Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

TESE DE DOUTORADO:

INSUFICIÊNCIA HEPÁTICA AGUDA INDUZ REATIVIDADE ASTROCITÁRIA, ALTERAÇÕES BIOENERGÉTICAS CEREBRAIS E PERMEABILIDADE DA BARREIRA HEMATOENCEFÁLICA EM RATOS; AVALIAÇÃO DOS EFEITOS NEUROPROTETORES DA GUANOSINA

Aluno Pedro Arend Guazzelli

Orientador Diogo Onofre Gomes de Souza

Porto Alegre, Rio Grande do Sul, 2020

Início: 15 de agosto de 2015

Término: 02 de setembro de 2020

Pedro Arend Guazzelli

CIP - Catalogação na Publicação

Arend Guazzelli, Pedro
INSUFICIÊNCIA HEPÁTICA AGUDA INDUZ REATIVIDADE ASTROCITÁRIA, ALTERAÇÕES BIOENERGÉTICAS CEREBRAIS E PERMEABILIDADE DA BARREIRA HEMATOENCEFÁLICA EM RATOS; AVALIAÇÃO DOS EFEITOS NEUROPROTETORES DA GUANOSINA / Pedro Arend Guazzelli. -- 2020.
111 f.
Orientador: Diogo Onofre Gomes de Souza.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Porto Alegre, BR-RS, 2020.

1. Insuficiência Hepática Aguda. 2. Encefalopatia Hepática. 3. Excitotoxicidade Glutamatérgica. 4. Barreira Hematoencefálica. 5. Guanosina. I. Onofre Gomes de Souza, Diogo, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

Pedro Arend Guazzelli

INSUFICIÊNCIA HEPÁTICA AGUDA INDUZ REATIVIDADE ASTROCITÁRIA, ALTERAÇÕES BIOENERGÉTICAS CEREBRAIS E PERMEABILIDADE DA BARREIRA HEMATO-ENCEFÁLICA EM RATOS; AVALIAÇÃO DOS EFEITOS NEUROPROTETORES DA GUANOSINA

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção de título de Doutor em Bioquímica

Porto Alegre, Rio Grande do Sul, Brasil

2020

Professor Diogo Onofre Gomes de Souza

Orientador

Agradecimentos

Ao concluirmos etapas de nossas vidas, olhamos tanto para as dificuldades superadas como as conquistas realizadas, percebendo todo o suporte que nos proporcionou chegar até este momento. Não é possível expressar apenas com palavras o agradecimento que tenho a todos que contribuíram de alguma maneira para a minha formação acadêmica e científica, a qual tem como grande marco, até o momento, o desenvolvimento desta tese. Não obstante, o mínimo que posso fazer é registrar aqui minha gratidão a essas pessoas por me proporcionarem essa experiência desafiadora e enriquecedora. Espero um dia poder retribuir todo o apoio e incentivo que me foi dado.

Agradeço à minha família, amigos e minha amada Luísa pelo apoio e suporte, tanto material quanto emocional, que me proporcionaram incondicionalmente ao longo dos anos. Por compreenderem as peculiaridades da ciência, a necessidade de se trabalhar mesmo em domingos, feriados e madrugadas para completar os experimentos, dando não só o apoio emocional como logístico;

Agradeço à Universidade Federal do Rio Grande do Sul, seus professores e funcionários, assim como os órgãos de fomento do Governo Brasileiro, por acreditarem e investirem na ciência, possibilitando o desenvolvimento deste e de tantos outros trabalhos, mesmo em momentos de escassez financeira e ataques ideológicos;

Agradeço aos colegas da bioquímica por toda ajuda e ensinamentos ao longo dos últimos 9 anos em que estive presente no Laboratório 28. Por proporcionarem uma rotina prazerosa e por contribuírem com seu conhecimento para o enriquecimento dos experimentos aqui apresentados. Sem dúvidas este trabalho é fruto de todas as pessoas incríveis com quem compartilhei a bancada, ideias, projetos e bons churrascos ao longo desse período;

Agradeço ao apoio dos colegas da faculdade de medicina, sem os quais seria impossível completar essa jornada simultânea de graduando e pós-graduando. Agradeço, também, aos professores da Faculdade de Medicina que estimularam a pesquisa e apoiaram seu desenvolvimento, relevando eventuais faltas e atrasos nas atividades curriculares;

Agradeço ao professor Alessandro Osvaldt por acreditar na proposta ambiciosa de dois alunos de iniciação científica e dedicar seu tempo a ensiná-los a realizar uma cirurgia complexa, a qual se tornou a base de todo o trabalho;

Agradeço aos colegas da faculdade que trabalharam comigo durante o desenvolvimento do projeto como alunos de Iniciação Científica. Pedro, Carol e, em especial, Anderson e Felipe. Espero que tenham aproveitado o tempo que compartilhamos no laboratório e que esta experiencia tenha sido benéfica para o crescimento pessoal e profissional de vocês;

Agradeço aos meus mestres Lucas e Giordano por me acolherem no laboratório, por me incorporarem à sua pesquisa e por estimularem o meu crescimento acadêmico, proporcionando inúmeros ensinamento. Mais importante que isso, agradeço a excelente convivência e amizade que desenvolvemos ao longo destes anos e agradeço por saber que posso sempre contar com vocês;

Agradeço ao professor Adriano de Assis por todos os ensinamentos, conselhos e companheirismo ao longo da minha vida acadêmica. Por investir integralmente nos seus colegas e alunos, participando ativamente de todo o processo científico de desenvolvimento desta tese. Por manter-se fundamental para a conclusão de meus estudos, sempre conseguindo tempo para revisar e contribuir com seus conhecimentos, mesmo que já como professor em outra universidade;

Por fim, agradeço ao excelentíssimo professor senhor doutor Diogo Souza por toda sua sabedoria, que parece não ter fim. Agradeço por ser um professor ímpar, que estimula os alunos a saírem da sala de aula para trabalhar nos laboratórios, preferindo a discussão científica e a leitura de artigos às aulas monótonas e previsíveis. Por acreditar irredutivelmente nos seus alunos desde o primeiro momento, lutando junto aos órgãos de fomento por oportunidades de crescimento, incluindo a bolsa de Doutorado Especial em Pesquisa Médica que proporcionou a realização desta tese. Por compartilhar seu contagiante “priapismo existencial” pela ciência, tornando instigante e satisfatório o processo científico. E, por fim, agradeço ao mestre Diogo por todos os ensinamentos sobre a vida e o amor, por ser um exemplo de ser humano a ser seguido, me ensinando e inspirando pelo modo como lida com seus alunos, colegas, amigos e companheira.

Sumário

Parte 1

1. **Apresentação** 07
2. **Resumo** 08
3. **Abstract** 09
4. **Introdução** 10

4.1 **Insuficiência Hepática Aguda** 10

4.2 **Encefalopatia Hepática** 13

4.3 **Fisiopatologia da Encefalopatia Hepática** 15

4.4 **Tratamento da Encefalopatia Hepática** 20

4.5 **Guanosina** 22

5. **Objetivos** 24

5.1 Objetivos Gerais 24

5.2 Objetivos Específicos 24

Parte 2

6. **Materiais, Métodos e Resultados** 26

6.1 Capitulo I: 26

6.2 Capitulo II: 41

6.3 Capitulo III: 66

Parte 3

7. **Discussão** 95
8. **Conclusão** 101
9. **Perspectivas** 101

Parte 4

10. **Lista de Abreviaturas** 104
11. **Palavras Chave** 104
12. **Referências** 105

Parte 1

1. Apresentação

Esta tese é dividida em quatro partes, conforme abaixo descritas:

Inicialmente, a primeira parte se destina a apresentação dos principais conceitos, dados e teorias sobre os temas centrais discutidos nesta tese, sem os quais não seria possível o entendimento e desenvolvimento do trabalho. Nesta parte é apresentado um pequeno resumo sobre os principais achados da pesquisa desenvolvida ao longo dos últimos 5 anos na forma de Resumo/Abstract. Os objetivos gerais e específicos dos estudos realizados neste período também são expostos em detalhe nesta sessão.

Na segunda parte, são descritas detalhadamente as metodologias utilizadas e os resultados obtidos pelo grupo referentes aos temas Insuficiência Hepática Aguda e Encefalopatia Hepática no período de 2015 a 2020. Os capítulos I e II são apresentados como forma de Artigo Científico conforme publicados ou submetidos às revistas científicas internacionais. O Capítulo III é também apresentado como forma de Artigo Científico, embora ainda não tenha sido submetido à publicação devido a atrasos ocasionados pela pandemia de COVID-19 e problemas com os equipamentos necessários para a realização de técnicas de imuno-histoquímica. Uma vez findada a pandemia e concluídas as análises de dados, o artigo será prontamente submetido para publicação em revista científica internacional.

Na terceira parte, são discutidos os principais resultados obtidos pelo grupo durante a realização desta tese. Além disso, é apresentada a conclusão do grupo sobre a importância destas descobertas para o entendimento e tratamento da Encefalopatia Hepática, assim como apresentadas as perspectivas de novos estudos que podem ser desenvolvidos com base nos dados aqui encontrados.

Por fim, na quarta parte, são apresentadas a lista de abreviatura e as palavras chave, assim como as referências utilizadas como embasamento para o desenvolvimento desta tese.

2. Resumo

Introdução: Insuficiência Hepática Aguda (IHA) é uma síndrome rara, porém potencialmente fatal e que ocorre em pacientes vítimas de uma perda rápida e extensa da função hepática. Além diminuição da capacidade de síntese (clinicamente expressa como INR > 1.5), a IHA leva ao surgimento de Encefalopatia Hepática (EH). A EH consiste no surgimento de distúrbios neurológicos secundários ao acúmulo de toxinas no sangue e cérebro de pacientes com doença hepática. A fisiopatologia da EH é multifatorial, envolvendo o acúmulo de amônia, a excitotoxicidade glutamatérgica, as alterações de bioenergética cerebral, dano à barreira hematoencefálica (BHE), entre outros. A modulação do sistema glutamatérgico vem sendo testada com sucesso para o controle dos sintomas da EH, em especial com o uso da Guanosina (GUO). **Objetivos:** i) estudar os efeitos da EH no metabolismo energético cerebral e na BHE, com foco no metabolismo de aminoácidos e ii) testar os efeitos neuroprotetores da GUO em um modelo animal de IHA. **Métodos:** utilizou-se modelo animal de IHA induzida por hepatectomia subtotal (remoção de 92% da massa hepática). Em uma coorte de animais, amostras de líquido cefalorraquidiano (LCR) e córtex cerebral foram colhidos 24 horas após a cirurgia para ensaios bioquímicos. Em um segundo grupo, a administração de guanosina foi testada por via intraperitoneal (i.p.) por 60 horas após a cirurgia para avaliar o desempenho neurológico e, sobretudo, a mortalidade dos animais hepatectomizados. **Resultados:** animais com EH apresentaram diversas alterações bioenergéticas como i) aumento do consumo de oxigênio mitocondrial; ii) aumento da atividade enzimática do ciclo de Krebs e produção de ATP; iii) aumento da oxidação de glutamato; e iv) diminuição da oxidação de glicose e lactato. Paralelamente, aumento de parâmetros de estresse oxidativo e reação astrocitária difusa em imagens de imuno-histoquímica foram encontrados no córtex parietal de animais com IHA. Em relação à BHE, animais com EH apresentaram níveis elevados de albumina e de amino ácidos no LCR. O aumento de amino ácidos no LCR foi relacionado ao grau de declínio neurológico e se apresentou como um biomarcador para identificar animais com prognóstico desfavorável. A administração de GUO foi capaz reduzir a mortalidade e melhorar parâmetros neurológicos e comportamentais. A GUO também reduziu os níveis de albumina no LCR, aumentou a captação de glutamato pelos astrócitos e reverteu as alterações histológicas provocadas pela reatividade astrocitária. **Discussão:** animais com IHA apresentaram intensa resposta celular astrocitária com aumento de volume celular, alterações no metabolismo energético cerebral, aumento de estresse oxidativo e aumento de albumina e amino ácidos no LCR, sugestivos de danos à BHE. O uso de GUO foi capaz de reverter muitos dos parâmetros avaliados, além de trazer claro benefício na mortalidade dos animais tratados. Os resultados apresentados nesta tese esclarecem aspectos da fisiopatologia da EH e reforçam os potenciais efeitos neuroprotetores do uso da GUO no contexto da IHA.

3. Abstract

Introduction: Acute Liver Failure (ALF) is a rare medical condition that occurs in patients with rapid and extensive loss of hepatic function, presenting high mortality rates. It is characterized by the reduction of the liver's synthetic capacity and the development of Hepatic Encephalopathy (HE). HE consists of a spectrum of neurological symptoms caused by the accumulation of toxins in blood and brain of patients with liver disease. HE's physiopathology is complex, multifactorial and involves ammonia accumulation, glutamatergic excitotoxicity, brain bioenergetic alterations and increased blood brain barrier (BBB) permeability. Modulation of the glutamatergic system is being tested with success to treat this condition, especially using the nucleoside Guanosine (GUO). **Objectives:** i) to study the effects of HE on brain bioenergetics and BBB permeability, with emphasis in amino acid metabolism; ii) to test the neuroprotective effects of GUO. **Methods**: the studies were performed using an animal model of ALF induced by subtotal hepatectomy (removal of 92% of the hepatic mass). Samples of cerebrospinal fluid (CSF) and cerebral cortex were harvested 24 hours after surgery for biochemical essays. A second cohort of animal had GUO administered via intraperitoneal (i.p.) injection for 60 hours after surgery to study the neurological performance and mortality of the animals. **Results:** animals with HE presented several bioenergetic alterations such as i) increased mitochondrial oxygen consumption; ii) increased TCA enzyme activity and ATP production; iii) increased glutamate oxidation; iv) decreased glucose and lactate oxidation. HE also increased free reactive oxygen species, which were associated with a diffuse astrocytic reactivity evidenced by immunohistochemical images of parietal cortex. Regarding BBB permeability, animals with HE presented increased levels of albumin and amino acids in the CSF, which were associated to the severity of encephalopathy. Elevated CSF amino acids also accurately predicted the mortality prognosis. The administration of GUO was able to reduce ALF's mortality and to improve neurological and behavioral function. GUO also improved biochemical parameters, as it normalized albumin levels in the CSF, increased glutamate uptake and prevented glial reactivity. **Discussion:** ALF induced a significant astrocytic reactivity with cellular expansion, which was associated with alterations in brain energy metabolism, increased oxidative stress and increased levels of albumin and amino acids in the CSF (suggesting increased BBB permeability). The administration of GUO prevented most of the evaluated biochemical parameters and presented a clear benefit by reducing mortality and improving neurological function of treated animals. The results of these studies bring new evidence for the understanding of HE's physiopathology and strengthen the potential neuroprotective effects of GUO under ALF.

4. Introdução

4.1 Insuficiência Hepática Aguda

Insuficiência Hepática Aguda (IHA) é a manifestação clínica de uma perda grave da função hepática em um período inferior a 26 semanas. Sua definição mais aceita é o desenvolvimento de doença hepática severa que produza diminuição da capacidade de síntese molecular (expressas como distúrbios de coagulação com *International Normalized Ratio* [INR] >1.5) e alterações no estado mental, conhecidas como encefalopatia hepática (EH), em um paciente sem doença hepática prévia [1-3]. Em relação a velocidade de progressão dos sintomas, a classificação mais utilizada é a proposta por O'Grady e colegas em 1993 [4]. Esta classificação se baseia nas características clínicas e no prognóstico dos pacientes com IHA, dividindo-os em três subgrupos conforme o tempo de desenvolvimento de encefalopatia a partir do surgimento de icterícia: *hiperaguda* (de 0 a 7 dias), *aguda* (de 8 a 28 dias) e *subaguda* (de 28 dias a 12 semanas) [4].

