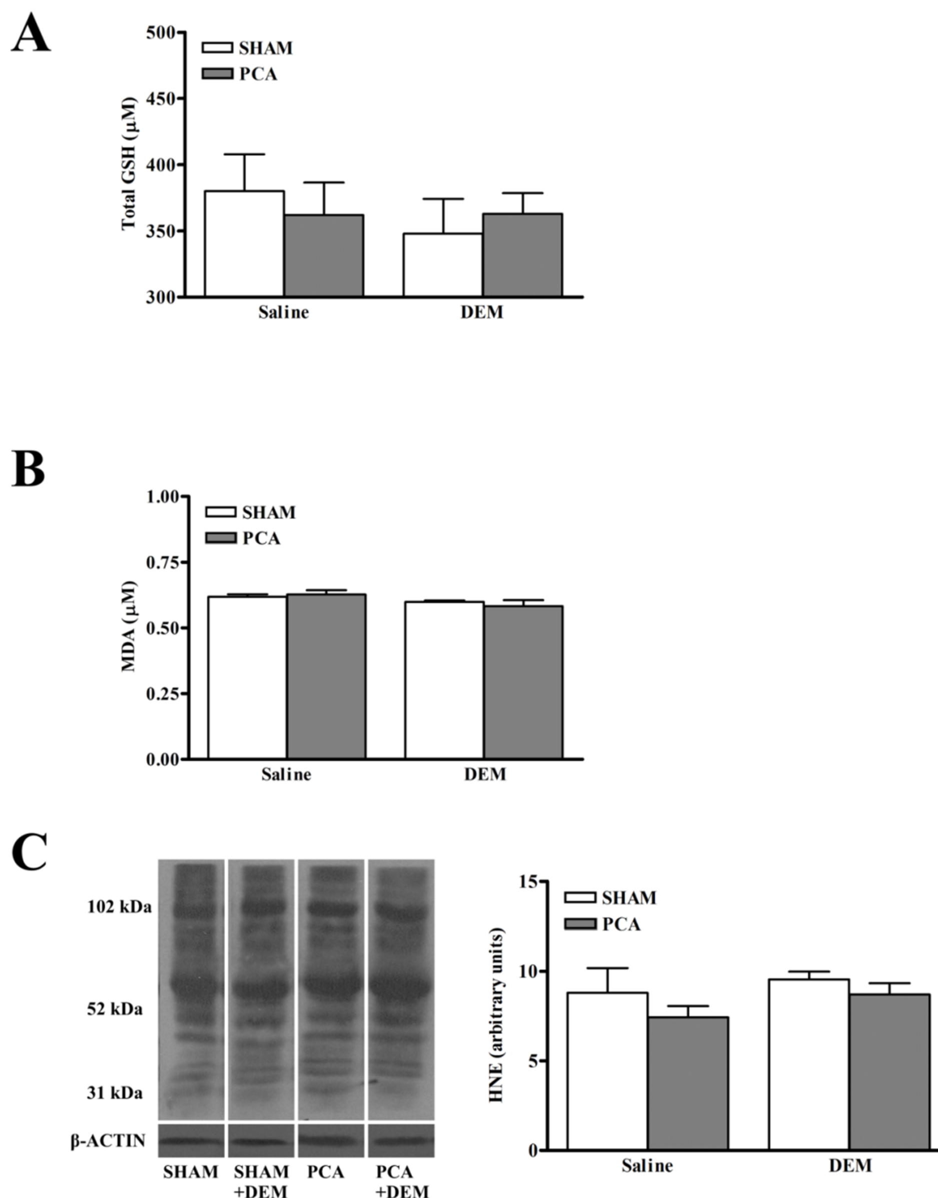

Figure 3. A) Cerebral glutathione (GSH); B) malondialdehyde (MDA) and C) 4-hydroxy-2-nonenal (HNE; representative immunoblotting images for each group along with densitometric quantification are shown) in rats with portacaval anastomosis (PCA) and sham-operated controls treated and not treated with diethyl maleate (DEM) at a dose of 1 mg/kg/day for 10 days. Data are expressed as mean±SEM.

DEM does not affect ammonia levels and liver function

DEM treatment did not induce hyperammonemia in sham-operated controls, nor influence the blood ammonia levels in PCA rats. In addition, DEM treatment did not increase the levels of AST or ALT in both PCA and sham-operated controls (table I).

Table I. Hepatic function markers aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and ammonia levels in in rats with portacaval anastomosis (PCA) and sham-operated controls treated and not treated with diethyl maleate (DEM) at a dose of 1 mg/kg/day for 10 days. Data are expressed as mean±SEM. ***p<0.001, significantly versus sham-operated rats.

	SHAM	PCA	SHAM+DEM	PCA+DEM
AST (U/l)	73.00 ± 5.02	85.50 ± 3.01	66.00 ± 1.03	80.00 ± 6.69
ALT (U/l)	45.60 ± 3.26	48.20 ± 2.43	41.00 ± 5.00	49.75 ± 6.39
Ammonia (μM)	44.04 ± 8.81	189.01 ± 17.84***	64.07 ± 9.56	152.10 ± 27.15***

Systemic oxidative stress independently does not lead to brain edema

Contrary to doses 0.4, 1, 2 and 3 mg/kg, DEM at dose 4 mg/kg provoked a significant increase in plasma ROS in sham-operated controls, which is similar to the increase in levels of ROS as those seen in PCA rats treated with DEM at a dose of 1 mg/kg. However, the former was not accompanied with the apparition of brain edema, as was observed in the DEM-treated PCA rats (fig. 4).

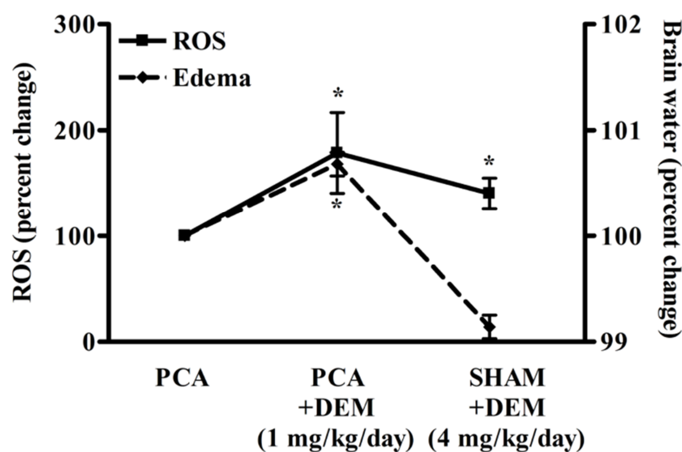

Figure 4. Circulating reactive oxygen species (ROS) and frontal cortex water in rats with portacaval anastomosis (PCA) treated and not treated with diethyl maleate (DEM) at a dose of 1 mg/kg/day for 10 days and sham-operated controls treated with diethyl maleate (DEM) at a dose of 4 mg/kg/day for 10 days. Data are expressed as percent change compared to non-treated sham-operated controls. ROS percent change is represented on the left axis and traced with a full line, while brain edema is represented on the right axis and traced with a dotted line. *$p<0.05$, significantly versus non treated PCA rats.

Discussion

DEM treatment significantly resulted in a reduction in circulating levels of GSH in hyperammonemic PCA rats, which in turn led to the induction of systemic, but not central, oxidative stress, and triggered the apparition of brain edema. DEM-induced systemic oxidative stress, in the absence of hyperammonemia, was not accompanied with presence of brain edema sustaining that both hyperammonemia and systemic oxidative stress are synergistically implicated in the pathogenesis of brain edema in CLD.

The aim of this study was to induce oxidative stress in hyperammonemic PCA rats by targeting and diminishing the antioxidant GSH, a tripeptide formed of glutamate, cysteine and glycine, whose cysteine residue acts as an electron donor. By scavenging ROS, GSH is converted into oxidized glutathione and reduced back to GSH by glutathione reductase. GSH, primarily produced by the liver, has been found to be increased in early stages of cirrhosis (36) but is evidently diminished during late stages of liver disease (28–30). However, in PCA rats, where the liver function is preserved, GSH levels are not affected (28). To target the inhibition of GSH, we used DEM, a direct GSH inhibitor that acts by irreversibly conjugating the cysteine's sulfhydryl group. It has been previously demonstrated that a single dose of DEM (600 mg/kg) administered to naïve rats induces a transient (12 h) decrease in hepatic and cerebral GSH (37). In order to simulate a chronic condition of oxidative stress (as observed during cirrhosis), daily injections of smaller doses of DEM were administered. Ten-day treatment of DEM, as low as 1 mg/kg, significantly diminished GSH levels in PCA rats. This resulted in the induction of oxidative stress in plasma, reflected with an increase in ROS and MDA. This same dose of DEM did not lead to similar results in sham-operated treated rats. Instead, a higher dose of DEM (4 mg/kg) was needed to attain a similar significant increase in ROS. This suggests that PCA rats demonstrate a higher susceptibility to a DEM-induced oxidative stress insult compared to sham-operated controls. The mechanisms underlying this susceptibility are unresolved, but the effect of chronic moderate elevated levels of ammonia in PCA rats may render the brain sensitive to insults. The mechanisms underlying increased brain

sensitivity following chronic ammonia exposure merits to be further investigated.

Ammonia has long been considered to play a major role in the pathogenesis of brain edema and HE; however, its weak relationship with severity of HE suggests that other factors are involved. Our results suggest that systemic oxidative stress is a key factor, in addition to ammonia that precipitates brain edema, since i) hyperammonemic PCA rats do not develop brain edema and ii) DEM-induced oxidative stress in sham-operated rats, which do not present hyperammonemia, does not lead to brain edema. This implies that neither oxidative stress nor ammonia independently induces brain edema. PCA in the rat represents a type B HE model resulting from portal-systemic shunting in the absence of parenchymal liver disease.

Therefore, in the absence of liver-derived factors like inflammation and oxidative stress, brain edema does not develop as previously demonstrated (12,13,28). Since DEM-induced oxidative stress in hyperammonemic PCA rats lead to the development of brain edema, this implies that a synergistic effect between ammonia and oxidative stress is required to cause an increase in brain water.

These observations support our previous studies demonstrating that a synergistic effect between systemic oxidative stress and ammonia is imperative in the development of brain edema in CLD (9,28). Furthermore, the clinical implications of this important synergy is sustained by a study from Montoliu et al., who elegantly demonstrated in cirrhotic patients with similar degrees of hypermamonemia, that the detection of 3-nitrotyrosine in plasma distinguished covert HE cirrhotic patients from those without HE (38). During liver disease, oxidative stress represents a systemic phenomenon (39) however the pathophysiological significance of whether oxidative stress vs ammonia is the “first” or “second” remains to be defined.

Swelling of the brain in DEM-treated PCA rats occurred in the absence of any indications of oxidative stress. It has been demonstrated that ammonia levels > 1mM lead to

the generation of ROS in cultured astrocytes and cell swelling (40,41). Similarly, *in vivo*, acute ammonia toxicity also leads to oxidative stress in the brain by increasing ROS production and diminishing antioxidant enzymes activities (42,43). Nevertheless, ammonia concentrations < 500 μM have been shown not to induce oxidative stress in both astrocyte cultures and brain slices (44,45). Furthermore, in both PCA rats and cirrhotic rats with brain ammonia levels < 250 μM, no signs of oxidative stress in the brain have been identified (28). However, signs of nitrosative stress have been demonstrated in the brains of PCA rats (46) which suggests nitrosative and oxidative stress may be implicated in different stages of HE and/or disease. Furthermore, there is strong evidence demonstrating that oxidative stress in the brain is associated with severe (overt) HE. Both acute ammonia intoxication and acute liver leading to severe HE and coma in rats (46–48), as well as cirrhotic patients who died with severe HE (grades III or IV) (49), markers of oxidative stress were detected in the brain. Overall, this suggests that the appearance of oxidative stress in the brain may be a vital stimulating factor in the pathogenesis of severe, overt HE.

The blood-brain barrier (BBB) is a structural barrier which impedes the influx of neurotoxic compounds from blood to brain. It has been previously demonstrated that circulating ROS can induce BBB oxidative damage and lead to tight junction protein modifications, resulting in BBB breakdown, as observed in stroke or sepsis (50). However, in different animal models of covert HE, the BBB has been proven to be anatomically intact (11,28). This implies, in the setting of CLD, that ROS may not be causing structural breakdown to the BBB, but rather, through oxidative modifications of proteins and lipids, may stimulate changes in BBB signalling and permeability. These pathophysiological mechanisms merit to be investigated in relation systemic oxidative stress and the development of brain edema in HE.

In the present study, we assessed oxidative stress and brain edema in the frontal cortex; an important region for cognitive function. However, the heterogeneity of the brain does not insinuate all areas of the brain react similarly. In fact, the cerebellum has

demonstrated to have an enhanced susceptibility to oxidative stress in hyperammonemic rats (47). Therefore we do not exclude the possibility that other cerebral regions are affected.

During end stage liver disease, multiple factors other than ammonia and oxidative stress may also be contributing to the pathogenesis of brain edema. Inflammation is an important factor, demonstrated to play an important role in inducing brain edema (51) and known to play an important role in the cognitive and motor alterations in PCA rats (13,52). Although inflammation is believed to be tightly associated with oxidative stress, their relationship remains unresolved. Other factors such as alterations of energy metabolism, osmotic changes triggered by increases in glutamate, glutamine, lactate or expression in water or ion channels have also been demonstrated to play a role in brain edema during liver disease, as reviewed by Bosoi and Rose (5). Neither DEM-treated or untreated PCA rats demonstrated any affects in liver function, which excludes the roles of the above-mentioned factors, which have been found to be induced from the ailing liver during disease (53).

In conclusion, our study confirms that chronic hyperammonemia and systemic (not central) oxidative stress independently do not lead to the development of brain edema; however, when both factors are present, they act synergistically to induce an increase in brain water. The presence of brain edema in covert HE may be a predisposing factor leading to a higher risk of triggering oxidative stress in the brain and developing overt HE. These results support that in addition to targeting ammonia producing/removing pathways, antioxidants or pro-oxidant inhibitors should be considered for the treatment of HE.

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Fourth paper: Increased brain lactate is central to the development of brain edema in rats with chronic liver disease

Aim of fourth publication

This paper aims to define the roles of lactate and glutamine and their relationship with ammonia in the pathogenesis of brain edema in CLD. To attain this objective, nuclear magnetic resonance spectroscopy was used to determine the *de novo* synthesis of lactate and glutamine from ^{13}C-labelled glucose in BDL rats. AST-120 and DCA were used as research tools to lower ammonia and lactate respectively. Their effects on blood ammonia, cerebral lactate and glutamine and brain edema were investigated along with the relationship between ammonia, lactate and glutamine. Furthermore, changes in other cerebral osmolytes implicated in the pathogenesis of brain edema in CLD were evaluated.

