Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

ESTUDO SOBRE OS EFEITOS DA GUANOSINA NA ENCEFALOPATIA HEPÁTICA CRÔNICA E NA HIPERAMONEMIA AGUDA EM RATOS

TESE DE DOUTORADO

Lucas Guazzelli Paim Paniz

Porto Alegre, RS, 2014.

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Tese apresentada ao programa de Pós-graduação em Ciências Biológicas: Bioquímica, como requisito parcial para a obtenção do título de Doutor em Bioquímica

Prof. Dr. Diogo Onofre Gomes de Souza

(Orientador)

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LISTA DE ABREVIATURAS

ADA – adenosine deaminase

ADP – adenosina-5'-difosfato

AMP – adenosina-5'-monofosfato

AMPc – adenosina-3',5'-monofosfato-cíclico

ATP – adenosina-5'-trifosfato

EEG- Eletroencefalograma

EH- Encefalopatia Hepática

GABA – ácido gama-aminobutírico

GMP – guanosina-5'-monofosfato

GMPc – guanosina 3',5'-monofosfato-cíclico

GDP – guanosina-5'-difosfato

GTP – guanosina-5'-trifosfato

i.p. – intraperitoneal

MK-801 – (+)-10,11-dihidro-5-metil-5H-dibenzo[a,d]cicloheptено-5,10 imina

NMDA – N-metil-D-aspartato

SNC- Sistema Nervoso Central

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APRESENTAÇÃO

Os resultados desta tese de doutorado estão apresentados sob a forma de artigos científicos. As seções Materiais e Métodos, Resultados, Discussão e Referência bibliográficas encontram-se nos próprios artigos. Os itens Introdução, Discussão e Conclusões encontradas nesta tese apresentam interpretações e comentários gerais sobre todos os artigos científicos contidos neste trabalho. As Referências Bibliográficas referem-se somente às citações que aparecem nos itens Introdução e Discussão desta tese. Detalhes técnicos mais precisos sobre a metodologia empregada em cada trabalho podem ser encontrados nos artigos científicos correspondentes.

PARTE I

INTRODUÇÃO E OBJETIVOS

RESUMO

A encefalopatia hepática (EH) é um conjunto de manifestações neuropsiquiátricas associada a um quadro de insuficiência hepática. Essas manifestações são atribuídas sobretudo à elevação nos níveis de amônia no Sistema Nervoso Central (SNC), causando distúrbios na homeostase dos neurotransmissores, edema cerebral, neuroinflamação e aumento do estresse oxidativo. A perda de função do fígado pode acontecer de forma aguda ou insidiosa, acarretando diferentes respostas no SNC relacionadas à elevação aguda ou crônica da amônia. Um dos neurotransmissores envolvidos na fisiopatologia desta síndrome é o glutamato, o principal neurotransmissor excitatório do SNC. A excitotoxicidade glutamatérgica está presente em ambas as apresentações da encefalopatia hepática. A Guanosina, um nucleosídeo derivado da guanina, é conhecida por apresentar uma função neuroprotetora no sistema nervoso central, agindo como um antagonista do sistema glutamatérgico através do aumento da captação de glutamato, além de possuir efeitos tróficos e antioxidantes em células neurais. Desta forma, nesta tese, avaliaram-se um modelo de EH crônica (ligação do ducto biliar de ratos); e um de hiperamonemia aguda (administração intra peritoenal de amônia em ratos). No quadro de EH crônica, o tratamento com Guanosina resultou em uma melhora no teste comportamental Y maze, diminuição do glutamato no líquor e redução dos marcadores de estresse oxidativo. Já no modelo de hiperamonemia aguda, quadro muto semelhante à EH aguda, a Guanosina diminuiu o tempo em coma, normalizou as alterações do eletroencefalograma, aumentou a captação de glutamato, normalizou os níveis de glutamato e amônia no líquor, atenuou o estresse oxidativo e diminuiu a mortalidade. Para o nosso conhecimento, os resultados dessa tese são a primeira descrição do efeito neuroprotetor da Guanosina na EH, com importante contribuição para o estudo da doença. Além disso, surge como potencial alternativa terapêutica a ser estudada para quadros de insuficiência hepática grave em que muitas vezes somente o transplante hepático pode evitar uma deterioração neurológica irreversível .

ABSTRACT

Hepatic encephalopathy (HE) is a neuropsychiatric syndrome associated with liver disease. Increasing of ammonia in the Central Nervous System (CNS) is the main implicated mechanism, resulting in cerebral edema, neurotransmitters disturbance, neuroinflamation and oxidative stress damage. Hepatic insufficiency may be developed suddenly or subtle, resulting in acute or chronic hyperammonemia. Glutamate, the main excitatory neurotransmitter in the CNS plays a major role in the physiopathology of this syndrome. Glutamatergic excitotoxicity is described in both presentations: acute and chronic encephalopathy. As Guanosine plays an important neuroprotective role in the CNS, exerting glutamatergic system antagonism, antioxidant activity and trophic effects on neural cells, in this study, we evaluated the neuroprotective effects of Guanosine on an animal model of hepatic encephalopathy. Initially, we demonstrated that Guanosine was neuroprotective against chronic hepatic encephalopathy (rats were submitted to bile duct ligation) once it has improved a behavioral test performance (Ymaze) and normalized cerebrospinal fluid (CSF) glutamate and brain oxidative stress parameters. Additionally, it was also investigated the Guanosine effects on acute hyperammonemia (rats were injected with ammonium acetate). The treatment was able to decrease oxidative stress damage, reduce glutamate and ammonia levels on CSF, increase glutamate uptake and improve neurological status in rats with severe acute hyperammonemia. Besides, Guanosine prevents death against acute hyperammonemia. Token altogether, we argue that Guanosine neuroprotective effect is related to its ability to antagonizing glutamatergic excitotoxicity by increasing glutamate uptake. Overall, to our knowledge, this is the first study presenting the neuroprotective effects of Guanosine on hepatic encephalopathy. Furthermore it could eventually be developed as a drug useful for helping severe hepatic encephalopathy, which, in many cases, liver transplant is the only alternative.

Introdução

A Encefalopatia Hepática (EH) é uma complicação de pacientes com doença no fígado que se manifesta no Sistema Nervoso Central (SNC). Caracteriza-se por um espectro de manifestações neuropsiquiátricas que variam desde leves alterações na atenção, podendo progredir para distúrbios motores, letargia, estupor e coma. A deterioração clínica da EH pode progredir para a morte se não for adequadamente manejada, sendo o transplante hepático a única opção terapêutica em alguns casos (Bernau, 2013; Butterworth et al., 2009; Córdoba, 2001; Ferenci et al., 2002; Mohammad, 2012).

A EH é classificada de acordo com o cenário em que acontece: pacientes que apresentam um quadro de insuficiência hepática aguda – EH tipo A; como complicação de um quadro de insuficiência hepática crônica – EH tipo C; ou ainda como conseqüência de shunts entre a circulação portal e sistêmica, nesses casos sem que haja uma doença no parênquima hepático - EH tipo B. A insuficiência hepática crônica, ou cirrose, é uma doença resultante de várias etiologias (álcool, vírus, autoimune, esteatose hepática), sendo assim, a EH tipo C aparece como a forma mais comum das EH, ocorrendo em até 48% dos pacientes com cirrose (Ferenci et al., 2002; Schulz et al., 2009).

As manifestações neuropsiquiátricas da doença no fígado podem variar em sua gravidade, conforme dito anteriormente, sendo os critérios de West Haven os mais usados para classificação da encefalopatia hepática de acordo com a gravidade:

→ Grau I: Alterações leves de comportamento e de funções biorregulatórias, como alternância do ritmo do sono, distúrbios discretos do comportamento como riso e choro "fácil", hálito hepático

→ Grau II: Letargia ou apatia, lentidão nas respostas, desorientação no tempo e espaço, alterações na personalidade e comportamento inadequado, presença de "flapping".

→ Grau III: Sonolência e torpor com resposta aos estímulos verbais, desorientação grosseira e agitação psicomotora, desaparecimento do flapping.

→ Grau IV: Coma não responsivo aos estímulos verbais e com resposta flutuante à dor.

O diagnóstico da EH é baseado na história clínica, exclusão de outras causas para os sintomas no SNC e emprego dos critérios de West Haven. Para quadros de EH mínima ou ainda para acompanhar a evolução de casos mais graves, pode-se ainda lançar mão do estudo eletrofisiológico – Eletroencefalograma (EEG) (Ferenci et al., 2002; Sociedade Brasileira de Hepatologia, 2011; Weissenborn et al., 2001 e 2005).

Fisiopatologia.

As manifestações neuropsiquiátricas observadas na EH são conseqüência do edema cerebral, inflamação, estresse oxidativo e de alterações no equilíbrio dos neurotransmissores. A principal toxina responsável por essas alterações é a amônia, que tem no fígado sua principal forma de metabolização. A amônia é proveniente da digestão de proteínas no trato gastrointestinal e, através da circulação portal, entra no parênquima hepático para ser transformada em uréia e eliminada pelos rins. Desta forma, pessoas com

fígado sadio não apresentam valores elevados de amônia na circulação sistêmica (Butterworth, 1987; Lemberg, 2009).

Por outro lado, doenças que acometam o fígado acarretam uma diminuição na metabolização da amônia, aumentando seus níveis na circulação sistêmica e fazendo com que outros sistemas assumam esse papel. Em especial, destacam-se os músculos e o cérebro com repercussão clínica significativa: pacientes com atrofia muscular apresentam com maior freqüência quadro de EH do que aqueles com boa massa muscular, uma vez que metabolizam menos a amônia por possuírem menor massa muscular (Merli, 3013; Rose, 2012). Já o cérebro consegue metabolizar a amônia através da enzima astrocitátia Glutamina Sintetase (reação: glutamato→glutamina), mas a hiperamonemia no SNC acaba gerando alterações patológicas na homeostase dos neurotrasmissores (Butterworth, 2010; Desjardins et al., 2012; Kosenko et al., 2003; Lamberg 2009).

As manifestações neurológicas do excesso de amônia são decorrentes do edema cerebral, alterações nos sistemas gabaérgico e glutamatérgico, inflamação, aumento do estresse oxidativo e no nível de neuroesteróides no SNC (Ahboucha et al. , 2009; Brück,2011; Cordoba et al., 2001; Felipo, 2002; Mendez, 2009). É um consenso nos estudos desta síndrome que não há uma causa única para as manifestações, sendo os sintomas decorrentes de disfunções em múltiplos sistemas neuronais.

De interesse especial para esta tese, o sistema glutamatérgico tem relação direta com as manifestações da EH aguda e crônica.

EH aguda: em um cenário de insuficiência hepática aguda, os níveis de amônia elevam-se de forma súbita e o sistema glutamatérgico responde a esse insulto com ativação dos receptores NMDA e elevação nos níveis de glutamato extracelullar e glutamina. Há edema astrocitário e edema cerebral, possivelmente por um aumento na conversão de glutamato→ glutamina em uma tentativa de diminuir os níveis de amônia. A reação glutamato→glutamina, mediada pela glutamina sintetase, acontece dentro dos astrócitos. No entanto, a hiperamonemia diminui a captação de glutamato pelo astrócito, acumulando glutamato no extracelular. A hiperativação dos receptores NMDA e o aumento do glutamato geram a excitotoxicidade glutamatérgica, estado de aumento no influxo de cálcio intracelular com ativação de mecanismos intracelulares que podem culminar com a morte celular (Desjardins et al., 2012; Hermenegildo, 2000; Rose, 2002; Rodrigo et al. 2009).

EH crônica→ a elevação insidiosa no níveis de amônia faz com que o SNC se adapte à hiperamonemia, diminuindo a atividade dos receptores NMDA em uma tentativa de evitar a excitotoxicidade glutamatérgica. No entanto, esta resposta adaptativa é uma das responsáveis pelas alterações comportamentais vistas nos casos de insuficiência hepática crônica- cirrose (Cauli 2007; Llansola et al., 2007 e 2013; Monfort et al. 2002; Yoden et al., 2012).

Tratamento

O tratamento da EH envolve essencialmente estratégias para diminuir os níveis de amônia, sendo a lactulose, laxativo que diminui a absorção de amônia pelo trato gastrointestinal, a primeira escolha. Como alternativa, pode-se usar antibióticos para

eliminar bactérias produtoras de amônia. A terapia ainda envolve medidas de suporte (diuréticos, hipotermia, sedação) para impedir a progressão do edema cerebral nos casos mais graves. Paciente irresponsivos se tornam candidatos ao transplante hepático como útima alternativa antes que ocorra herniação da amígdalas cerebrais e morte nos casos de EH aguda ou antes que as alterações cognitivas se tornem irreversíveis nos casos de EH crônica (Frontera, 2014; Mohammad, 2012; Rose, 2012; Sociedade Brasileira de Hepatologia, 20011).

Nos casos de EH tipo C, quando o doente apresenta uma doença hepática crônica, é comum existir algum fator precipitante do quadro de EH, tal como infecção, hemorragia, insuficiência renal ou outra descompensação metabólica. Nestes casos, deve-se também tratar o fator desencadeante ao mesmo tempo em que iniciam-se estratégias para diminuir os níveis de amônia.

Sistema Purinérgico- Guanosina.

O Sistema Purinérgico

O sistema purinérgico compreende as bases purínicas, como adenina e guanina, e seus derivados nucleotídeos e nucleosídeos que são moléculas amplamente distribuídas dentro e fora das células. As purinas são classificadas em derivados da adenina (ATP, ADP, AMP, adenosina, adenina) e derivados da guanina (GTP, GDP, GMP, Guanosina e guanina). Ainda compõem as purinas os metabólitos diretos dos derivados da adenina e da guanina: inosina, xantina, hipoxantina e ácido úrico. Historicamente as purinas são relacionadas a diversas funções biológicas como construção do DNA e RNA (adenina e

guanina), metabolismo energético celular (ATP) e reguladoras de mecanismos intracelulares de transdução de sinal como mensageiros secundários (AMPc e GMPc) (Schmidt, 2007).

Nos últimos anos, diversos trabalhos demonstraram o papel fundamental destas moléculas no espaço extracelular. Os derivados da adenina, principalmente o nucleotídeo ATP e o nucleosídeo adenosina são os principais efetores do sistema purinérgico em nível extracelular, tendo um papel de neurotransmissores, neuromoduladores, fatores tróficos e neuroprotetores endógenos contra estímulos nocivos (Burnstock, 2011).