Múltiplos fatores etiológicos estão envolvidos no desenvolvimento da IHA, englobando eventos comuns como a toxicidade do paracetamol (acetaminofeno), hepatites virais, reações adversas a medicamentos, doença de Wilson e síndromes de hipoperfusão causadas por insuficiência cardíaca, sepse, doenças veno-oclusivas (Budd-Chiari ou trombose de veia hepática) ou uso de drogas vasoconstritoras como cocaína e metanfetamina [5, 6].

Nos países desenvolvidos, a principal causa de IHA é a ingesta de altas doses de paracetamol. Williams e colegas demonstraram que cerca de 60% dos transplantes de fígado realizados para o tratamento de IHA na Inglaterra tem como causa a toxicidade do acetaminofeno, frequentemente ingerido como tentativa de suicídio [7]. Estes números estão de acordo com dados americanos, onde 46% dos casos de IHA entre 1998 e 2007 tiveram como etiologia a intoxicação por este medicamento [8]. Os dados deste estudo são trazidos em detalhe na Figura 1, apresentando todas as principais etiologias envolvidas no surgimento de IHA nos EUA durante este período.

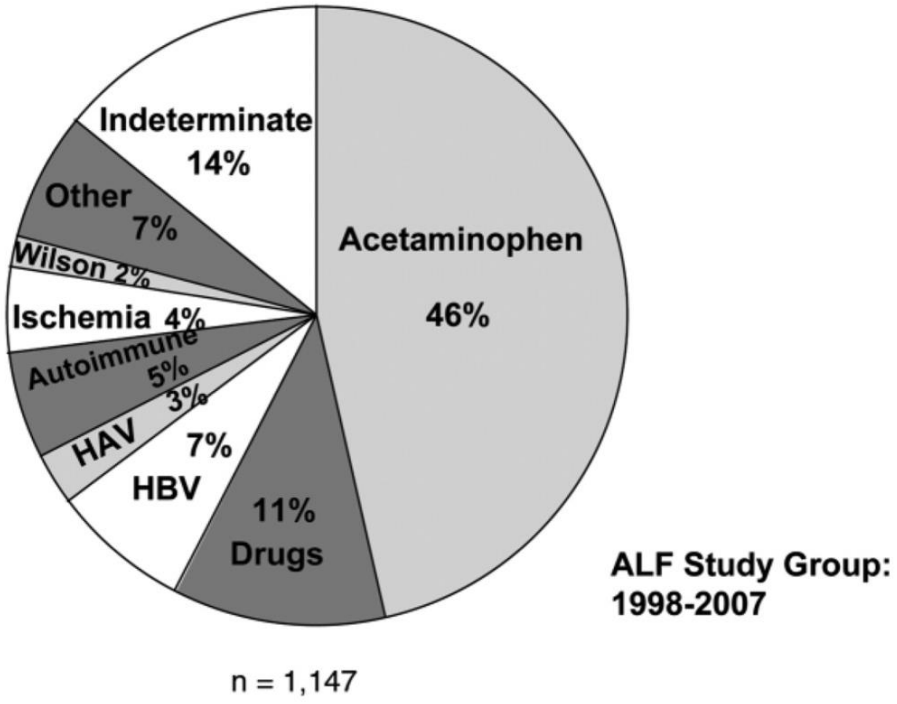


Figura 1: Etiologia da Insuficiência Hepática Aguda em adultos

Figura extraída de Lee, *et al,* 2008 [8]

Acetaminophen: paracetamol; Drugs: medicamentos; HBV: hepatite por vírus B; HAV: hepatite por vírus A; Autoimmune: autoimune; Ischemia: isquemia; Indeterminate: causas indeterminadas; Other: outras causas; n: número de pacientes. ALF Study Group: Grupo de estudos de Insuficiência Hepática Aguda.

As hepatites virais também correspondem a um percentual importante dos casos de IHA. O total de casos atribuídos às infecções virais flutua entre 10 a 30% nos diferentes estudos sobre o tema, sendo os vírus das hepatites B e A os mais comuns no ocidente [7-9]. Acredita-se que em países em desenvolvimento as taxas de falência hepática por infecções virais sejam ainda maiores, especialmente na África e Ásia, pois o vírus da hepatite E é endêmico nestas regiões [10]. Curiosamente, apesar dos esforços e dos avanços da medicina para o estabelecimento do diagnóstico etiológico da IHA, um percentual importante dos casos (14-43%) não tem sua causa corretamente definida, como demonstrado em uma série de casos analisados de centros hospitalares de países nórdicos [9].

Na perspectiva epidemiológica, a IHA é, felizmente, uma doença pouco frequente. Weiler e colegas calcularam a incidência de IHA na Alemanha em

1,13 casos /100.000 habitantes-ano, tomando como base os dados do maior plano de saúde alemão, com cerca de 25 milhões de pacientes [11]. Bower e colegas encontraram uma incidência de 1.600 casos por ano nos Estados Unidos (aproximadamente 0,48 casos /100.000 habitantes-ano) [12]. No entanto, a incidência de IHA em países subdesenvolvidos é mais elevada. Análises de dados da Tailândia e Taiwan apresentam incidências de 6,3 casos /100.000 habitantes-ano e 8,0 casos /100.000 habitantes-ano, respectivamente [13, 14]. Esta variação se deve, provavelmente, a maior incidência de hepatites virais nos países subdesenvolvidos, como comentado anteriormente.

O manejo dos pacientes com quadro de IHA demanda, como regra, leito em unidades de terapia intensiva (UTI), implicando, assim, altos custos para os pacientes e sistemas de saúde. Além das complicações neurológicas conhecidas como encefalopatia hepática (a qual será discutida com detalhes nas sessões 4.2 a 4.4), a IHA cursa com a perda de capacidade de síntese dos hepatócitos. Isto faz com que fatores de coagulação e moléculas do sistema imune tenham sua produção comprometida, tornando os pacientes suscetíveis a sangramentos e infecções [1, 15, 16]. O equilíbrio hemodinâmico destes pacientes também é afetado, pois a falência hepática leva à hipotensão arterial por diminuição do tônus vascular. Este efeito predispõe ao choque e, consequentemente, à falência múltipla de órgãos devido à má perfusão tecidual [1, 16]. É importante destacar que, apesar das medidas de suporte oferecidas nas UTIs, o transplante hepático é, com frequência, a única medida capaz de reverter a progressão da doença e assim prevenir a morte dos pacientes com IHA [1, 17]. De fato, cerca de 7% dos transplantes de fígado realizados na Europa tem como finalidade o tratamento de pacientes com IHA [18].

Em relação ao prognóstico de pacientes com IHA, a perspectiva de recuperação espontânea é bastante reduzida. Entre as primeiras análises da mortalidade da IHA, destacam-se os dados trazidos por O'Grady e colegas. Este grupo de pesquisa encontrou que pacientes que desenvolveram encefalopatia hepática nos primeiros 7 dias de sintomas (quadros hiperagudos) apresentaram pequenas taxas de recuperação espontânea, com mortalidade de aproximadamente 64%. Paradoxalmente, formas mais lentas da doença (IHA aguda e subaguda) apresentaram gravidade ainda maior, com taxas de

mortalidade de 93% e 86%, respectivamente [4]. Desde então, diversos modelos para a avaliação do prognóstico dos pacientes com IHA foram desenvolvidos ao longo dos anos, os quais são discutidos na revisão recente de Mishra e Rustgi [19]. Felizmente, com o melhor manejo clínico nas UTIs, assim como o aperfeiçoamento das técnicas e cuidados com o transplante hepático, o percentual de pacientes com IHA que se recupera vem aumentando ao longo dos anos [20].

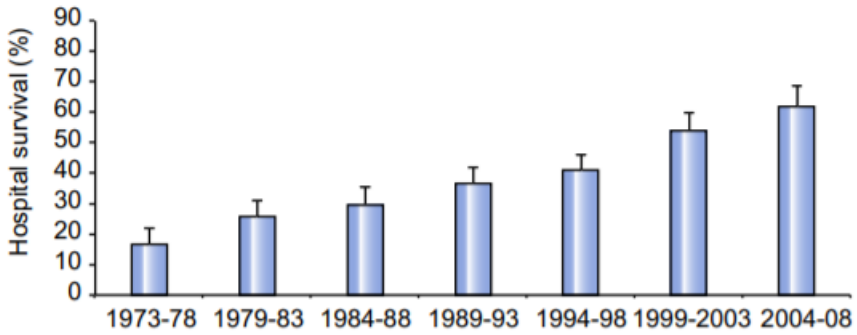


Figura 2: Taxa de sobrevivência de pacientes hospitalizados com IHA no King's College Hospital, Inglaterra, 1973-2008

Hospital survival: Sobrevida intra-hospitalar. Figura extraída de Bernal, *et al*, 2015 [20].

4.2 Encefalopatia Hepática

O fígado é um órgão vital responsável não só por produzir componentes essenciais ao funcionamento do organismo, como por metabolizar inúmeras substâncias potencialmente tóxicas, tanto as endógenas quanto as ingeridas. A circulação sanguínea proveniente do intestino converge diretamente para o fígado através do sistema da veia Porta, com o propósito de se realizar a filtragem inicial das substâncias ingeridas pelo indivíduo antes que este sangue proveniente da circulação entérica tenha contato com os demais tecidos do organismo. Assim sendo, nas situações em que o funcionamento celular hepático é comprometido ou em que haja desvio do fluxo sanguíneo entérico de modo a ultrapassar o fígado, toxinas se acumulam no organismo, gerando repercussões deletérias em diversos órgãos e, em especial, no sistema nervoso central (SNC). As alterações cerebrais secundárias ao mal funcionamento do fígado compõe uma síndrome conhecida como Encefalopatia Hepática (EH).

A apresentação clínica da EH compreende um grande espectro de manifestações neurológicas e psiquiátricas. Esta condição afeta diferentes perfis de pacientes e com variada duração e intensidade de sintomas. Na sua forma mais branda, a EH produz discretas alterações na atenção, memória de trabalho e agilidade psicomotora, sendo praticamente imperceptível sem os devidos testes diagnósticos [21, 22]. Esta etapa da doença é conhecida como Encefalopatia Hepática Mínima. Com a progressão da doença, os sintomas se tornam clinicamente evidentes, surgindo tremores, alterações na fala, mudanças de humor e distúrbios de ciclo sono-vigília [21, 23, 24]. Por fim, os estágios mais graves da doença levam à letargia, coma e, eventualmente, morte [21, 23, 24]. Especialistas no estudo da EH propuseram diferentes definições e classificações para padronizar o entendimento dessa síndrome. Ao longo desta tese utilizaremos duas definições estabelecidas pela *American Association for the Study of Liver Disease* em conjunto com a *European Association for the Study of the Liver*, publicadas em 2014 [21] :

1) Referentes à doença de base:

- Tipo A: EH resultante de Insuficiência Hepática Aguda
- Tipo B: EH resultante de *shunt* porto-sistêmico
- Tipo C: EH resultante de cirrose hepática

2) Referentes à severidade da manifestação clínica (critérios de West Haven):

- Mínima: Alterações em teste neuro-psicométricos, sem sintomas aparentes;
- Grau I: desatenção, dificuldade em executar tarefas complexas ou motoras finas, ansiedade;
- Grau II: tremores, alteração de sono-vigília, alterações de humor, desorientação leve em tempo e espaço;
- Grau III: letargia, confusão mental severa, comportamento bizarro, sonolência;
- Grau IV: coma.

Critérios de West Heaven

Mímima ou Subclínica	Mímima	Testes neuropsicométricos alterados. Sem manifestação clínica evidente;
	Grau I	Desatenção, euforia, ansiedade, alterações de sono, dificuldade com contas e tarefas complexas;
Descompensada ou Manifesta	Grau II	Tremores, alteração de personalidade, apatia, desorientaçãoem tempo e/ou espaço;
	Grau III	Sonolência, letargia, confusão mental grave, comportamento bizarro;
	Grau IV	Coma.

Figura 3: critérios de West Heaven

Figura adaptada de American Association for the Study of Liver, 2014 [21].

A encefalopatia do tipo B e C tem apresentações clínicas, evolução e manejo muito semelhantes, embora estes se diferem significativamente da encefalopatia tipo A. Detalhes do manejo e tratamento destas condições serão revisados na sessão 4.4.

Em relação à epidemiologia da EH, verificam-se dois cenários bastantes distintos. Em relação ao tipo A (decorrente de insuficiência hepática aguda), a incidência desta patologia é, por definição, igual ao número de casos de IHA. Estes dados foram discutidos e podem ser encontrados na sessão 4.1. Já nos pacientes com hepatopatia crônica, a prevalência da EH não é tão bem estabelecida. É sabido que, no momento do diagnóstico de cirrose hepática, cerca de 10% dos pacientes apresentam sinais de encefalopatia [25, 26]. Em conjunto, de 30 a 40% dos pacientes com cirrose estão sob o risco de apresentar um episódio de EH clinicamente evidente no curso da doença, sendo que uma parcela significativa destes indivíduos terá episódios recorrentes ao longo da vida [27]. Uma análise de dados dos Estados Unidos estimou o custo médio de uma internação por EH entre U$ 46.663,00 e U$ 63.108,00, totalizando um custo estimado de 7 bilhões e 244 milhões de dólares dedicados ao manejo hospitalar da EH apenas no ano de 2009 [28]. No entanto, o verdadeiro impacto da EH em

pacientes cirróticos é difícil de ser mensurado, pois a EH mínima afeta grande parcela desses indivíduos e, com frequência, passa despercebida pelo paciente, seus familiares e equipe médica, não sendo diagnosticada ou tratada [29, 30].

4.3 Fisiopatologia da Encefalopatia Hepática

As alterações celulares e moleculares que culminam na encefalopatia hepática, conhecidos como a fisiopatologia da doença, ainda não são completamente compreendidos. Isto se deve, em grande parte, por se tratar de uma sequência de acontecimentos complexos e multifatoriais [31, 32]. Alguns mecanismos já foram propostos para explicar essa sequência de eventos, entre eles o aumento nos níveis sanguíneos de amônia (hiperamonemia) e a ativação excessiva e patológica do sistema de neurotransmissão do glutamato (excitotoxicidade glutamatérgica). Além disso, alterações em outros sistemas de neurotransmissão, no metabolismo energético cerebral e na homeostase redox (estresse oxidativo), assim como alterações na permeabilidade da barreira hematoencefálica (BHE), parecem estar envolvidos no surgimento da EH [31, 32].