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Increased brain lactate is central to the development of brain edema in rats with chronic liver disease

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Authors contributions to the present publication

CRB contributed to the study design, performed the animal experiments (surgeries, therapeutic interventions, brain water content measurement) and part of the assays (ammonia, glutamine), analyzed the data, and wrote the manuscript. CZ performed the NMR experiments. HM performed the lactate assay. JH and CPR assisted to the animal experiments. MT participated to the study design, data analysis and corrected the manuscript. CFR conceived the study, supervised the research group and approved the final manuscript.

Abstract:

Background & Aims: The pathogenesis of brain edema in patients with chronic liver disease (CLD) and minimal hepatic encephalopathy (HE) remains undefined. This study evaluated the role of brain lactate, glutamine and organic osmolytes, including myo-inositol and taurine, in the development of brain edema in a rat model of cirrhosis.

Methods: Six-week bile-duct ligated (BDL) rats were injected with ^{13}C-glucose and *de novo* synthesis of lactate, and glutamine in the brain was quantified using ^{13}C nuclear magnetic resonance spectroscopy (NMR). Total brain lactate, glutamine, and osmolytes were measured using ^{1}H NMR or high performance liquid chromatography. To further define the interplay between lactate, glutamine and brain edema, BDL rats were treated with AST-120 (engineered activated carbon microspheres) and dichloroacetate (DCA: lactate synthesis inhibitor).

Results: Significant increases in *de novo* synthesis of lactate (1.6-fold, $p<0.001$) and glutamine (2.2-fold, $p<0.01$) were demonstrated in the brains of BDL rats vs. SHAM-operated controls. Moreover, a decrease in cerebral myo-inositol ($p<0.001$), with no change in taurine, was found in the presence of brain edema in BDL rats vs. controls. BDL rats treated with either AST-120 or DCA showed attenuation in brain edema and brain lactate. These two treatments did not lead to similar reductions in brain glutamine.

Conclusions: Increased brain lactate, and not glutamine, is a primary player in the pathogenesis of brain edema in CLD. In addition, alterations in the osmoregulatory response may also be contributing factors. Our results suggest that inhibiting lactate synthesis is a new potential target for the treatment of HE.

Introduction

Hepatic encephalopathy (HE) is a neuropsychiatric disorder, a major complication of both acute liver failure and chronic liver disease (CLD). Brain edema is a neuropathological feature of HE that contributes to intracranial hypertension (a fatal complication of acute liver failure, (Clemmesen et al., 1999)); but is also associated with minimal HE (MHE) and CLD (Häussinger, 2006; Lodi et al., 2004; Shah et al., 2008; Sugimoto et al., 2008). Characterized by impairment in concentration, attention, memory, vigilance, reaction time and behavior, MHE is detected using sensitive neuropsychometric and neurophysiological tests (Córdoba, 2011). As much as 80% of patients with end-stage liver disease are affected by MHE, which severely impacts on the patients' capability to drive a car, to continue working, and their ability to function daily, overall affecting their health-related quality of life (Stewart and Smith, 2007).

Brain edema is an accumulation of water within the cerebral tissue (intracellular and/or extracellular). It occurs as a result of an increase in osmolarity and/or compromised volume regulatory responses. Impairment in the efflux of brain organic osmolytes, such as polyols (myo-inositol) and amino acids (taurine, glutamate, glutamine), fails to compensate for the increased osmolarity and, therefore, an increase in brain water content prevails (McManus et al., 1995).

Ammonia is considered a major pathogenic factor in the development of HE (Felipo and Butterworth, 2002). The brain solely relies on the amidation of glutamate catalyzed by the enzyme glutamine synthetase (GS) to detoxify ammonia. However, because the ailing liver is incapable of efficiently clearing it, the increase in blood ammonia leads to neurotoxic levels of ammonia. Therefore, during hyperammonemia, elevated brain ammonia leads to an increase in brain glutamine (Pasantes-Morales and Cruz-Rangel, 2010), a pathway believed to be involved in the development of brain edema and HE.

Lactate is another pathogenic factor demonstrated to be implicated in HE [11]. Lactate is

a product of anaerobic glycolysis, but also a metabolite used by neurons as an energetic substrate (Pellerin and Magistretti, 2012). An increase in cerebral lactate, due to increased glycolytic activity and/or energy failure, can osmotically induce an increase in water influx in the brain, and thus lead to brain edema, as demonstrated in numerous neuropathies, including cerebral ischemia (Helbok et al., 2011). However, the role of lactate in the pathogenesis of brain edema in HE due to CLD remains undetermined.

The present study aims to explore the pathophysiological mechanisms implicated in brain edema in cirrhotic rats with MHE, with an emphasis on the role of lactate and glutamine and brain osmolytes, including myo-inositol and taurine. The 6-week bile-duct ligated (BDL) rat is a well-characterized animal model of liver fibrosis and necrosis (Bataller et al., 2005), which develops both brain edema and MHE (Bosoi et al., 2011; Davies et al., 2009). In this model, we investigated the metabolic fluxes of ^{13}C-labelled glucose, and focused on *de novo* synthesis of lactate and glutamine using nuclear magnetic resonance (NMR) spectroscopy, an excellent technique to quantify cellular metabolic fluxes (fig. 1A). As ^{13}C represents only 1.1% of natural carbon, sample enrichment following injection of ^{13}C-labelled glucose permits the evaluation of glucose metabolic fluxes through the glycolytic pathway and the tricarboxylic acid (TCA) cycle (Zwingmann, 2007). In order to evaluate the interplay between lactate, glutamine and organic osmolytes, BDL rats were treated with AST-120 (oral ammonia adsorbent engineered activated carbon microspheres) and dichloroacetate (DCA), a lactate synthesis inhibitor.

Materials and methods

Animal models

Male Sprague-Dawley rats (250-275 g) (Charles River, St-Constant, QC) were randomly selected to either bile-duct ligation (BDL) or SHAM operation, and studied 6 weeks following surgery (Bosoi et al., 2011); for cerebral ammonia measurement, cerebrospinal fluid was collected from the cisterna magna, as previously described (Bosoi et al., 2012). All the experiments were performed following the Guidelines of the Canadian Council on Animal Care, and were approved by the Animal Protection Committee of the CRCHUM.

Nuclear magnetic resonance

Administration of [U-^{13}C]-Glucose: 6 weeks after surgery BDL and SHAM rats received [U-^{13}C]-glucose (500 mg/kg, intraperitoneally; Cambridge Isotope Laboratories, Andover, MA) and were sacrificed exactly 30 minutes later by decapitation. Arterial blood glucose levels were monitored 3 days before sacrifice; however, no glycaemia differences were found between SHAM and BDL operated rats. Therefore, there was no need to correct glucose levels. [U-^{13}C]-glucose is transformed through glycolysis in[U-^{13}C]-pyruvate. This can either enter the TCA cycle or form [U-^{13}C]-lactate through lactate dehydrogenase. Upon entering the TCA cycle, glucose-derived pyruvate is metabolized either by pyruvate dehydrogenase (PDH), the key enzyme for mitochondrial energy found in both astrocytes and neurons, or by pyruvate carboxylase (PC), an important anaplerotic enzyme that replenishes TCA cycle intermediates, found exclusively in astrocytes. The [U-^{13}C]-pyruvate formed from [U-^{13}C]-glucose metabolised through PDH results in [1,2-^{13}C]-acetyl-CoA, and further via α-ketoglutarate to [4,5-^{13}C]-labelled glutamate and glutamine, whereas [U-^{13}C]-pyruvate metabolised through PC results in [1,2,3-^{13}C]-oxaloacetate and [2,3-^{13}C]-labelled metabolites. These reactions allowed for the quantification of fluxes through these pathways (Zwingmann, 2007) (fig.1A).

NMR spectroscopy (Zwingmann et al., 2004)**:** Immediately after sacrifice, the brains were snap frozen in liquid nitrogen and stored at -80°C until measurements were performed. Water-soluble metabolites were extracted with 7% perchloric acid. The lyophilized water-soluble samples were dissolved in D_2O, centrifuged, and adjusted to pH 7.2. NMR spectra were recorded on a DRX-600 Bruker spectrometer. ^{1}H-NMR spectra were recorded with a 5-mm H,C,N-inverse-triple-resonance probe, flip angle 40°, repetition time 15 s, spectral width 7,183 Hz. ^{13}C-NMR spectra were recorded with a 5-mm ^{1}H/^{13}C dual probe, repetition time 2.5 s, flip angle 27°, composite pulse decoupling with WALTZ-16, spectral width 47,619 Hz. Total brain glutamine, glutamate, myo-inositol and taurine were determined on ^{1}H-NMR spectra, while ^{13}C-labelled lactate and glutamine were determined on ^{13}C-NMR spectra.

Therapeutic interventions

AST-120: AST-120 (Ocera Therapeutics, San Diego, CA), engineered carbon microspheres, were administered by gavage (1 g/kg/day; concentration 1g/10 ml of 2% methylcellulose) every 12 hours, for a period of 6 weeks, beginning day 1 after surgery in both SHAM and BDL rats (Bosoi et al., 2011). As controls, another group of SHAM and BDL rats were treated with equivalent volumes of methylcellulose.

Dichloroacetate: Dichloroactate (DCA; Sigma-Aldrich), a lactate synthesis inhibitor, was administered by intraperitoneal injection at a dose of 25 mg/kg/day (concentration of 25 mg/ml saline) for 7 days (starting day 35 after surgery) in both SHAM and BDL rats. This PDH kinase inhibitor results in a dephosphorylation of PDH and hence increases its activity. As a result, the flux of pyruvate into the TCA cycle increases, consequently decreasing lactate synthesis by shifting lactate dehydrogenase activity from lactate to pyruvate production (Stacpoole et al., 1998).

At 6 weeks, brain tissue was collected to measure lactate, glutamine and brain edema as described below.

Ammonia measurement

Ammonia was assessed in cerebrospinal fluid using a commercial kit (Sigma-Aldrich). The kit is based on the reaction of ammonia with α-ketoglutarate and reduced nicotinamide adenine dinucleotidephosphate in the presence of L-glutamate dehydrogenase. Oxidation rate of reduced nicotinamide adenine dinucleotide phosphate was recorded by the absorbance decrease at 340 nm. Ammonia concentration was calculated according to the manufacturer's protocol.

Lactate measurement

The frontal cortex was dissected and homogenized in lysis buffer (50 mM Tris pH 7.5, 1 mM EDTA, 1/500 cold Protease Inhibitor Cocktail (Roche, Indianapolis, IN)). Lactate levels were assessed following its oxidation by lactate oxidase to pyruvate and hydrogen peroxide, which reacts with AmplexRed (10-acetyl-3,7-dihidroxyphenoxazine) and releases resorufin, a fluorescent oxidation product. Fluorescence was read at 530 nm excitation and 590 nm emission wavelengths, and lactate levels were calculated based on a standard curve of known lactate concentrations.

Glutamine measurement

Brain frontal cortex samples were analyzed using the Agilent 1100 Chemstation reverse-phase HPLC system (Agilent Technologies, Germany) with fluorescence detection as previously described (Bélanger et al., 2006). Glutamine concentration was calculated by peak area analysis with an automated integrator (Agilent Technologies, Germany), based on standard curves and internal standards.

Brain water content

Frontal cortex brain water content was measured using the sensitive densitometry technique described by Marmarou *et al*., as previously reported by our group (Bosoi et al., 2011; Marmarou et al., 1982).

Statistical analysis

Data are expressed as mean±standard error of the mean (SEM). Significance of difference was tested with Student t test or ANOVA, followed by Newman-Keuls post-test; correlation was calculated with Spearman test using GraphPad Prism4 (La Jolla, CA). Probability values $p<0.05$ were considered to be statistically significant.

Results

Cerebral lactate and glutamine in BDL rats

Six week BDL rats demonstrated a significant increase in brain lactate (254.6±11.1 µM/µg protein vs. SHAM: 111.7±7.1 µM/µg protein, $p<0.001$), and brain glutamine (8.42±12.77 µmol/g tissue vs. SHAM: 4.42 ±3.38 µmol/ g tissue, $p<0.01$).

De novo *synthesis of cerebral lactate and glutamine from ^{13}C-labelled glucose in BDL rats*

Following ^{13}C-labelled glucose administration, *de novo* synthesis of lactate and glutamine significantly increased 1.6- and 2.2-fold in BDL vs. SHAM-operated control rats (fig. 1B,C). Determination of the position of the ^{13}C-labelled carbon in *de novo* synthetized glutamine showed that the flux through PDH (2.3-fold increase) and PC (1.8-fold increase) was higher in BDL vs SHAM rats (fig. 1C).

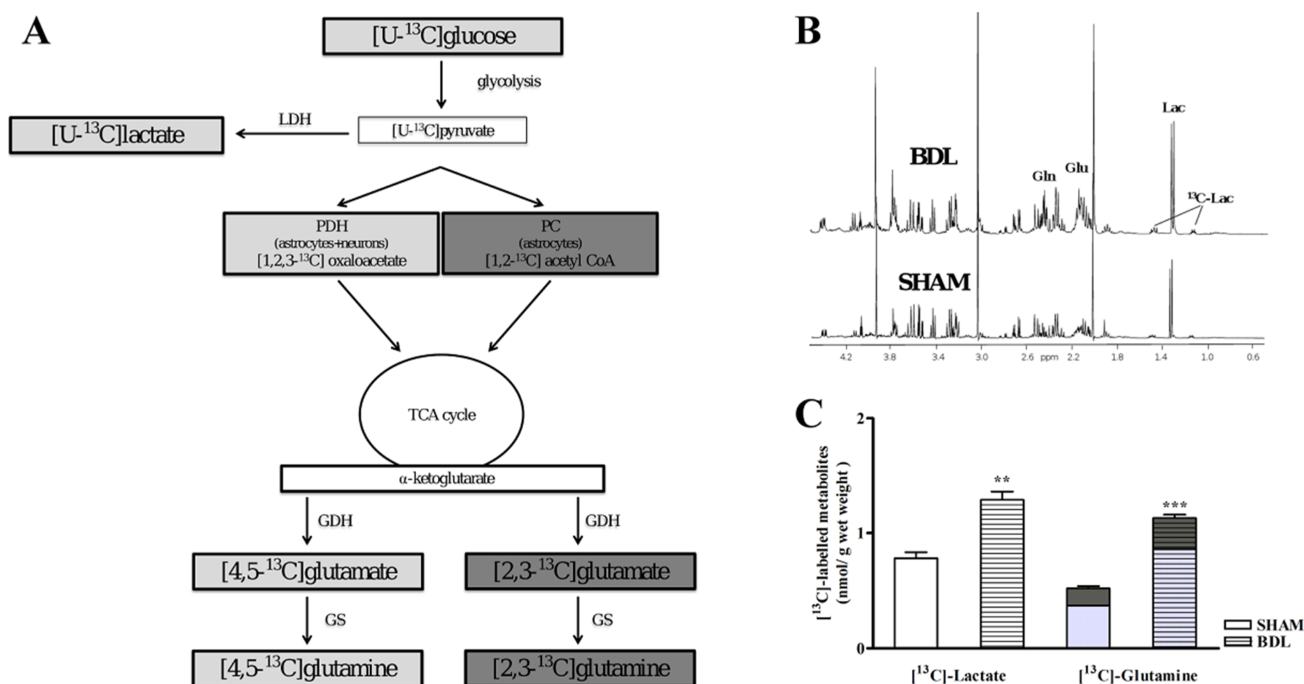

Figure 1: ^{13}C nuclear magnetic resonance spectroscopy. (A) Schematic representation of ^{13}C-labelled metabolites formation from [U-^{13}C]-glucose. Glucose is metabolized via glycolysis into pyruvate which either produces lactate through lactate dehydrogenase (LDH) or enters the TCA cycle. Upon entering the TCA cycle, pyruvate is metabolized via pyruvate dehydrogenase (PDH), found in both astrocytes and neurons, or via pyruvate carboxylase (PC), found exclusively in the astrocytes. The [U-^{13}C]-pyruvate formed from [U-^{13}C]-glucose through PDH results in [1,2-^{13}C]-acetyl-CoA, and further via α-ketoglutarate to [4,5-^{13}C] labelled glutamate and glutamine; through PC it results in [1,2,3-^{13}C]-oxaloacetate and [2,3-^{13}C] labelled metabolites. Glutamate is produced from the TCA intermediate α-ketoglutarate via α-ketoglutarate dehydrogenase and via amidation by glutamine synthetase (GS) can form glutamine. LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase; TCA cycle, tricarboxylic acid cycle; GS, glutamine synthetase. B) Representative ^{13}C nuclear magnetic resonance spectra in rats with bile-duct ligation (BDL) compared to SHAM-operated controls. C) Cerebral *de novo* synthesis from ^{13}C-glucose of lactate and glutamine in rats with bile-duct ligation (BDL) compared to SHAM-operated controls. Light grey: [4,5-^{13}C]-glutamine formed through PDH; dark grey: [2,3-^{13}C]-glutamine formed through PC. Data are expressed as mean±SEM. **$p<0.01$, ***$p<0.001$, significantly different from SHAM.