Efeitos das purinas derivadas da guanina sobre o SNC

Apesar dos derivados do ATP e da adenosina serem considerados os principais efetores do sistema purinérgico em nível extracelular, mais recentemente os derivados da guanina, principalmente os nucleotídeos GTP e GMP e o nucleosídeo Guanosina, têm demonstrado diversos efeitos biológicos extracelulares como efeitos tróficos em células neurais e antagonismo do sistema glutamatérgico. De fundamental importância para esta tese, a atividade antiglutamatérgica da Guanosina é decorrente principalmente do aumento da captação de glutamato pelos astrócitos e de sua atividade antioxidante (Frizzo et al., 2001 & 2003; Petronilho et al., 2012) . Trabalhos in vivo demonstraram efeitos anticonvulsivantes da Guanosina contra agonistas glutamatérgicos como o ácido quinolínico em ratos. (Schmidt et al., 2000; Oliveira et al., 2004). A Guanosina ainda demonstrou possuir efeito neuroprotetor em eventos hipóxicos e com potencial antinociceptivo em eventos de dor aguda e crônica, além de efeitos amnésicos em

diversos testes de memória, como a esquiva inibitória em roedores (Rathbone et al., 2011; Schmidt et al., 2009 2010-a, 2010-b, 2010-c & 2010-d; Lara et al., 2001; Vinadé et al., 2004).

Baseado nesta propriedade antiglutamatérgica da Guanosina, e no exposto anteriormente sobre o papel do sistema glutamatérgico na fisiopatologia da encefalopatia hepática, esta tese estudou a ação da Guanosina sobre um modelo de encefalopatia hepática aguda e encefalopatia hepática crônica.

Objetivos desta Tese

1) Estudar o efeito neuroprotetor da Guanosina em um modelo de encefalopatia hepática crônica em ratos.

Objetivos específicos:

- *Estudar efeitos comportamentais da Guanosina.*

- *Estudar o efeito eletrofisiológico da Guanosina*

- *Avaliar o perfil das purinas e do glutamato no líquor dos animais com EH*

2) Estudar o efeito neuroprotetor da Guanosina em um modelo de hiperamonemia aguda em ratos.

Objetivos específicos:

- *Estudar o efeito neuroprotetor da Guanosina sobre o desfecho morte.*

- *Estudar o efeito eletrofisiológico da Guanosina*

- *Estudar a captação de glutamato no modelo de hiperamonemia aguda*

- *Avaliar o perfil das purinas e do glutamato no líquor dos animais com hiperamonemia aguda.*

PARTE II

RESULTADOS

CAPÍTULO 1

The modulatory effects of allopurinol on N-methyl D-aspartate receptors in the central nervous system

Lucas Guazzelli Paniz, Antre Prato Schmidt e Diogo Onofre Souza

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Letter to the Editor

To the Editor,

We read with great interest the elegant manuscript by Yonden *et al.*,[1] showing that allopurinol is capable of normalizing *N*-methyl D-aspartate (NMDA) pathway functions altered by ammonia either by activation of NMDA receptors or by enhancing the expression of NMDA receptor subunits, particularly the NR2A subunit. The authors argue that this modulatory effect may be due to allopurinol's antioxidant effect by inhibiting the xanthine oxidase (XO) pathway and by reducing the generation of free radicals. They also emphasize that allopurinol can also act as a free radical scavenger and the prevent modification of the NMDA receptor, attenuating brain cell membrane injury. However, we propose here a supplementary explanation for their results.[1]

Chronic hyperammonemia leads to a depression of excitatory neurotransmission by the impairment of NMDA receptor function and by the inhibition of NMDA receptor-mediated signal transduction pathways, possibly as an adaptative response to the increased extracellular glutamate levels found in hepatic encephalopathy.[2] The impairment of glutamatergic pathways may prevent some of the toxic effects of ammonia on the overactivation of NMDA receptors but also reduces normal physiological functions, potentially contributing to the clinical symptoms displayed in chronic hyperammonemia.[3] Although Yoden *et al.*[1] pointed out that the main effect of allopurinol in the context of chronic hyperammonemia is by acting as an antioxidant, the indirect contribution of other neurotransmitters or neuromodulators to these findings could not be fully ruled out. This might be especially important when interpreting allopurinol effects on chronic hyperammonemia. Therefore, we speculate that allopurinol may present these neuroprotective effects due to an increase in the levels of purines in the central nervous system (CNS). This might be especially important when interpreting some of the effects of the nucleosides guanosine and adenosine on the glutamatergic system.[4]

Allopurinol is a structural analogue of hypoxanthine and a potent inhibitor of the enzyme XO that catalyzes the transformation of hypoxanthine to xanthine and uric acid, reducing both uric acid formation and purine degradation.[5] Although allopurinol is used primarily in the treatment of hyperuricemia and gout,[6] more recently, some evidence has emerged showing that allopurinol may present several effects on the CNS, being effective in the treatment of seizures,[7] pain[8] and psychiatric disorders.[9]

The effects of allopurinol as a neuromodulator may be secondary to its inhibitory effect on purine degradation, causing extracellular purine accumulation and enhanced purinergic activity. We hypothesize that allopurinol may cause a decrease in the systemic concentration of uric acid and an increase in the concentration of oxypurines such as hypoxanthine and xanthine. These precursors, particularly hypoxanthine, can be converted to inosine, IMP and consequently to adenosine and guanosine.[8] Thus, the primary effect of allopurinol is the inhibition of uric acid production, and the overall result is the inhibition of the metabolism of xanthine and hypoxanthine, leading to a greater salvage of the nucleosides inosine, adenosine and guanosine. These findings, both in the CNS and periphery, have been extensively demonstrated after the systemic administration of allopurinol in animals and humans.[8,10,11]

The purinergic system involves adenosine and ATP as the major endogenous effectors, acting on P_1 and P_2 receptors, respectively.[12] In the past few years, the antiglutamatergic effects of purines and their derivatives have been extensively demonstrated, as recently reviewed in detail elsewhere.[4,12] Adenosine is essentially an inhibitory neuromodulator, regulating synaptic activity and release of several neurotransmitters, including glutamate.[12] In addition, we and others have shown that guanine-based purines may be neuroprotective endogenous compounds released under excitotoxic conditions and have been shown to prevent NMDA-induced toxicity in neurons.[13] Moreover, we have demonstrated that guanine-based purines, mainly the nucleoside guanosine, are antinociceptive against several models of acute and chronic pain[14,15] and prevent seizures and toxicity induced by glutamatergic agents.[16,17] These effects may be due to guanosine's interaction with specific binding sites on brain cell membranes[18] and to an increase in astrocytic glutamate uptake under excitotoxic conditions.[19]

Altogether, these findings indicate that allopurinol prevent the hyperammonemia-induced down-regulation of NMDA receptors due to an increase of CNS purines, mainly adenosine and guanosine. The attenuation of some behavioural effects

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of NMDA by allopurinol may be related to a decrease in the glutamate release induced by adenosine and/or an increase of glutamate uptake by astrocytes promoted by guanosine. Hence, the overall result of allopurinol in chronic hyperammonemia may be the decrease of extracellular glutamate levels at the synaptic cleft, leading to a less tonic activation of NMDA receptors and preventing pathologic adaptative responses such as the down-regulation of NMDA receptor subunits. Although the mechanism of action of allopurinol and its modulatory effects on NMDA receptors are not completely elucidated, these findings point to a potential neuroprotective property of allopurinol. Caffeine and theophylline are the classic P_1 adenosine antagonists currently used in humans, but adenosine or guanosine agonists for human use are still lacking. We hypothesized that the inhibition of XO by allopurinol, thereby reducing purine degradation, could be a valid strategy to enhance purinergic activity, which is in line with the anticonvulsant, neuropsychiatric and antinociceptive effects observed with allopurinol treatment.[7,8,20,21] Further experiments investigating the effects of allopurinol, purines and their derivatives in chronic hyperammonemia are currently being carried out in our laboratory, and new evidences may emerge in next future.

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CAPÍTULO 2

Neuroprotective effects of Guanosine administration on behavioral, brain activity, neurochemical and redox parameters in a rat model of chronic hepatic encephalopathy

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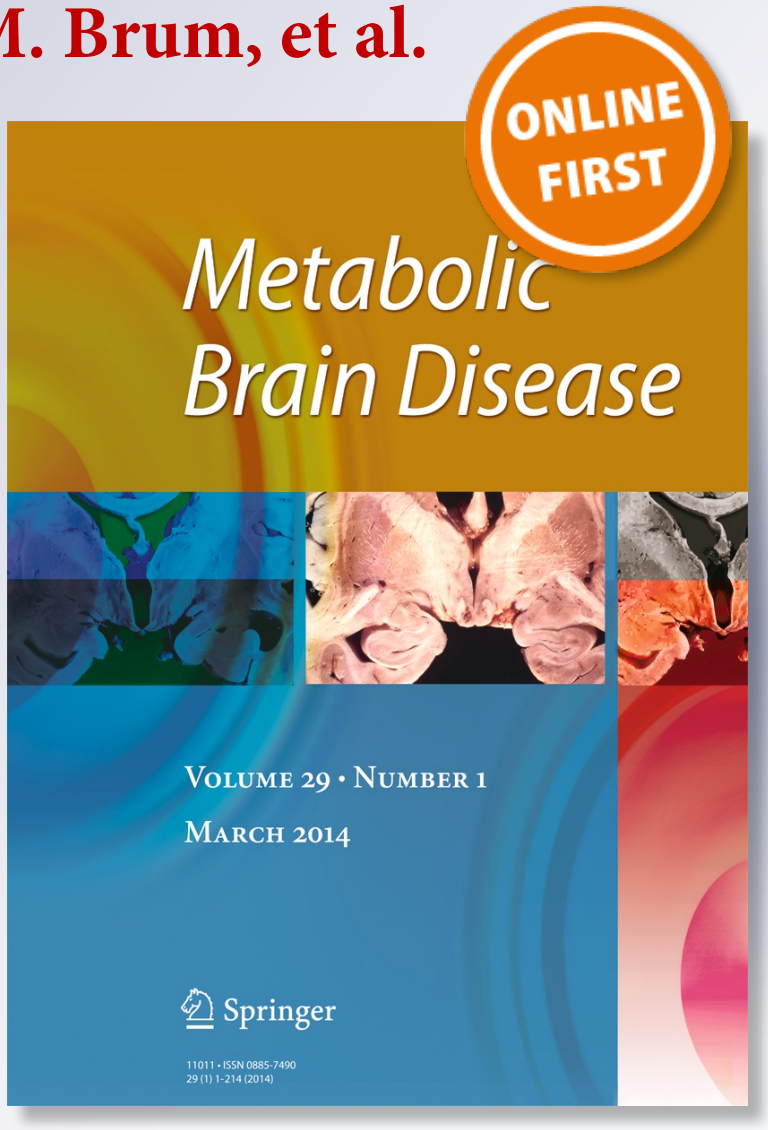

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RESEARCH ARTICLE

Neuroprotective effects of guanosine administration on behavioral, brain activity, neurochemical and redox parameters in a rat model of chronic hepatic encephalopathy

L. G. Paniz · M. E. Calcagnotto · P. Pandolfo · D. G. Machado · G. F. Santos · G. Hansel · R. F. Almeida · R. S. Bruch · L. M. Brum · F. V. Torres · A. M. de Assis · E. P. Rico · D. O. Souza

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Abstract It is well known that glutamatergic excitotoxicity and oxidative stress are implicated in the pathogenesis of hepatic encephalopathy (HE). The nucleoside guanosine exerts neuroprotective effects through the antagonism against glutamate neurotoxicity and antioxidant properties. In this study, we evaluated the neuroprotective effect of guanosine in an animal model of chronic HE. Rats underwent bile duct ligation (BDL) and 2 weeks later they were treated with i.p. injection of guanosine 7.5 mg/kg once a day for 1-week. We evaluated the effects of guanosine in HE studying several aspects: a) animal behavior using open field and Y-maze tasks; b) brain rhythm changes in electroencephalogram (EEG) recordings; c) purines and glutamate levels in the cerebral spinal fluid (CSF); and d) oxidative stress parameters in the brain. BDL rats presented increased levels of glutamate, purines and metabolites in the CSF, as well as increased oxidative damage. Guanosine was able not only to prevent these effects but also to attenuate the behavioral and EEG impairment induced by BDL. Our study shows the neuroprotective effects of systemic administration of guanosine in a rat model of HE and highlights the involvement of purinergic system in the physiopathology of this disease.

Keywords Guanosine · Glutamate · Oxidative stress · Neuroprotection · Electroencephalogram · Hepatic encephalopathy

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Introduction

Hepatic encephalopathy (HE) is a severe neuropsychiatric syndrome associated with liver disease, impairment of cognitive function, disturbance of sleep – waking cycle and decrease of motor activity (Butterworth et al.1987; Mohammad et al. 2012). These neuropsychiatric alterations secondary to liver failure may lead to coma and death. Although the pathophysiology of HE is not completely understood, it is clear that ammonia is the main implicated toxin. It seems that, in animal models of chronic liver failure, ammonia affects neurotransmission, leading to increased extracellular levels of glutamate in the central nervous system (CNS) (Butterworth et al. 1991; Mohammad et al. 2012; Tossman et al. 1987). Besides the undoubted epidemiologic relevance of HE, there are no effective strategies to prevent or treat this disease (Mohammad et al. 2012).

Although glutamate is the most important excitatory neurotransmitter in CNS of mammals, its high concentration at the synapses leads to neurotoxicity (Beart and O'Shea 2007; Butterworth 2010; Llansola et al. 2013; Sheldon and Robinson 2007). The glutamate uptake by transporters located mainly in cell membranes of astrocytes, is the most important mechanism responsible for maintaining the synaptic glutamate levels below toxic levels (Coulter and Eid 2012; Kim et al. 2011). Interestingly, in chronic liver disease, the overall excitatory neurotransmission in the CNS decreases, possibly

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as an adaptive response to the increased extracellular levels of glutamate (Llansola et al. 2013; Monfort et al. 2002; Rodrigo et al. 2007; Yonden et al. 2010). This impairment in the excitatory system may prevent some of the toxic effects of glutamatergic hyperactivity. On the other hand, it could also contribute to the clinical symptoms of chronic encephalopathy (Llansola et al. 2007).

Protecting the brain against glutamatergic excitotoxicity could help to decrease the deleterious effect of HE over the CNS (Llansola et al. 2013). It has been shown that the nucleoside guanosine exhibits several neuroprotective effects in experimental models of brain injuries involving excitotoxicity, such as seizures, brain ischemia and glutamatergic pain (Connell et al. 2013; Rathbone et al. 2011; Schmidt et al. 2007, 2010a; Schmidt and Souza 2010). The antioxidant properties of guanosine (Petronilho et al. 2012; Schmidt et al. 2007) and its ability to increase the astrocytic glutamate uptake (Frizzo et al. 2003a; Schmidt et al. 2007; Schmidt and Souza 2010) seem to be related to the neuroprotective effects.

Therefore, it is reasonable to postulate that guanosine could play an important role in neuroprotection in HE. To address this issue we examined the effects of guanosine administration on behavioral tasks, neurochemical and redox parameters, and electroencephalogram (EEG) profile of an effective rat model of chronic HE induced by Bile Duct Ligation (BDL) (Butterworth et al. 2009; García-Ayllón et al. 2008; Jover et al. 2006).

Materials and methods

All procedures with animals followed the ARRIVE guidelines (Kilkenny et al. 2010).

Ethics statement

All procedures with animal subjects have been approved by the Ethic Committee for Use of Animals – CEUA (n.20121) from Universidade Federal do Rio Grande do Sul. All surgery was performed under ketamine and xylazine anesthesia and all efforts were made to minimize suffering.