Antes de discutirmos os mecanismos específicos pelo qual a doença hepática pode interferir no funcionamento cerebral, é necessário compreender brevemente a fisiologia do sistema glutamatérgico (SG). O sistema de neurotransmissão do glutamato (ou SG) é o principal sistema de neurotransmissão excitatório dos cérebros de mamíferos, podendo corresponder a até 80% de suas sinapses [33, 34]. Como todo mecanismo de neurotransmissão, o SG é necessita cumprir quatro etapas essenciais para manter seu adequado funcionamento:

- Síntese e estoque da substância ativa em vesículas no neurônio pré-sináptico;
- Liberação do neurotransmissor na fenda sináptica após um estímulo neuronal (potencial de ação);
- Identificação da liberação do neurotransmissor e produção de uma resposta neuronal pós-sináptica através de receptores de membrana

- Remoção sistemática do neurotransmissor da fenda sináptica após sua liberação e ativação de receptores

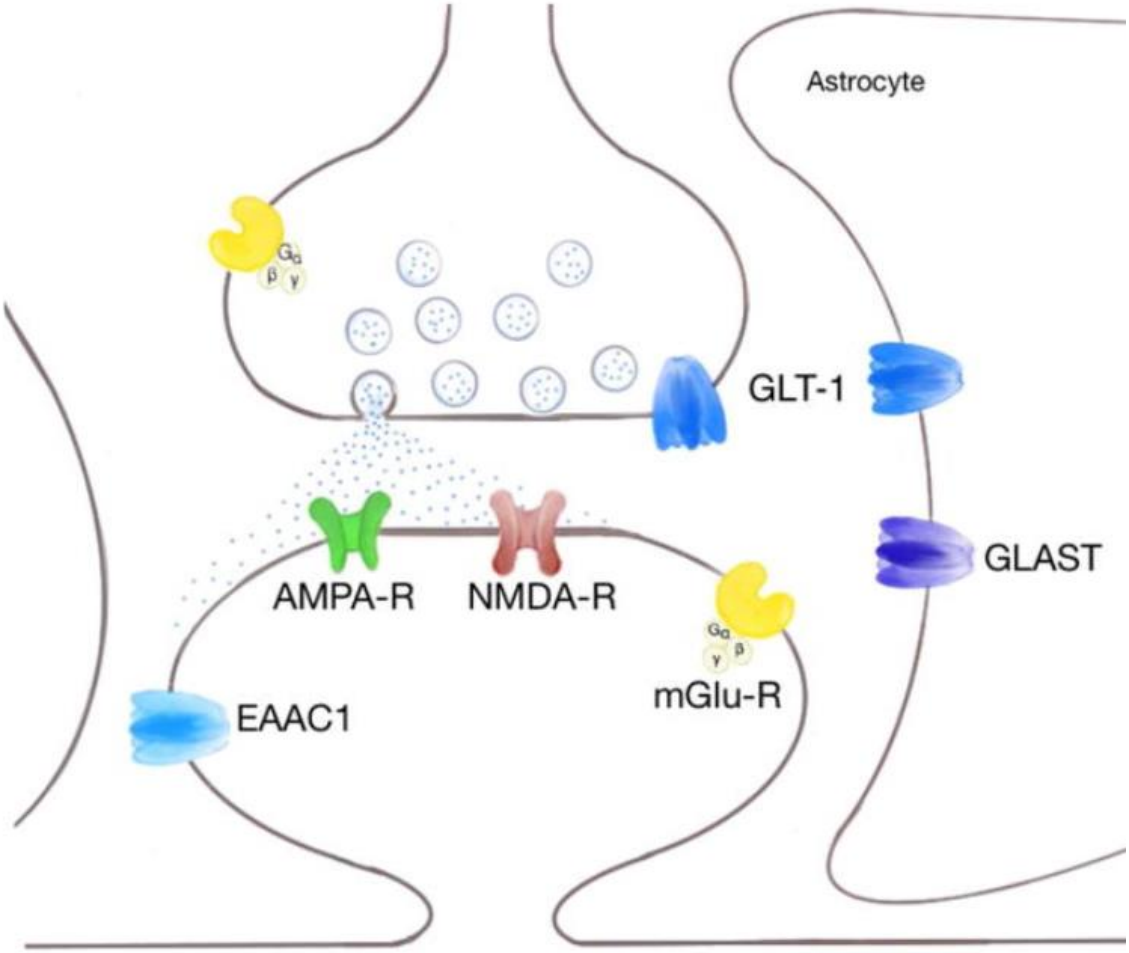


Figura 4: representação de sinapse tripartida de neurônios glutamatérgicos.

Figura adaptada de Rimmele, *et al*, 2016 [33]

A figura 4 representa uma sinapse tripartida (neurônio pré-sináptico, neurônio pós-sináptico e astrócito), esquematizando os principais componentes do SG. De fato, o neurônio pré-sináptico sintetiza moléculas de glutamato e as conserva em vesículas próximas às membranas celulares para que, após um potencial de ação, os neurotransmissores sejam liberados na fenda sináptica. Uma vez liberado na fenda, o glutamato exercerá seu papel excitatório ao entrar em contato com os seus principais receptores de membrana, os receptores AMPA e NMDA, os quais estimularão um potencial de ação no neurônio pós sináptico. A seguir, os astrócitos, retirarão o glutamato da fenda sináptica através dos transportadores de membrana (GLAST e GLT-1) num processo chamado de

recaptação de glutamato [33, 34]. Uma vez dentro dos astrócitos, o glutamato é convertido em glutamina através da enzima glutamina sintase (GS). A glutamina é então secretada de volta para o neurônio pré-sináptico, onde é convertida novamente em glutamato e estocada em vesículas, completando o ciclo [35].

Neste fino estado autorregulado da atividade excitatória glutamatérgica, o acúmulo patológico de toxinas como a amônia pode induzir desequilíbrios e, consequentemente, alterar no correto funcionamento neurológico [36-38]. Estudos em modelos animais de EH demonstraram que a amônia não só ocasiona a hiperativação direta de receptores NMDA [39-41], como também reduz a recaptação de glutamato da fenda sináptica, aumentando a exposição do neurônio pós-sináptico ao estímulo excitatório [42, 43]. A reduzida expressão e funcionamento dos transportadores de membrana astrocitária (GLAST e GLT-1) encontrados nestes modelos são uma hipótese para explicar a diminuição da capacidade de recaptação de glutamato no contexto de hiperamonemia [44-47]. Ademais, evidências da interferência da hiperamonemia no funcionamento da enzima glutamina sintase também já foram descritos [48, 49]. No SNC, esta enzima está presente exclusivamente nos astrócitos, o que reforça o papel central destas células nos processos de metabolismo do glutamato e de detoxificação de amônia [50].

Em suma, a literatura científica referente ao sistema glutamatérgico converge no sentido de que, no contexto de hiperamonemia, há um excessivo estímulo excitatório glutamatérgico que interfere negativamente no funcionamento neuronal [38, 51]. Em concordância com estes dados, estudos prévios do nosso grupo de pesquisa encontraram evidências que reforçam o envolvimento do SG na fisiopatologia da EH. Com efeito, demonstramos que a hiperamonemia induziu aumento nos níveis de glutamato livre no líquido cefalorraquidiano (LCR), reduziu a capacidade de recaptação de glutamato por astrócitos e inibiu a atividade da enzima GS [48]. Ademais, demonstramos o aumento patológico de glutamato e glutamina livres no LCR de animais com EH induzida por hepatectomia subtotal, os quais também apresentaram uma importante redução na expressão proteica dos transportadores GLAST e GLT-1 [45].

Já em relação ao metabolismo energético cerebral, estudos sobre os efeitos da amônia na bioenergética vêm sendo realizados e discutidos há décadas. De fato, em 1955, Bessman e Bessman levantaram a hipótese de que, no contexto de hiperamonemia, o α-cetoglutarato (um dos componentes do Ciclo do Ácido Cítrico [TCA] ou ciclo de Krebs) seria utilizado pelas células para absorver moléculas de amônia ao formar glutamato e glutamina [52]. Nesse sentido, a produção celular de Adenosina Trifosfato (ATP) em um indivíduo com hiperamonemia estaria comprometida devido ao deslocamento do α-cetoglutarato do TCA para uma rota de detoxificação de amônia, ocasionando um relevante déficit energético [52]. A diminuição no consumo de oxigênio cerebral, a qual seria um marcador indireto da redução no metabolismo energético cerebral, já foi demonstrada no contexto de EH [53, 54]. No entanto, diferentes estudos encontraram apenas alterações sutis ocasionadas pela amônia ou EH na produção de ATP, não caracterizando, portanto, alterações bioenergéticas significativas [55-59]. Uma discussão mais aprofundada sobre esses achados controversos foi realizada recentemente por Schousboe e colegas [60].

Outro mecanismo possivelmente associado ao surgimento da EH é o aumento de permeabilidade da barreira hematoencefálica. A BHE é uma estrutura multicelular complexa que reveste as estruturas vasculares que permeiam o sistemas nervoso central, servindo não só como um obstáculo à entrada de patógenos, como modulando a entrada e saída de inúmeras substâncias no líquor, neurônios e glia [61]. Um dos componentes integrantes da BHE é o astrócito, que reveste estruturas vasculares com seus prolongamentos celulares. O aumento da permeabilidade da BHE está associado ao desenvolvimento de patologias do SNC [62, 63]. Alterações no funcionamento da BHE foram descritas, também, no contexto de hiperamonemia e insuficiência hepática [64-68]. Uma hipótese para explicar o aumento da permeabilidade da BHE secundária a hiperamonemia é o mal funcionamento astrocitário.

Tendo em vista os dados apresentados acima, retomamos o conceito de que a fisiopatologia da EH é complexa e multifatorial. De fato, as variadas etiologias do dano hepático, assim como as diferentes formas de desenvolvimento de encefalopatia (tops A, B e C) podem envolver mecanismos

fisiopatológicos distintos. Além disso, a amônia provavelmente não é a única toxina responsável pelo dano neurológico no contexto de insuficiência hepática. De fato, um estudo realizado em 2011 demonstrou que a administração exclusiva de amônia endovenosa em adultos saudáveis resultou apenas em sensação de fadiga, sem alterar testes motores ou cognitivos [69]. Estes achados reforçam a noção de que a EH se desenvolve a partir de um sinergismo de múltiplas toxinas às quais o SNC fica exposto no contexto de insuficiência hepática [70, 71].

4.4 Tratamento da Encefalopatia Hepática

O tratamento da encefalopatia hepática é dependente da forma de apresentação da doença, assim como da sua gravidade, devendo ser individualizado para cada paciente. Casos iniciais da EH do tipo A tem como objetivo a interrupção e manejo das condições que proporcionaram o dano hepático. Já o manejo dos casos graves tem como foco o controle da pressão intracraniana, pois, devido à rápida progressão da doença, estes pacientes têm elevado risco de morte por herniação cerebral [72]. O estrito controle do balanço hídrico se faz necessário para evitar a piora da hipertensão intracraniana, porém grande parte dos pacientes com encefalopatia em graus avançados tem como única opção terapêutica o transplante hepático [73]. O tratamento com redução da temperatura corporal para 33-34ºC (hipotermia terapêutica) demonstrou resultados promissores em modelos animais [74-76] e estudos preliminares em humanos [77]. No entanto, esta estratégia terapêutica não demonstrou benefício clínico quando testada em ensaio clínico randomizado de maior porte [78]. Até o presente momento, não há terapia medicamentosa específica para combater os efeitos neurológicos da EH em quadros de IHA.

Já o manejo da EH dos tipos B e C, os quais serão abordados em conjunto por sua semelhança clínica, apresenta outros princípios terapêuticos. Estas formas de EH ocorrem em pacientes com hepatopatia crônica ou alterações vasculares que desviam o fluxo sanguíneo hepático (*shunt* ou *by-pass*), apresentando variabilidade na apresentação de sintomas, conforme discutido na sessão 4.2 (Figura 3). Quando clinicamente manifesta (Graus II, III ou IV) o manejo da EH tem dois pilares essenciais: o controle do fator de

descompensação da função hepática e a redução dos níveis de amônia sanguínea do paciente [21, 79-82]. O controle dos fatores desencadeantes se refere ao tratamento de possíveis infecções, sangramentos, constipação, abuso de álcool e medicamentos ou quaisquer outros fatores que possam piorar agudamente a função de um fígado previamente doente. Já a redução dos níveis de amônia se dá pelo controle da dieta e uso de medicamentos. Entre as diversas opções terapêuticas com esse propósito [80], discutiremos brevemente três estratégias com grande impacto clínico:

- Lactulose: a lactulose é um dissacarídeo não absorvível que, sem dúvidas, constitui o principal e mais antigo tratamento para a encefalopatia hepática [80]. Esta molécula apresenta mais de um mecanismo de ação no intestino de pacientes cirróticos, entre eles a aceleração do trânsito intestinal e a acidificação do bolo fecal, a qual favorece o crescimento de flora bacteriana benéfica (*Lactobacillus spp*). Esses efeitos se somam para reduzir significativamente os níveis de amônia intestinal absorvidos pelos pacientes cirróticos. Seus efeitos benéficos são bem estabelecidos na EH [83], porém os efeitos adversos como náuseas e diarreia podem dificultar seu uso.
- Rifaximina: a rifaximina é um antibiótico com amplo espectro de ação (gram-positivos, gram-negativos e, em especial, anaeróbios) e que apresenta excelente atividade contra a flora bacteriana intestinal [84]. Desta maneira, seu uso visa reduzir a inflamação e a produção de amônia ocasionados por esses microrganismos. A rifaximina é recomendada em associação a lactulose [21, 85]. Uma metanálise de 2015 evidenciou que este fármaco tem o maior potencial de redução dos níveis séricos de amônia entre as diversas opções terapêuticas para a EH [86].
- Aminoácidos de cadeia ramificada (BCAA): acredita-se que a administração oral de BCAA possa trazer bons resultados em pacientes com hepatopatia crônica por aumentar a síntese proteica muscular e aumentar a atividade da enzima glutamina sintase, facilitando a detoxificação de amônia. Uma metanálise recente de 16 estudos com o uso de BCAA comprovou melhora na sintomatologia e qualidade de vida

dos pacientes cirróticos ao minimizar a EH [87]. Não houve, no entanto, alteração na mortalidade desses pacientes.

É importante frisar que, quando as opções terapêuticas para a EH falham em prover os resultados adequados ou quando o quadro se torna muito recorrente, está indicada a realização de transplante hepático para o manejo destes pacientes [21]. Atualmente não se dispõe de tratamentos com foco específico no SNC para manejar os distúrbios provocados pela EH.

Em relação à EH mínima, os *guidelines* vigentes não trazem indicação clara para se iniciar terapia medicamentosa nestes pacientes [21]. No entanto, alguns ensaios clínicos demonstraram benefício na qualidade de vida destes pacientes com o uso de lactulose [88, 89] e rifaximina [90, 91]. Ademais, medidas gerais de cuidado para prevenção de descompensação da cirrose, assim como o esclarecimento dos riscos quanto à condução de veículos, devem ser estimulados pelo médico assistente [92].

4.5 Guanosina

Guanosina (GUO) é um nucleosídeo endógeno derivado da guanina que faz parte do conjunto de substâncias conhecidas como sistema purinérgico. O sistema purinérgico engloba todas as moléculas derivadas da guanina e da adenosina, as quais exercem diversos papeis no funcionamento celular. De fato, estas moléculas são fundamentais para o organismo, pois estão presentes na composição do DNA, atuam como coenzimas em processos metabólicos e exercem papéis de mensageiros intracelulares. Dentre os exemplos mais conhecidos destas moléculas podemos citar a adenosina trifosfato (ATP), a qual consiste na principal fonte energética celular.

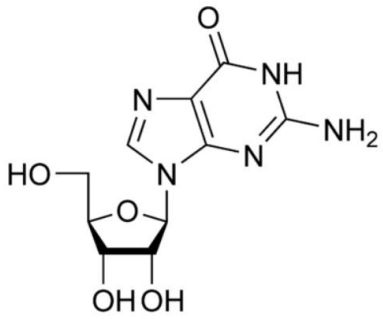


Figura 5: Estrutura molecular da Guanosina

O interesse em particular pelos efeitos da GUO surge a partir de descobrimentos de que este nucleosídeo apresenta propriedades neuroprotetoras. Acredita-se que seus efeitos sejam decorrentes da modulação do sistema glutamatérgico e do seu potencial de otimizar o funcionamento dos astrócitos em situações de excitotoxicidade glutamatérgica [93]. Efeitos desta

substância já foram demonstrados em diversos estudos com modelos animais de convulsão, isquemia cerebral, doença de Alzheimer e doença de Parkinson, entre outros [94-98]. Lanznaster e colegas da Universidade Federal de Santa Catarina recentemente publicaram uma excelente revisão que detalha os achados neuroprotetores da guanosina em diversos modelos animais e celulares estudados até o momento [99].