Brain osmolytes in BDL rats

^{1}H is naturally present in tissues and through its detection, numerous molecules can thus be simultaneously quantified by ^{1}H NMR spectroscopy. The cerebral osmolyte pool, obtained by adding all measured osmolytes, was 1.3-fold higher in BDL vs. SHAM-operated control rats (table I). In BDL rats, a significant increase in brain glutamine (128%; $p<0.01$ vs. SHAM) and glutamate (26%; $p<0.01$ vs. SHAM) was accompanied by a significant decrease in myo-inositol (23%; $p<0.001$ vs. SHAM). Brain taurine remained unchanged.

Table I. ^{1}H-NMR concentration of brain osmolytes in rats with bile-duct ligation (BDL) compared to respective SHAM-operated controls. Data are expressed as mean±SEM. **p<0.01, ***p<0.001, significantly different from SHAM.

	BDL-SHAM	BDL
Glutamine (µmol/g ww)	4.67 ± 0.28	10.67 ± 0.98**
Glutamate (µmol/g ww)	11.55 ± 0.48	14.56 ± 0.99**
Myo-inositol (µmol/g ww)	4.31 ± 0.14	3.34 ± 0.14***
Taurine (µmol/g ww)	4.71 ± 0.43	5.48 ± 1.73
Total	25.20 ± 1.33	34.00 ± 2.95

Effect on cerebral lactate and glutamine following ammonia reduction

Administration of AST-120, engineered activated carbon microspheres with a high nonspecific adsorptive surface area acting within the gut, lead to a significant decrease in ammonia in the brain (cerebrospinal fluid) (fig. 2A) and to an attenuation in brain edema (fig. 2B). Moreover, AST-120-treated BDL rats resulted in a significant reduction in brain lactate versus non-treated BDL rats. However, lactate remained significantly high in AST-120 treated BDL rats compared to SHAM-operated controls (p<0.001) (fig. 2C). Contrary to lactate, the lowering of ammonia by AST-120 did not reduce brain glutamine levels (fig. 2D).

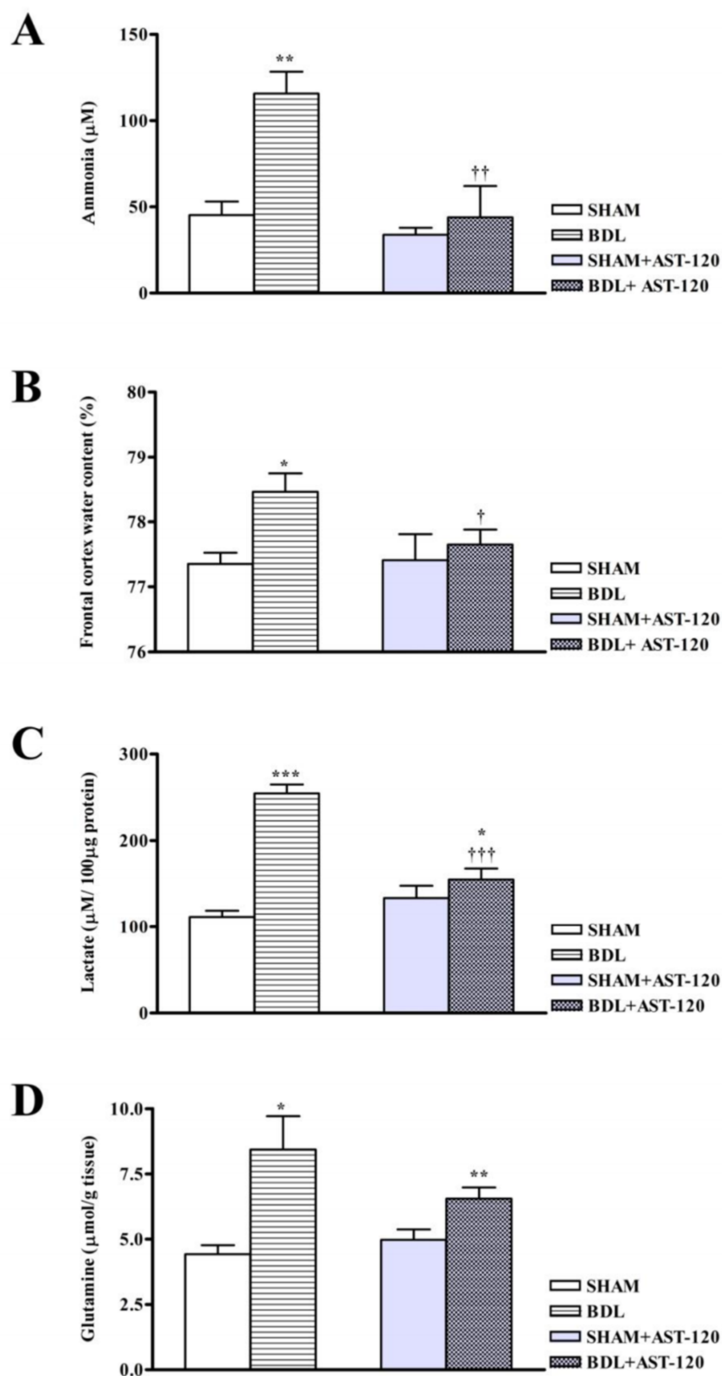

Figure 2. Effect of AST-120 (spherical carbon adsorbent): (A) cerebrospinal fluid ammonia; (B) frontal cortex brain water content; (C) frontal cortex lactate and (D) frontal

cortex glutamine levels in bile-duct ligation (BDL) rats compared to treated and non-treated SHAM-operated controls and non-treated BDL rats. Data are expressed as mean±SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significantly different from SHAM; ††$p<0.01$, †††$p<0.001$, significantly different from non-treated BDL.

Effect on brain edema following lactate reduction

In order to identify the precise role of lactate in the pathogenesis of brain edema, BDL rats were treated with DCA, a lactate synthesis inhibitor. The DCA treatment normalized brain lactate levels (fig. 3A) and reduced brain water content in BDL rats ($p<0.05$) (fig. 3B). Cerebral glutamine decreased following DCA treatment to levels that were not significantly different versus either non-treated BDL rats or SHAM-operated controls (fig. 3C). To verify if these modifications are not due to a direct effect of DCA on ammonia levels, those were assessed and were found to be similar in non-treated and DCA-treated BDL rats (fig.4D). In addition, DCA treatment did not have a beneficial effect on the liver function as no change in liver enzymes (aspartate aminotransferase, AST and alanine aminotransferase, ALT) was found between non-treated and DCA-treated BDL rats (AST: DCA-treated: 301.3±141.7 U/l vs. non-treated:364.4±64.7 U/l, $p>0.05$; ALT: DCA-treated: 65.2±11.8 U/l vs. non-treated:73.4±7.1 U/l, $p>0.05$).

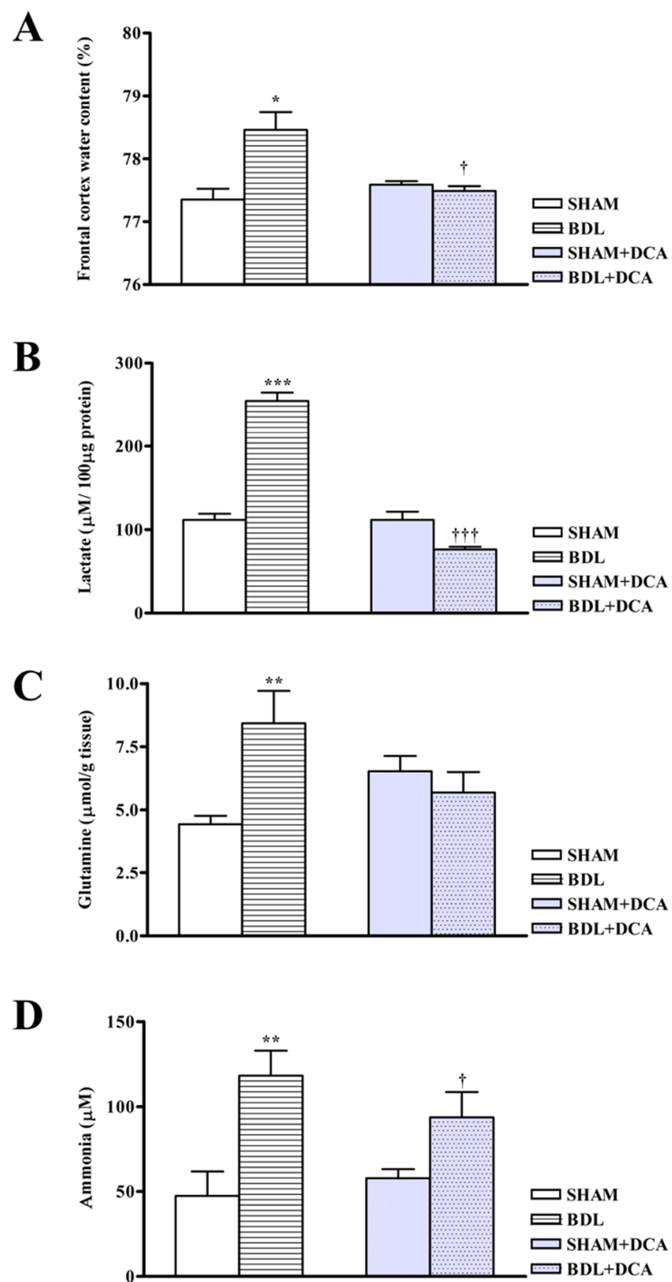

Figure 3. Effect of dichloroacetate (DCA) (lactate synthesis inhibitor): (A) frontal cortex water content; (B) frontal cortex lactate; (C) frontal cortex glutamine and D) cerebrospinal fluid ammonia levels in bile-duct ligation (BDL) rats compared to treated and non-treated SHAM-operated controls and non-treated BDL rats. Data are expressed as mean±SEM. *$p<0.05$,

**p<0.01, ***p<0.001, significantly different from SHAM; †p<0.05,†††p<0.001, significantly different from non-treated BDL.

Cerebral lactate temporal resolution

To thoroughly understand the relationship between ammonia, lactate and glutamine, we monitored the changes of these three pathogenic factors at weeks 2, 4 and 6, and characterized their temporal resolution in relation to the appearance of brain edema. No differences were found between SHAM-operated controls sacrificed at 2, 4 and 6 weeks. After 2 weeks of BDL, neither brain lactate, brain ammonia or brain water content was significantly elevated. However, brain glutamine levels were significantly higher compared to SHAM-operated controls. After 4 weeks of BDL, along with no evidence of brain edema, a significant increase in brain lactate and ammonia was observed (vs. 2 weeks), with a similar increase in the levels of glutamine as at 2 weeks. Six weeks following BDL, brain edema appeared, along with a significant further increase in brain lactate and ammonia, compared to 4 weeks. No further increase in glutamine levels was demonstrated in comparison to weeks 2 and 4 (fig. 4A). Using the data obtained at 2, 4 and 6 weeks after BDL and SHAM-operated controls, a significant correlation was calculated between cerebrospinal fluid ammonia and lactate ($r = 0.5447$, $p<0.05$; fig.4B). Ammonia levels did not significantly correlate with cerebral glutamine ($r = -0.1255$, fig.4C).

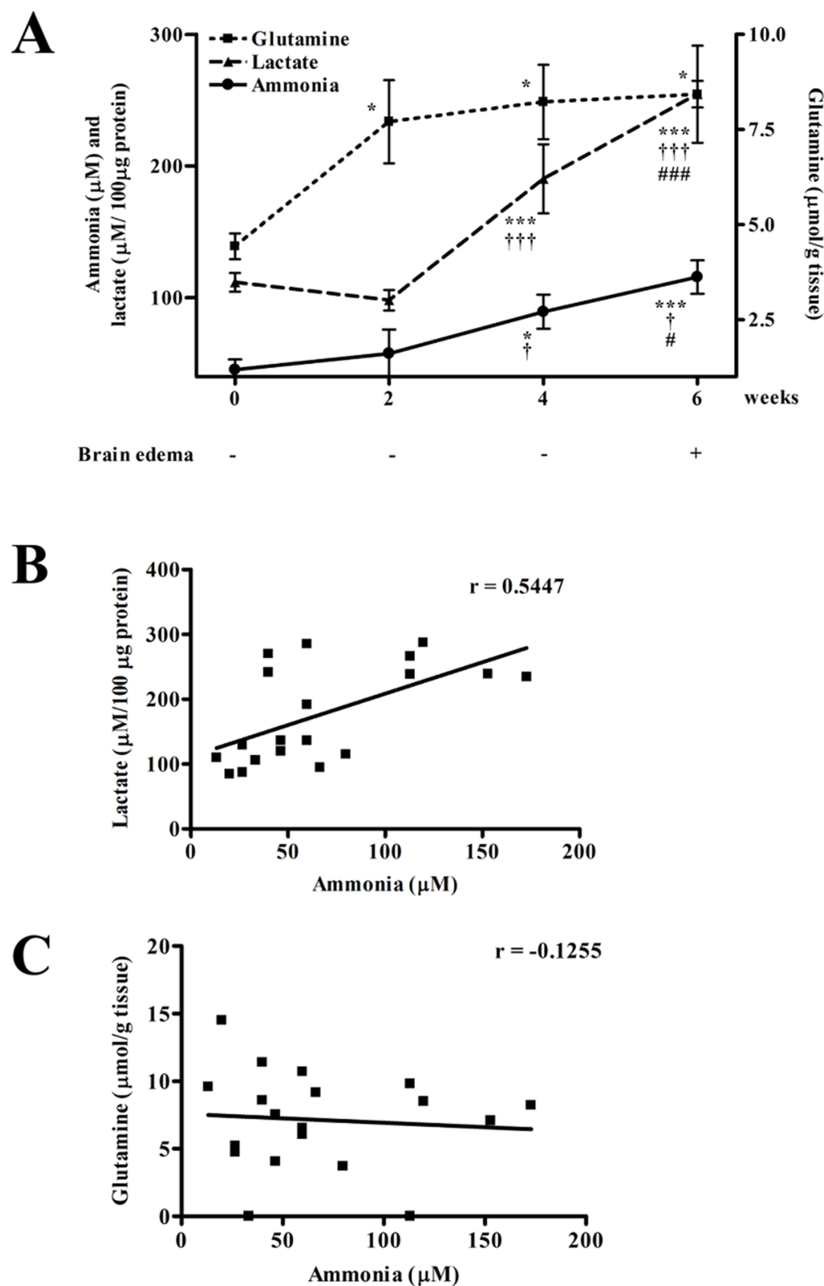

Figure 4. Correlations between lactate, glutamine, ammonia and brain edema. (A) Temporal resolution of lactate, glutamine, ammonia and brain edema over 6 weeks in rats with bile-duct ligation (BDL) compared to respective SHAM-operated controls. The time point 0 represents the value for SHAM-operated controls sacrificed 6 weeks after surgery. (B) Correlation between

changes in cerebrospinal fluid ammonia and brain lactate in BDL rats following 2, 4 and 6 weeks after the intervention. (C) Correlation between changes in cerebrospinal ammonia and cerebral glutamine in BDL rats following 2, 4 and 6 weeks after the intervention. Data are expressed as mean±SEM. *p<0.05, ***p<0.001, significantly different from SHAM; †p<0.05, †††p<0.001, significantly different from BDL 2 weeks; #p<0.05, ###p<0.001, significantly different from BDL 4 weeks.