Animals

Male adult Wistar rats (3-months-old, 300–350 g) were kept on a 12-h light/dark cycle (lights on at 7:00 am) at temperature of 22 °C, housed in Plexiglas's cages (41 cm×34 cm×16 cm: LxWxH)) (5 per cage) with tap water and commercial food ad libitum.

Drugs

Guanosine was purchased from Sigma Chemicals (St Louis, MO) and dissolved in 0.1 mM NaOH. Vehicle solution was 0.1 mM NaOH. Both solutions were buffered to pH 7.4 with HCl 0, 1 N. Animals were treated with an Intraperitoneal (i.p.) injection of either guanosine (7.5 mg/kg) or vehicle. All other reagents were of scientific grade.

Surgical procedure

Animals were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) i.p. To clamp the bile duct, a midline abdominal incision was made. The common bile duct was localized, doubly ligated, and cut in between, separating the two ends. In sham animals, a midline incision was performed, with no BDL (Jover et al. 2006). Another group of animals were anesthetized as described above, and underwent surgery for both BDL and implantation of epidural electrodes for posterior EEG recordings. After BDL procedure, the head of the animal was fixed in a stereotaxic instrument, and the skin covering the skull was cut with a 3-cm-long rostro-caudal incision in the midline. After skull exposure 3 stainless steel screw electrodes (1.0 mm diameter) were placed over the dura-mater through holes in the skull made with a dental drill. Two of them were used as positive electrodes (2.0 mm lateral, right or left, 1.0 mm posterior to bregma) and the reference (negative) electrode was placed in the midline of the occipital bone and kept in contact with cerebrospinal fluid. The electrodes were fixed with dental acrylic cement, and a screw used for fixation of the dental acrylic helmet to the skull was used as ground (Torres et al. 2012).

Experimental design

After the surgery, sham and BDL rats were housed under standard conditions for 2 weeks, then randomized into 4 groups: Sham + Vehicle (S + V); Sham + Guanosine 7.5 mg/kg (S + G); BDL + Vehicle (BDL + V); BDL + Guanosine 7.5 mg/kg (BDL + G). Guanosine (or vehicle) was administered for the first time 2 weeks after the surgery, once a day, for 7 day. The guanosine dose was chosen based on several previous in vivo studies (Lara et al. 2001; Schmidt et al. 2000, 2007; Vinadé et al. 2004) and others (Connell et al. 2013; Rathbone et al. 2011), which demonstrated the optimal effect of guanosine at 7.5–8 mg/kg in rats.

Two groups of animals were independently used: I) one for behavioral studies and neurochemical and redox analysis and II) other for EEG evaluation. This specific group was not used in behavioral tasks because the animals were submitted to

additional surgery procedure that could affect the behavioral performance.

Behavioral studies

Based on previous in vivo studies, animals received i.p. injection of guanosine (or vehicle) 15 min before each task (Connell et al. 2013; Lara et al. 2001; Rathbone et al. 2011; Schmidt et al. 2007, 2010b). The person performing the experiment was blind for groups and treatments applied.

Open field The Open Field Test provides simultaneous measures of locomotion, exploration and anxiety (Walsh and Cummins 1976). The experiment consisted of 2 trials separated by 24 h. In the first trial it was analyzed the total distance travelled, which indicated the motor activity. In the second trial it was analyzed the habituation to the environment (Apelqvist et al. 1999; Butterworth et al. 2009; Jover et al. 2006). During the trials, each animal was placed in a MDF cage (50 cm×50 cm×50 cm) covered with synthetic water proof material. The distance travelled was recorded for 10 min and analyzed using the ANY-Maze video-tracking system (Stoelting, CO). The apparatus was cleaned after each animal with 70 % alcohol and let dry.

Y maze Mazes are experimental devices used for global evaluation of spatial memory in rodents, among other purposes (Paul et al. 2009). Here, 24 h after the open field task, each animal was evaluated in an apparatus made of wood covered with Formica; with 3 arms. Each arm was 50 cm long, 12 cm wide and 30 cm height. The experiment consisted of 2 trials separated by 30 min. In the first trial, 1 (of 3) arm of the Y-maze was closed. Rats were placed in an arm, their head oriented in the opposite direction to the center of the maze and they were allowed to visit the 2 open arms for 5 min. In the second trial (30 min later), they had free access to the 3 arms and were allowed to explore the maze for 5 min. The total time exploring the novel arm was used for evaluation of short-term habituation (Conrad et al. 1996).

Tissue preparation

After 3 days of behavioral studies, animals continued receiving guanosine or vehicle once a day up to complete 1 week of treatment. Twenty-four hours after the last administration, the rats were anesthetized with sodium thiopental (40 mg/kg, 1 mL/kg i.p.) and placed in a stereotaxic apparatus, to take a sample of cerebrospinal fluid (CSF) (40 to 80 μL per rat) by direct puncture of the cisterna magna with an insulin syringe (27 gauge × 1/2-in. length) (Schmidt et al. 2007). The CSF was centrifuged at 10,000 × g for 10 min; the supernatant was stored at −80 °C for further evaluation of purines and glutamate CSF levels. The blood was taken from cardiac puncture, followed by centrifugation at 5,000 g for 10 min, and the plasma was stored at −80 °C for further evaluation. They were then decapitated, the brain was removed and the brain structures were immediately dissected and stored at −80 °C for further neurochemical and redox analysis. Just before the neurochemical analysis, the cerebral cortex (to measure ammonia levels), hippocampus and striatum (to evaluate redox parameters) were homogenized in PBS (Phosphate Buffered Saline, 20 mM, pH 7.4).

Blood biochemical parameters

Plasmatic alanine aminotransferase (ALT) (Cat. No. 20764957 322 System-ID 07 6495 7), bilirubin (Cat. No. 03146022 122 System-ID 07 65872), alkaline phosphatase (Cat. No. 03333752 190 System-ID 07 6761 1), γ-glutamyl transpeptidase (GGT) (Cat. No. 03002721 122 System-ID 07 6598 8), creatinine (Cat. No. 04810716 190 System-ID 07 6928 2) and urea (Cat. No. 04460715 190 System-ID 07 6303 9) were measured using commercial kits (Labtest, MG, Brazil).

Ammonia concentration in cerebral cortex

Ammonia levels were measured in the homogenized cerebral cortex, using a commercial kit (Sigma, St. Louis, MO, USA, Cat. No. AA0100) according to the manufacturer's protocol.

Redox parameters

The oxidative stress parameters were evaluated in hippocampus and striatum as the involvement of these structures on the behavioral tasks here modulated by BDL and guanosine has been well documented in the literature (Eichenbaum et al. 1999; Izquierdo et al. 2006; Paul et al. 2009; Packard and Knowlton 2002; Packard 2009).

Dihydrodichlorofluorescein (DFCH) oxidation To assess the levels of reactive oxygen species (ROS), 2′,7′-dichlorofluorescin (DCFH-DA, Sigma) was used as a probe (Lebel et al. 1992). Inside the cell, esterases cleave acetate groups of DCFH-DA, trapping the reduced form of the probe (DCFH). ROS inside the cells may lead to DCFH oxidation, resulting in the fluorescent product DCF. An aliquot of the sample was incubated with DCFH-DA (100 μM) at 37 °C for 30 min. The formation of the oxidized fluorescent derivative (DCF) was monitored at excitation and emission wavelengths of 488 and 525 nm, respectively, using a fluorescence spectrophotometer. The ROS

content was quantified using a DCF standard curve and results were expressed as nmol DCF formed/mg protein.

Thiobarbituric acid reactive species (TBARS) In order to assess the extent of lipoperoxidation, the levels of TBARS were measured through a heated and acidic reaction. This is widely adopted as a method for the measurement of lipid redox states, as previously described (Draper and Hadley 1990). Aliquots of structures homogenate (200 μg) were mixed with 0.3 mL of 20 % TCA and 0.1 mL of 0.82 % thiobarbituric acid and heated in a boiling water bath for 45 min. The level of TBARS was determined by measuring the absorbance at 532 nm. The concentration of TBARS was determined from a calibration curve using 1,1,3,3-tetramethoxypropane as standard. Results are expressed as nmol of TBARS per milligram of protein.

CSF parameters

Purines (Schmidt et al. 2010c) and Glutamate (Schmidt et al. 2009) CSF levels were measured by High-Performance Liquid Chromatography (HPLC). Purines levels were evaluated due the fact that guanine and adenine purines are involved in the pathophysiology of brain injuries (Burnstock et al. 2011; Schmidt et al. 2007). The following purines were evaluated: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine, guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine, Inosine monophosphate (IMP), Inosine, hypoxanthine, xanthine and uric acid.

EEG analysis

The animals that were implanted with epidural electrodes for EEG recordings were not submitted to behavioral studies or neurochemical analysis. Two weeks after the surgery, they received guanosine 7.5 mg/kg or vehicle for 7 days, once daily. EEG was performed 24 h after the last administration. Each animal was then individually transferred to an observation cage (Plexiglas chambers). Electrophysiological signals were recorded with a standard data acquisition system (Multichannel Plexon Acquisition Processor System). After ensuring a good signal-to-noise ratio and usual characteristics (with no suppression or abnormal electrical discharges) animals were transferred to a square-shaped black arena (50×50×50 cm). They were kept on this arena to perform the continuous EEG recording for 15 min. EEG signals were filtered at 0.01–100 Hz followed by digitization at 1 kHz for posterior analysis. All analyses were done using a built in and custom written routines in MATLAB (Mathworks, Inc). The power spectra density was obtained and the EEG left index was calculated as the logarithm of the ratio between power of the low frequency (1–7.4 Hz) and the high frequency (13.5–26.5 Hz). Normal rats have an EEG left index of approximately 0.60, and rats in coma approximately 0.80–0.90 (Vogels et al. 1997a). After EEG rerecording the animals were sacrificed.

Statistical analysis

Data are expressed as means ± S.E.M. To compare different groups, we used a one-way ANOVA followed by Duncan post-hoc, Kruskal Wallis test followed by Dunn's Multiple Comparison Test and student *t* test, when mentioned, using GraphPad Prism4 and SPSS (La Jolla, CA, USA). Significance level was taken as $P<0.05$.

Specifically for EEG experiment, we use a small number of animals of S + G group based on previous experience showing that at this dose, guanosine does not affect any parameter on EEG of sham animals.

Results

After 3 weeks of the surgery, all BDL rats (vehicle or guanosine treated) exhibited clinical signs of chronic liver disease: jaundice, ascites and nodular liver. All animals of the 4 groups exhibited similar weight. As the mortality rate was zero, all animals were sacrificed for blood and brain biochemical analysis.

Biochemical parameters in the blood

Plasmatic levels of ALT, bilirubin, alkaline phosphatase, and GGT were higher in BDL groups when compared to Sham groups, characterizing the hepatic disorder caused by BDL (one-way ANOVA, $P<0.05$). There was no difference in blood levels of urea among groups. Creatinine levels decreased in (one-way ANOVA, $P<0.05$) BDL animals, which could be attributed to a skeletal muscle wasting commonly found in cirrhosis (Table 1). Guanosine had no effect on these parameters, except on Alkaline Phosphatase (one-way ANOVA,$P<0.05$).

Behavioral tasks

Open Field In order to evaluate the motor activity of the animals we performed the open field task (Fig. 1).

At the first trial, the total distance traveled (in m) was significantly lower in BDL + V (T1: 16.53 ± 1.62, $n=12$) compared to S + V (25.54 ± 1.38, $n=13$) (one-way ANOVA, $P<0.05$), these data indicating the efficacy of BDL, as reported previously (Butterworth et al. 2009; Jover et al. 2006). Besides, in

Table 1 Plasma biochemical measurements

	S + V	S + G	BDL + V	BDL + G
PLASMA				
ALT (U/L)	23.57 ± 1.87	17.50 ± 0.90	55.27 ± 6.74*	46.50 ± 4.00*
GGT (U/L)	0	0	34.50 ± 4.12*	43.30 ± 7.99*
Alkaline Phosphatase (U/L)	100.90 ± 8.43	82 ± 3.80	220.30 ± 22.14*	305.30 ± 27.41*#
Bilirubin (mg/dl)	0.09 ± 0.01	0.12 ± 0.01	7.70 ± 0.34*	7.50 ± 0.58*
Urea (mg/dl)	52.20 ± 1.14	48.40 ± 1.39	48.90 ± 2.31	54.38 ± 1.33
Creatinine (mg/dl)	0.33 ± 0.01	0.34 ± 0.01	0.20 ± 0.01*	0.20 ± 0.01*

*$p<0.05$ when compared to S + V # $p<0.05$ when compared to BDL + V. N between 8 and 13 animals

S + V group, the total distance traveled (in meters) significantly decreased (*t* test, $P<0.05$) from the 1st (T1) (25.54 ± 1.38, $n=13$) to the 2nd session (T2) (19.4 ± 1.51, $n=13$), indicating habituation in the sham group, while, in the BDL + V there was no difference between the sessions, pointing the absence of habituation (T1: 16.53 ± 1.62, $n=12$; T2: 17.82 ± 1.53,$n=13$). With Guanosine there no difference between the sessions in the sham group, S + G (T1: 28.28 ± 1.85, $n=8$; T2 24.83 ± 1.36, $n=8$), indicating the amnesic effect as previously demonstrated in other behavioral tasks (Schmidt et al. 2007). Also, Guanosine had no effect on BDL group, BDL + G (T1: 18.30 ± 1.80, $n=10$; T2 17.71 ± 1.05).

Y Maze Animals from BDL + V group (56 ± 7, $n=11$) spent less time (in seconds) exploring the novel arm (short-term memory), compared to S + V and BDL + G: S + V (82 ± 5, $n=15$), BDL + G (77 ± 7, $n=11$) and S + G (69 ± 5, $n=7$) (one-way ANOVA, $P<0.05$), Fig. 2. Note that guanosine reversed this BDL effect, without affecting the Sham group.

Ammonia levels in the cerebral cortex

The ammonia levels (μmol/g) in the cerebral cortex were higher in BDL + V (0.108 ± 0.010, $n=6$), compared to S + V (0.074 ± 0.010, $n=8$), (Kruskal Wallis test, $P<0.05$). Guanosine had no effect on ammonia levels in the brain of both BDL and S groups (Fig. 3).

EEG parameters

EEG recordings were performed 24 h after the last administration of guanosine or vehicle. As showing in the representative EEG traces in Fig. 4a, by simple visual inspection it was observed prominent theta (4–10 Hz) oscillations in the epidural EEG of Sham animals, while in the BDL animals, the EEG basal theta activity was interposed by periods of intermittent slow delta activity (0–4 Hz). By this simple visual inspection there was no clear difference in slow activity between BDL + V and BDL + G. To better analyze the EEG, the EEG left index calculation was performed, Fig. 4b.