Em relação à encefalopatia hepática, a modulação do sistema glutamatérgico tem se demonstrado promissora para reduzir os impactos da doença. Estudos com MK-801 (bloqueador de receptores glutamatérgicos) e MSO (bloqueador da enzima glutamina sintase) demonstraram redução de mortalidade em animais com IHA [100-102]. Em paralelo, nosso grupo recentemente publicou dois estudos demonstrando os efeitos da administração da GUO em modelos animais de encefalopatia hepática. Demonstramos que o uso de GUO em modelo de EH crônica induzida pela ligadura das vias biliares (BDL) resulta em melhora de parâmetros comportamentais, eletroencefalográficos (EEG) e bioquímicos [103]. No segundo trabalho, o grupo demonstrou os efeitos neuroprotetores da GUO em um modelo de hiperamonemia aguda induzida pela administração de acetato de amônia [48]. Foi observado, no grupo de animais que recebeu o tratamento com GUO, a diminuição da mortalidade, diminuição do tempo de coma, melhora de parâmetros de EEG e normalização de parâmetros bioquímicos, entre eles o aumento de recaptação de glutamato e diminuição de estresse oxidativo [48].

Considerando as informações expostas acerca da insuficiência hepática aguda e encefalopatia hepática, é urgente a necessidade de compreender os fatores desencadeantes da EH, assim como desenvolver novas medidas para o tratamento desta condição. Neste contexto, a modulação do sistema glutamatérgico e os efeitos neuroprotetores da guanosina surgem como uma possibilidade terapêutica a ser explorada.

5. Objetivos

5.1 Objetivos gerais

Estudar os mecanismos fisiopatológicos da encefalopatia hepática em ratos com insuficiência hepática aguda induzida por hepatectomia subtotal;

Testar os potenciais efeitos neuroprotetores da guanosina em ratos com insuficiência hepática aguda induzida por hepatectomia subtotal.

5.2 Objetivos específicos

Todos os objetivos específicos listados abaixo têm como objeto de estudo a encefalopatia hepática em ratos com insuficiência hepática aguda induzida por hepatectomia subtotal:

1) Estudar os efeitos da EH sobre a bioenergética cerebral, com ênfase no consumo de oxigênio mitocondrial, produção de ATP, atividade enzimática e oxidação de substratos;
2) Avaliar os efeitos da EH sobre a produção de espécies reativas de oxigênio e atividade de enzimas essenciais ao equilíbrio redox;
3) Estudar os efeitos da EH sobre a morfologia astrocitária através de técnicas de imuno-histoquímica;
4) Caracterizar uma escala neurológica para a avaliação e classificação de animais com EH;
5) Estudar os efeitos da EH sobre o metabolismo de aminoácidos, com ênfase nos seus níveis liquóricos e potencial como biomarcadores;
6) Estudar os efeitos da EH sobre a permeabilidade da barreira hematoencefálica, com ênfase nos níveis liquóricos de albumina;
7) Estudar os efeitos da administração de guanosina em animais com IHA, visando a avaliação de mortalidade, testes comportamentais (campo aberto), parâmetros bioquímicos (recaptação de glutamato, níveis liquóricos de albumina e expressão proteica de enzimas) e imuno-histoquímica de córtex cerebral.

Parte 2

6. Materiais, Métodos e Resultados

Capítulo I

Acute liver failure induces glial reactivity, oxidative stress and impair brain energy metabolism in rats

Guazzelli PA, Cittolin-Santos GF, Meira-Martins LA, Grings M, Nonose Y, Fontella FU, Lazzarotto GS, Nogara DA, Wajner M, Leipnitz G, Souza DO, de Assis AM

Manuscrito publicado na revista científica Frontiers in Neuroscience

10 de janeiro de 2020

https://www.frontiersin.org/journals/neuroscience

28 frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
published: 10 January 2020
doi: 10.3389/fnmol.2019.00327

Acute Liver Failure Induces Glial Reactivity, Oxidative Stress and Impairs Brain Energy Metabolism in Rats

Pedro Arend Guazzelli[1,2†], Giordano Fabricio Cittolin-Santos[1,2†], Leo Anderson Meira-Martins[1], Mateus Grings[1], Yasmine Nonose[1], Gabriel S. Lazzarotto[1], Daniela Nogara[2], Jussemara S. da Silva[1], Fernanda U. Fontella[1], Moacir Wajner[1,2], Guilhian Leipnitz[1], Diogo O. Souza[1,2] and Adriano Martimbianco de Assis[1,3]*

[1]Post-graduate Program in Biological Sciences: Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul—UFRGS, Porto Alegre, Brazil, [2]Department of Biochemistry, Universidade Federal do Rio Grande do Sul—UFRGS, Porto Alegre, Brazil, [3]Post-graduate Program in Health and Behavior, Health Sciences Centre, Universidade Católica de Pelotas—UCPel, Pelotas, Brazil

OPEN ACCESS

Edited by:
Michele Papa,
University of Campania
Luigi Vanvitelli, Italy

Reviewed by:
Xiaolu Zhang,
Northern Jiangsu People's Hospital (NJPH), China
Yu-Feng Wang,
Harbin Medical University, China

***Correspondence:**
Diogo O. Souza
diogo@ufrgs.br

[†]These authors have contributed equally to this work

Received: 12 September 2019
Accepted: 18 December 2019
Published: 10 January 2020

Citation:
Guazzeli PA, Cittolin-Santos GF, Meira-Martins LA, Grings M, Nonose Y, Lazzarotto GS, Nogara D, da Silva JS, Fontella FU, Wajner M, Leipnitz G, Souza DO and de Assis AM (2020) Acute Liver Failure Induces Glial Reactivity, Oxidative Stress and Impairs Brain Energy Metabolism in Rats. Front. Mol. Neurosci. 12:327. doi: 10.3389/fnmol.2019.00327

Acute liver failure (ALF) implies a severe and rapid liver dysfunction that leads to impaired liver metabolism and hepatic encephalopathy (HE). Recent studies have suggested that several brain alterations such as astrocytic dysfunction and energy metabolism impairment may synergistically interact, playing a role in the development of HE. The purpose of the present study is to investigate early alterations in redox status, energy metabolism and astrocytic reactivity of rats submitted to ALF. Adult male Wistar rats were submitted either to subtotal hepatectomy (92% of liver mass) or sham operation to induce ALF. Twenty-four hours after the surgery, animals with ALF presented higher plasmatic levels of ammonia, lactate, ALT and AST and lower levels of glucose than the animals in the sham group. Animals with ALF presented several astrocytic morphological alterations indicating astrocytic reactivity. The ALF group also presented higher mitochondrial oxygen consumption, higher enzymatic activity and higher ATP levels in the brain (frontoparietal cortex). Moreover, ALF induced an increase in glutamate oxidation concomitant with a decrease in glucose and lactate oxidation. The increase in brain energy metabolism caused by astrocytic reactivity resulted in augmented levels of reactive oxygen species (ROS) and Poly [ADP-ribose] polymerase 1 (PARP1) and a decreased activity of the enzymes superoxide dismutase and glutathione peroxidase (GSH-Px). These findings suggest that in the early stages of ALF the brain presents a hypermetabolic state, oxidative stress and astrocytic reactivity, which could be in part sustained by an increase in mitochondrial oxidation of glutamate.

Keywords: acute liver failure, brain energy metabolism, hepatic encephalopathy, redox homeostasis, mitochondria, glial reactivity

INTRODUCTION

Acute liver failure (ALF) is a syndrome characterized by sudden hepatic injury and dysfunction in patients with a previously healthy liver and is associated with high lethality and morbidity (Craig et al., 2010; Bernal, 2017). The characteristic features of this condition are impaired liver synthetic function (expressed as coagulopathy), hepatic encephalopathy (HE) and, in severe cases, multi-organ failure (Craig et al., 2010; Scott et al., 2013). The manifestation of the neurological impairment under ALF varies from minor confusion, disorientation and sleep disorders to severe agitation, delirium and, in most advanced stages, coma and death (Blei and Larsen, 1999; Bernal, 2017). Indeed, the final stages of HE reach 20–25% of lethality due to cerebral edema and high intracranial pressure (Larsen and Wendon, 2008; Stravitz and Larsen, 2009) which demands rapid and aggressive treatment strategies such as liver transplantation (Acharya and Bajaj, 2018; Rajaram and Subramanian, 2018).

The molecular basis involved in the development of HE is complex and still a matter of debate and controversy. Nonetheless, ammonia appears to be the main factor in the progress of this syndrome (Bjerring et al., 2009; Hadjihambi et al., 2018). Ammonia is mostly metabolized in the liver *via* the urea cycle, and thus, ammonia bloodstream levels increase in the context of liver insufficiency (Bjerring et al., 2009; Scott et al., 2013). Ammonia can cross the blood-brain barrier by diffusion (Cooper et al., 1985) and numerous studies have shown a positive correlation between its arterial concentration and intracranial hypertension in humans (Clemmesen et al., 1999; Bernal et al., 2007).

Astrocytes occupy around one-third of the cerebral cortex volume and are involved in various neurochemical and cellular regulatory processes (Souza et al., 2019), including AFL (Scott et al., 2013). Astrocytes are the only brain cells that contain glutamine synthetase (GS), an essential enzyme of the glutamatergic system. Therefore, when ammonia concentration increases in the brain, these glial cells start to detoxify it by converting glutamate to glutamine catalyzed by GS (Martinez-Hernandez et al., 1977). Albrecht and Norenberg (2006) proposed the "Trojan Horse" hypothesis which suggests that glutamine works as a carrier of ammonia into the astrocytes' mitochondria once it is metabolized back to glutamate and ammonium, leading to oxidative stress and cell dysfunction (Albrecht and Norenberg, 2006).

Previous studies demonstrated that cultured astrocytes treated with ammonia increased reactive oxygen species (ROS) levels, such as superoxide (Murthy et al., 2001), and the same effect was seen in a hyperammonemia rat model (Kosenko et al., 1997) and clinical studies (Montes-Cortes et al., 2019). Another study showed that ammonia increased mRNA levels of heme-oxygenase-1 (HO-1)—a typical marker of oxidative stress—in rats with HE (Warskulat et al., 2002). Furthermore, the administration of antioxidants such as vitamin E, catalase (CAT), and superoxide dismutase (Ulm et al., 2007) reduced ammonia-induced astrocyte swelling in rats (Jayakumar et al., 2006).

Oxidative stress is known to induce mitochondrial permeability transition (Crompton et al., 1987), which then causes the opening of the permeability transition pore (PTP), a non-selective channel in the inner mitochondrial membrane. The PTP leads to swelling of the mitochondrial matrix, defective adenosine triphosphate (ATP) production, and oxidative phosphorylation, increasing the formation of free radicals and creating a vicious cycle that results in cellular dysfunction (Zoratti et al., 2005). Furthermore, hyperammonemia has been reported to impair energy metabolism not only due to PTP but also directly affecting enzymes involved in energy metabolism (Heidari, 2019). In this regard, previous studies demonstrated that ammonia inhibits α-ketoglutarate dehydrogenase (α-KGDH) and isocitrate dehydrogenase activities (Walker, 2014) and decreases oxygen consumption in the brain (Alman et al., 1956; Strauss et al., 2003; Iversen et al., 2009; Dam et al., 2013). Nonetheless, studies with animal models of ALF have shown that brain ATP levels were only moderately decreased and the TCA cycle was not inhibited under acute HE (Hindfelt and Siesjö, 1971; Hindfelt et al., 1977), implying that the effects of hyperammonemia on brain energy metabolism are still a matter of debate. Considering the above stated, it is urgent to expand the knowledge regarding the mechanisms that lead to astrocyte dysfunction in acute HE in order to establish innovative therapeutic strategies.

The surgical resection of the liver is a well-established and extensively studied animal model of ALF and presents the fundamental features of this disease (Eguchi et al., 1996; Madrahimov et al., 2006; Detry et al., 2013). Indeed, subtotal hepatectomy (resection of 92% of the liver mass) is a reproducible model that induces death by intracranial hypertension and brain herniation and presents a therapeutic window for assessing new therapy strategies (Eguchi et al., 1996; Detry et al., 2013; Cittolin-Santos et al., 2019). We performed in this study subtotal hepatectomy in rats and evaluated astrocyte morphology, neurochemical parameters, redox homeostasis and brain energy metabolism. The objective of the present study is to elucidate early cerebral metabolic disturbances in acute HE.

MATERIALS AND METHODS

Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glucose-D, [^{14}C(U)] (ARC0122H) and Lactic acid, L-[11-^{4}C] sodium salt were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Glutamic acid, L-[^{14}C(U)] (#NEC290E250UC) and Optiphase "Hisafe" 3 (−437) scintillation liquid were purchased from PerkinElmer (Boston, MA, USA). Protein quantification was performed with the BCA Protein Assay kit from Thermo Fisher Scientific (#23227, Rockford, IL, USA), using bovine serum albumin (BSA) as standard.

Animals

Experiments were performed on 90-day-old male Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, at the Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. The animals were maintained in a 12:12 h light/dark cycle (lights on 07:00–19:00 h) and in

an air-conditioned constant temperature (22 ± 1°C) colony room with free access to water and standard commercial chow (SUPRA, Porto Alegre, RS, Brazil). The experimental protocol was approved by the Ethics Committee for Animal Research of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, under the project number 29468, and followed the NIH Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1996). All efforts were made to minimize the number of animals used and their suffering.

Surgical Procedure

Subtotal hepatectomy was performed according to previous descriptions, with minor modifications (Kieling et al., 2012; Detry et al., 2013; Cittolin-Santos et al., 2019). Anesthesia was induced and maintained with 3% isoflurane and an oxygen flow of 0.8 L/min during the whole procedure. The animals were placed on a warmed operating table and a midline laparoscopy was performed to expose the liver. Hepatic ligaments were resected and then pedicles of the anterior lobes were ligated with a 4–0 silk thread to interrupt the blood flow to allow lobe resection. The same procedure was then performed on the right lobes. Only the omental lobes (8% of the liver mass) remained functional. The abdominal wall was sutured with 4–0 nylon thread. Sham group was submitted to the same protocol, except none of the liver lobes pedicles were ligated nor resected.

The animals received intramuscular lidocaine in the abdominal wound to reduce postoperative pain and were kept in a warmed box until full recovery from the anesthesia before being returned to their home cages. Animals had free access to 20% glucose in the drinking water during the whole experiment. Also, three glucose injections of the same glucose solution were administered (2 ml/kg, i.p.) after the surgery to avoid hypoglycemia at the time marks 0, 6 and 12 h.

Tissue Preparation

Twenty-four hours after the surgery, the rats were euthanized by decapitation, and blood was immediately collected in heparinized tubes. The blood samples were then centrifuged at 2,500× *g* for 10 min at 20°C to yield the plasma fraction for subsequent biochemical analyses. Also, the same animals had samples of the cerebral cortex dissected and separated to evaluate the following parameters: (I) astrocytic reactivity; (II) oxygen consumption; (III) metabolic enzyme activities; (IV) substrates oxidation to CO_2; and (V) redox homeostasis.