Discussion

Results of the present study reveal for the first time in the setting of CLD that increased cerebral lactate, and not increased glutamine, is a key factor in the pathogenesis of brain edema. NMR spectroscopy revealed an increase in both lactate and glutamine *de novo* synthesis in the brain from ^{13}C-glucose in cirrhotic rats with brain edema and MHE. The importance of lactate in the development of brain edema was established following the treatment with AST-120. These orally administered carbon microspheres, in addition to attenuating hyperammonemia and normalizing brain water content in BDL rats, also decreased lactate levels in the brain. To confirm the crucial role of lactate, following the reduction of brain lactate levels in BDL rats treated with DCA (lactate synthesis inhibitor), the cerebral content of water was attenuated. Furthermore, following the same treatment regimens, no change in brain glutamine levels was found, suggesting glutamine does not contribute to an increase in cerebral water in cirrhotic rats. Taken together, these findings underscore the importance of lactate over glutamine in the development of brain edema in CLD.

There is substantial evidence that links increased cerebral lactate to severe HE. In patients with fulminant hepatic failure, it has been shown that increases in extracellular lactate correlate with rises in intracranial pressure (Tofteng et al., 2002). These findings have been supported in rats with acute liver failure, whereby using ^{13}C-NMR spectroscopy, it was found that an increase *de novo* synthesis of lactate from glucose correlated with severe HE (Chatauret et al., 2002; Zwingmann et al., 2003), and that the progression from pre-coma to coma stage is associated with the development of brain edema and a marked increase in cerebral lactate (Chavarria et al., 2010). Moreover in acute liver failure, therapeutic interventions, such as mild hypothermia and albumin dialysis, have shown to reduce cerebral lactate along with brain edema and the development of severe HE (coma and intracranial hypertension) (Chatauret et al., 2002; Rose et al., 2007; Sen et al., 2006). Furthermore, in the setting of CLD, a 1.37-fold increase in lactate in the cerebrospinal fluid has been found in patients with end-stage liver disease and overt/severe HE (grades 3 and 4) (Yao et al., 1987).

Chronic hyperammonemic rats (induced following 4-week portacaval anastomosis) injected with a toxic dose of ammonia precipitates severe HE (coma), which is accompanied with brain edema and an increase in brain lactate (Hindfelt et al., 1977; Therrien et al., 1991). However, notwithstanding concrete evidence associating elevated concentrations of brain lactate and severe HE (intracranial hypertension, coma), the role of lactate in the pathogenesis of MHE remains elusive.

MHE is a clinically important entity that affects up to 80% of patients with end-stage liver disease, placing them at a 4-times higher risk of developing overt HE (Hartmann et al., 2000). For this, our study describes, for the first time, the implications of lactate in the pathogenesis of brain edema and cirrhosis-induced MHE. Our results demonstrate that increased cerebral lactate due to *de novo* synthesis from glucose plays a vital role in the development of brain edema in cirrhotic rats. Interestingly, the overall increase in ^{13}C-labelled *de novo* synthesis of lactate in BDL rats with brain edema and MHE is 1.7-fold, compared to the 4.0-fold increase observed in acute liver failure rats with brain edema and severe HE (coma) (Zwingmann et al., 2003). Moreover, brain edema is attenuated following DCA treatment.

These results, together with previous data arising from rats with acute liver failure, may suggest brain lactate not only plays a significant role in the development of brain edema, but might also correlate with the severity of HE. It has been proposed in the setting of CLD, where intracranial hypertension is rarely observed, that the degree of brain edema is of "low-grade" (Häussinger, 2006). This implies a strong relationship between lactate levels, degree of brain edema and severity of HE.

Liver failure leads to a significant reduction in the capacity to detoxify ammonia and, as a result, the developing hyperammonemia causes a rise in brain ammonia levels. Ammonia toxicity has been demonstrated to lead to an increase in lactate by inhibiting enzyme α-ketoglutarate dehydrogenase in the TCA cycle (Lai and Cooper, 1986). This in turn stimulates

glutamate dehydrogenase, an alternative pathway to remove ammonia through the amidation of α-ketoglutarate to glutamate and subsequently to glutamine. Our results demonstrate an increase in glucose-derived glutamine in BDL rats, supporting stimulation of this pathway. In addition, we observed an increase in glucose-derived lactate, and an increase in glycolysis flux, possibly a result of ammonia-stimulated phosphofructokinase activity (Lowry and Passonneau, 1966). However, in spite of these TCA cycle alterations (inhibition of α-ketoglutarate dehydrogenase), ATP levels were maintained (Fitzpatrick et al., 1988; Mans et al., 1994). Therefore, ammonia-induced increase in brain lactate is a not a result of energy failure (activated anaerobic metabolism); rather, the increase in lactate synthesis may occur as a compensatory mechanism to maintain ATP levels.

It is well documented that the shuttling of lactate between astrocytes and neurons plays an important role in brain physiology. Astrocyte-derived lactate is used by surrounding neurons as an energy substrate, coupling cerebral glucose metabolism to neuronal activity (Pellerin and Magistretti, 2012). Hence, dysregulation of the astrocyte-neuron lactate shuttle due to changes in lactate metabolism results in altered lactate homeostasis and leads to brain edema and cerebral dysfunction. Indeed, affecting lactate homeostasis in the brain can lead to differential lactate compartmentalization and changes in osmolarity. It has previously been shown that exposure of astrocytes (cell type shown to selectively exhibit swelling in HE) to pathophysiologically relevant concentrations of lactate can lead to significant swelling (Staub et al., 1990). Furthermore, increased lactate production not only leads to osmotic stress, but also generates more water per ATP formed than oxidative phosphorylation (Preuss, 2012). This supports our results that, in the brains of BDL rats, an increase in lactate synthesis is a pivotal factor in the development of brain edema.

In an effort to remove ammonia from the brain, the brain depends on the enzyme glutamine synthetase (GS), which is specifically found in astrocytes (Martinez-Hernandez et al., 1977). Using NMR and administering ^{13}C-labelled glucose, the *de novo* synthesis of metabolites from glucose can be quantified by evaluating the position of ^{13}C. This helps

distinguish if glutamine is synthetized from ^{13}C-labelled glucose via PDH (oxidative pathway found in both neurons and astrocytes) or via PC (anaplerotic pathway found exclusively in astrocytes) (Zwingmann, 2007). In BDL rats, an increase in *de novo* synthesis of glutamine via both PDH and PC was demonstrated, which suggests that both oxidative and anaplerotic pathways were upregulated. This provides evidence that neuronal glucose-derived glutamate is synaptically released (excitatory neurotransmission), and is subsequently captured by astrocytes and expended to detoxify ammonia through GS.

During conditions of hyperammonemia, intracellular glutamine trapping (accumulation) is believed to contribute to astrocyte hypertonicity, astrocyte swelling and brain edema (Brusilow and Traystman, 1986). It has been previously shown that exposing primary cultured astrocytes to ammonia results in cell swelling, an outcome that is abolished following the inhibition of GS with methionine-sulfoximine (MSO) (Norenberg and Bender, 1994). Moreover, in ammonia-infused portacaval shunted rats, pre-treatment with MSO leads to an attenuation of cerebral glutamine levels and ameliorated brain edema (Master et al., 1999). However, in addition to its osmotic influence, glutamine is believed to be toxic and has demonstrated to impair mitochondrial function through opening of the mitochondrial transition pore, thus causing astrocyte swelling (Albrecht et al., 2010). In the present study, we found glutamine levels to be persistently high following brain edema resolution with treatments AST-120 and DCA. This suggests glutamine accumulation is not an important pathogenetic factor in the development of brain edema in HE. Interestingly, this observation has also been described in animal models of acute liver failure, in which high cerebral glutamine levels persisted following ammonia-lowering treatments and resolution of ICP, brain edema and HE (Chatauret et al., 2002; Zwingmann et al., 2004). Moreover, 4-week hyperammonemic portacaval-shunted rats, with an increase in brain glutamine, do not develop brain edema (Bosoi et al., 2012). Therefore, taken together, there is strong, accumulating evidence dictating that increased cerebral glutamine does not play a vital role in the development of brain edema in liver failure.

Myo-inositol and taurine, considered to be major organic osmolytes, play an important role in cell volume regulation. They are released into the extracellular space, compensating for intracellular hypertonicity, thus preventing cell swelling and the development of brain edema (Heins and Zwingmann, 2010). In hyperammonemic portacaval shunted rats, it is stated that the reason brain edema does not develop, even in the presence of increased brain glutamine, is a result of a substantial compensatory decrease in myo-inositol, taurine and glutamate (Cordoba et al., 1996). Interestingly, also in portacaval shunted rats, lack of brain edema is accompanied with no increase in brain lactate (Therrien et al., 1991). In the present study, 6-week BDL rats developed brain edema, along with increases in both brain glutamine and lactate. A decrease in brain myo-inositol, but no significant change in taurine, was observed. As a result, higher osmolarity was calculated in BDL rats compared to SHAM-operated controls. This suggests that impaired brain osmoregulation, possibly the result of exhaustive release of osmoregulators, cannot compensate for the increase in both brain glutamine and lactate. This osmolyte profile has been similarly observed in rats with acute liver failure, where the sum of all brain osmolytes (including glutamine) exceeded the decrease in myo-inositol and taurine at coma stage (in the presence of brain edema).

To further understand the relation and interplay between lactate, glutamine and ammonia in the development of brain edema, we studied the temporal resolution of these factors in 6-week cirrhotic rats. Two weeks following BDL, brain edema was not present. A surge in cerebral glutamine was observed with no significant elevation in ammonia or lactate. At 4 weeks, with brain edema still not present, a significant increase in ammonia and lactate was detected with no further increase in glutamine observed. At 6 weeks, the appearance of brain edema was associated with an additional rise in ammonia and lactate levels, but no further rise in glutamine levels. Taken all together, a significant correlation was found between brain ammonia and lactate, but not between brain ammonia and glutamine. The sudden rise in glutamine at 2 weeks, followed by no significant additional increase at weeks 4 and 6 may be the result of saturation or inhibition of GS activity (Cooper et al., 1985; Desjardins et al.,

1999; Kanamori et al., 1996; Schliess et al., 2002a). A reduction in GS activity is not due to glutamate being a limiting factor, since increases in brain glutamate were found in BDL vs. SHAM-operated rats (Table I). Our results sustain that increased brain lactate (and not brain glutamine) is a consequence of hyperammonemia-induced increased brain ammonia which leads to brain edema. In the context of HE, increased glutamine levels are commonly observed even when brain edema is not present, as observed in portacaval-shunted rats (Cordoba et al., 1996). However, in other neurological diseases such as cerebral ischemia, increased brain lactate levels have been associated with the development of brain edema, while brain glutamine levels were decreased (Kamiya et al., 1993; Nonaka et al., 1998; van der Zijden et al., 2008). Although our study was performed in frontal cortex, it is possible other cerebral regions may be affected dissimilarly. For example, it was demonstrated in cerebellum of rats with acute liver failure that brain edema precedes an increase in lactate (Cauli et al., 2011). Different regions within the brain in relation to lactate levels and water content remain to be investigated.

In conclusion, the present study demonstrates for the first time that an increase in brain lactate, and not brain glutamine, is a pivotal factor involved in the pathogenesis of brain edema in end-stage liver disease. In addition, the results of the present study also suggest that impaired compensatory osmoregulatory mechanisms may be a contributing factor in the development of brain edema in CLD. Currently, lowering ammonia levels represents the primary treatment strategy in patients with HE and CLD (Rose, 2012). The present results demonstrate AST-120 is an efficient ammonia-lowering strategy as has been previously demonstrated (Bosoi et al., 2011). However, in addition, the results of the present study also reveal DCA as a potential treatment of HE. This lactate synthesis inhibitor has previously demonstrated a long-term safety profile in patients with congenital lactic acidosis (Abdelmalak et al., 2013) and beneficial effects with no adverse reactions in other diseases such as cancer (Strum et al., 2013) and chronic obstructive pulmonary disease (Calvert et al., 2008). Therefore, DCA can rapidly provide a promising therapeutic approach for the

management of patients with end-stage liver disease.

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Chapter 3: Discussion

3.1 Role of ammonia in the pathogenesis of brain edema in MHE during CLD

Ammonia and HE

Ammonia has been long considered the main pathogenic factor involved in the pathogenesis of HE. Since the liver represents the main ammonia detoxifying organ, a decrease in liver function results in increased circulating ammonia levels. Increased ammonia has been documented in both ALF and CLD along with neurological dysfunction characterized by an increased ICP (in ALF) leading to coma and death, with varying symptoms defining grade of HE based on the West Haven criteria (tableI – page 4) in CLD (Bernal et al., 2007; Clemmesen et al., 1999; Ong et al., 2003; Quero et al., 1996). Interestingly, in these studies blood ammonia levels are around 200 µM in both ALF and CLD. Furthermore, healthy individuals receiving a continuous ammonium acetate infusion for 4h reached a plateau of ~ 200 µM of blood ammonia after 1h, peaking at 225 µM after 4h. This concentration of ammonia did not affect their performance of neuropsychological tests (Wilkinson et al., 2011). These evidences confirm that ammonia levels are poorly correlated with severity of HE and that other pathogenic factors are involved in the onset of HE.