Normal rats have an EEG left index of approximately 0.60, and rats in coma approximately 0.80–0.90 (Vogels et al. 1997a). Here, the EEG left index was abnormally higher in BDL + V group (0.70 ± 0.04, $n=9$) when compared to normal values of S + V (0.56 ± 0.02, $n=10$), (Kruskal Wallis, $P<0.05$). Notably, the BDL + G animals have values of EEG left index (0.62 ± 0.03, $n=9$) slightly lower than BDL + V, and similar to S + G (0.56 ± 0.03, $n=3$) and S + V. Thus, guanosine had no significant effect on EEG left index in BDL animals.

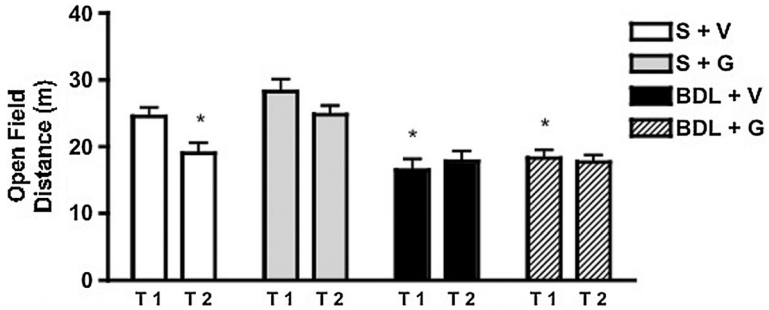

Fig. 1 Open field task. Motor activity was evaluated by the travelled distance (*m*) performed in an open field session for 10 min. *$P<0.05$ when compared to S + V trial 1 (*T1*)

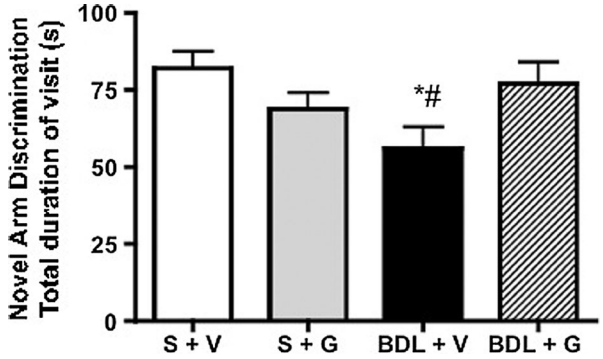

Fig. 2 Y maze task. Short-term habituation was evaluated by the time (*s*) spent exploring the Novel Arm in a Y maze session, which last 300 s. * $P<0.05$ when compared to S + V. # $P<0.05$ when compared to BDL + G

Levels of glutamate and purines in CSF

Levels of glutamate (Fig. 5) and purines - Inosine, GMP, xanthine and uric acid - (Fig. 6) in CSF of BDL + V animals were higher, when compared to all other groups (one-way ANOVA or Kruskal Wallis, $P<0.05$). Guanosine prevented the increment of glutamate and purine CSF levels, without affecting the levels in Sham groups.

Redox analysis in the brain

The levels of reactive oxygen species (DCFH) and lipoperoxidation (TBARS) were measured in hippocampus and striatum. The levels of DCFH in the hippocampus were similar for all groups (Fig. 7a), while in the striatum, levels of DCFH were higher in BDL + V when compared to S+V, (one-way ANOVA, $P<0.05$), Fig. 7b. Moreover, the levels of TBARS in hippocampus and striatum were higher in the BDL + V when compared to S+V (one-way ANOVA, $P<0.05$), Fig. 7c-d. Therefore, only on DCFH in hippocampus, neither BDL nor Guanosine had any effect.

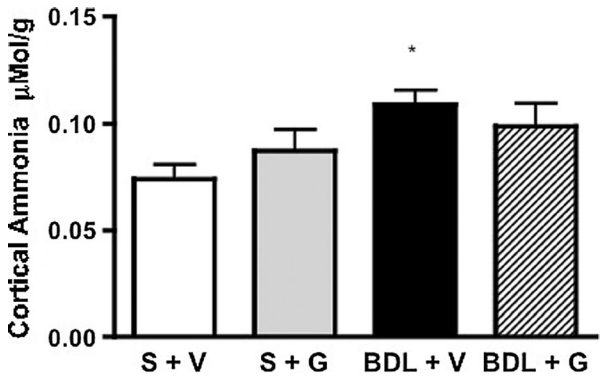

Fig. 3 Ammonia levels in cerebral cortex. Ammonia levels are expressed in µmol/g. * $P<0.05$ when compared to S + V

Discussion

We used an effective experimental model of chronic Hepatic Encephalopathy (BDL), exhibiting behavioral, biochemical and EEG pattern alterations seen in human HE.

The blood biomarkers of hepatic disease (Table 1) measured in the experimental model of HE indicated that BDL definitely affected the liver. Guanosine had no effect on blood biomarkers, except on Alkaline Phosphatase. This hepatic dysfunction altered various neurological parameters: I) increased the levels of ammonia in the brain; II) increased the levels of reactive oxygen species and the activity of brain lipoperoxidation; III) increased the levels of glutamate and some purines and metabolites in the CSF; IV) impaired the behavioral performance observed in the open field and Y maze task; and V) altered EEG pattern. To our knowledge, this is the first report indicating the participation of purinergic system in the CNS of rats with HE.

Furthermore, we were able to demonstrate the neuroprotective effects of guanosine in the BDL model of HE. This data reinforces previous findings indicating that guanosine plays an important role in neuroprotection of several in vivo and in vitro animal models of glutamate excitotoxicity, such as seizures, brain ischemia and glutamatergic nociception (Connell et al. 2013; Rathbone et al. 2011; Schmidt et al. 2007; Schmidt and Souza 2010). Guanosine did not affect the levels of blood biomarkers of hepatic disease, suggesting that it did not affect the liver activity. However, guanosine reverted, totally or partially, some brain effects of HE, such as the increase of CSF levels of glutamate and purines, the increase in brain oxidative stress and the impairment of the performance in Y maze task.

The EEG analysis is considered as an effective method to evaluate HE (Amodio and Gatta 2005; Vogels et al. 1997b). In this study, the EEG analysis showed an intermittent slow activity and an increased EEG left index in BDL animals, indicating a decline in the conscious level (Amodio and Gatta 2005; Vogels et al. 1997b). Guanosine has no significant effect on these EEG parameters. Although a clear effect of guanosine on the EEG pattern was not observed by the qualitative visual inspection, the guanosine was able to slightly decrease (no statistical difference, compared to S + V and S + G groups) the EEG left index values in BDL treated animals.

BDL reduced the motor activity and abolished the habituation in an open field task (Fig. 1), similar to previous findings observed in this model of HE (Apelqvist et al. 1999; Butterworth et al. 2009; Jover et al. 2006). This strengthens the effectiveness of our experimental model.

Cauli and colleagues (Cauli et al. 2007) have already described an impaired Y maze performance in rats with HE. In our study, BDL animals presented an impairment of short-term habituation (memory) in the Y maze task (Fig. 2) and guanosine was able to restore it, without affecting the sham

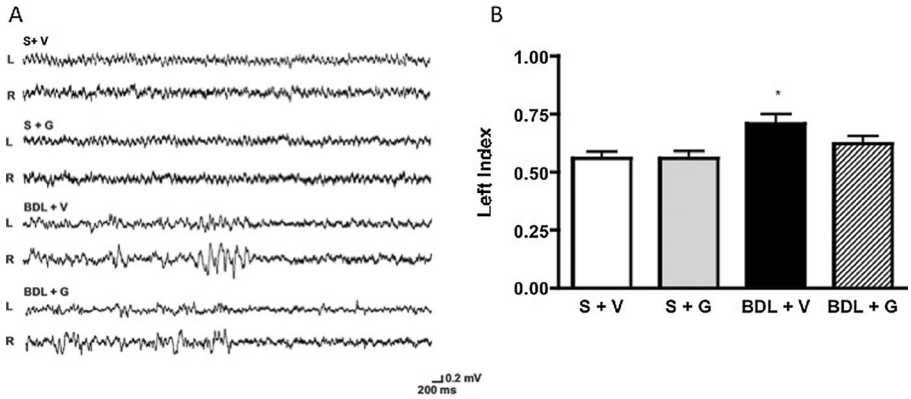

Fig. 4 EEG. A: Samples of EEG traces obtained from S + V; S + G; BDL + V; BDL + G; B: Spectral EEG left index. *$P<0.05$ when compared to S + V

group performance. This result may reflect a behavioral manifestation of a neuroprotective effect of guanosine.

It has been demonstrated that both in vitro and in vivo administration of guanosine had neuroprotective effect against the glutamate excitotoxicity (Frizzo et al. 2001; Gottfried et al. 2002; Lara et al. 2001; Oliveira et al. 2004; Schmidt et al. 2007; Schmidt and Souza 2010). We found increased levels of glutamate in the CSF levels of BDL animals, which was notably reversed by guanosine, Fig. 5. This data supports previous evidence of glutamatergic system alterations in HE (Butterworth 2010) and indicates the ability of guanosine to decrease glutamate extracellular levels under pathologic conditions - an effect that was previously correlated with an increase of glutamate uptake by astrocytes (Frizzo et al. 2003; Schmidt et al. 2007, 2009).

The analyses of CSF also demonstrated increased levels of Inosine, GMP, xanthine and uric acid, Fig. 6. The purinergic system, including the adenine- and guanine-based purines, is able to modulate glutamate excitotoxicity (Burnstock et al. 2011; Schmidt et al. 2007; Schmidt and Souza 2010). It has been shown that adenosine decreases glutamate release in the CNS, while guanosine increase astrocytic glutamate uptake (Burnstock et al. 2011; Frizzo et al. 2001, 2003; Schmidt et al. 2007; Schmidt and Souza 2010; Sheldon and Robinson 2007).

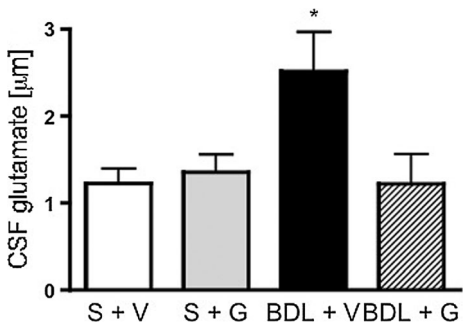

Fig. 5 Extracellular levels of glutamate in the CSF. Levels of glutamate are expressed in µM. All results are presented as the mean + S.E.M. (n=8 per group). * $P<0.05$, when compared to all other groups

Surprisingly, the levels of these nucleosides in the CSF (adenosine and guanosine) were not enhanced in the BDL + V group, despite the increased levels of Inosine and GMP. As the exogenous administration of guanosine normalized the purinergic parameters in the CSF, we can suggest that the pathophysiological implications of changes in endogenous purines may represent an attempt of CNS to enhance levels of adenosine and guanosine. Our hypothesis is based on the ability of guanosine to prevent, in BDL + G group, the increment of GMP levels in the CSF, a nucleotide that can be converted to guanosine. Noteworthy, we cannot rule out that the increased levels of purines in CSF of rat with HE could be simply a sign of the CNS dysfunction, with no physiological relationship to the glutamate excitotoxicity.

Additionally, the increased xanthine and uric acid levels in CSF, Fig. 6c-d, caused by BDL procedure, may represent an indirect generation of free oxygen radicals through xanthine dehydrogenase/oxidase (XDH) enzyme complex (Stover et al. 1997; Yonden et al. 2010). This can be corroborated by the increased levels of DCFH and TBARS, Fig. 7c-d in BDL + V rats. Oxidative stress is one of the key points in the pathogenesis of HE that could be related to the glutamate excitotoxicity (Brück et al. 2011) and increased lipoperoxidation and ROS. It has been shown a strong linkage among behavioral alterations in HE and dysfunctions in hippocampus and striatum (Arias et al. 2013; Méndez et al. 2008, 2009). Moreover, the involvement of these brain structures and the behavioral performance of the rats were modulated by the BDL procedure and guanosine in vivo administration (Eichenbaum et al. 1999; Izquierdo et al. 2006; Paul et al. 2009; Packard and Knowlton 2002; Packard 2009). Therefore, hippocampus and striatum were chosen for oxidative stress analysis. Our results suggest that the guanosine-induced reduction of oxidative stress in HE could be attributed, at least in part, to the decreased glutamate extracellular levels (Fig. 5) consequent to the enhanced glutamate uptake by astrocytes caused by guanosine. Accordingly, it has been previously demonstrated a protective effect of guanosine in vivo in a rat model of sepsis (Petronilho et al.

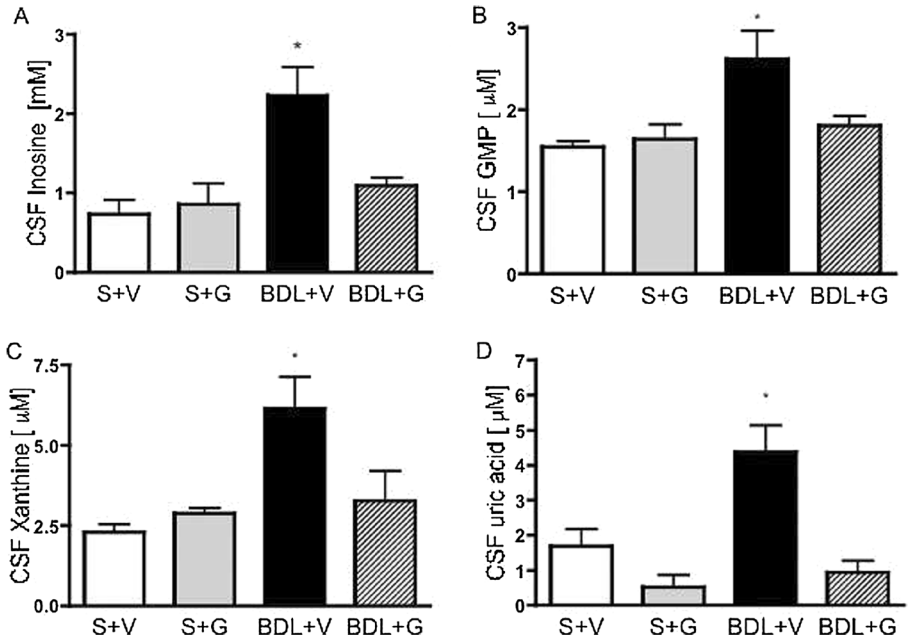

Fig. 6 Levels of purines and metabolites in the CSF. (**a**, **b**) Levels of Purines and (**c**, **d**) levels of metabolites in the CSF. Levels of Inosine **a** are expressed in mM and GMP **b**, Xanthine **c** and Uric Acid **d** are expressed in µM. All results are presented as the mean + S.E.M. (n=8 per group). * P<0.05 when compared to S + V. * P<0.05 when compared to all other groups

2012). Thus, it can be hypothesized that the improvement in the oxidative stress parameters, in hippocampus and striatum are associated with the improvement in short memory shown in the Y maze task.