Immunohistochemistry and Astrocyte Morphological Analysis

Immunohistochemistry for glial fibrillary acidic protein (GFAP) positive astrocyte was performed to evaluate morphological parameters. After decapitation, brains were fixed by immersion for 24 h in 4% PFA diluted in phosphate buffer saline (PBS, pH 7.4), cryoprotected through immersion in sucrose solution (gradually, 15% to 30% until sinking) and frozen at −20°C. Coronal brain slices of 30 μm, approximately +2.20 mm rostrally from bregma, were obtained using a cryostat (MEV, SLEE Medical GMBH, Mainz, Germany). Brain slices were post-fixed with 4% PFA-PBS for 15 min., permeabilized in 0.1% Triton X-100 diluted in PBS (PBS-Tx), and then blocked for 1 h with 5% fetal goat serum diluted in PBS-Tx. The samples were incubated for 24 h at 4°C with polyclonal rabbit anti-GFAP (Z0334, 1:500 in PBS-Tx, Dako, Glostrup, Denmark), followed by 2 h incubation with goat anti-rabbit AlexaFluor® 555 secondary antibody (1:1,000 in PBS-Tx, Invitrogen, Carlsbad, CA, USA). Images were obtained in Leica TCS SP5 II laser-scanning confocal microscopy and acquired at 8-bit gray-scale (256 gray levels) using the Leica Application Suite Advanced Fluorescence software (Leica Microsystems, Munich, Germany). The Sholl's mask creation (virtual concentric circles and orthogonal lines) and all analyses were performed using the ImageJ software, a public domain Java Image processing program[1].

Plasma Biochemical Parameters Evaluation

Plasma ammonia, glucose, lactate, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using commercial kits (Lab test, MG, Brazil) and a SpectraMax M5 microplate reader (Molecular Devices, CA, USA; de Assis et al., 2009).

Preparation of Mitochondrial Fractions

Twenty-four hours after the surgery, cerebral cortex mitochondria were isolated as previously described (Rosenthal et al., 1987) with slight modifications (Mirandola et al., 2008). Immediately after decapitation, the brain was rapidly removed, the cerebral cortex was dissected and placed into an ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1% (BSA; fatty acid-free) and 10 mM HEPES, pH 7.2. The tissue was cut into small pieces using surgical scissors and extensively washed to remove the blood and then homogenized with 1.5 ml of isolation buffer. The homogenate was centrifuged for 3 min at 2,000× *g*. After centrifugation, the supernatant was centrifuged again for 8 min at 12,000× *g*. The pellet was resuspended in 1 ml of isolation buffer containing 4 μl of 10% digitonin and centrifuged for 10 min at 12,000× *g*. The final pellet containing the mitochondria was gently washed and suspended in isolation buffer devoid of EGTA, at an approximate protein concentration of 8 mg/ml.

Determination of Mitochondrial Respiratory Parameters by Oxygen Consumption

Oxygen consumption rate was measured using an OROBOROS Oxygraph-2k (Innsbruck, Austria) in a thermostatically controlled environment (37°C) and magnetically stirred in an incubation chamber (2 ml of standard reaction medium) in respiring medium containing 0.3 M sucrose, 5 mM KH2PO4, 1 mM EGTA, 1 mg/ml BSA, 5 mM 3-[N-morpholino] propane sulfonic acid (MOPS), pH 7.4, using glutamate plus malate (2.5 mM each) as substrates. State 3 respiration was measured

[1] http://rsb.info.nih.gov/ij/

after the addition of 1 mM ADP to the incubation medium. To measure resting (state 4) respiration, 1 μg/ml oligomycin A was added to the incubation medium. The respiratory control ratio (RCR: state 3/state 4) was then calculated. The uncoupled respiration was induced by the addition of carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 2 μM). States 3, 4 and uncoupled respiration were calculated as nmol O_2 consumed/min/mg protein, and the results were expressed as a percentage of control.

Determination of ATP Concentration

In order to measure brain ATP levels in frontoparietal cortex, animals were euthanized by decapitation and the whole head was immediately submerged in liquid nitrogen. Once the head was utterly frozen, brain tissue was quickly harvested with a hammer and chisel through a median craniectomy, and the brain tissue (while still frozen) was submerged in 200 μl of 0.7 N perchloric acid at 4°C. The samples were then homogenized and centrifuged (16,000× *g* for 10 min at 4°C). The supernatants were neutralized with 4.0 N KOH and clarified with second centrifugation (16,000× *g* for 30 min. at 4°C). After the second centrifugation, the supernatants were collected and centrifuged a third time (16,000× *g* for 30 min. at 4°C). ATP analysis was performed by HPLC, as previously described (Voelter et al., 1980). Aliquots of 20 μl were applied to a reversed-phase HPLC (Shimadzu, Japan) using a C18 column (Ultra C18, 25 cm × 4.6 mm × 5 μm, Restek Corporation, Bellefonte, PA, USA). The elution was carried out by applying a linear gradient from 100% solvent A (60 mM KH2PO4 and 5 mM of tetrabutylammonium chloride, pH 6.0) to 100% of solvent B (solvent A plus 30% methanol) over a 30-min period (flow rate at 1.4 ml/min). The amounts of purines were measured by absorption at 254 nm. The retention times of standards were used as parameters for identification and quantification.

Determination of Glutamate Dehydrogenase (GDH) Activity

Glutamate dehydrogenase (GDH) activity was assayed according to Colon et al. (1986). The reaction mixture contained mitochondrial preparations (60 μg of protein), 50 mM triethanolamine buffer, pH 7.8, 2.6 mM EDTA, 105 mM ammonium acetate, 0.2 mM NADH, 10 mM α-ketoglutarate and 1.0 mM ADP. The reduction of NADH absorbance was monitored spectrophotometrically at 340 nm. GDH activity was calculated as nmol NADH/min/mg protein.

Determination of Malate Dehydrogenase (MDH) Activity

Malate dehydrogenase (MDH) activity was measured according to Kitto et al. (1970). The incubation medium consisted of mitochondrial preparations (1 μg of protein), 10 μM rotenone, 0.1% Triton X-100, 0.14 mM NADH, 0.3 mM oxaloacetate and 50 mM potassium phosphate, pH 7.4. MDH activity was determined following the reduction of NADH fluorescence at wavelengths of excitation and emission of 366 and 450 nm, respectively. MDH activity was calculated as nmol NADH/min/mg protein.

Determination of α-Ketoglutarate Dehydrogenase (α-KGDH) Complex Activity

The α-KGDH complex activity was evaluated according to Lai and Cooper (1986) and Tretter and Adam-Vizi (2004) with some modifications. The incubation medium contained mitochondrial preparations (250 μg of protein), 1 mM $MgCl_2$, 0.2 mM thiamine pyrophosphate, 0.4 mM ADP, 10 μM rotenone, 0.2 mM EGTA, 0.12 mM coenzyme A-SH, 1 mM α-ketoglutarate, 2 mM NAD^+, 0.1% Triton X-100 and 50 mM potassium phosphate, pH 7.4. The reduction of NAD^+ was recorded at wavelengths of excitation and emission of 366 and 450 nm, respectively. The α-KGDH activity was calculated as nmol NADH/min/mg protein.

Determination of Citrate Synthase (Kaplan et al., 2015) Activity

CS activity was measured according to Shepherd and Garland (1969), by determining 5,5-dithio-bis (2-nitrobenzoic acid; DTNB) reduction at λ = 412 nm. The incubation medium contained mitochondrial preparations (2 μg of protein), 5 mM potassium phosphate buffer, pH 7.4, 300 mM sucrose, 1 mM EGTA, 0.1% BSA, 5 mM MOPS, 0.1% Triton X-100, 0.1 mM DTNB, 0.1 mM acetyl-CoA and 0.2 mM oxaloacetate. CS activity was calculated as nmol TNB/min/mg protein.

Determination of Succinate Dehydrogenase (SDH) Activity

Succinate dehydrogenase (SDH) activity was measured according to Fischer et al. (1985) by determining 2,6-dichloroindophenol (DCIP) reduction at λ = 600 nm. The incubation medium contained tissue supernatant (30 μg of protein), 40 mM potassium phosphate buffer pH 7.4, 16 mM sodium succinate, 4 mM sodium azide, 7 μM rotenone, 8 μM DCIP and 1 mM phenazine methosulfate. SDH activity was calculated as nmol reduced DCIP/min/mg protein.

Redox Assays

Reactive Oxygen Species (ROS) Levels

To assess ROS levels, DCFH-DA was used as a probe (LeBel et al., 1992). An aliquot of the parietal cortex homogenate (100 μg–30 μl) was incubated with DCFH-DA (100 μM) at 37°C for 30 min. The formation of fluorescent DCF was monitored at excitation and emission wavelengths of 488 and 525 nm, respectively, using a fluorescence spectrophotometer. ROS contents were quantified using a DCF standard curve. The results are expressed as nmol DCF formed/mg protein.

Antioxidant Enzymes Activities

Superoxide dismutase (Ulm et al., 2007; EC 1.15.1.1) activity was assessed on parietal cortex samples by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation at 480 nm, as previously described, and the results were expressed as units SOD/mg protein (Boveris, 1984). Glutathione peroxidase (GSH-Px; EC 1.11.1.9) activity was measured according to Wendel (1981). One unit of GSH-Px activity was defined as 1 μmol NADPH consumed/min and the specific activity is expressed as units/mg protein.

Substrates Oxidation to $^{14}CO_2$

Cerebral cortex slices (300 μm, 100–120 mg) were obtained as described above, transferred into flasks and pre-incubated in Dulbecco's buffer for 30 min. Before incubation with substrates, the reaction medium was gassed with a 95% O_2:5% CO_2 mixture for 30 s. Slices were incubated in 1 ml of Dulbecco's buffer containing either: (i) 5 mM D-Glucose + 0.2 μCi D-[^{14}C(U)]glucose (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA); (ii) 10 μM L-glutamic Acid + 0.2 μCi L-[^{14}C(U)] Glutamate (PerkinElmer Boston, MA, USA); and (iii) 10 μM sodium L-lactate + 0.2 μCi L-[U-^{14}C]lactate (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA). Then, flasks containing the slices were sealed with rubber caps and parafilm and incubated at 37°C for 1 h in a Dubnoff metabolic shaker (60 cycles/min) as described previously (Dunlop et al., 1975; Ferreira et al., 2007). The incubation was stopped by adding 0.2 ml of 50% trichloroacetic acid (TCA) through the rubber cap into the flask while 0.1 ml of 2 N NaOH was injected into the central wells. Thereafter, flasks were shaken for an additional 30 min. at 37°C to trap CO_2. Afterward, the content of the central well was transferred to vials and assayed for $^{14}CO_2$ radioactivity in a liquid scintillation counter. All the results are expressed as nmol of substrate oxidized per mg of tissue and the initial specific activity of the incubation medium was considered for calculations (Müller et al., 2013).

Statistical Analysis

The data are expressed as mean ± SEM. All analyses were performed with Prism GraphPad (Version 6.01 for Windows, GraphPad Software, San Diego, CA, USA[2]). Differences among the groups were analyzed by *t*-test with levels of significance below $p < 0.05$ indicated in the following section.

RESULTS

An initial cohort of 20 animals was operated on and observed to evaluate the efficiency of the surgical procedure and compare it to previous reports (Cittolin-Santos et al., 2019). Our findings were similar to previous studies demonstrating 80% lethality from 30 to 60 h after the surgical procedure (**Supplementary Figure S1**; Cittolin-Santos et al., 2019). Considering the above stated, a 24-h post-surgery time mark was chosen to collect blood and brain samples, because at this point all animals presented signs of encephalopathy although most were still alive. The second cohort of animals was operated on to obtain samples of blood or cerebral cortex (frontoparietal), and the third cohort of animals were later operated on to perform brain ATP measurement.

Plasma Biochemical Parameters

In **Table 1**, we observed that the hepatectomy group presented several plasma alterations that are consistent with ALF, as previously described (Eguchi et al., 1996; Detry et al., 2013; Cittolin-Santos et al., 2019). Hepatectomized animals presented higher levels of ammonia (HEPATEC: 48.4 ± 3.9 vs. SHAM: 22.4 ± 2.1; μmol/L, $p < 0.001$), lactate (HEPATEC: 5.2 ± 1.1 vs. SHAM: 1.5 ± 0.4; mg/dL, $p < 0.001$), ALT (HEPATEC: 61.6 ± 3.1 vs. SHAM: 38.1 ± 1.8 U/L, $p < 0.001$) and AST (HEPATEC: 54.9 ± 4.5 vs. SHAM: 32.6 ± 2.7 U/L, $p < 0.001$) than the controls. The hepatectomy group presented lower levels of glucose (HEPATEC: 71 ± 9 vs. SHAM: 99 ± 8; mg/dL, $p < 0.05$) compared to the sham group.

TABLE 1 | Plasma biochemical parameters.

	Sham	Hepatectomy
Ammonia (μmol/L)	22.4 ± 2.1	48.4 ± 3.9***
Glucose (mg/dL)	99 ± 8	71 ± 9*
Lactate (mg/dL)	1.5 ± 0.4	5.2 ± 1.1***
ALT (U/L)	38.1 ± 1.8	61.6 ± 3.1***
AST (U/L)	32.6 ± 2.7	54.9 ± 4.5***

*Twenty-four hours after hepatectomy, animals with acute liver failure (ALF) presented higher levels of ammonia (48.4 ± 3.9 vs. 22.4 ± 2.1; μmol/L), lactate (5.2 ± 1.1 vs. 1.5 ± 0.4; mg/dL), ALT (61.6 ± 3.1 vs. 38.1 ± 1.8 U/L) and AST (54.9 ± 4.5 vs. 32.6 ± 2.7 U/L) than the controls. The hepatectomy group presented lower levels of glucose (71 ± 9 vs. 99 ± 8; mg/dL) compared to the sham-operated group. *$p < 0.05$ and ***$p < 0.001$ indicate a significant difference from the sham-operated group (t-test).*

Immunohistochemistry and Astrocyte Morphological Analysis

Confocal images of parietal cortex stained with GFAP showed that animals with ALF (**Figure 1A**) presented an increase in the number of astrocytes/mm^3 (HEPATEC: 3.05 ± 0.20 vs. SHAM: 2.44 ± 0.16, **Figure 1B**, $p < 0.05$) as well as in regional optical density (HEPATEC: 4.99 ± 1.12 vs. SHAM: 3.41 ± 1.16, **Figures 1C,D**, $p < 0.05$) and in cellular optical density (HEPATEC: 87.60 ± 7.39 vs. SHAM: 57.22 ± 15.21, **Figure 1E**, $p < 0.001$) when compared to the control animals. The area and the volume occupied by astrocytes was also increased when compared to the sham group (HEPATEC: 36.57 ± 2.42 vs. SHAM: 29.24 ± 1.90 and HEPATEC: 24.38 ± 1.61 vs. SHAM: 19.49 ± 1.27, respectively, **Figures 1F,G**, $p < 0.05$). Nonetheless, the number of brain cells was equal in both groups (data not shown). Regarding cellular morphology (**Figure 2A**), the astrocytes of hepatectomized animals presented a general increase in the ratio of Central processes/lateral processes (LP; HEPATEC: 0.82 ± 0.03 vs. SHAM: 1.09 ± 0.04, **Figure 2B**, $p < 0.05$), number of primary processes (HEPATEC: 3.74 ± 0.23 vs. SHAM: 2.97 ± 0.21, **Figure 2C**, $p < 0.05$) and secondary processes (HEPATEC: 1.46 ± 0.43 vs. SHAM: 0.46 ± 0.26, **Figure 2D**, $p < 0.001$). Consequentially, the number of intersections between these cellular processes was also significantly increased in animals with ALF when compared to the sham group (HEPATEC: 24.74 ± 2.13 vs. SHAM: 13.85 ± 5.62, **Figure 2E**, $p < 0.01$). Additional analysis of astrocytic morphology is also expressed in **Supplementary Figure S2** .