Since ammonia can easily cross membranes, blood-derived ammonia enters into the brain and exerts several neurotoxic effects: the direct effects of ammonia on pH, membrane potential and metabolic reactions lead to a cascade of events including energy metabolism alterations, glutamine increase, OS induction and others not evaluated in the present thesis. These effects were demonstrated *in vitro* and in animal models of ALF where ammonia concentrations are high (1-5 mM, much higher than those seen in CLD patients). However the consequences of lower ammonia in CLD (150-250 µM, 2-3-fold higher vs normal (Ong et al., 2003)) remain elusive.

Ammonia and brain edema

In our study, moderate hyperammonemia (125–250 µM) in PCA rats without extensive liver failure, does not lead to brain edema (Bosoi et al., 2012). However, it has been shown in the same model that by increasing blood ammonia to 1 mM (using iv infusion of ammonia), brain edema and severe HE develop (Cordoba et al., 1996). Whereas in BDL rats with hyperammonemia, brain edema, and hepatic dysfunction, lowering arterial ammonia levels with AST-120 (from 180 to 81 µM) leads to attenuation of brain edema and improvement of locomotor activity (Bosoi et al., 2011). In this model, the temporal resolution of ammonia following BDL surgery is the following: after 2 weeks there is no significant change in blood ammonia vs SHAM-operated controls; 4 weeks there is a significant increase and after 6 weeks there is a further significant rise (vs 4 weeks) (Bosoi et al., 2012). Brain edema appears at 6 weeks and not at 4 weeks however it has been reported at 5 weeks (Davies et al., 2009). In the 4-6 weeks interval, the further increase in ammonia along with the concomitant increase in other liver disease related factors contribute to the development of brain edema.

We used AST-120 as a tool to reduce ammonia and evaluate the effect of brain edema in 3 different settings. First, AST-120 was administered for 6 weeks, starting at day 1, (when hyperammonemia was not present) and therefore ammonia levels did not significantly rise throughout the 6-week model. Second, AST-120 treatment was administered for 2 weeks, starting 4 weeks after surgery, at a moment where ammonia levels were high but brain edema not yet present. Third, a 3 day treatment started at 39 days, a time point where both hyperammonemia and brain edema were present. Following all 3 treatment regimens, ammonia was reduced and the apparition of brain edema was prevented. These results sustain the importance of ammonia in inducing brain edema and also demonstrate the efficacy of AST-120 both as a long and short-term treatment (Bosoi et al., 2011).

Conclusion

All these evidences indicate ammonia independently does not induce brain edema however it plays an important role in the pathogenesis of brain edema in MHE during CLD. As explained above, the level of ammonia is important, but the period needed to attain high ammonia levels, may allow defense mechanisms to develop differently, therefore differences between animal models exists. This is most probably due to a synergistic action with other factors ensued as a consequence of liver disease. Moreover, the nature and severity of HE depends on the degree and acuteness of hyperammonemia.

3.2 Role of lactate in the pathogenesis of brain edema in MHE during CLD

Lactate and HE

Lactate is produced from pyruvate by LDH. Although this reaction was believed to occur mostly during hypoxic conditions, new data suggest that lactate is a preferred metabolic fuel by the brain over glucose under normoxic conditions. Elevated systemic lactate is a prognostic marker of ALF (Bernal et al., 2002) and cerebral lactate is consistently found increased in ALF patients but also in animal models of acute hyperammonemia or ALF (Fitzpatrick et al., 1989; Rose et al., 2007; Tofteng et al., 2002; Yao et al., 1987).

Increased cerebral lactate is related to the development of brain edema and ICP as manifestations of severe HE in ALF patients (Tofteng et al., 2002) and in ALF animals (Chatauret et al., 2002; Chavarria et al., 2010; Zwingmann et al., 2003). In patients with cirrhosis, only one study has shown an increase in brain (cerebrospinal fluid) lactate, in patients with HE grade 3-4 (Yao et al., 1987). Brain edema was not evaluated in this study.

Our study is the first to describe an increase in cerebral lactate in an animal model of MHE. Moreover, we demonstrated that increased cerebral lactate is central in the

development of brain edema in CLD as by directly inhibiting lactate synthesis with DCA, brain edema is prevented. In ALF rats with coma, lactate levels are increased to ~20-fold vs SHAM-operated controls (Zwingmann et al., 2003). In our CLD model of MHE, lactate levels were ~3-fold increased vs SHAM-operated controls (Bosoi et al., 2014), therefore suggesting a strong relationship between lactate levels, degree of brain edema and severity of HE.

Lactate and ammonia

In ALF patients, a causal link between hyperammonemia and the elevation of cerebral lactate was suggested (Bjerring et al., 2010, 2008). Here, we demonstrated this cause-effect relationship is also valid in CLD (Bosoi et al., 2014). Ammonia rises progressively throughout the 6 weeks of the BDL model and cerebral lactate follows a similar pattern. Moreover, cerebral lactate levels respond promptly and reduce following AST-120 treatment (Bosoi et al., 2014). It is important to note that when lactate synthesis was inhibited following DCA treatment, no effect on ammonia levels was observed, suggesting the ammonia-lactate relationship is unidirectional and increased brain lactate is a consequence of ammonia neurotoxicity.

Cerebral lactate metabolism in MHE

Lactate does cross the BBB through the lactate transporter MCT1 on endothelial cells (Dalsgaard et al., 2004; Gerhart et al., 1997; Oldendorf, 1973; Smith et al., 2003). However, in BDL rats circulating lactate is not increased vs SHAM-operated controls (data not published), therefore cerebral lactate is BDL rats not a consequence of hyperlactatemia. In addition, using nuclear magnetic resonance, we demonstrated that increased cerebral lactate is the result of local cerebral production (Bosoi et al., 2014).

According to the ANLS hypothesis, lactate is primary produced by astrocytes, released extracellularly and taken up by neurons where it is used a fuel for the TCA cycle

(Pellerin et al., 2007; Schurr, 2006). According to this hypothesis, glucose taken up by astrocytes from the systemic circulation is metabolized to lactate by the LDH5 enzyme and released extracellularly by MCT1 and MCT4. From here, lactate is taken up by neuronal MCT2 and metabolized back to pyruvate by LDH1 in order to fuel the TCA cycle.

There is scarce evidence about any alterations of the ANLS in HE or hyperammonemia. Only one study evaluated LDH activity in ALF pigs treated with albumin dialysis (MARS). LDH activity increased in ALF pigs along with cerebral ammonia and lactate; following MARS treatment a decrease in extracellular ammonia and lactate (using cerebral microdialysis) was observed, however LDH activity remained high (as observed in non-treated ALF) compared to sham-operated controls (Rose et al., 2007). This implies a decrease of lactate from the extracellular space may be due to increased neuronal utilization of lactate facilitated following ammonia reduction. Modifications of LDH and MCT isoforms in different settings of hyperammonemia and HE merit to be investigated.

Lactate and brain edema

Brain edema during HE is defined by astrocyte swelling. Lactate is known to induce astrocyte swelling *in vitro* (Staub et al., 1990). In addition, lactate plays a major role in the development of brain edema in ALF (Chavarria et al., 2010). In our study, the inhibition of lactate synthesis in CLD rats prevented the apparition of brain edema (Bosoi et al., 2014). However, the underlying mechanisms by which lactate induces brain edema or cell swelling remain unknown. It has been speculated that alterations in lactate homeostasis (and/or altered ANLS) can lead to lactate-induced osmotic changes and hence cell swelling (Preuss, 2012).

Conclusion

These evidences demonstrate that increased cerebral lactate is a direct effect of hyperammonemia and that cerebral lactate plays an important role in the pathogenesis of

brain edema in MHE during CLD.

3.3 Role of glutamine in the pathogenesis of brain edema in MHE during CLD

Glutamine and HE

Glutamine is an important ammonia detoxification product following its incorporation to glutamate by the enzyme GS found in the brain exclusively in astrocytes. Therefore, an increase in cerebral glutamine is present during hyperammonemia (Chatauret et al., 2002; Fries et al., 2014; Hourani et al., 1971; McConnell et al., 1995).

Glutamine and brain edema

Glutamine is an osmotic molecule and its cerebral accumulation leads to an increase in cerebral water (Brusilow and Traystman, 1986). It is believed that increased astrocytic GS and hence an accumulation of intracellular glutamine leads to development of astrocyte swelling and brain edema. However, the evidences of glutamine playing a role in brain edema development are contradictory. The following studies support glutamine plays an important role in the onset of brain edema. Administration of the GS inhibitor MSO attenuates the increase in glutamine as well as the swelling both in cultured astrocytes and PCA rats treated with ammonia (Master et al., 1999; Norenberg and Bender, 1994; Willard-Mack et al., 1996). Also, few studies demonstrated a positive correlation of glutamine with severity of HE (Hourani et al., 1971; Laubenberger et al., 1997). Evidences suggesting glutamine does not have a role in the development of brain edema are: in ALF rats following liver devascularization, cerebral glutamine levels do not vary between precoma and coma stages in spite of an increase in brain water content (Chavarria et al., 2010; Zwingmann et al., 2003). Moreover, different ALF treatments such as hypothermia, albumin dialysis or L-ornithine

phenylacetate lead to a significant attenuation of brain edema but not to a decrease of cerebral glutamine levels (Sen et al., 2006; Ytrebø et al., 2009; Zwingmann et al., 2004). Moreover, PCA rats present increased glutamine levels, but no brain edema (Master et al., 1999). In BDL rats the MSO treatment results in persisting high glutamine levels, however brain edema was not evaluated in this study, therefore the effect of MSO remains elusive (Fries et al., 2014).

The results of the present thesis add to the data questioning the correlation of glutamine with brain edema and HE severity. In our study lowering ammonia or lactate levels did not normalize brain glutamine levels, in spite of attenuating brain edema (Bosoi et al., 2014), implying that cerebral glutamine does not play a vital role in the development of brain edema in CLD.

Cerebral osmolytes and brain edema

A cell where a pathological process leads to an increase in osmolytes is capable of compensating this increase by eliminating others, therefore maintaining an osmotic equilibrium and preventing swelling. *In vitro*, in ammonia exposed astrocytes the increase in glutamine is followed by a decrease in other osmolytes such as myo-inositol and taurine (Zwingmann, 2007). It is believed that in hyperammonemic PCA rats brain edema does not develop due to a decrease in myo-inositol, taurine and glutamate which compensates the increase in glutamine, re-balancing the osmotic equilibrium as reflected by a constant sum of all osmolytes compared to controls (Cordoba et al., 1996). In PCA rats, osmolyte levels were maintained in tissue and in CSF, suggesting osmolytes are released not only from astrocytes but also from the brain. In ALF, brain edema ensues because these mechanisms are overwhelmed and the sum of all brain osmolytes significantly increases at coma stage in the presence of brain edema compared to controls (Zwingmann et al., 2004). We describe a similar effect in rats with CLD where increases in brain glutamine and lactate were not compensated by decrease in brain myo-inositol, thus resulting in a higher osmolarity and consequently brain edema (Bosoi et al., 2014). This mechanism does not suffice to fully

maintain the osmotic equilibrium (table I).

Table I. Variation of cerebral organic osmolytes and their sum in relation to brain edema in rats with chronic hyperammonemia induced by portacaval anastomosis (PCA), acute liver failure following liver devascularization (ALF) and cirrhosis induced by bile-duct ligation (BDL).

Metabolite	PCA rats (Cordoba et al., 1996)	ALF rats (coma stage, Zwingmann et al., 2004)	BDL rats (Bosoi et al., 2014)
Glutamine	↑	↑	↑
Glutamate	↓	↓	↑
Taurine	↓	↓	=
Myo-inositol	↓	↓	=
Sum	=	↑	↑
Brain edema	-	+	+

Conclusion

Increased cerebral glutamine does not play an important role in the pathogenesis of brain edema in MHE during CLD. Moreover, the compensatory release of other cerebral osmolytes fails to maintain osmotic equilibrium.

3.4 Role of oxidative stress in the pathogenesis of brain edema in MHE during CLD

OS during CLD has been stated as a systemic phenomenon (Chen et al., 1997; Ljubuncic et al., 2000) which results as an imbalance between an increase in liver-disease related oxidant mechanisms (XO) and a decrease in hepatic antioxidant production (GSH and albumin).

Cerebral OS and ammonia

Numerous studies have demonstrated an important role of OS in the pathogenesis of brain edema in HE. Ammonia (>500 µM) induces OS and swelling in cultured astrocytes, however lower concentrations (100-200 µM) do not (Görg et al., 2008; Murthy et al., 2001). This increase in ROS leading to astrocyte swelling is reduced following antioxidant treatments (Jayakumar et al., 2006), suggesting that ammonia-induced astrocyte swelling is linked to the generation of ROS. *In vivo*, acute ammonia intoxication (intraperitoneal injection of 12 mmol/kg of ammonium acetate in naïve rats) induces central OS by decreasing antioxidant defense (Kosenko et al., 2003). In ALF rats both systemic and central OS are present along with high ammonia levels (reaching up to 1 mM, Jiang et al., 2009a; Sathyasaikumar et al., 2007). In CLD, cerebral OS markers were found in brains of patients that died with severe HE (grade 3 and 4, (Görg et al., 2010)). Interestingly, in this study the mean blood ammonia concentration before death was 150 µM, suggesting that cerebral OS might be a consequence of another factor related to liver failure rather than a result of hyperammonemia. In our study, only a significant increase in the activity of glutathione reductase was found in brains of both hyperammonemic and cirrhotic rats (Bosoi et al., 2012). Since all the other markers were not changed, this increase may suggest subtle OS modifications which are well controlled, preserving an oxidant-antioxidant balance. We concluded that, in MHE due to CLD, cerebral OS is not present. The above observations indicate that cerebral OS is strongly related to

severe HE and coma. One possible explanation is that in severe HE due to ALF ammonia levels rise higher and faster versus CLD (to values higher than 1 mM versus 200 µM), therefore cerebral OS develops as a direct effect of hyperammonemia.

Systemic OS and ammonia

We assessed OS markers both systemically and centrally. Moreover, in order to fully understand the origins and effects of OS, we studied all aspects of OS. We assessed ROS per se as well as antioxidant defense mechanisms, but also oxidant triggers related to liver and cirrhosis as well as the effects of OS on proteins and lipids. We used PCA and BDL rats, 2 MHE animal models where ammonia reaches on average 200 µM.

In PCA rats, no evidence of systemic OS was found in association with the absence of central OS. However, in cirrhotic BDL rats, systemic OS was evidenced in the absence of central OS. ROS increased within 2 weeks following BDL and remained constantly high, while ammonia levels increased progressively throughout the time-line of the model. In addition, treating BDL rats with the XO inhibitor allopurinol decreased circulating ROS without affecting ammonia levels (Bosoi et al., 2012). Moreover, following ammonia reduction with AST-120, circulating ROS did not decrease (Bosoi et al., 2011). This sustains there is no direct relationship between systemic OS and hyperammonemia, the former being a consequence of hepatic necrosis and the consequent release of oxidant enzymes such as XO.