Glutamatergic dysfunction and oxidative stress are clearly implicated in the pathogenesis of HE. It is well known that the behavioral manifestations of this syndrome are related to the glutamate excitotoxicity. We showed that guanosine was able to prevent the increase in the levels of glutamate in CSF and to improve short-term memory in HE. Guanosine also normalized the levels of endogenous purines in the CSF, and the brain levels of biomarkers of oxidative damage, such as TBARS and DCFH.

To our knowledge, this study, besides to indicating the well known involvement of the glutamatergic system and the oxidative stress in HE, is the first to show: (1) the neuroprotective effects of systemic administration of guanosine in a rat model of HE; and (2) the participation of the purinergic system in the pathophysiology of HE. Further studies should be performed to clarify the role of purinergic system on this disease and the neuroprotection mechanisms of guanosine.

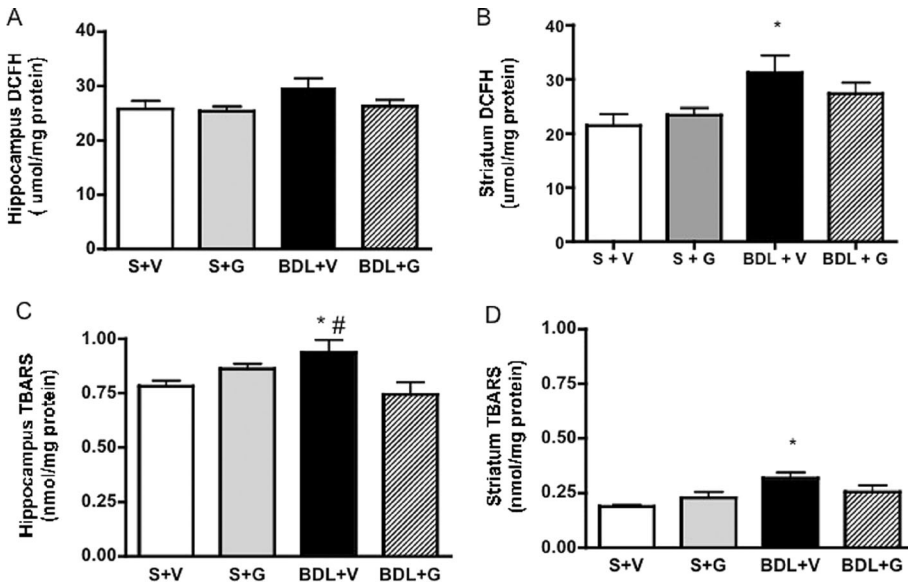

Fig. 7 Brain oxidative damage analysis in hippocampus and striatum. DCFH oxidation (**a**, **b**) and TBARS levels (**c**, **d**) measured in hippocampus and striatum. DCFH (**a**, **b**) and TBARS (**c**, **d**) are expressed in nmol/mg protein. All results are presented as the mean + S.E.M. (n=6 per group).* P<0.05 when compared to S + V. # P<0.05 when compared to BDL+G

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Conflict of interests The authors declare that they have no conflict of interest.

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CAPÍTULO 3

Guanosine prevents death in a rat model of acute hyperammonemia

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Artigo em preparação para ser submetido no periódico Hepatology

Guanosine prevents death in a rat model of acute hyperammonemic encephalopathy

Abstract: It is well known that glutamatergic excitotoxicity and oxidative stress are implicated in the pathogenesis of acute hyperammonemia. The nucleoside Guanosine exerts neuroprotective effects through the antagonism against glutamate neurotoxicity and antioxidant properties. In this study, we evaluated the neuroprotective effect of Guanosine in an animal model of acute hyperammonemia. Rats received a pretreatment with Guanosine (60mg/kg) or vehicle and after that were injected with ammonium acetate (7mM/kg) or distilled water. All the drugs were administered intra-peritoneal. We evaluated the effects of Guanosine in acute hyperammonemia studying several aspects: a) neurological status; b) mortality c) brain rhythm changes in electroencephalogram (EEG) recordings; d) glutamate and ammonia levels in the cerebral spinal fluid (CSF); e)glutamate uptake and f) oxidative stress parameters in the brain. Ammonium acetate caused neurological deterioration and presented increased levels of glutamate and ammonia on CSF, as well as increased oxidative damage. Guanosine was able not only to prevent these effects but also to prevent death and EEG impairment induced by hyperammonemia. Our study shows the neuroprotective effects of systemic administration of Guanosine in a rat model of acute hyperammonemia.

Introduction

Acute hyperammonemic encephalopathy is associated with high morbidity and mortality unless promptly treated. Acute hepatic dysfunction leading to deficiencies in enzymes of the urea cycle is the most frequent etiology, called hepatic encephalopathy (HE), but other metabolic disorders can be implicated [1,2,3,4,5]. The encephalopathy is attributed to ammonia neurotoxicity leading to brain edema, coma and death [6, 7], and liver transplantation is the only treatment to avoid brain herniation in several cases [8]. This deleterious effect on the brain involves the glutamatergic system excitotoxic mechanism through elevation of extracellular glutamate, decreased glutamate transporters and hyperactivation of NMDA receptor and increase in brain oxidative stress damage [8,9]. Previous studies demonstrated that neuroprotection against glutamatergic excitotoxicity helps to decrease the deleterious effect of hyperammonemia over the CNS [10]. This can be achieved through MK801 antagonism on NMDA receptors, for instance [10]. The nucleoside Guanosine also exhibits several neuroprotective effects in experimental models of brain injuries involving glutamatergic excitotoxicity, such as seizures, brain ischemia and pain [11,12,13,14]. The antioxidant properties of Guanosine [15] and its ability to increase the astrocytic glutamate uptake [11,16,17] seem to be related to its neuroprotective effects. Recently, we has shown that \ Guanosine has neuroprotective effects in chronichepatic encephalopathy in rats [18]

However, it remains unclear whether Guanosine could play an important role in neuroprotection in acute hyperammonemic encephalopathy. Therefore, we examined the effects of Guanosine in a rat model of acute hyperammonemia evaluating mortality rate,

coma duration by clinical and electroencephalogram (EEG) profile, brain redox parameters and brain glutamate uptake

Matherial and Methods

Ethics Statement

All experiments were approved by the local Ethics Commission (CEUA/UFRGS) under project number 23980, and they all followed the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (NIH publication N°. 80-23, revised 1996).

Animals

Adult male Wistar rats (60 days old) from the Central Animal House of the Department of Biochemistry were maintained under a standard dark/light cycle (the lights were on between 7:00 a.m. and 7:00 p.m.) at room temperature (22 ± 2°C), housed in plastic cages (5 per cage) with tap water and commercial food ad libitum. These conditions were maintained constant throughout the experiments.

Drugs

Guanosine and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Guanosine was dissolved in 0.1mM NaOH. Vehicle solution was 0.1mM NaOH. Both solutions were at pH 7.4. Either Guanosine (30, 60 and 120mg/kg) or vehicle were administered with an intraperitoneal (i.p) injection, 2ml/kg.

Ammonia administration: Ammonium acetate (2.5-7mmol/kg) was dissolved in distilled water and injected intraperitoneally. The concentrations of the solutions were adjusted to reach the desired dose by injecting 3 μL per gram of body weight as previous described [10]. For neurochemical evaluation we used distilled water i.p. at the control group.

Neurological Evaluation. Following ammonia intoxication, rats suffered a progressive deterioration of neurological status. Three stages (precoma, coma, and death) were observed, as described [10]: at precoma (loss of righting ability), rats could no longer right themselves after being placed on their backs; at coma, a corneal reflex could not be elicited, sometimes followed by seizures; and death.. The neurological evaluation was made every 3 minutes, during 60 minutes after the ammonium acetate i.p. injection.

Experimental design.

Protocol standardization (Figure 1):

Ammonium acetate: A response dose curve as performed and the dose established was 7mmol/kg i.p. (dose that caused 85% of coma and death in this model) (Figure 2A).

Guanosine: Animals were pretreated different doses of Guanosine (i.p.) 20 min before ammonium acetate injection (7mmol/kg i.p.). We evaluated the effect of Guanosine at different concentrations on mortality rate (Figure 2B). (N between 10-12 per group). Once we observed a clearly dose dependent effect of Guanosine, we chose the lower dose (60mg/kg) to study other outcomes such as: duration of coma, EEG alterations, glutamate uptake, CSF glutamate and purines and REDOX parameters.

Protocol 1: For clinical and EEG analysis animals were divided into 2 groups: Vehicle + Ammonium Acetate; Guanosine + Ammonium acetate. The rats were monitored for 60 minutes after ammonia injection.

Protocol 2: For biochemical analysis animals were divided into 4 groups: Distilled water; Guanosine + Distilled water; Ammonium Acetate; Guanosine + Ammonium acetate. The rats were sacrificed 20 minutes after ammonia or distilled water injection for biochemical analysis.

We used ammonium acetate 7mmol/kg and Guanosine 60 mg/kg based on our previous response dose curve. Vehicle was used as Guanosine control group.

In both protocols Ammonium Acetate was injected 20 minutes after Guanosine or Vehicle. We used distilled water as ammonia acetate control group at Protocol 2 (biochemical analysis).

Electrode implantation for EEG

The animals were anesthetized with with ketamine (80mg/kg) and xylazine (10mg/kg) i.p. Each animal was fixed in a stereotaxic instrument to implant the electrodes. , The skin covering the skull was cut with a 3-cm-long rostro-caudal incision in the midline. After skull exposure, 3 stainless steel screw electrodes (1.0mm diameter) were placed over the dura-mater through holes in the skull made with a dental drill. Two of them were used as positive electrodes (2.0mm lateral, right or left, 1.0mm posterior to bregma) and the reference (negative) electrode was placed in the midline of the occipital bone and kept in contact with cerebrospinal fluid. The electrodes were fixed with dental acrylic cement,

and a screw used for fixation of the dental acrylic helmet to the skull was used as ground [17]. Electrodes were implanted 1 week before ammonia injection and EEG analysis.

EEG analysis

The animals that were implanted with epidural electrodes for EEG recordings were not submitted to clinical evaluation or neurochemical analysis. One week after electrodes implantation, each animal was individually transferred to an observation cage to perform the continuous EEG recording for 15 min (Plexiglas chambers). After, Guanosine (60mg/kg) or vehicle was i.p. injected, and 20 minutes later ammonium acetate was i.p. injected . Electrophysiological signals were recorded with a standard data acquisition system (Multichannel Plexon Acquisition Processor System). EEG signals were filtered at 0.01-100 Hz followed by digitization at 1 kHz for posterior analysis. All analyses were done using a built in and custom written routines in MATLAB (Mathworks, Inc). The power spectra density was obtained to calculate EEG left index during the baseline, after Guanosine injection, after ammonium acetate infection and at the end of the EEG recording. EEG left index was calculated as the logarithm of the ratio between power of the low frequency (1-7.4 Hz) and the high frequency (13.5-26.5 Hz). Normal rats have an EEG left index of approximately 0.60, and rats in coma approximately 0.80-0.90 [19]. After 70 minutes of EEG recording the animals who survived after coma were sacrificed.

Biochemical Parameters

After 20 minutes of ammonium acetate or distilled water injection the rats were anesthetized with sodium thiopental (40mg/kg, 1mL/kg i.p.) and were placed in a stereotaxic apparatus, to take a sample of cerebrospinal fluid (CSF) (40 to 80μL per rat) by direct puncture of the cisterna magna with an insulin syringe (27 gauge × 1/2-inch length) [6]. The CSF was centrifuged at 10000 x g for 10 minutes; the supernatant was stored at -80°C for further evaluation of purines and glutamate CSF levels. The blood was taken from cardiac puncture, followed by centrifugation at 5000 x g for 10 minutes, and the plasma was stored at -80°C for further evaluation. They were then decapitated, the brain was removed and the brain structures were immediately dissected and stored at -80°C for further neurochemical analysis.

Serum Biochemical Characterization. Plasmatic alanine aminotransferase (ALT), bilirubin, alkaline phosphatase, γ-glutamyl transpeptidase (GGT), creatinine and urea were measured using routine biochemistry techniques.

Ammonia. Ammonia levels were measured in the homogenized cerebral cortex, using a commercial kit (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol.

Glutamate Uptake

Animals were immediately decapitated 10 or 20 minutes after ammonium acetate or distilled water administration. The frontoparietal cortex immediately dissected on ice

(4°C). Slices from frontoparietal cortex (0.2 mm thick) were rapidly prepared using a McIlwain Tissue Chopper, separated in HBSS (in mM: 137 NaCl, 0.63 Na2HPO4, 4.17 NaHCO3, 5.36 KCl, 0.44 KH2PO4, 1.26 CaCl2, 0.41 MgSO4, 0.49 MgCl2 and 1.11 glucose, pH 7.2) at 4°C. Frontoparietal cortical slices were preincubated with HBSS at 37°C for 15 min, followed by the addition of 0.33 μCi mL−1 L-[3H] glutamate (PerkinElmer®). Incubation was stopped after 7 min with 2 ice-cold washes of 1 mL HBSS. After washing, 0.5N NaOH was immediately added to the slices and they were stored over-night. Na+ independent uptake was measured using the above-described protocol with alterations in the temperature (4°C) and the composition of the medium (N-methyl-D-glucamine instead of NaCl). Results (Na+ dependent uptake) were measured as the difference between the total uptake and the Na+-independent uptake. Each incubation was performed in quadruplicate [20]. Incorporated radioactivity was measured using a liquid scintillation counter (Hidex 300 SL).

Oxidative Stress

The oxidative stress parameters were evaluated in cortex. The structures were homogenized in PBS (Phosphate Buffered Saline, 20 mM, pH 7.4) for analysis of redox parameters.

Dihydrodichlorofluorescein oxidation. Levels of reactive oxygen/nitrogen species were measured as LeBel [21]. Briefly, DCFH-DA was incubated for 30 min at 37°C with an aliquot of cortex homogenate (5μL per mg of tissue). Fluorescence was measured using excitation and emission wave length of 488nm and 525nm, respectively. A calibration

curve was performed with standard DCF and the levels of reactive species were expressed as μmol of DCF formed per mg protein.

Thiobarbituric acid reactive species (TBARS). In order to assess the extent of lipoperoxidation, the levels of TBARS were measured through a heated and acidic reaction [22]. Aliquots of structures homogenate were mixed with 0.6mL of 10% TCA and 0.5mL of 0.67% thiobarbituric acid and heated in a boiling water bath for 25 min. The level of TBARS was determined by measuring the absorbance at 532 nm. The concentration of TBARS was determined from a calibration curve using 1,1,3,3-tetramethoxypropane (which had been subjected to the same treatment as the supernatants) as a standard. Results are expressed as nmol of TBARS per milligram of protein.

Antioxidant enzyme activities

The superoxide dismutase (EC 1.15.1.1) (SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described, and the results are expressed as units of SOD/mg of protein [23]. The glutathione peroxidase (EC 1.11.1.9) (GSH-Px) activity was measured according to [24]. One unit of GSH-Px activity was defined as 1 μmol of NADPH consumed/min and the specific activity was expressed as unit/mg of protein.