Oxygen Consumption

Mitochondrial oxygen consumption was mostly increased in animals with HE compared to the control group. Indeed, an elevated oxygen consumption level was found in state 3 (HEPATEC: 118.20 ± 9.58 vs. SHAM: 100.00 ± 7.90, **Figure 3A**, $p < 0.01$), in-state 4 (HEPATEC: 112.80 ± 8.63 vs. SHAM: 100.00 ± 9.73 m, **Figure 3B**, $p < 0.05$) and in uncoupled respiration (CCCP; HEPATEC: 116.40 ± 13.09 vs. SHAM: 100.00 ± 10.31,

[2]www.graphpad.com

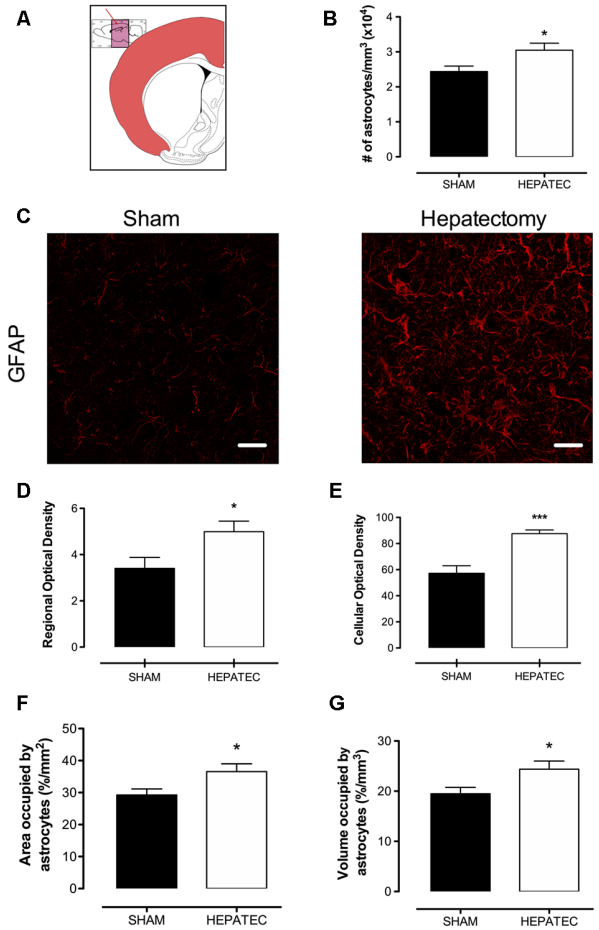


FIGURE 1 | Immunofluorescence for glial fibrillary acidic protein (GFAP). Panel **(A)** represents the region of the brain that was analyzed—parietal cortex; panel **(B)** represents the number of astrocytes/mm^3; panel **(C)** represents immunofluorescence for the GFAP in the parietal cortex. The hepatectomy group presented an increase in regional immunofluorescence for GFAP **(D)** and cellular optical density (87.60 ± 7.39 vs. 57.22 ± 15.21) **(E)**. The area occupied by astrocytes (36.57 ± 2.42 vs. 29.24 ± 1.90) **(F)** and the volume occupied by astrocytes (24.38 ± 1.61 vs. 19.49 ± 1.27) **(G)** were also augmented. Differences between groups were analyzed by *t*-test and are indicated as $^*p < 0.05$ and $^{***}p < 0.001$.

Figure 3C, $p < 0.05$). No alterations were found in the RCR (HEPATEC: 102.10 ± 7.47 vs. SHAM: 100.00 ± 7.15, **Figure 3D**). Results are expressed as a percentage of control.

ATP Levels

Parietal cortex ATP levels were significantly elevated in animals with ALF (HEPATEC: 1.20 ± 0.13 vs. SHAM: 0.63 ± 0.05, **Figure 3E**, $p < 0.05$), as expressed in **Figure 3E**. Results are expressed as μmol/mg of tissue.

Enzyme Activities of Brain Energy Metabolism

Hepatectomized animals presented increased activity in all evaluated metabolic enzymes when compared to the sham group (**Figure 4**). Enzyme activity in hepatectomized and control group was, respectively: citrate synthase (HEPATEC: 201.05 ± 16.11 vs. SHAM: 171.10 ± 20.03, **Figure 4A**, $p < 0.05$); MDH (HEPATEC: 133,954 ± 8,690 vs. SHAM: 98,149 ± 30,939, **Figure 4B**, $p < 0.05$); SDH (HEPATEC: 12.22 ± 1.46 vs. SHAM: 10.65 ± 0.39, **Figure 4C**, $p < 0.05$); α-KGDH (HEPATEC: 7.29 ± 0.59 vs. SHAM: 5.85 ± 0.71, **Figure 4D**, $p < 0.05$); and GDH (HEPATEC: 339.1 ± 8.24 vs. SHAM: 299.90 ± 46.46, **Figure 4E**, $p < 0.01$). The results are expressed as nmol of substrate/min/mg of protein.

Substrates Oxidation to $^{14}CO_2$

Animals submitted to hepatectomy presented an increase in glutamate oxidation to $^{14}CO_2$ (HEPATEC: 9.36 ± 0.82 vs. SHAM: 6.11 ± 0.45, **Figure 5A**, $p < 0.01$) while presenting lower oxidation to $^{14}CO_2$ of glucose (HEPATEC: 511.30 ± 38.86 vs. SHAM: 591.80 ± 80.97, **Figure 5B**, $p < 0.05$) and lactate (HEPATEC: 2,602.0 ± 228.90 vs. SHAM: 3,142.0 ± 266.50, **Figure 5C**, $p < 0.05$). The results are expressed as pmol of substrate/min/mg of tissue.

Redox Assays

Several alterations in the redox homeostasis were induced by hepatectomy. Acute HE presented elevated levels of ROS (HEPATEC: 757.80 ± 36.82 vs. SHAM: 417.00 ± 22.54, **Figure 6A**, $p < 0.001$). Accordingly, it caused a decrease in the activity of two essential antioxidant enzymes: SOD (HEPATEC: 26.62 ± 1.19 vs. SHAM: 33.54 ± 1.38, **Figure 6B**, $p < 0.001$) and GSH-Px (HEPATEC: 21.90 ± 1.32 vs. SHAM: 27.55 ± 1.89, **Figure 6C**, $p < 0.05$). PARP-1 immunocontent in the cerebral cortex of hepatectomized animals was elevated when compared to the sham group (HEPATEC: 0.089 ± 0.002 vs. SHAM: 0.078 ± 0.001, respectively, **Figure 6D**, $p < 0.01$).

DISCUSSION

ALF is a meaningful and potentially life-threatening syndrome that is caused by liver damage and substantial neurotoxin accumulation in the brain, such as ammonia (Bernal, 2017). Indeed, blood ammonia elevation plays an essential role in the development of encephalopathy and leads to glutamatergic excitotoxicity, oxidative stress and astrocytic dysfunction (Cieńko-Michalska et al., 2012; Butterworth, 2015). In the present study, we used an experimental model of ALF induced by subtotal hepatectomy to investigate astrocyte reactivity, brain redox status, energy metabolism and mitochondrial function in rodents. To our knowledge, this is the first study describing evidence of a brain hypermetabolic state induced by ALF, as previous similar results had been found only using cell cultures or acute ammonium intoxication models (Kosenko et al., 1994; Xue et al., 2010). The hypermetabolic state involved the increase in brain oxygen consumption and activities of mitochondrial enzymes, elevated ATP levels, and increased glutamate oxidation. We also postulate a new link between the hypermetabolic state of HE and the increase of ROS with astrocytic reactivity, suggesting a new understanding of the early (24 h) brain metabolic profile in acute HE.

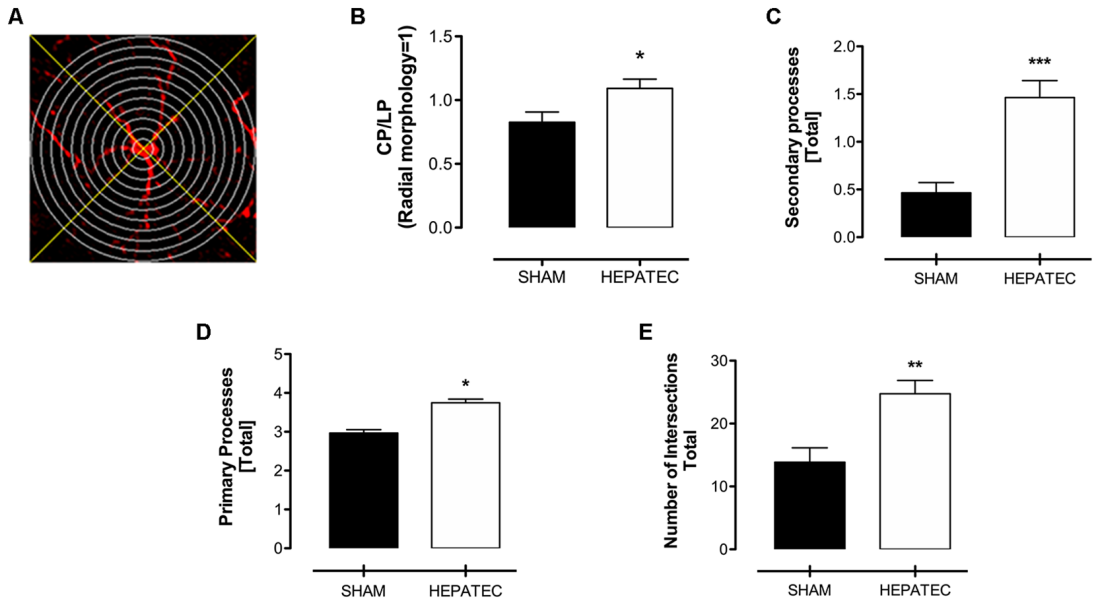


FIGURE 2 | Sholl's mask creation—virtual concentric circles and orthogonal lines **(A)** and astrocytic processes and intersections. Panel **(B)** expresses the ratio between central processes (McPhail et al., 2010) and lateral processes (LP; 1.09 ± 0.07 vs. 0.83 ± 0.08). Hepatectomy group presented an increase in the size of both cellular primary processes **(C)** (19.68 ± 1.41 vs. 12.90 ± 1.31) and secondary processes **(D)** (1.46 ± 0.43 vs. 0.46 ± 0.26). The number of intersections **(E)** was also increased in animals operated on (24.74 ± 2.13 vs. 13.85 ± 5.62). Differences between groups were analyzed by *t*-test and are indicated as $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$.

For this discussion, it is crucial to emphasize that hepatectomy causes up to 80% lethality in the interval from 30 to 60 h after surgery (**Supplementary Figure S1**), as demonstrated in the previous work of our group (Cittolin-Santos et al., 2019). Therefore, the 24-h time mark after surgery was chosen to harvest blood and brain samples as most animals were still alive and already showing marked signs of HE such as ataxia, lethargy and diminished response to pain (Cauli et al., 2008). At this point, animals also presented elevated plasma liver enzymes and alterations in blood glucose, lactate and ammonia levels (**Table 1**) that are well described in this animal model of ALF (Eguchi et al., 1996; Detry et al., 2013; Fusco et al., 2013; Cittolin-Santos et al., 2019).

As mentioned before, elevated intracranial pressure is an essential contributor to HE's high lethality rates (Larsen and Wendon, 2008) and understanding the mechanisms that contribute to this process may be vital in establishing better patient care. Previous studies with magnetic resonance imaging performed in humans with ALF have shown a reduction in the brain's apparent diffusion coefficient, indicating an increase in cellular volume and brain edema (Keiding and Pavese, 2013). Indeed, ALF induces an accumulation of glutamine in the astrocyte in an attempt to detoxify ammonia which has been linked to astrocyte swelling and dysfunction due to glutamine's osmolyte effect (Scott et al., 2013; Rama Rao et al., 2014). In parallel, astrocytic reactivity may be a defense mechanism to modulate brain homeostasis by increasing astrocytic workload, and it may contribute to the elevation of brain pressure due to increased cellular volume. This process has been described in several cerebral diseases, including experimental and human HE (Pilbeam et al., 1983; Kimura et al., 2008). In this manuscript, we describe severe astrocytic morphological changes that characterize a state of diffuse astrocytic reactivity. Indeed, we encountered a significant increase in cellular optical density and astrocytic volume consequential to the increase of astrocytic processes. No alterations were found in the number of astrocytes, indicating that the increase in GFAP density was due to the proliferation in size and number of astrocytic processes. Previous work using the same ALF experimental model that our study used demonstrated an increase in the intracranial pressure following hepatectomy in rats (Detry et al., 2013). Although we did not directly measure intracranial pressure, we demonstrate a state of diffuse astrocytic growth early in the development of HE which could indicate that cytotoxic edema may not be the only mechanism involved in the expansion of the total astrocytic volume and resulting increased intracranial pressure.

Regarding mitochondrial function and brain bioenergetics, there are controversial data about the influence of hyperammonemia on TCA enzymatic activity and energy production (Schousboe et al., 2014; Heidari, 2019). Classic *in vitro* studies has shown that ammonium intoxication inhibits critical enzymes in brain energy metabolism (Bessman and Bessman, 1955). On the other hand, excess ammonium leads to a disturbance in glutamatergic homeostasis which has been linked to increased glycolysis (increased activity of phosphofructokinase and aldolase) and increased activity of TCA enzymes (Zwingmann et al., 2003). Normal brain ATP levels and TCA activity have been described in hyperammonemia

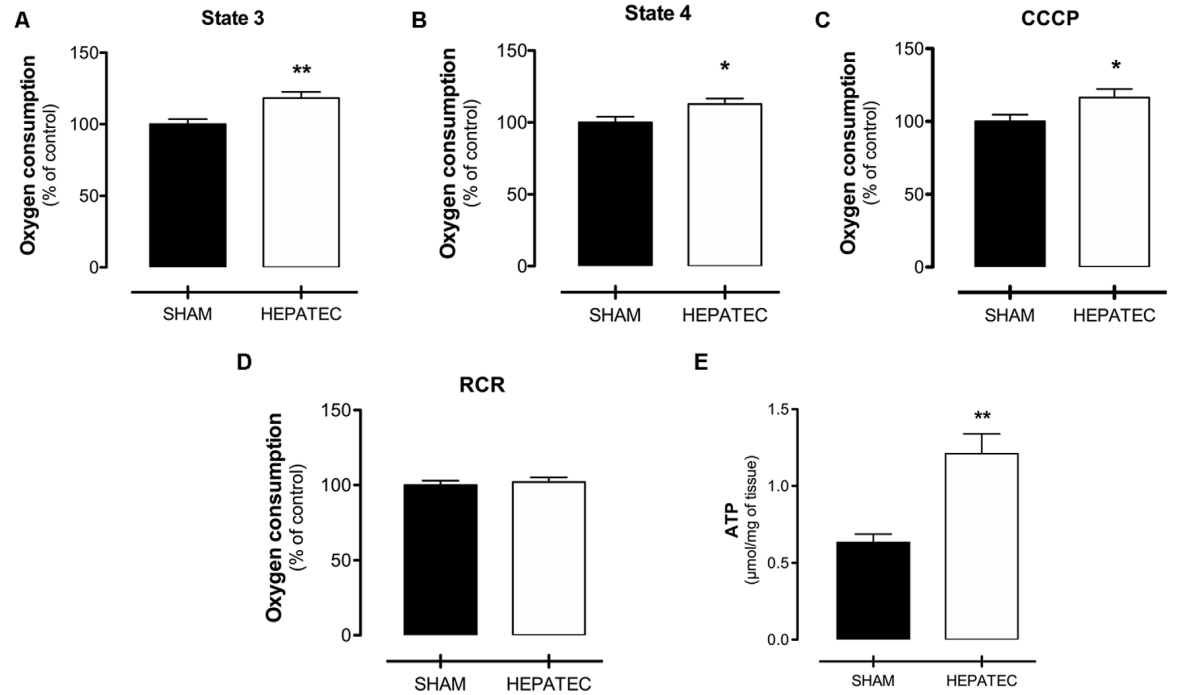


FIGURE 3 | Mitochondrial oxygen consumption. Animals with acute liver failure (ALF) presented higher oxygen consumption in mitochondrial state 3 (118.20 ± 9.58 vs. 100.00 ± 7.90 as % of control and 833.91 ± 93.51 vs. 753.91 ± 79.42 as pmol/s/mg) **(A)**; mitochondrial state 4 (112.80 ± 8.63 vs. 100.00 ± 9.73 as % of control and 121.26 ± 14.68 vs. 111.83 ± 10.88 as pmol/s/mg) **(B)** and CCCP (116.40 ± 13.09 vs. 100.00 ± 10.31 as % of control and 858.81 ± 115.82 vs. 792.22 ± 99.09 as pmol/s/mg) **(C)**. No differences were found in respiratory control ratio (RCR) oxygen consumption (102.10 ± 7.47 vs. 100.00 ± 7.15 as % of control and 6.90 ± 0.50 vs. 6.76 ± 0.48 as pmol/s/mg) **(D)**. Brain ATP was also augmented in animals with ALF [1.20 ± 0.13 vs. 0.63 ± 0.05, **(E)**, $p < 0.05$, µmol/mg]. **(E)** The results in the figure are expressed as (% of control). Differences between groups were analyzed by *t*-test and are indicated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

and soon after experimental liver devascularization (Holmin et al., 1983; Fitzpatrick et al., 1989; Mans et al., 1994). In our current study, we found that the enzymatic activity of pathways involved in bioenergetics metabolism and oxygen consumption were elevated 24 h after hepatectomy, which was accompanied by elevated brain cortical ATP levels and thus indicating a significant alteration in the energy metabolism homeostasis. Since the alterations in brain bioenergetics follow the rapid rise in ammonium levels due to liver insufficiency, we hypothesize that the astrocytes require high levels of energy consumption to remove glutamate from the synaptic cleft and metabolize it in the TCA cycle. Thus, the increase in oxygen consumption and enzymatic activity could be a reactive mechanism of the neural cells trying to provide enough energy to enable the neural tissue to respond to brain injury, which characterizes a hypermetabolic state. This effect, however, is probably time-dependent, occurring only in the early stages (acute phase) of HE. Indeed, previous evaluations of brain ATP levels found that early HE presented mild elevations of brain ATP 4 h after total hepatectomy (Holmin et al., 1983) while ATP measurement of animals with chronic exposure to high ammonia levels (4 weeks after bile duct ligation) have demonstrated decreased ATP levels (Dhanda et al., 2018).