Systemic OS and brain edema

OS independently did not lead to brain edema as demonstrated following AST-120 treatment in BDL rats which lowered ammonia and attenuated brain edema, but did not alter ROS (Bosoi et al., 2011). The role of systemic OS was confirmed following allopurinol treatment in BDL rats which attenuated ROS and brain edema, but ameliorated only partially the locomotor activity, suggesting remaining factors contribute to neurological dysfunction

(Bosoi et al., 2012).

Since, ROS levels are increased throughout the 6 weeks of the BDL time line, we proposed systemic OS to be an important "first hit" which sensitizes the brain. Hyperammonemia develops 4 weeks after surgery and acts as a "second hit" therefore leading to the development of brain edema (Bosoi et al., 2012).

Synergistic effect of systemic OS and ammonia in the pathogenesis of brain edema

The facts that i) hyperammonemic PCA rats do not develop brain edema; ii) ammonia and OS are associated with brain edema in BDL rats; iii) following AST-120 treatment in BDL rats, OS independently did not induced brain edema, and iv) following allopurinol treatment in BDL rats, ammonia independently did not induced brain edema strongly suggest a synergistic relationship between ammonia and OS in the pathogenesis of brain edema in MHE during CLD. The synergistic relationship was confirmed following OS induction in PCA rats. After a chronic (10 days) treatment with DEM, only systemic, not central markers of OS were increased and consequently, brain edema was present (Bosoi et al., 2013). In SHAM-operated rats, a 4 times higher dose of DEM was needed in order to overcome the antioxidant defense and induce the same amount of ROS as in PCA rats. Following OS induction by DEM treatment, brain edema developed only in PCA rats and not SHAM-operated controls, proving that hyperammonemia renders the brain more susceptible to oxidative stress. The underlying mechanisms remain to be explored.

This synergistic effect explains the poor correlation of ammonia with MHE, as sustained by a study in cirrhotic patients which investigated the role of systemic OS in the development of MHE (Montoliu et al., 2011). It was performed in two different Spanish centers on 212 subjects either controls, cirrhotics without MHE and cirrhotics with MHE. The study established the plasmatic OS marker 3-nitrotyrosine as being able to dissociate cirrhotic

patients with MHE from those without MHE, in spite of similar ammonia levels (152±9 in the MHE vs 139±7 μM in the non-MHE cohort). Plasmatic 3-nitrotyrosine was 8.6 fold increased in patients with MHE (figure 1). Although this study did not evaluate brain edema, its association with MHE was already proven by numerous studies (Córdoba et al., 2001; Häussinger, 2006; Häussinger et al., 2000; Kale et al., 2006; Shah et al., 2008; Sugimoto et al., 2008), strongly suggesting a role of systemic OS in the pathogenesis of brain edema in CLD patients with MHE.

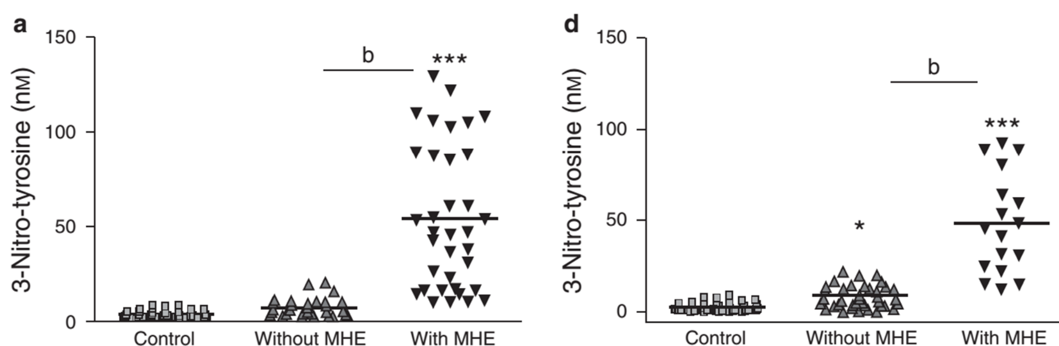

Figure 1. Individual data of serum 3-nitro-tyrosine concentrations for each patient and control. a) Data for patients from the Hospital Clinico; d) Data for the Hospital Arnau de Vilanova. These tables show the mean values and statistical analysis. Values significantly different from controls: *$p < 0.05$; ***$p < 0.001$. Values significantly different between patients with or without MHE: b$P < 0.001$. After Montoliu et al., 2011 with the publisher's permission.

Conclusion

The above evidence establishes that ammonia and OS independently do not lead to brain edema during MHE due to CLD, however together they have a synergistic effect. We can state that the degree and/or the acuteness of the onset of hyperammonemia is important for the induction of OS and that an oxidatively stressed brain is associated with severe HE (figure 2).

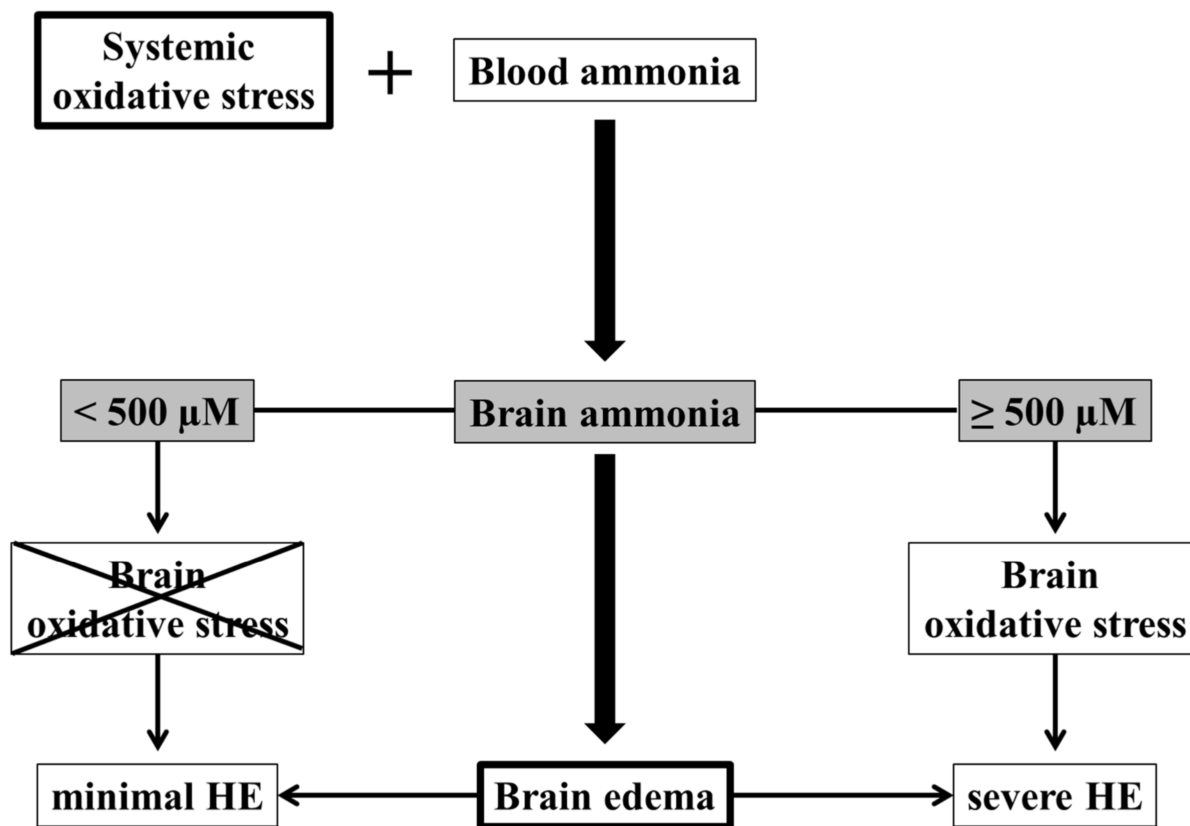

Figure 2. A synergistic effect between ammonia and systemic oxidative stress is proposed in the pathogenesis of brain edema in hepatic encephalopathy. Ammonia concentrations <500 µM in the brain do not induce cerebral oxidative stress, and lead to minimal hepatic encephalopathy. Ammonia concentrations ≥500 µM provoke oxidative stress in the brain, causing severe hepatic encephalopathy. After Bosoi and Rose, 2013 with the publisher's permission.

Multifactorial pathogenesis of brain edema during HE due to CLD

In conclusion, brain edema in HE has a multifactorial pathogenesis. Other factors aside ammonia have been shown to contribute to its pathogenesis. Inflammation is an important factor, demonstrated to play an important role in inducing brain edema and to act synergistically with ammonia (Wright et al., 2007). Inflammation is strongly associated with

OS, but this relationship remains poorly described. This thesis establishes that during CLD induces numerous other factors such as alterations of lactate metabolism, or systemic OS released as a consequence of liver failure also play a role in the pathogenesis brain edema; on the other hand osmotic changes triggered by increases in glutamine are not important as reviewed by Bosoi and Rose (Bosoi and Rose, 2013).

3.5 Role of the BBB in the pathogenesis of brain edema in MHE during CLD

The BBB is a highly selective structure whose role is to control the flux of molecules between the systemic circulation and the brain. Endothelial cells connected by tight junctions, resting on a basal lamina, as well as astrocytes and pericytes contribute to providing neurons with the demanded molecules.

BBB breakdown in MHE following CLD

We believe that the BBB plays an important role in protecting the brain from systemic ROS. ROS are known as key mediators of BBB breakdown, by inducing oxidative damage, modifying tight junctions and activating matrix metalloproteinases (Pun et al., 2009). However, we demonstrated there is no breakdown of the BBB since no brain extravasation of Evans blue and sodium fluorescein in BDL rats was observed (Bosoi et al., 2012). This fact is sustained by Wright et al., who also demonstrated the anatomical integrity of the BBB using electron microscopy (Wright et al., 2007). Moreover, we measured tight junctions expression in BDL rats and found no difference vs SHAM-operated controls (Huynh et al., 2011).

BBB permeability in MHE following CLD

An intact BBB allows the passage of molecules transcellularly and not paracellularly. Many transporters and channels exist along the BBB which regulate cellular ionic homeostasis and volume control and therefore disturbances in this well-regulated water homeostasis leads to deleterious effects on brain function. Since ROS's main role is as signaling molecules, the hypothesis arises that ROS by activating different pathways induce posttranslational modifications of such transporters or channels, thus leading to BBB hyperpermeability.

For example, the function of water channels AQP4 is coupled with K^+channels (Nagelhus et al., 1999; Rao et al., 2011). As mentioned in chapter 3.1.1, the latter can transport ammonia (NH_4^+), therefore together these mechanisms could contribute to brain edema. An increase in expression of AQP4 in the brain, in association with brain edema, has been demonstrated in both ALF mice (Eefsen et al., 2010) and cirrhotic BDL rats (Wright et al., 2010). However, the last study reported inconsistent results in other animal models since brain edema was not related with an increase in brain AQP4 in galactosamine treated or chronic hyperammonemic rats (induced by hyperammonemic diet) (Wright et al., 2010). Further investigations on the role of AQP4 in brain edema related to MHE are needed.

Another transporter at the BBB is NKCC, a Na^+-K^+-$2Cl^-$ co-transporter, a protein positively regulated by a number of kinases such as OSR1; oxidative stress response1 which is upregulated during OS conditions (Anselmo et al., 2006). Our group found an increase in NKCC1 mRNA in relation to brain edema in BDL rats (Huynh et al., 2011). The sulfonylurea receptor 1 regulated $NC_{Ca\text{-}ATP}$ channel is implicated in oncotic cell swelling and oncotic (necrotic) cell death. Only one study investigated the role of this channel in HE and found an increase in sulfonylurea receptor 1 in rats with ALF (Jayakumar et al., 2014). A definitive role of NKCC, $NC_{Ca\text{-}ATP}$ and other similar transporters in BBB permeability related to HE remains to be investigated.

A temporal resolution of BBB permeability during the time course of the BDL

model may also differently affect the development of brain edema. A decreased blood-to-brain transfer constant of [^{14}C]α-aminoisobutyric acid and [^{14}C]dextran was evidenced in several brain regions in BDL rats 5 days after surgery (Wahler et al., 1993). This parameter reflects a decrease in membrane fluidity which may be translated into a nonspecific decrease in BBB permeability.

Conclusion

The BBB remains anatomically intact in MHE during CLD, therefore the origin of brain edema is of cytotoxic rather than of vasogenic origin. An increase in transcellular permeability by the action of blood-derived OS on different channels and transporters remains an interesting pathway to investigate.

3.6 Role of brain edema in MHE during CLD

Brain edema and MHE

Brain edema is a constant finding in animal models or patients with HE due to CLD. However, the role of brain edema in the clinical picture of MHE, whether it represents a neuropathological feature of HE or a cause of HE, remains a controversial topic. Brain edema may inflict differential effects (physical stress as well as metabolic alterations leading to dysregulated astrocyte-neuronal communication) on cerebral function and since MHE patients have a 4-fold increased risk of developing overt HE (Hartmann et al., 2000), brain edema may be predisposing to this risk. Moreover, it may also represent a pathogenic factor of HE.

Evidence proving brain edema is associated to neurological dysfunction in HE during MHE

Numerous studies indicate a strong association between brain edema and HE. Several studies in cirrhotic patients have showed a positive correlation between brain edema (evaluated by different magnetic resonance techniques) and scoring in neuropsychological tests (Kumar et al., 2008; Sugimoto et al., 2008). Following liver transplantation in patients with MHE, a decrease in brain edema was associated with an improvement in cognitive function (Rovira et al., 2007). This association is sustained by the results of this thesis. In our studies, both ammonia lowering (AST-120) and antioxidant (allopurinol) treatments in BDL rats attenuate the development of brain edema, along with amelioration of neurological status confirmed by improvement of locomotor activity (Bosoi et al., 2012, 2011). Interestingly, following both treatments, locomotor activity did not normalize, but improved partially, thus strengthening the synergistic effect of ammonia and oxidative stress in the pathogenesis of MHE.

In ALF, brain edema determines an increase in brain volume and ICP, leading to brain stem herniation which represents an important cause of death. Contrary, in CLD and MHE brain edema is present without leading to an increase in ICP. However whether brain edema itself has a direct metabolic contribution to the severe deterioration of neurological function remains unknown.

Evidence questioning that brain edema is associated to neurological dysfunction in HE during MHE

This controversy is based on different studies that demonstrated various effects of brain edema in HE. First, brain edema is present in BDL (type C HE), not in PCA (type B HE), rats, although both are recognized as valid MHE animal models. Increased ammonia in PCA rats leading to HE is a proof of its neurotoxicity. The fact that PCA rats present normal liver function values, while BDL not suggests that several factors induced by liver failure are implicated in the pathogenesis of brain edema and that HE exists even in the absence of the

latter. These facts suggest HE should be classified rather by the type of neurological dysfunction and associated symptomatology, rather by the type of liver dysfunction.