CSF Parameters

Purines [25] and Glutamate [26] CSF levels were measured by High-Performance Liquid Chromatography (HPLC). Purines levels were evaluated due the fact that guanine and adenine purines are involved in the pathophysiology of brain injuries [11,27]. The

following purines were evaluated: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine, Guanosine triphosphate (GTP), Guanosine diphosphate (GDP), Guanosine monophosphate (GMP), Guanosine, inosine monophosphate (IMP), inosine, hypoxanthine, xanthine and uric aid.

Statistical analysis

Data are expressed as means ± SEM. To compare different groups, we used a one-way ONE-WAY ANOVA followed by Newman–Keuls post-test or Kruskal Wallis test followed by Dunn's Multiple Comparison Test, and student t test, when mentioned, using GraphPad Prism4 (La Jolla, CA, USA). Significance level was taken as $p < 0.05$.

Results

Clinical assessment

We observed that 69% (n=42) of Guanosine pretreated rats and control animals 75% (n=41) developed coma (Figure 2) No statistical difference was observed. On the other hand, , the coma state duration in the Guanosine group (n=20) was 22.80+1.95 minutes while in the Control group (n=18) was 29.17 +1.54 minutes, (Figure 3) ($P<0.005$, t test). Importantly, the mortality rate (Figure 4) in Guanosine group was lower (15,5% ; n=52) than the Control group (35.9% ; n=78), $p<0.05$, fisher test.

EEG

EEG recordings were performed during 70 minutes. We observed that the EEG left index (Figure 5A) was abnormally higher 10 minutes after ammonia administration in both groups, but Guanosine group (n=8, no deaths) return to normal values after 20 minutes while Control group (n=10, but 5 animals dead and we didn't consider for left index analysis) remain with low left index values until the end of recording time The representative EEG traces in Figure 5B.

Neurochemical analysis

Cerebrospinal Fluid (CSF) Ammonia (Figure 5A) and Glutamate (Figure 5B) were increased in animals that received ammonia injection and it was reversed by Guanosine ($P<0.05$, one-way ANOVA).

Glutamate Uptake Ammonium acetate decrease glutamate uptake 10 minutes after injection in both groups (to 77.14% +3.37 in ammonium acetate and to 74.84% + 4.94 Guanosine+ ammonium acetate). On the other hand, 20 minutes after ammonia injection animals that received vehicle + ammonium acetate keep decreasing the uptake (to 70.86 % + 1.90) while animals that received Guanosine+ ammonium acetate increased the uptake (to 81.72%+3.04) ($P<0.005$, one-way ANOVA) Figure 6.

Redox Parameters Ammonium acetate has no effect on DCFH and TBARS (Figure 7C and 7D). On the other hand, when we analyzed antioxidan enzymes (glutatione peroxidase and superoxide dismutase) we found that animals that received Guanosine +

ammonium acetate shown its levels similar to control animals while animals that received vehicle + ammonium acetate shown a decrease in superoxide dismutase and an increase in glutathione peroxidase, which points to brain oxidative stress damage (Figure 7A and 7B).

Discussion

We used an effective experimental model of acute hyperammonemic encephalopathy, exhibiting behavioral and EEG pattern alterations similar to human with acute hepatic encephalopathy.

This ammonia intoxication altered various neurological parameters: I) impaired conscious levels leading to coma and death; II) altered EEG pattern III) decrease glutamate uptake IV) altered antioxidant enzymes activity.

Besides, to our knowledge, this is the first report indicating the effect of Guanosine on Acute Hyperammonemic encephalopathy, which reinforces previous data from our and other groups indicating that Guanosine is neuroprotective in various animal models of glutamate excitotoxicity, such as seizures, brain ischemia, in the glutamatergic nociception and in chronic hepatic encephalopathy [11,13,14,18]. Pretreatment with Guanosine before ammonium acetate injection decreased the mortality rate, induced a reduction in coma duration, normalized the EEG pattern, increased glutamate uptake, normalized glutamate and ammonia on CSF and attenuated the brain oxidative stress damage observed in this model.

Hermenegildo and colleagues [10] had already described death induced by ammonium acetate in rats and it was totally prevented with MK-801, pointing that the toxicity of acute hyperammonemia was directly related do glutamatergic excitotoxicity. Furthermore, Rose [8] has described that the physiopathology of hyperammonemic encephalopathy in acute hepatic dysfunction could be related to a decrease in glutamate uptake in CNS. In this context, our study demonstrated that Guanosine reduce coma duration (Figure 3), prevents animals death (Figure 3), normalized CSF parameters (Figure 5) and increase glutamate uptake (Figure 6), which reflect a behavioral and neurochemical manifestation of a neuroprotective effect in acute hyperammonemia. Besides, as it is already known that glutamine synthetase is the mais pathway of ammonia metabolization in the brain, we can speculate that Guanosine enchance glutamine synthetase activity, once we found a decrese in ammonia levels on CSF.

The EEG analysis is considered as an effective method to evaluate neurological alterations seen in metabolic encephalopathy [28,29]. In this study, the EEG analysis showed an intermittent slow activity and an increased EEG left index in animals that received ammonium acetate injection, indicating a decline in the conscious level [28,29]. On the other hand, Guanosine pretreated animals that shown a shorter period of EEG abnormalities associated with coma state and a subsequent normalization of EEG profile (Figure 4). Token altogether it indicates an important neuroprotective effect of Guanosine in our experimental model.

It has been demonstrated that both, in vitro and in vivo administration of Guanosine have effect against the glutamate excitotoxicity [11, 16, 30] . This effect has been attributed to ability of Guanosine to stimulate glutamate uptake by astrocytes. Recently, Petronilho et

al. (2012) demonstrated a neuroprotective effect of Guanosine in vivo in a rat model of sepsis by improving oxidative stress parameters. Our study shown that animals injected with ammonium acetate had antioxidant enzymes dysfunction, while the Guanosine pretreated animals presented values similar to control group (Figure 7). Therefore, Guanosine may have additional mechanisms for neuroprotection besides stimulating gluatamate uptake, such as, by decreasing brain oxidative stress damage. Noteworthy, we could detect decrease in brain glutamate uptake in 10 minutes after ammonium acetate injection (figure 6), with no changes in oxidative stress parameters (data not shown) . On the other hand, 20 minutes after ammonium acetate injection both parameters were altered: decrease in glutamate uptake and dysfunction on antioxidant enzymes. Therefore, it seems that the decreasing in glutamate uptake occur early in the physiopathology of acute hyperammonemia. Moreover, we can speculate that the antioxidant effect of Guanosine may be a consequence of its ability to enhance glutamate uptake.

In conclusion, this study shows the well known involvement of the glutamatergic system and the oxidative stress in the physiopathology of acute hyperammonemia. Most important, our data suggests a remarkable neuroprotective effect of Guanosine in acute hyperammonemic encephalopathy with a important decrease in mortality rate, considering that this disease has high morbidity and mortality unless promptly treated

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Figure Legends

Figure 1. Experimental protocol.

Figure 2. Dose election: A: Ammonium acetate curve B: Guanosine dose-effect curve

Figure 3: Clinical assessment: (A) Coma rate after ammonium acetate injection in animals that received pretreatment with Vehicle or Guanosine. (B) Coma duration after ammonium acetate injection in animals that received pretreatment with Vehicle or Guanosine. $P<0.05$, t test. (C) Death rate after ammonium acetate injection in animals that received pretreatment with Vehicle or Guanosine. $P<0.05$, Fischer test.

Figure 4: EEG. A: Spectral EEG left index. *$P<0.05$ when compared animals that received pretreatment with Guanosine with animals that received vehicle. B: Samples of EEG traces obtained. (1), baseline (2) 10-20 min post vehicle or Guanosine (3) 10-20 min post ammonium acetate (d) 20-30 min post ammonium acetate.

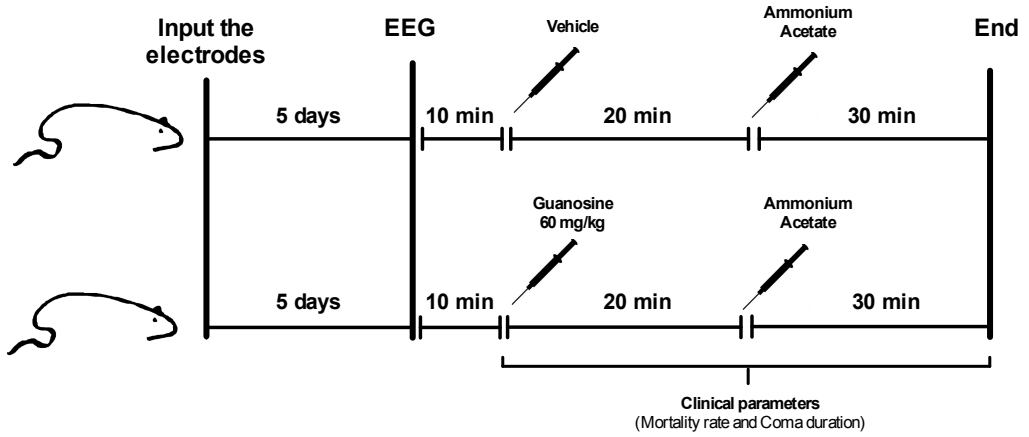
Figure 5. Extracellular levels of glutamate and ammonia in the CSF. (*$p < 0,05$, one-way ANOVA when compared to all other groups)

Figure 6: Glutamate uptake: Glutamate Uptake levels (% of control) in 10 min and 20 minutes after ammonium or distilled water acetate injection. Ammonium acetate decreased glutamate uptake levels in 10 and 20 minutes; animals that received pretreatment with Guanosine shown an increased uptake levels in 20 minutes when compared with those that received vehicle ($p < 0,05$, one-way ANOVA).

Figure 7. Brain oxidative damage and antioxidant enzymes analysis. A) TBARS, B) DCFH, C) SOD and D) GPx are expressed in % of control group. (*$p < 0,05$, one-way ANOVA when compared to all other groups).

Figure 1.

A) Protocol 1 - Clinical and electrophysiological parameters



B) Protocol 2 - Plasmatic, liquoric and neurochemical parameters

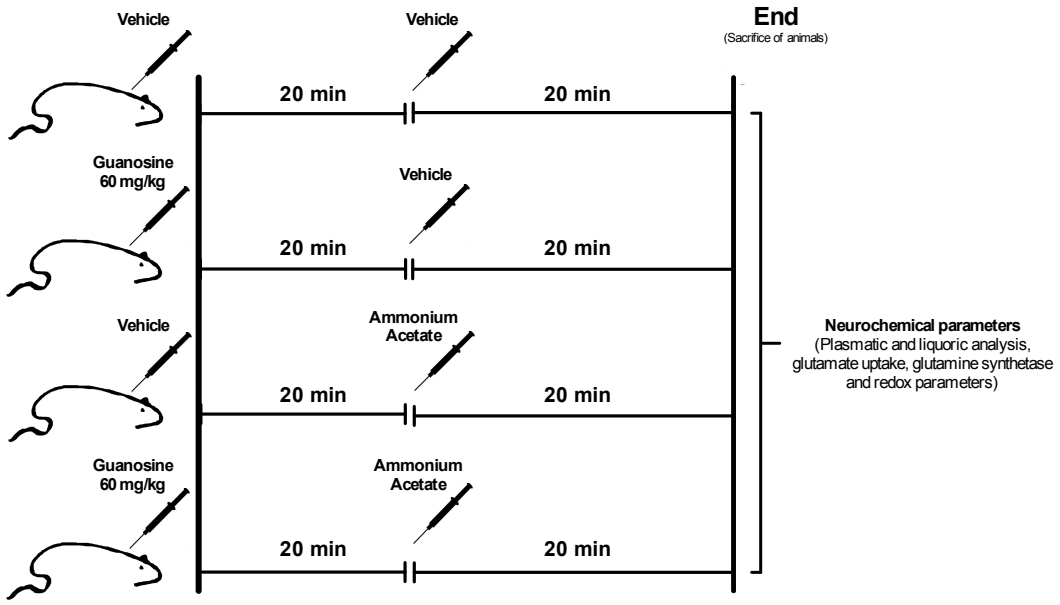


Figure 2.

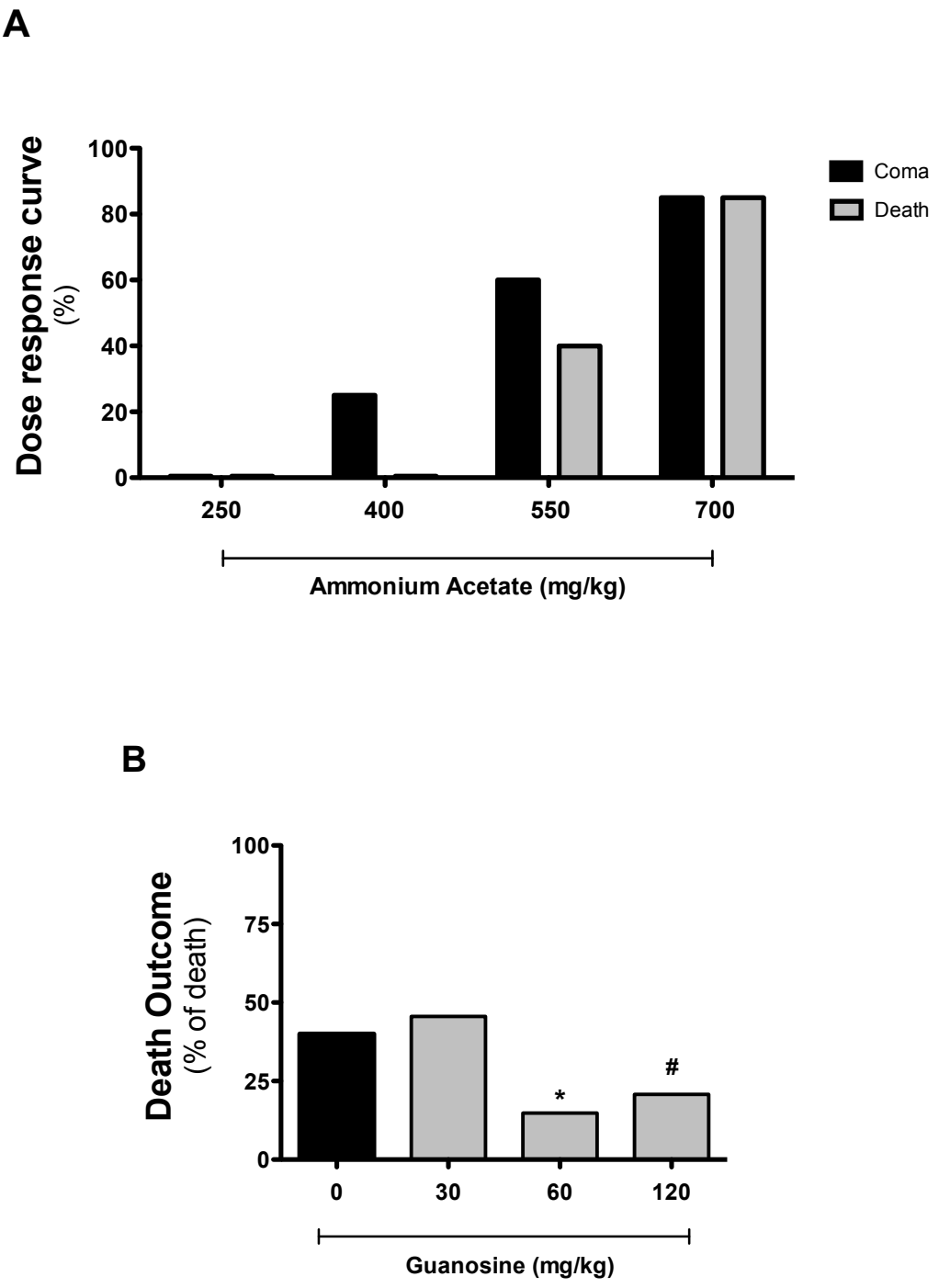

A
Dose response curve
(%)
100
80
60
40
20
0
250
400
550
700
Ammonium Acetate (mg/kg)
Coma
Death
B
Death Outcome
(% of death)
100
75
50
25
0
*
#
0
30
60
120
Guanosine (mg/kg)

Figure 3.