As stated above, astrocytes are involved in the progression of HE and some authors even propose that HE is primarily a glyopathy (Norenberg, 1996; El Khiat et al., 2019) as these cells are responsible for most of the ammonium and glutamate detoxification. This process, however, consumes a significant amount of glucose to produce glutamine and absorb two ammonia molecules which can lead to a decrease in glucose oxidation to CO_2. Indeed, Sibson et al. (2001) demonstrated that up to 80% of brain glucose is utilized for ammonia detoxification by glutamine formation in rats with hyperammonemia, while healthy animals utilize around 30% of glucose in this process (Sibson et al., 2001). In our study, both glucose and lactate oxidation to CO_2 decreased in animals with HE (**Figure 5**). Nonetheless, several reports of experimental models with acute rises in ammonia levels failed to demonstrate a brain energy deficit (Lin and Raabe, 1985; Fitzpatrick et al., 1989; Mans et al., 1994), and our data indicate elevated mitochondrial oxygen consumption as well as no ATP deficit. This could mean that some compensatory mechanisms are activated during hyperammonemia to sustain energy balance. Johansen et al. (2007) discuss the role of branched-chain amino acids in the reposition of carbon skeletons and glutamate production under HE. In this study, we found that the oxidation of glutamate to CO_2 was significantly increased in animals 24 h after hepatectomy (**Figure 5**). We propose that the increase in ammonia levels due to ALF augments the utilization of glucose for glutamine production contributing to the decrease of glucose

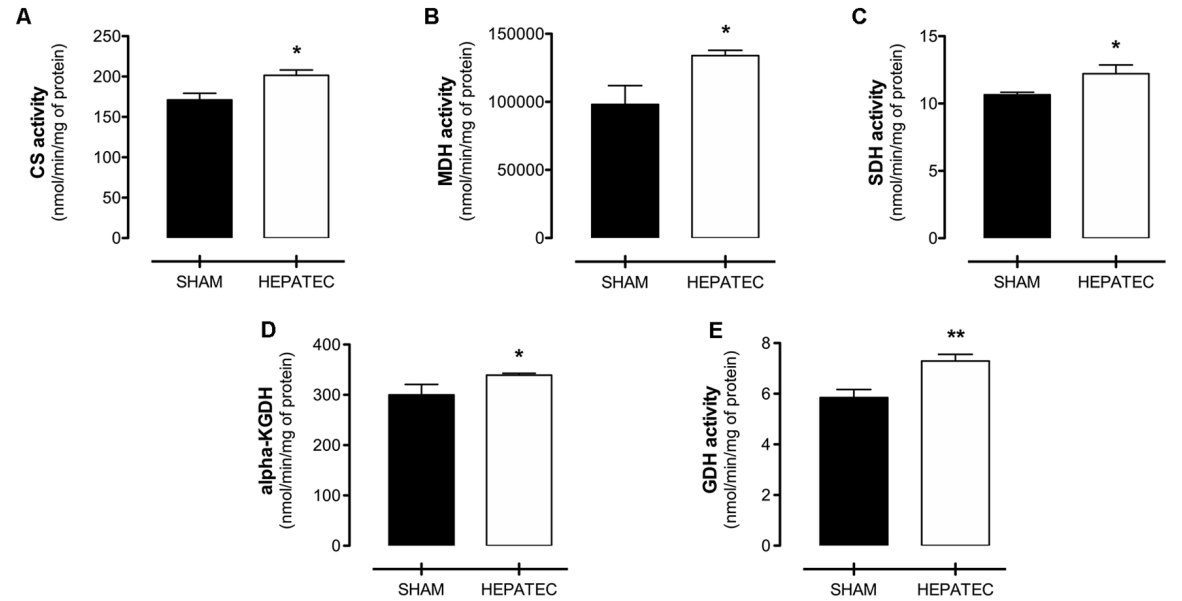


FIGURE 4 | Enzyme activities of brain energy metabolism. Hepatectomy group was found to have an increase in the activity of all analyzed enzymes: **(A)** Citrate synthase (201.05 ± 16.11 vs. 171.10 ± 20.03); **(B)** Malate dehydrogenase (MDH; 133,954 ± 8,690 vs. 98,149 ± 30,939); **(C)** Succinate dehydrogenase (SDH; 12.22 ± 1.46 vs. 10.65 ± 0.39); **(D)** Alpha-ketoglutarate dehydrogenase (α-KGDH; 7.29 ± 0.59 vs. 5.85 ± 0.71); and **(E)** Glutamate dehydrogenase (GDH; 339.1 ± 8.24 vs. 299.90 ± 46.46). The results are expressed as nmol/min/mg of protein. Differences between groups were analyzed by *t*-test and are indicated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

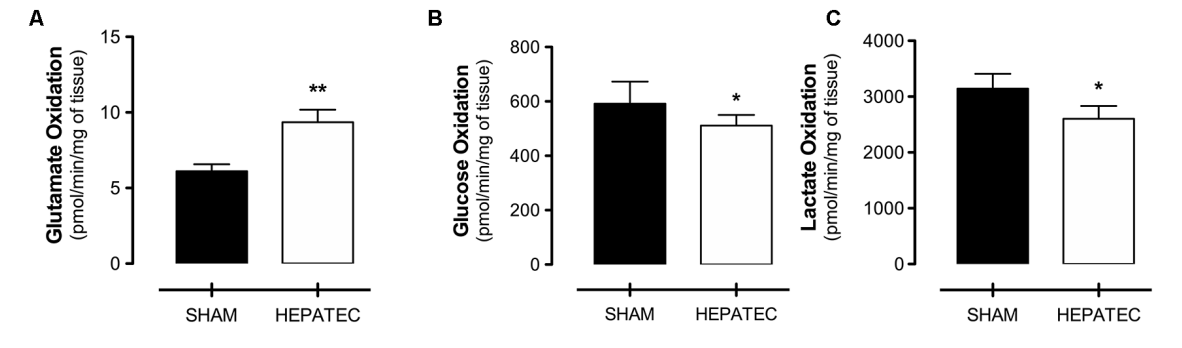


FIGURE 5 | Substrate oxidation to CO_2. Animals submitted to hepatectomy presented elevated glutamate oxidation (9.36 ± 0.82 vs. 6.11 ± 0.45) **(A)**. However, the hepatectomy group had lower oxidation of glucose (511.30 ± 38.86 vs. 591.80 ± 80.97) **(B)** and lactate (2,602.0 ± 228.90 vs. 3,142.0 ± 266.50) **(C)** when compared to control animals. Results are expressed in pmol/min/mg of tissue. Differences between groups were analyzed by *t*-test and are indicated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

oxidation to CO_2. Moreover, the increased glutamate oxidation could, in part, contribute to sustaining brain energy homeostasis as well as removing excess glutamate from the synaptic cleft and potentially attenuating the glutamatergic excitotoxicity and NMDA overstimulation.

Ammonium accumulation in the central nervous system, as described in experimental models of ammonium intoxication and ALF, causes overstimulation of NMDA receptors (Montana et al., 2014; Oja et al., 2017; Dabrowska et al., 2018). This happens both by an increase in extracellular glutamate levels and by direct ammonium activation of NMDA receptors. The NMDA receptor overstimulation is a crucial factor in the development of oxidative stress (Sathyasaikumar et al., 2007; Cittolin-Santos et al., 2017) due to the increase of calcium influx into the cell, which in turn increases ROS production (Hermenegildo et al., 2000; Montes-Cortes et al., 2019). The hepatectomy group presented elevated levels of ROS and decreased activity of SOD and GSH-Px (**Figures 6B,C**). Similar results regarding SOD activity were previously described in acute ammonia intoxication in rats by our group and others (Kosenko et al., 1998; Görg et al., 2013; Cittolin-Santos et al., 2017). This means that ammonia may cause an imbalance in brain redox status through antioxidant enzymes inhibition as well as glutamatergic overstimulation. Indeed, our group and others have shown that by modulating glutamatergic

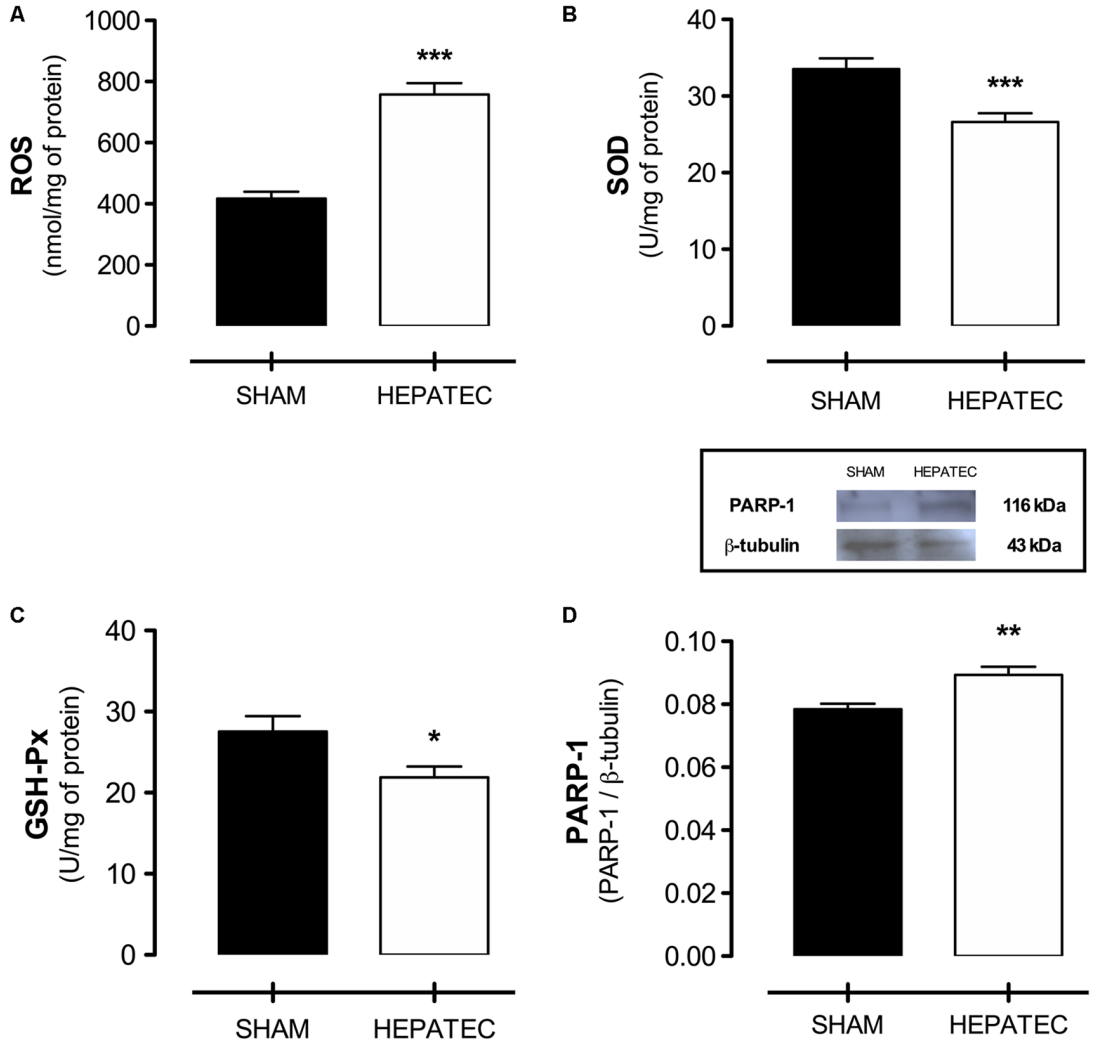


FIGURE 6 | Redox homeostasis and PARP-1 expression. The hepatectomy group presented elevated levels of reactive oxygen species (ROS) **(A)** (757.80 ± 36.82 vs. 417.00 ± 22.54) as well as decreased SOD **(B)** (26.62 ± 1.19 vs. 33.54 ± 1.38) and GSH-Px **(C)** (21.90 ± 1.32 vs. 27.55 ± 1.89) activity. An increased expression in PARP-1 content was also encountered in this group of animals **(D)** (0.089 ± 0.002 vs. 0.078 ± 0.001). ROS are expressed as nmol/mg of protein; the enzyme activity is expressed as U/mg of protein and PARP-1 expression as PARP-1 / b-tubulin ratio. Differences between groups were analyzed by *t*-test and are indicated as $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$.

excitotoxicity there is a normalization of brain redox status and a decrease in lethality under acute ammonia intoxication (Cauli et al., 2014; Paniz et al., 2014; Cittolin-Santos et al., 2017).

Poly (ADP-ribose) polymerase 1 (PARP-1) is a nuclear enzyme involved in critical cellular processes such as DNA repair and cell death (Jubin et al., 2016). The enzymatic activity of PARP-1 is stimulated significantly in the presence of a wide range of activators like damaged DNA, nucleosomes and various protein-binding partners (Eustermann et al., 2011; Langelier et al., 2011). It is noteworthy that oxidative stress induces DNA damage in neural cells (Guo et al., 2013; Narciso et al., 2016) which may act as the signal to activate PARP-1. PARP-1 upregulation has already been linked to increased lethality in experimental models of ALF induced by acetaminophen intoxication, just as PARP-1 inhibition was related to a diminished lethality rate (Dönmez et al., 2015). Considering the above stated and the elevated levels of ROS presented in this manuscript, we hypothesize that oxidative stress is an early event in the development of acute HE that may induce DNA genic activation and PARP-1 upregulation in ALF.