However, a few other well-designed studies question the association between brain edema and HE. Three weeks after the BDL intervention, rats present without brain edema, but with impaired behaviour tests performance (Jover et al., 2006). Whether brain edema is a consequence of these impairments or its appearance later in the model course induces further impairments remains unknown. In the study by Wright and colleagues challenged both BDL (4 week after surgery, before development of brain edema) and sham-operated controls with LPS. Both groups developed brain edema; however, only BDL rats presented alterations of neurological status (Wright et al., 2007). Here, this fact indicates brain edema may act along with other factors in inducing overt HE. Another study demonstrated that treating brain edema in the cortex and brain stem of ALF rats with mannitol treatment did not improve motor tract function (Oria et al., 2010). However, in this study, the authors only evaluated brain water in the cortex and brain stem; therefore, it is possible that the regions implicated in the modulation of the motor tract function (red nucleus, substantia nigra, basal ganglia) were not edematous and therefore would not respond to mannitol treatment. Another study demonstrated the persistence of brain edema for 5 days following clinical resolution of overt HE in cirrhotic patients (Poveda et al., 2010). Whether these patients regressed from overt to MHE remains unknown, however this could explain the persistence of brain edema.

Implication of different cerebral regions

Brain edema was assessed in this study in the frontal cortex, an important region for cognitive function, therefore a region of interest for HE. The brain is a heterogeneous organ, composed of many regions and areas with different tasks and furthermore not all areas of the brain react similarly. The AST-120 study evidenced that white matter is more amenable to therapy than gray matter (Bosoi et al., 2011), an observation previously demonstrated in

pigs with ALF (Sen et al., 2006). Numerous imaging studies observed differential swelling of various brain regions in patients with different HE grades (Qi et al., 2013; Shah et al., 2008; Sugimoto et al., 2008). These studies conclude that grey matter water content is much less altered in HE as compared to white matter and, with increasing grade of HE, brain edema affects also the gray matter. Also, some grey matter containing regions such as the basal ganglia, the putamen or the globus pallidus exhibit significant changes in water content in HE patients compared to other regions. This difference in becoming edematous between white and grey matter may be explained by the different regulation of neurotransmitters in different cerebral regions. As detailed in chapter 3.1.4, neurotransmitter changes differ widely in HE among cerebral regions. Of the above mentioned neurotransmitters, acetylcholine seems to have an important effect on white matter, as its metabolism is more intense in white matter regions than the metabolism of other neurotransmitters such as glutamate or GABA (Hassel et al., 2008). Moreover, the striatum, a region containing high quantities of white matter known to be affected in HE (Rose et al., 1999a), also contains high concentrations of acetylcholine. Exactly how the cholinergic system affects HE in white matter remains to be investigated.

Implication of different types of astrocytes

Possibly different astrocyte types found in grey/white matter contribute to these differential findings in water content and amenability to treatment. Protoplasmic astrocytes were mainly described in gray matter, with rich branched processes, each surrounding a large number of synapses (~20,000–120,000 in rodents and ~270,000–2 million in humans) (Bignami and Dahl, 1974). In white matter, astrocytes are significantly larger than protoplasmic astrocytes, with equally spaced cell bodies and unbranched straight processes orientated in the direction of axon bundles; they were named fibrous astrocytes (Oberheim et al., 2009). Moreover, protoplasmic and fibrous astrocytes differential pathological implications were described in Parkinson’s disease (Song et al., 2009) and cerebral ischemia

(Lukaszevicz et al., 2002) and the evidence presented in the above paragraph suggest this is also valid for HE. Therefore, this differential implication of cerebral regions might explain the multitude of symptoms present in HE patients.

Importance of imaging studies in defining the role of brain edema in MHE

Evidencing brain edema in MHE patients has been possible with the development of new magnetic resonance imaging (MRI) techniques, which became an important tool in the diagnosing and monitoring the syndrome (McPhail et al., 2012). These techniques evolved from detecting structural into evidencing functional changes, however at present they remain mostly a research tool due to their high cost.

MRI techniques can assess not only the quantity of water in the brain, but also its movement through cellular barriers (the diffusion weighted imaging technique) or distinguish between bound and free water (the magnetization transfer ratio technique). The first technique demonstrated differences in regional (frontal/parietal white matter) water movement between patients with MHE and patients with cirrhosis without neurosychometric impairment. These changes predicted the progression to future overt HE (Sugimoto et al., 2008). Moreover, this technique established that brain edema in HE is of cytotoxic origin and water accumulation affects the intracellular compartment of the brain (Chavarria et al., 2010). The second technique estimates brain water content better than a simple MRI and evidenced brain edema in cirrhotic patients (Rovira et al., 2001).

Functional MRI (fMRI) assesses function of different cerebral regions by measuring local blood flow and the amount of reduced deoxyhaemoglobin during resting states or preset tasks, therefore correlating brain blood utilization and with cognitive impairment. This technique demonstrated aberrant brain activity in different cerebral regions in patients with MHE, especially the occipital lobe, the left frontal lobe and the left cerebellum posterior lobe (Chen et al., 2012; Qi et al., 2012). Moreover, fMRI was able to evidence fatigue, a common symptom in

cirrhotic patients, by evidencing reduced functional brain connectivity in cerebral areas related to fatigue (basal ganglia, putamen, frontal gyrus) in primary biliary cirrhosis patients (Mosher et al., 2014).

Magnetic resonance spectroscopy permits the analysis of metabolites and metabolic processes. The most utilized nuclei is ^{1}H, which is naturally found in tissues and helps quantify choline, creatine, N-acetyl aspartate, glutamine, glutamate, myoinositol and taurine. Artificially synthetized nuclei, injected in the body help track different metabolic pathways. One such nuclei is ^{13}C, which incorporated in glucose identifies metabolic pathways such as glycolysis or the TCA cycle (Zwingmann, 2007). In the present thesis, this technique helped identify an important role of lactate synthesis and reject a role for glutamine in the pathogenesis of brain edema in HE (Bosoi et al., 2014).

Positron emission tomography (PET) also allows the quantification of metabolic processes, using intravenously administered tracers of natural substances or analogs such as ^{15}O-water, ^{15}O-oxygen or ^{13}N-ammonia. This technique brought new insights into the study of cerebral oxygen uptake and blood flow which were found to be reduced cirrhotic patients with clinically overt HE but not in cirrhotic patients with MHE or no HE compared to healthy subjects or into cerebral ammonia metabolism which was shown to be enhanced due to increased blood ammonia in cirrhotic patients (as reviewed by (Keiding and Pavese, 2013)).

Conclusion

In conclusion, brain edema, if present, leads to neurological dysfunction in MHE due to CLD. Since HE is described also in the absence of brain edema (as in hyperammonemic PCA rats), different types of MHE may be described. More sensitive tests for learning and memory, attention, anxiety, motor and sensory function need to be developed both for humans and animal models in order to depict different types of MHE. Also, magnetic resonance studies may help link the modifications of cerebral metabolism to clinical symptoms

and different MHE types.

3.7 Potential emerging therapies for MHE during CLD

The mechanisms explained above led to the emergence of new potential therapies for MHE during CLD.

3.7.1 AST-120

AST-120 consists of engineered activated carbon microspheres (0.2-0.4 mm in diameter) with high nonspecific adsorptive surface area (>1600 m^2/g) for low-molecular-weight compounds (<10 kDa) present in the bowel (Shen 2009). Therefore it specifically targets gut-derived ammonia. It lowered plasma ammonia levels in dogs with PCA (Hiraishi, 1987) and attenuated systemic OS in uremic rats (Owada et al., 2010). Also, preliminary studies in humans have shown neurocognitive improvements in patients with low-grade HE after AST-120 treatment (Pockros et al., 2009).

AST-120 treatment efficiently removed ammonia *in vitro* and *in vivo.* Moreover, *in vivo* it was given as prevention but also as short-term therapy and it adsorbed not only gut-derived but also blood-derived ammonia. The latter is an interesting finding in that it proves ammonia diffuses not only from the gut to the portal system, but also from systemic circulation to the gut following its concentration gradient; in addition it strengthens AST-120 as a gut ammonia sink, capable of clearing blood-derived ammonia. In our rat model, AST-120 was well tolerated and no adverse effects were observed; as sustained by unaltered weight curves and daily protein intake measurements. Moreover, the protective effect on brain edema was not a result of liver function amelioration (Bosoi et al., 2011). AST-120 may be considered a gut ammonia sink which significantly lowers ammonia thus protecting against

the development of brain edema.

Following the results presented in this thesis, AST-120 was evaluated in a multi-center, randomized, double-blind, placebo-controlled phase II clinical trial in the management of MHE during CLD (Mulllen et al., presented at ISHEN 2010). Although this study concluded that AST-120 was safe and well tolerated and efficiently reduced ammonia in MHE patients with elevated ammonia levels, the reduction in ammonia did not correlate with a clinical benefit. The repeatable battery for the assessment of neuropsychological status (RBANS) was used to evaluate MHE. These results remain debated because a learning effect of the required tasks for the test was observed in all study groups; therefore a correct assessment for MHE was not possible. Further studies in humans may prove AST-120 as a gut ammonia-sequestering agent and with potential benefit in the management of MHE during CLD.

3.7.2 Allopurinol

Allopurinol is a structural isomer of the XO substrates hypoxanthine and xanthine. It is oxidized by XO to a more active metabolite, oxypurinol, which acts by irreversibly binding to the active site of the enzyme (Smith et al., 2009). Since XO is a major source of ROS in cirrhosis (Battelli et al., 2001) and an increase in the activity of this enzyme was found in BDL rats, its inhibition seemed a reasonable antioxidant therapeutic target.

Allopurinol treatment in BDL rats indeed attenuated OS and brain edema. This protective effect was not due to an improvement in liver function or hyperammonemia (Bosoi et al., 2012). Therefore, an antioxidant treatment targeting a specific pro-oxidant such as XO may be more beneficial in HE than a treatment directed toward improving antioxidant defense or ROS scavengers. Moreover, allopurinol is already used in patients with gout at an

affordable price. However, the use of allopurinol has been associated with asymptomatic elevations of serum transaminase and alkaline phosphatase levels reversible once treatment is stopped. Isolated cases of hypersensitivity reaction to allopurinol have also been reported. These manifest by high fever, eosinophilia, exfoliative dermatitis and fulminant hepatic failure usually having a fatal outcome (Yaylacı et al., 2012). Therefore, therapy with allopurinol should be administered cautiously in patients with hepatic impairment.

3.7.3 Dichloroacetate

The PDH kinase phosphorylates and inhibits PDH activity, thus resulting in decreased entry of pyruvate in the TCA cycle and increased shift of pyruvate to lactate. DCA is a PDH kinase inhibitor, therefore PDH is dephosphorylated and presents an increased activity. As a consequence, the flux of pyruvate into the TCA cycle increases, LDH activity shifts from lactate to pyruvate production and the synthesis of lactate decreases (Stacpoole et al., 1998, figure 3). DCA has been previously used and proven beneficial in other diseases such as congenital lactic acidosis, cancer and chronic obstructive pulmonary disease (Abdelmalak et al., 2013; Calvert et al., 2008; Strum et al., 2013). In these patients, DCA maintained normal lactate levels without side effects.

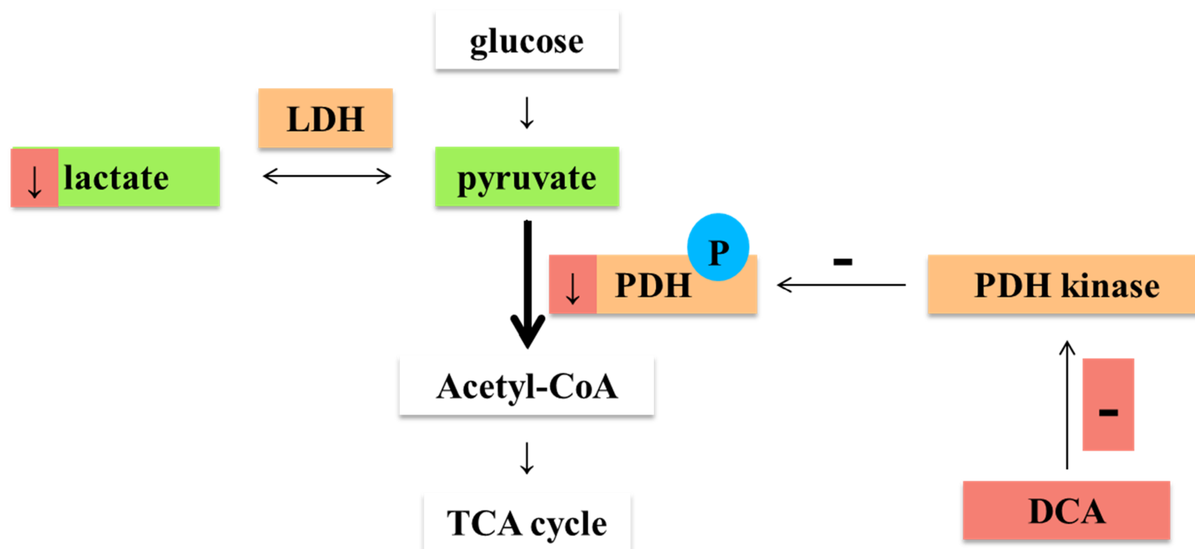

Figure 3. Mechanism of action of dichloroacetate. Explanations are given in the text. DCA, dichloroacetate; PDH, pyruvate dehydrogenase; TCA cycle, tricarboxylic acid cycle; LDH, lactate dehydrogenase.

In the present study, DCA reduced brain water content in BDL rats as a consequence of brain lactate levels normalization. This effect was not due to a decrease in cerebral glutamine levels or amelioration of ammonia levels or liver function (Bosoi et al., 2014). DCA may prove itself as a new therapeutic approach for the management of patients with end-stage liver disease.

Research in the field of treating HE has made major avances in the last 10 years. Some new products/therapeutic strategies are presently in clinical trials for the treatment of HE, therefore they are important in the current context and will be discussed below.

3.7.4 Probiotics

Although the use of probiotics raised some concerns regarding variations of the live microorganisms, lack of standardization or optimal dosage, VSL#3 has shown some promising results. This formulation is a mix of 8 bacterial species (four *Lactobacillus*, three *Bifidobacterium* and one *Streptococcus* species) and contains the highest available concentration of probiotic bacteria. Two recent publications demonstrated a beneficial effect in cirrhotic patients and HE. In the first, after a treatment period of 6 months in cirrhotic patients, VSL#3 reduced the risk of hospitalization for HE along with the severity of cirrhosis as evaluated by the Child-Pugh score (Dhiman et al., 2014). The second study, proved VSL#3 as efficient as lactulose in improving MHE after 2 months of treatment (Pratap Mouli et al., 2014). Moreover, the mechanism of action of VSL#3 has been studied in cirrhotic mice, where it was shown to reduce cerebral and systemic inflammation, therefore improving behaviour tests, while not having significant effects on gut microbiota or liver function (D'Mello et al., 2014). As they lack harmful or side effects, probiotics represent an important player in the future therapy of HE.