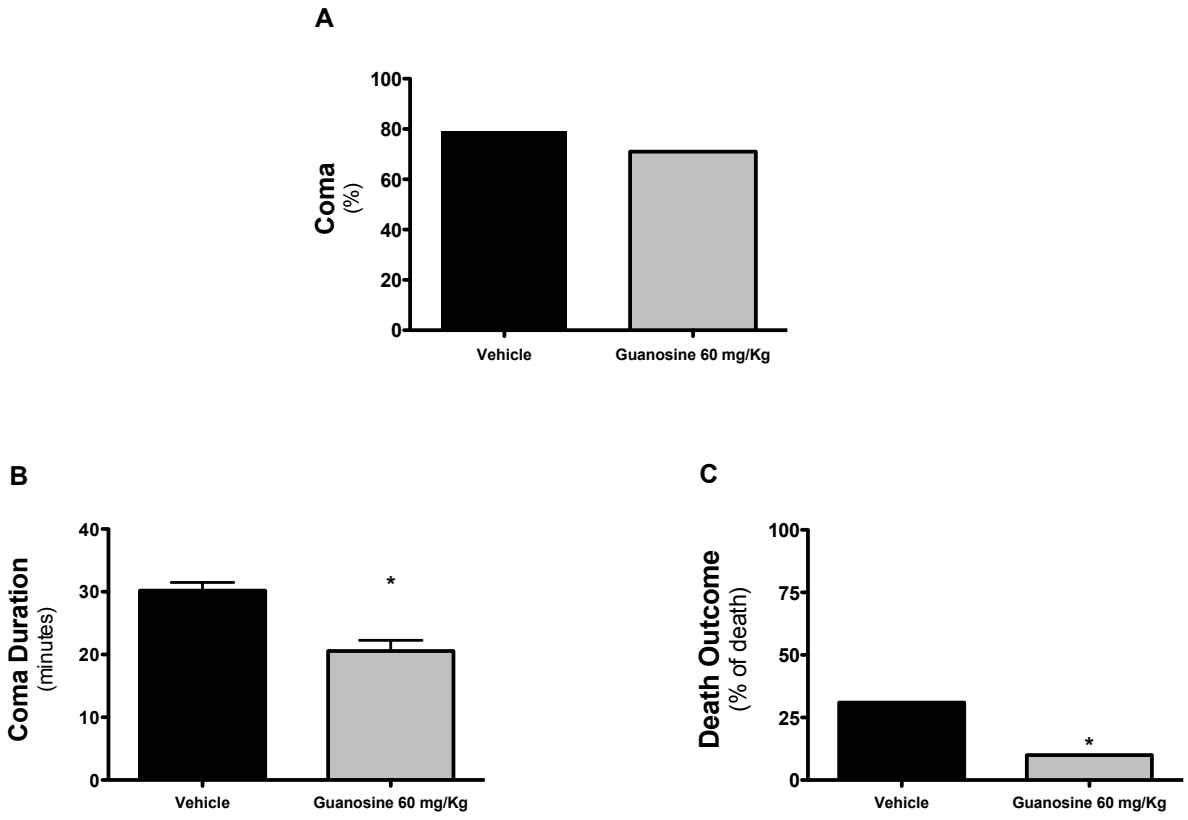

A
Coma
(%)
100
80
60
40
20
0
Vehicle
Guanosine 60 mg/Kg
B
Coma Duration
(minutes)
40
30
20
10
0
*
Vehicle
Guanosine 60 mg/Kg
C
Death Outcome
(% of death)
100
75
50
25
0
*
Vehicle
Guanosine 60 mg/Kg

Figure 4.

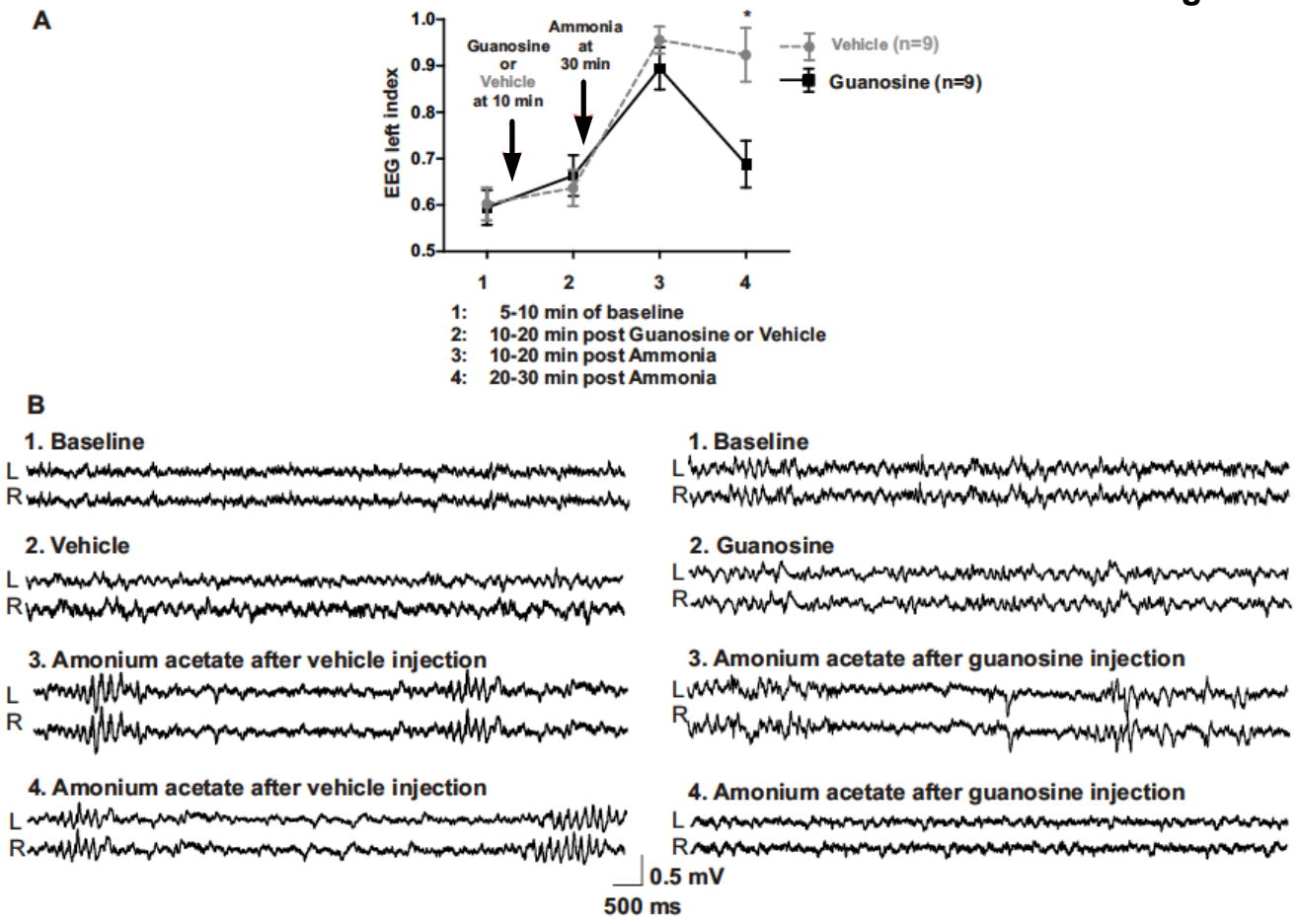

A
EEG left index
1.0
0.9
0.8
0.7
0.6
0.5
Guanosine or Vehicle at 10 min
Ammonia at 30 min
*
Vehicle (n=9)
Guanosine (n=9)
1
2
3
4
1: 5-10 min of baseline
2: 10-20 min post Guanosine or Vehicle
3: 10-20 min post Ammonia
4: 20-30 min post Ammonia
B
1. Baseline
2. Vehicle
3. Amonium acetate after vehicle injection
4. Amonium acetate after vehicle injection
1. Baseline
2. Guanosine
3. Amonium acetate after guanosine injection
4. Amonium acetate after guanosine injection
L
R
0.5 mV
500 ms

Figure 5.

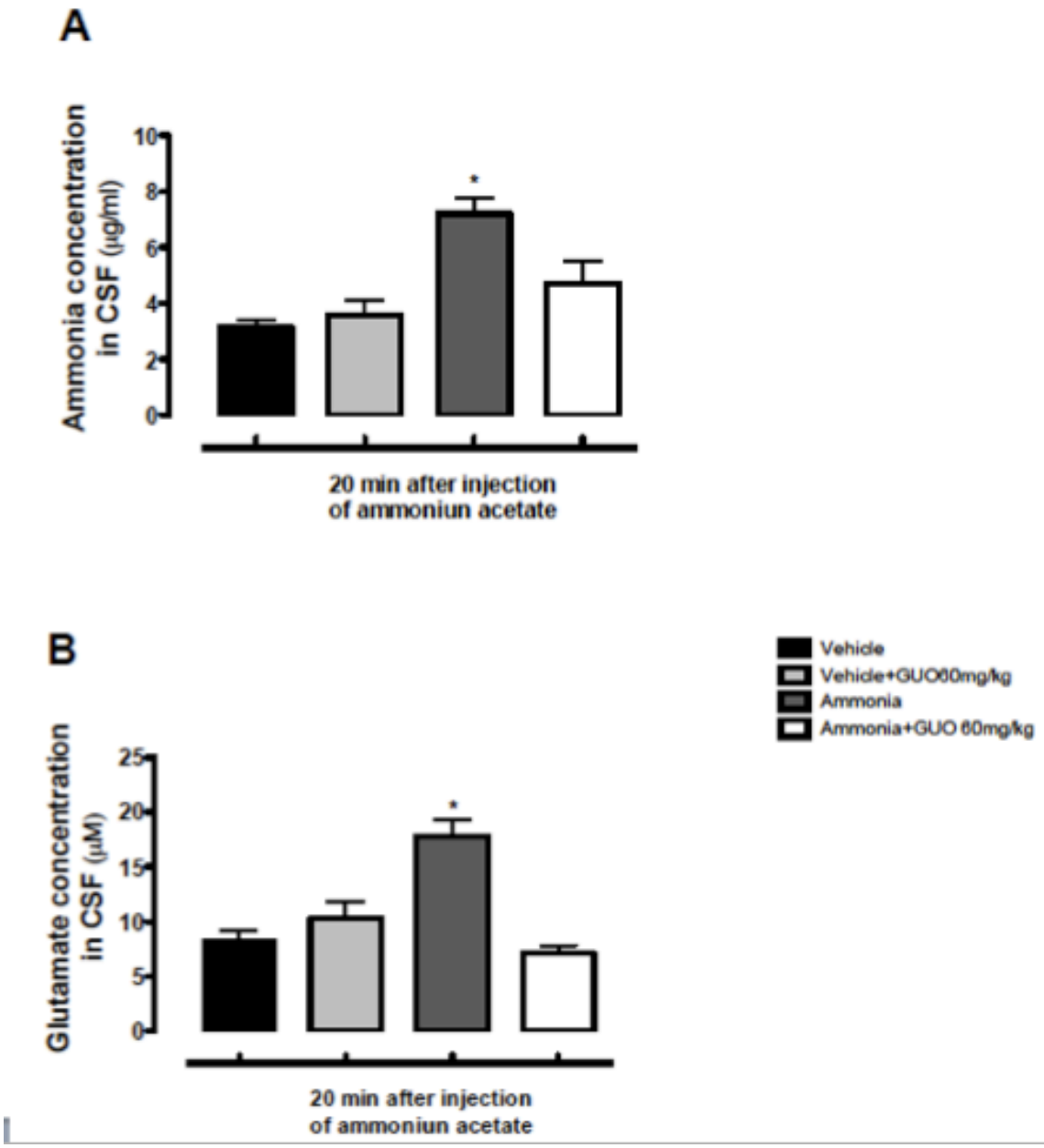

A
Ammonia concentration in CSF (μg/ml)
10
8
6
4
2
0
*
20 min after injection of ammoniun acetate
B
Vehicle
Vehicle+GUO60mg/kg
Ammonia
Ammonia+GUO 60mg/kg
Glutamate concentration in CSF (μM)
25
20
15
10
5
0
*
20 min after injection of ammoniun acetate

Figure 6

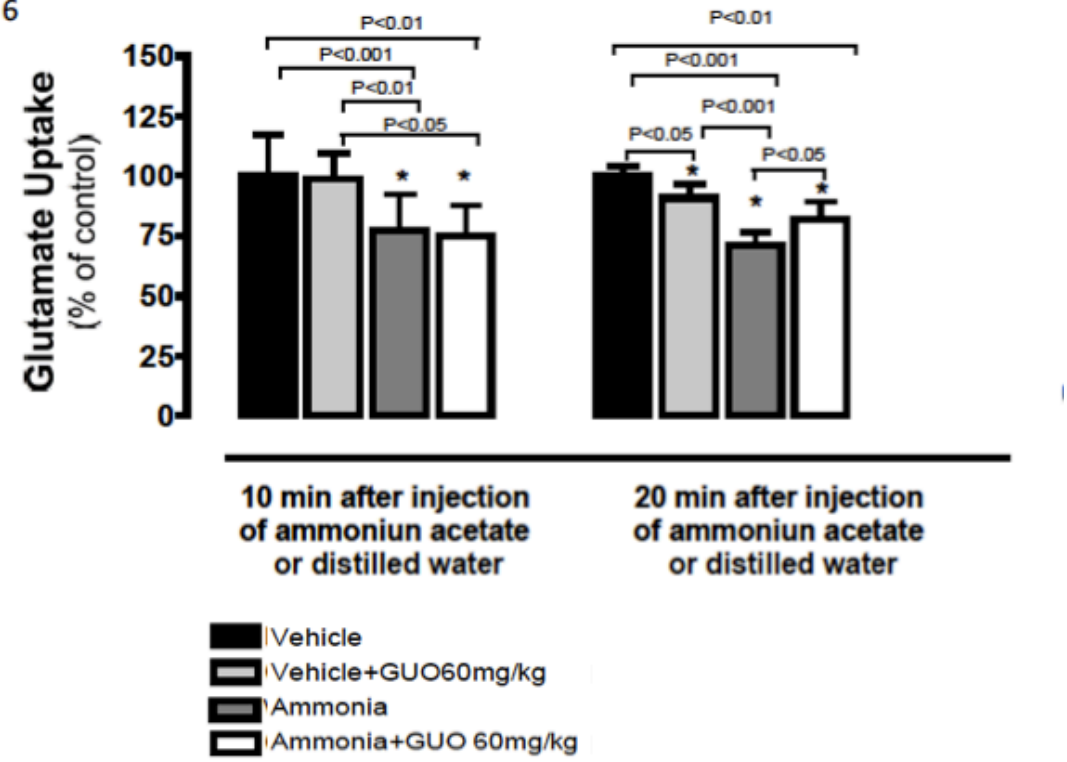
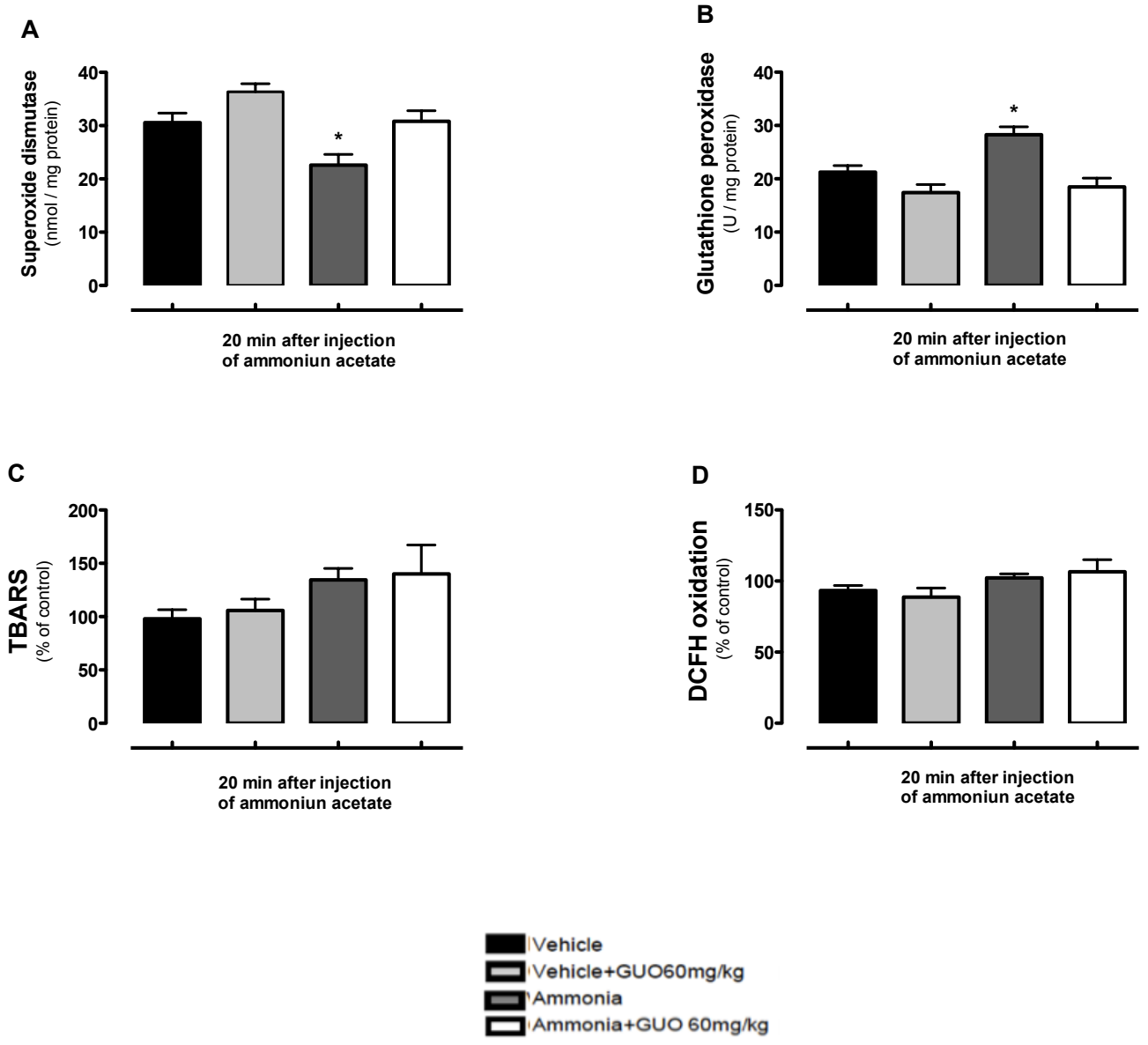

Glutamate Uptake
(% of control)
150
125
100
75
50
25
0
P<0.01
P<0.001
P<0.01
P<0.05
P<0.01
P<0.001
P<0.001
P<0.05
P<0.05
10 min after injection
of ammoniun acetate
or distilled water
20 min after injection
of ammoniun acetate
or distilled water
Vehicle
Vehicle+GUO60mg/kg
Ammonia
Ammonia+GUO 60mg/kg

Figure 7.


A
Superoxide dismutase
(nmol / mg protein)
40
30
20
10
0
*
20 min after injection
of ammoniun acetate
B
Glutathione peroxidase
(U / mg protein)
40
30
20
10
0
*
20 min after injection
of ammoniun acetate
C
TBARS
(% of control)
200
150
100
50
0
20 min after injection
of ammoniun acetate
D
DCFH oxidation
(% of control)
150
100
50
0
20 min after injection
of ammoniun acetate
Vehicle
Vehicle+GUO60mg/kg
Ammonia
Ammonia+GUO 60mg/kg

PARTE III

DISCUSSÃO DE RESULTADOS E PERSPECTIVAS

Discussão

A EH é uma síndrome neuropsiquiátrica decorrente da doença no fígado, podendo acontecer em situações de insuficiência hepática aguda ou insuficiência hepática crônica. Sua fisiopatologia está relacionada com o aumento nos níveis séricos da amônia, o que ocasiona alterações em diversos sistemas no SNC.

De maior importância para esta tese, o sistema glutamatérgico tem papel fundamental na patogenia da síndrome. Nos casos de insuficiência hepática aguda há uma hiperativação deste sistema, que ocorre através de um aumento na atividade dos receptores NMDA. Esse aumento da atividade NMDA acontece em paralelo com o aumento da liberação e diminuição da captação do glutamato, ocasionando edema e morte celular. Por outro lado, há uma resposta adaptativa cerebral nos casos de insuficiência hepática crônica: com o objetivo de evitar a excitotoxicidade glutamatérgica, há uma diminuição na atividade de receptores NMDA e um aumento na densidade de receptores metabotrópicos, alterações que estão relacionados aos estados de letargia e estupor observados na EH crônica.

Na seção 2 desta tese, nosso grupo discutiu os resultados de Yoden que descreveu efeitos neuroprotetores do alopurinol em um modelo de encefalopatia hepática crônica como sendo resultado exclusivo da ação antioxidante do alopurinol. O alopurinol é um inibidor da xantina oxidase que catalisa a transformação de hipoxantina à xantina e acido úrico, reduzindo tanto a formação de ácido úrico como a degradação das purinas. A acumulação de precursores purinérgicos, especialmente a hipoxantina, pode ser convertida à inosina, IMP e, finalmente à adenosina e Guanosina (Day et al., 2007). Esse potencial estimulador

do sistema purinérgico do alopurinol já foi relacionado a efeitos anticonvulsivantes, antinociceptivos e neuroprotetor em desordens psiquiátricas. (Lara, 2000; Schmidt 2009-B; Wada, 1992). Desta forma, é possível considerar que os efeitos neuroprotetores do alopurinol na hiperamonemia crônica também estejam relacionados ao aumento da atividade purinérgica, sobretudo Guanosina e adenosina, nucleosídeos com conhecida ação antiglutamatérgica. Sendo assim, há uma evidência indireta da ação neuroprotetora das purinas em um quadro estabelecido de encefalopatia hepática.

Nesse contexto, o nucleosídeo Guanosina, derivado da guanina, foi utilizado para avaliar um possível efeito neuroprotetor em dois modelos animais desta síndrome.

No primeiro trabalho experimental desta tese, utilizou-se o modelo de ligadura do ducto biliar em ratos (BDL, do inglês bile duct ligation). Após 2-3 semanas da cirurgia, os animais foram submetidos a testes comportamentais e à análise de EEG, identificando-se o quadro de EH. Foi realizada avaliação neuroquímica do líquor e do tecido cerebral destes animais. O modelo já descrito na literatura mostrou-se bem reprodutível, tendo os ratos apresentado alterações comportamentais (open Field e Y maze) e eletroencefalográficas compatíveis com humanos com EH. Os achados no líquor confirmaram outros estudos que descreveram um aumento no glutamato extracelular em casos de EH. Cauli e colaboradores (2007) já haviam descrito que o antagonismo do sistema glutamatérgico em casos de EH crônica em ratos resultava em melhora nos testes comportamentais. Abordando um outro ponto da fiosiopatogenia da EH, Bruck e colaboradores (2011) descreveram um aumento no estresse oxidativo no cérebro de ratos com EH crônica, e uma melhora em parâmetros comportamentais e neuroquímicos com o uso de anti inflamatórios que atenuassem esse estresse oxidativo. Nesse panorama, a

Guanosina se mostrou neuroprotetora nos ratos submetidos à cirurgia BDL ao melhorar a performance dos animais em um teste comportamental, diminuir os níveis de glutamato extracelular e melhorar parâmetros de estresse oxidativo.

Neste modelo crônico ainda foi possível caracterizar uma alteração no perfil purinérgico nos ratos com EH, mostrando elevação nos níveis de Inosina e GMP no líquor desses animais. Não se observou, no entanto, alteração nos níveis de adenosina e Guanosina. Por outro lado, os ratos com EH que foram tratados com Guanosina apresentaram níveis normais das purinas no líquor, o que pode significar que o aumento de Inosina e GMP seja uma tentativa de aumentar os níveis de Guanosina, levando em consideração que o GMP extracelular pode ser convertido a Guanosina. Outra hipótese para estes achados é que a alteração liquórica seja apenas uma sinalização da disfunção no SNC nos ratos com EH.

No segundo modelo experimental estudado nesta tese, utilizou-se o modelo de hiperamonemia aguda para simular alterações neurológicas decorrentes de insuficiência hepática aguda, como acontece em casos de hepatite viral fulminante ou hepatite medicamentosa. Neste modelo, embora o parênquima hepático dos ratos esteja normal, simula-se a elevação súbita nos níveis de amônia através de injeção intra peritoneal de acetato de amônio. A hiperamonemia aguda é um modelo bem padronizado na literatura, gerando alterações comportamentais e eletrofisiológicas compatíveis com humanos com EH aguda. O modelo já descrito na literatura mostrou-se bem reprodutível, tendo os ratos apresentado uma evolução clínica para coma e morte, além de um padrão de ondas trifásicas e lentificadas no EEG que é compatível com EH. Conforme já descrito na literatura (Jayakumar, 2006; Lemberg, 2009), a elevação súbita da amônia causa uma

diminuição na captação de glutamato, alteração que contribui para o edema cerebral e a deterioração clínica dos ratos com EH aguda, além de ocasionar um aumento no estresse oxidativo. Kosenko e colaboradoes (1999) descreveram que o antagonismo glutamatérgico através de MK-801(antagonista de receptor NMDA) diminuía o estresse oxidativo e melhorava desfechos clínicos frente à hiperamonemia aguda. Nessa perspectiva , nosso estudo mostrou que a Guanosina aumentou a captação de glutamato nos animais com hiperamonemia aguda, bem como atenuou o estresse oxidativo provocado pela hiperamonemia. Estes resultados apresentaram significado comportamental ao passo que os animais que receberam um pré tratamento com Guanosina antes da injeção de acetato de amônia tiveram uma menor taxa de mortalidade, menor duração do coma e rápida normalização no traçado do EEG. Esses resultados reforçam o papel da excitotoxicidade glutamatérgica na EH aguda e trazem a Guanosina como uma alternativa na modulação desses quadros.

O nucleosídeo Guanosina já foi estudado em inúmeros experimentos in vivo e in vitro com conhecida atividade neuroprotetora através de sua ação antiglutamatérgica com destaque especial para os modelos de dor, hipóxia, convulsão e remielinizaçao medular (Connell et al., 2013; Jiang et al., 2008; Rathbone, 2011; Schmidt et al., 2009 2010-a, 2010-b, 2010-c & 2010-d; Lara et al., 2001; Vinadé et al., 2004). A literatura mostra dados sólidos quanto a capacidade deste nucleosídeo em aumentar a captação de glutamato pelos astrócitos (Schmidt, 2007). De forma mais recente, Pentronilho (2012) publicou efeitos antioxidantes da Guanosina em um modelo de sepse em ratos, apresentando melhora em parâmetros neuroquímicos de estresse oxidativo e no desempenho de testes comportamentais de cognição. Deste modo, em ambos os modelos

estudados nesta tese, o nucleosídeo Guanosina confirmou efeito neuroprotetor, sendo que tanto de forma indireta com a diminuição do glutamato extracelular, quanto de forma direta através do aumento da captação de glutamato, houve inequívoca ação antiglutamatérgica. Os resultados apresentados também confirmaram resultados prévios de ação antioxidante da Guanosina através da diminuição da produção de espécies reativas no modelo crônico e normalização das enzimas antioxidantes endógenas no modelo agudo.

Cabe ressaltar ainda que o uso da Guanosina nos modelos estudados teve um benefício "clínico": melhora da cognição no modelo crônico; diminuição da mortalidade e do tempo em coma no modelo agudo.

Os resultados desta tese, então, reforçam o papel da excitotoxicidade glutamatérgica na fisiopatogenia da encefalopatia hepática e, para o nosso conhecimento, descreve pela primeira vez na literatura o efeito neuroprotetor da Guanosina na encefalopatia hepática, abrindo uma nova perspectiva no arsenal terapêutico para o tratamento desta síndrome.

Perspectivas da Pesquisa:

A conclusão deste estudo responde algumas perguntas, mas também, como é característico da ciência, traz outras tantas interrogações. Sabemos que a EH é uma síndrome complexa sendo necessário o estudo em vários modelos animais até que os resultados possam ser extrapolados para os pacientes. Diante disso, a avaliação da Guanosina frente a outros modelos de insuficiência hepática tais como hepatectomia, intoxicação por Galactosamina e Thioacetamide já estão sendo realizados pelo nosso grupo. Além disso, experimentos para esclarecer a interação da Guanosina com o sistema glutamato→*glutamina sintetase*→ glutamina também estão sendo conduzidos com o objetivo de ampliar o entendimento da síndrome e o caracterizar melhor a farmacodinâmica relacionada ao aumento da captação de glutamato provocado pela Guanosina.

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