3.7.5 Ornithine Phenylacetate

As mentioned in the introduction, Ornithine Phenylacetate (OP) attenuated the increase in arterial and cerebral ammonia, thus preventing the apparition of ICP in pigs with ALF (Ytrebø et al., 2009). Moreover, it also proved beneficial effects in BDL rats with MHE, where it improved arterial ammonia, its cerebral metabolism and normalized brain water (Davies et al., 2009). As a result, an ongoing clinical trial is investigating the ability of OP to reduce hyperammonemia and speed recovery from HE episodes in cirrhotic patients (ClinicalTrials.gov number: NCT01966419).

3.7.6 Glycerol phenylbutyrate

Glycerol phenylbutyrate represents a pathway to remove ammonia incorporated into glutamine by forming phenylacetyl glutamine which is excreted urinary, as explained in chapter 1.5.5. GPB is already approved for treatment of urea cycle disorders and inherited disorders manifested by hyperammonemia. In cirrhotic patients, glycerol phenylbutyrate significantly lowered plasma ammonia and reduced HE events, but adverse effects were present in similar proportion in glycerol phenylbutyrate (79%) and placebo groups (76%) (Rockey et al., 2013). Its potential as a therapeutic option remains to be established by future studies.

Chapter 4: Conclusion and future directions

4.1 Conclusions

The results presented in this thesis demonstrate for the first time that in cirrhotic rats with MHE:

- Hyperammonemia independently does not lead to brain edema.
- Systemic oxidative stress is a consequence of liver disease, not a result of hyperammonemia.
- Systemic oxidative stress independently does not lead to brain edema.
- Systemic oxidative stress represents an important “first hit “which acts synergistically with hyperammonemia as a “second hit “leading to the development of brain edema.
- Cerebral oxidative stress is not associated with brain edema.
- AST-120 significantly lowers arterial ammonia levels and protects against the development of brain edema.
- Allopurinol attenuates both systemic oxidative stress and brain edema, therefore an antioxidant directed towards the inhibition of oxidative stress sources specific to cirrhosis may be a new treatment option in HE.
- Hyperammonemia renders the brain more susceptible to oxidative stress.
- Increased cerebral lactate, not glutamine contributes to the pathogenesis of brain edema, therefore inhibiting lactate synthesis represents a potential therapeutic target in HE.

These conclusions respond to the aim of the project. An important role of systemic oxidative stress, as well as its synergistic effect with ammonia in the pathogenesis of brain edema during MHE was demonstrated. A key role of lactate in the pathogenesis of brain edema during MHE associated with CLD. These mechanisms led to the proposal of three different treatments as potential therapeutic options. Further studies will determine the validity

of the therapies.

HE is a heterogenous syndrome, with a multifactorial pathogenesis. Aside factors known to contribute to the syndrome, such as ammonia, inflammation, neurosteroids, this thesis establishes that alterations of lactate metabolism and systemic OS released as a consequence of liver failure acting synergistically with ammonia also play a role in the pathogenesis brain edema in MHE during CLD. Along with already used treatment options targeting different pathogenic aspects of HE, the one proposed herein merit to be further investigated.

The conclusions are resumed in fig. 1 below.

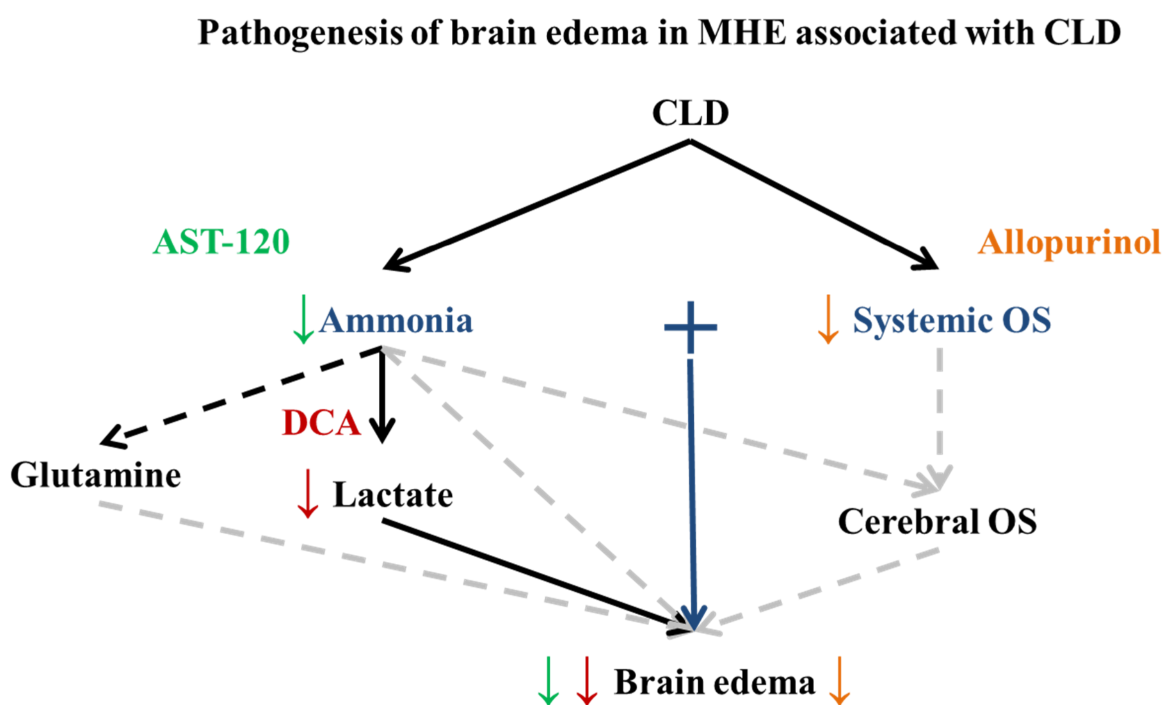

Figure 1: Pathogenesis of brain edema in minimal hepatic encephalopathy (MHE) during chronic liver disease (CLD). Roles and interrelations between ammonia (systemic and cerebral), systemic and cerebral oxidative stress, cerebral glutamine and cerebral lactate in the pathogenesis of brain edema in MHE during CLD. Effects of therapeutic strategies aimed to

decrease ammonia levels (AST-120), to reduce oxidative stress (allopurinol) or to inhibit lactate synthesis (DCA, dichloroacetate). Grey dashed lines = factors are not related; black dashed line = the relationship remains unclear; black continuous line = the relationship is proven; blue continuous line = a synergistic effect was proven.

4.2 Future directions

4.2.1 The mechanism of systemic ROS leading to brain edema

ROS have an important role in cell signaling (Devasagayam et al., 2004). Circulating ROS may signal cerebral endothelial cells and as a consequence induce posttranslational modifications of either protein implicated in the BBB or transport pathways through the BBB, leading to hyperpermeability. Such examples are the AQP, NKCC and NCCa-ATP, which were discussed in chapter 3.5. Therefore, only subtle changes in BBB permeability might be involved in the pathogenesis of brain edema in MHE. All the above aspects remain speculative and further studies directed on the role of ROS on endothelial cells may clarify them.

4.2.2 The link between systemic oxidative stress and increased cerebral lactate in the pathogenesis of brain edema

This thesis demonstrated that two factors are individually implicated in the pathogenesis of brain edema in MHE following CLD: systemic OS and cerebral lactate. Therefore the question is; are these factors interrelated?

In HE, increased in cerebral lactate is considered as impairment in cellular energy metabolism rather than an obvious sign of energy failure (Zwingmann, 2007). Numerous studies suggest a direct relation between OS and energy failure in the brain: in diseases like stroke, Parkinson's or Alzheimer's OS is linked to mitochondrial dysfunction and energy

failure (Jenner, 2003; Mamelak, 2007; Saeed et al., 2007). In spite of these evidences, there is only one paper linking OS and lactate metabolism *in vitro*. This shows a decreased rate of lactate production by cultured astrocytes exposed to hydrogen peroxide (Liddell et al., 2009), which is rather the contrary of increased cerebral lactate during hyperammonemia and CLD. Further studies will depict if OS has an effect on lactate metabolism in HE.

4.2.3 Describing different types of HE

As explained above, HE is classified by the type of liver function which induces it (Ferenci et al., 2002). Patients present with a variety of symptoms which define the grade of HE depending on their severity (the West Haven criteria – table I – page 4). These symptoms reflect the impairment of specific cortical functions such as learning and memory, attention, anxiety, sleep patterns, motor and sensory function. Therefore, a classification of HE by the type/types of dysfunction would probably better reflect the region/regions affected as well as the triggering factor, thus leading to the appropriate therapeutic strategy for each individual.

The insertion of a TIPS for the treatment of portal hypertension induces HE in about 40% of patients (Casadaban et al., 2014). As these patients make frequent hospital visits, they are an important population to clinically examine HE and its' pathogenesis. For example, TIPS patients were an important part in the study that validated the EncephalApp, a smartphone based test for the diagnosis of MHE, as a reliable test (Bajaj et al., 2014). More tests on pathogenic factors implicated in the pathogenesis of HE such as those described in this thesis, in relation to TIPS-induced HE in a cirrhotic population, may contribute to translating our findings into clinical practice.

However, establishing exactly which functions are impaired in each patient is difficult. For animal models, several behavioral tests are available, but most of those involve physical tasks which are challenging for an animal not physically fit (nutritional impairments and an

extra weight due to ascites). The tests included in the currently used batteries for clinical trials in patients often asses several functions at the same time, which are difficult to be scored separately. Therefore, a consensus on specific tests for HE in both patients and animal models are warranted in order to establish a classification of HE based on neurological dysfunction.

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CURRICULUM VITAE

ÉTUDES

Université de Montréal, Montréal, QC

- Études de doctorat en sciences biomédicales 05/2009 - 10/2014
- Études de maîtrise en sciences biomédicales 01/2008 - 04/2009

Université de Médecine et Pharmacie «Carol Davila», Bucarest, Roumanie

- Doctorat en médecine 10/1997 – 09/2003

Lycée «Colegiul National Mihai Viteazul», Bucarest, Roumanie

- Diplôme de baccalauréat en mathématique – physique 09/1993 – 06/1997

ACTIVITÉ SCIENTIFIQUE

A. Publications

1. **Bosoi, C. R.** and Rose, C. F. Elevated cerebral lactate: Implications in the pathogenesis of hepatic encephalopathy Metab Brain Dis. 2014 Dec;29(4):919-25. doi: 10.1007/s11011-014-9573-9. Epub 2014 Jun 11. Review (Impact Factor 2.39).
2. **Bosoi, C. R**., Tremblay, M. and Rose, C. F. Systemic oxidative stress induces brain edema in portacaval shunted rats. Liver Int. 2014 Oct;34(9):1322-9. doi: 10.1111/liv.12414. Epub 2013 Dec 17. (Impact Factor 4.41)
3. **Bosoi, C.R.**, Zwingmann, C., Marin, H., Parent-Robitaille, C., Huynh, J., Tremblay, M. and Rose, C. F. Increased brain lactate is central to the development of brain edema in rats with chronic liver disease: a 13C-nuclear magnetic resonance study. J Hepatol. 2014 Mar;60(3):554-60. doi: 10.1016/j.jhep.2013.10.011. Epub 2013 Oct 26. (Impact Factor 10.40)
4. **Bosoi, C. R.** and Rose, C. F. Oxidative stress: a systemic factor implicated in the pathogenesis of hepatic encephalopathy. Meta Brain Dis. 2013 Jun;28(2):175-8. doi: 10.1007/s11011-012-9351-5. Epub 2012 Nov 6. Review.(Impact Factor 2.39)
5. **Bosoi, C. R.** and Rose, C. F. Brain edema in acute liver failure and chronic liver disease: Similarities and differences. Neurochem Int. 2013 Mar;62(4):446-57. doi: 10.1016/j.neuint.2013.01.015. Epub 2013 Jan 31. Review. (Impact Factor 2.65)
6. **Bosoi, C. R.**, Yang, X., Huynh, J., Parent-Robitaille, C., Jiang, W., Tremblay, M. and Rose, C.F. Systemic oxidative stress is implicated in the pathogenesis of brain edema in rats with chronic liver failure. Free Radic Biol Med. 2012 Apr 1;52(7):1228-35. doi: 10.1016/j.freeradbiomed.2012.01.006. Epub 2012 Jan 24. (Impact Factor 5.71)
7. **Bosoi, C. R.**, Parent-Robitaille, C., Anderson, K., Tremblay, M. and Rose, C. F. AST-120 (spherical carbon adsorbent) lowers blood ammonia levels and attenuates brain edema in bile-duct ligated rats. Hepatology. 2011 Jun;53(6):1995-2002. doi: 10.1002/hep.24273. (Impact Factor 11.19)

8. Yang, X., **Bosoi, C. R.**, Jiang, W., Tremblay, M. and Rose, C. F. Portacaval anastomosis-induced hyperammonemia does not lead to oxidative stress. Metab Brain Dis. 2010 Mar;25(1):11-5. doi: 10.1007/s11011-010-9174-1. Epub 2010 Mar 2. (Impact Factor 2.39).
9. **Bosoi, C. R.** and Rose, C. F. Identifying the direct effects of ammonia on the brain. Metab Brain Dis.2009 Mar;24(1):95-102. doi: 10.1007/s11011-008-9112-7. Epub 2008 Dec 23. Review. (Impact Factor 2.39)

B. Présentations orales

- 2 présentations à titre d'invité (Séminaire du CRCHUM 2010; 10th Symposium on research topics in gastrointestinal disease 2011).
- 4 conférences internationales (AASLD 2009 et ISHEN 2012).
- 10 conférences nationales (CASL 2009, 2010, 2011, 2012, 2013; ACFAS 2012).
- 5 conférences du CRCHUM/Université de Montréal (2009, 2010, 2011, 2012, 2013).

C. Présentations par affiche:

- 17 conférences internationales (AASLD 2008, 2010, 2011, 2012, 2013; SFRBM 2008; EASL 2009, 2010, 2011, 2013; ISHEN 2010).
- 3 conférences nationales (CASL 2011, 2013).
- 3 conférences du CRCHUM (2011, 2012).

BOURSES ET PRIX

- Bourse de recherche au doctorat du FRQS (2012-2014).
- Bourse de passage accéléré au doctorat: Université de Montréal (2009).
- prix pour la meilleure présentation orale: 15th ISHEN Symposium, Grenaa, Danemark.
- prix du CIHR Training Program in Neurodegenerative Lipidomics Research (2014).
- 3 Student Research Prize du CASL (2009, 2011, 2012).
- 2 Bourses de complément CRCHUM (2011, 2013).
- 3 Bourses du programme du Département des Sciences Biomédicales de l'Université de Montréal (2009, 2010, 2011).
- 6 Bourses de voyage: 2 bourses de voyage de l'EASL (2009, 2010); 1 bourse de registration de l'EASL (2014); 2 bourses de perfectionnement du CRCHUM pour participer au congrès d'AASLD (2010, 2011); 1 bourse de voyage de l'ISHEN.
- 2 Affiches de distinction EASL 2010, CASL 2011.