Understanding the complexity of brain metabolic alterations, glial reactivity and cellular dysfunction are the first steps for the development of new treatment strategies for HE due to ALF. In this work, we described several astrocytic alterations that characterize a state of astrocytic reactivity, here observed as an increase in the astrocytic volume due to the proliferation of cellular processes that are known to take part in the pathophysiology of acute HE. We also associated these glial morphological alterations with significant brain metabolic abnormalities such as redox imbalance, increased brain energy

metabolism (increased oxygen consumption and enzymatic activities), increased brain ATP levels as well as alterations in substrate oxidation.

Furthermore, as discussed above, glutamine production for ammonia detoxification utilizes an increased amount of glucose in animals with HE. Thus, we propose that the increase in glutamate oxidation may contribute to sustaining brain ATP levels in the early stages of HE. We also found that the brain hypermetabolic state is associated with imbalances in redox homeostasis and upregulation of PARP-1. Finally, we bring new evidence to the literature regarding the association between astrocytic reactivity, oxidative stress and alterations in brain mitochondrial metabolic and in substrate oxidation under experimental ALF.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee for Animal Research of the Universidade Federal do Rio Grande do Sul (29468), Porto Alegre, Brazil.

AUTHOR CONTRIBUTIONS

PG and GC-S were responsible for the design, acquisition, analysis, interpretation, drafting, and approval of the final version of the manuscript. LM-M, MG, YN, GSL, DN, JS and FF were responsible for acquisition, analysis, interpretation, and approval of the final version of the manuscript. MW and GL were responsible for interpretation, drafting, critical revision, and approval of the final version of the manuscript. DS and AA were responsible for the design, interpretation, drafting, critical revision, and approval of the final version of the manuscript.

FUNDING

This work was supported by Brazilian agencies and grants: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/CSF 88881.030387/2013-01), INCT—Excitotoxicity, and Neuroprotection (465671/2014-4).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2019.00327/full#supplementary-material.

FIGURE S1 | Experimental protocol and subtotal hepatectomy lethality. Panel **(A)** represents the surgical protocol and time mark of 24 h post-surgery for sample harvesting. Panel **(B)** represents the overall mortality and of animals after the hepatectomy. The animals operated on presented 80% lethality within the first 30–60 h after the procedure.

FIGURE S2 | Astrocytic processes and intersections. The hepatectomy group presented an increase in the number of **(A)** central primary processes (1.75 ± 0.20 vs. 1.23 ± 0.08); **(C)** central secondary processes (0.76 ± 0.08 vs. 0.19 ± 0.08); and **(D)** lateral secondary processes (0.70 ± 0.14 vs. 0.27 ± 0.06). The number of **(B)** lateral primary processes was equal in both groups (1.99 ± 0.17 vs. 1.74 ± 0.10). The number of central **(E)** and lateral **(F)** intersections was also increased in animals with acute liver failure (11.79 ± 2.19 vs. 5.90 ± 0.98 and 13.42 ± 1.70 vs. 7.56 ± 1.44, respectively). Differences between groups were analyzed by *t*-test and are indicated as $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$.

REFERENCES

Acharya, C., and Bajaj, J. S. (2018). Current management of hepatic encephalopathy. *Am. J. Gastroenterol.* 113, 1600–1612. doi: 10.1038/s41395-018-0179-4

Albrecht, J., and Norenberg, M. D. (2006). Glutamine: a Trojan horse in ammonia neurotoxicity. *Hepatology* 44, 788–794. doi: 10.1002/hep.21357

Alman, R. W., Ehrmantraut, W. R., Fazekas, J. F., and Ticktin, H. E. (1956). Cerebral metabolism in hepatic insufficiency. *Am. J. Med.* 21, 843–849. doi: 10.1016/0002-9343(56)90098-5

Bernal, W. (2017). Acute liver failure: review and update. *Int. Anesthesiol. Clin.* 55, 92–106. doi: 10.1097/aia.0000000000000141

Bernal, W., Hall, C., Karvellas, C. J., Auzinger, G., Sizer, E., and Wendon, J. (2007). Arterial ammonia and clinical risk factors for encephalopathy and intracranial hypertension in acute liver failure. *Hepatology* 46, 1844–1852. doi: 10.1002/hep.21838

Bessman, S. P., and Bessman, A. N. (1955). The cerebral and peripheral uptake of ammonia in liver disease with an hypothesis for the mechanism of hepatic coma. *J. Clin. Invest.* 34, 622–628. doi: 10.1172/jci103111

Bjerring, P. N., Eefsen, M., Hansen, B. A., and Larsen, F. S. (2009). The brain in acute liver failure. A tortuous path from hyperammonemia to cerebral edema. *Metab. Brain Dis.* 24, 5–14. doi: 10.1007/s11011-008-9116-3

Blei, A. T., and Larsen, F. S. (1999). Pathophysiology of cerebral edema in fulminant hepatic failure. *J. Hepatol.* 31, 771–776. doi: 10.1016/s0168-8278(99)80361-4

Boveris, A. (1984). Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. *Methods Enzymol.* 105, 429–435. doi: 10.1016/s0076-6879(84)05060-6

Butterworth, R. F. (2015). Pathogenesis of hepatic encephalopathy and brain edema in acute liver failure. *J. Clin. Exp. Hepatol.* 5, S96–S103. doi: 10.1016/j.jceh.2014.02.004

Cauli, O., Gonzalez-Usano, A., Cabrera-Pastor, A., Gimenez-Garzo, C., Lopez-Larrubia, P., Ruiz-Sauri, A., et al. (2014). Blocking NMDA receptors delays death in rats with acute liver failure by dual protective mechanisms in kidney and brain. *Neuromolecular Med.* 16, 360–375. doi: 10.1007/s12017-013-8283-5

Cauli, O., Rodrigo, R., Boix, J., Piedrafita, B., Agusti, A., and Felipo, V. (2008). Acute liver failure-induced death of rats is delayed or prevented by blocking NMDA receptors in brain. *Am. J. Physiol. Gastrointest. Liver Physiol.* 295, G503–G511. doi: 10.1152/ajpgi.00076.2008

Ciećko-Michalska, I., Szczepanek, M., Slowik, A., and Mach, T. (2012). Pathogenesis of hepatic encephalopathy. *Gastroenterol. Res. Pract.* 2012:642108. doi: 10.1155/2012/642108

Cittolin-Santos, G. F., de Assis, A. M., Guazzelli, P. A., Paniz, L. G., da Silva, J. S., Calcagnotto, M. E., et al. (2017). Guanosine exerts neuroprotective effect in an experimental model of acute ammonia intoxication. *Mol. Neurobiol.* 54, 3137–3148. doi: 10.1007/s12035-016-9892-4

Cittolin-Santos, G. F., Guazzelli, P. A., Nonose, Y., Almeida, R. F., Fontella, F. U., Pasquetti, M. V., et al. (2019). Behavioral, neurochemical and brain oscillation abnormalities in an experimental model of acute liver failure. *Neuroscience* 401, 117–129. doi: 10.1016/j.neuroscience.2018.12.032

Clemmesen, J. O., Larsen, F. S., Kondrup, J., Hansen, B. A., and Ott, P. (1999). Cerebral herniation in patients with acute liver failure is correlated with arterial ammonia concentration. *Hepatology* 29, 648–653. doi: 10.1002/hep.510290309

Colon, A. D., Plaitakis, A., Perakis, A., Berl, S., and Clarke, D. D. (1986). Purification and characterization of a soluble and a particulate glutamate dehydrogenase from rat brain. *J. Neurochem.* 46, 1811–1819. doi: 10.1111/j.1471-4159.1986.tb08500.x

Cooper, A. J., Mora, S. N., Cruz, N. F., and Gelbard, A. S. (1985). Cerebral ammonia metabolism in hyperammonemic rats. *J. Neurochem.* 44, 1716–1723. doi: 10.1111/j.1471-4159.1985.tb07159.x

Craig, D. G., Lee, A., Hayes, P. C., and Simpson, K. J. (2010). Review article: the current management of acute liver failure. *Aliment. Pharmacol. Ther.* 31, 345–358. doi: 10.1111/j.1365-2036.2009.04175.x

Crompton, M., Costi, A., and Hayat, L. (1987). Evidence for the presence of a reversible Ca^{2+}-dependent pore activated by oxidative stress in heart mitochondria. *Biochem. J.* 245, 915–918. doi: 10.1042/bj2450915

Dabrowska, K., Skowronska, K., Popek, M., Obara-Michlewska, M., Albrecht, J., and Zielinska, M. (2018). Roles of glutamate and glutamine transport in ammonia neurotoxicity: state of the art and question marks. *Endocr. Metab. Immune Disord. Drug Targets* 18, 306–315. doi: 10.2174/1871520618666171219124427

Dam, G., Keiding, S., Munk, O. L., Ott, P., Vilstrup, H., Bak, L. K., et al. (2013). Hepatic encephalopathy is associated with decreased cerebral oxygen metabolism and blood flow, not increased ammonia uptake. *Hepatology* 57, 258–265. doi: 10.1002/hep.25995

de Assis, A. M., Rieger, D. K., Longoni, A., Battu, C., Raymundi, S., da Rocha, R. F., et al. (2009). High fat and highly thermolyzed fat diets promote insulin resistance and increase DNA damage in rats. *Exp. Biol. Med.* 234, 1296–1304. doi: 10.3181/0904-rm-126

Detry, O., Gaspar, Y., Cheramy-Bien, J. P., Drion, P., Meurisse, M., and Defraigne, J. O. (2013). A modified surgical model of fulminant hepatic failure in the rat. *J. Surg. Res.* 181, 85–90. doi: 10.1016/j.jss.2012.05.080

Dhanda, S., Sunkaria, A., Halder, A., and Sandhir, R. (2018). Mitochondrial dysfunctions contribute to energy deficits in rodent model of hepatic encephalopathy. *Metab. Brain Dis.* 33, 209–223. doi: 10.1007/s11011-017-0136-8

Dönmez, M., Uysal, B., Poyrazoğlu, Y., Öztas, Y. E., Türker, T., Kaldirim, Ü., et al. (2015). PARP inhibition prevents acetaminophen-induced liver injury and increases survival rate in rats. *Turk. J. Med. Sci.* 45, 18–26. doi: 10.3906/sag-1308-48

Dunlop, D. S., van Elden, W., and Lajtha, A. (1975). Optimal conditions for protein synthesis in incubated slices of rat brain. *Brain Res.* 99, 303–318. doi: 10.1016/0006-8993(75)90031-1

Eguchi, S., Kamlot, A., Ljubimova, J., Hewitt, W. R., Lebow, L. T., Demetriou, A. A., et al. (1996). Fulminant hepatic failure in rats: survival and effect on blood chemistry and liver regeneration. *Hepatology* 24, 1452–1459. doi: 10.1002/hep.510240626

El Khiat, A., Tamegart, L., Draoui, A., El Fari, R., Sellami, S., Rais, H., et al. (2019). Kinetic deterioration of short memory in rat with acute hepatic encephalopathy: involvement of astroglial and neuronal dysfunctions. *Behav. Brain Res.* 367, 201–209. doi: 10.1016/j.bbr.2019.03.046

Eustermann, S., Videler, H., Yang, J. C., Cole, P. T., Gruszka, D., Veprintsev, D., et al. (2011). The DNA-binding domain of human PARP-1 interacts with DNA single-strand breaks as a monomer through its second zinc finger. *J. Mol. Biol.* 407, 149–170. doi: 10.1016/j.jmb.2011.01.034

Ferreira, G. C., Tonin, A., Schuck, P. F., Viegas, C. M., Ceolato, P. C., Latini, A., et al. (2007). Evidence for a synergistic action of glutaric and 3-hydroxyglutaric acids disturbing rat brain energy metabolism. *Int. J. Dev. Neurosci.* 25, 391–398. doi: 10.1016/j.ijdevneu.2007.05.009

Fischer, J. C., Ruitenbeek, W., Berden, J. A., Trijbels, J. M., Veerkamp, J. H., Stadhouders, A. M., et al. (1985). Differential investigation of the capacity of succinate oxidation in human skeletal muscle. *Clin. Chim. Acta* 153, 23–36. doi: 10.1016/0009-8981(85)90135-4

Fitzpatrick, S. M., Hetherington, H. P., Behar, K. L., and Shulman, R. G. (1989). Effects of acute hyperammonemia on cerebral amino acid metabolism and pHi *in vivo*, measured by 1H and 31P nuclear magnetic resonance. *J. Neurochem.* 52, 741–749. doi: 10.1111/j.1471-4159.1989.tb02517.x

Fusco, S., Reitano, F., Gambadoro, N., Previti, M., Russo, G., Basile, G., et al. (2013). Severe hypoglycemia associated with levofloxacin in a healthy older woman. *J. Am. Geriatr. Soc.* 61, 1637–1638. doi: 10.1111/jgs.12436

Görg, B., Schliess, F., and Häussinger, D. (2013). Osmotic and oxidative/nitrosative stress in ammonia toxicity and hepatic encephalopathy. *Arch. Biochem. Biophys.* 536, 158–163. doi: 10.1016/j.abb.2013.03.010

Guo, C., Sun, L., Chen, X., and Zhang, D. (2013). Oxidative stress, mitochondrial damage and neurodegenerative diseases. *Neural Regen. Res.* 8, 2003–2014. doi: 10.3969/j.issn.1673-5374.2013.21.009

Hadjihambi, A., Arias, N., Sheikh, M., and Jalan, R. (2018). Hepatic encephalopathy: a critical current review. *Hepatol. Int.* 12, 135–147. doi: 10.1007/s12072-017-9812-3

Heidari, R. (2019). Brain mitochondria as potential therapeutic targets for managing hepatic encephalopathy. *Life Sci.* 218, 65–80. doi: 10.1016/j.lfs.2018.12.030

Hermenegildo, C., Monfort, P., and Felipo, V. (2000). Activation of N-methyl-D-aspartate receptors in rat brain *in vivo* following acute ammonia intoxication: characterization by *in vivo* brain microdialysis. *Hepatology* 31, 709–715. doi: 10.1002/hep.510310322

Hindfelt, B., Plum, F., and Duffy, T. E. (1977). Effect of acute ammonia intoxication on cerebral metabolism in rats with portacaval shunts. *J. Clin. Invest.* 59, 386–396. doi: 10.1172/jci108651

Hindfelt, B., and Siesjö, B. K. (1971). Cerebral effects of acute ammonia intoxication. II. The effect upon energy metabolism. *Scand. J. Clin. Lab. Invest.* 28, 365–374. doi: 10.3109/00365517109095711

Holmin, T., Agardh, C. D., Alinder, G., Herlin, P., and Hultberg, B. (1983). The influence of total hepatectomy on cerebral energy state, ammonia-related amino acids of the brain and plasma amino acids in the rat. *Eur. J. Clin. Invest.* 13, 215–220. doi: 10.1111/j.1365-2362.1983.tb00090.x

Iversen, P., Sorensen, M., Bak, L. K., Waagepetersen, H. S., Vafaee, M. S., Borghammer, P., et al. (2009). Low cerebral oxygen consumption and blood flow in patients with cirrhosis and an acute episode of hepatic encephalopathy. *Gastroenterology* 136, 863–871. doi: 10.1053/j.gastro.2008.10.057

Jayakumar, A. R., Rao, K. V., Murthy Ch, R., and Norenberg, M. D. (2006). Glutamine in the mechanism of ammonia-induced astrocyte swelling. *Neurochem. Int.* 48, 623–628. doi: 10.1016/j.neuint.2005.11.017

Johansen, M. L., Bak, L. K., Schousboe, A., Iversen, P., Sorensen, M., Keiding, S., et al. (2007). The metabolic role of isoleucine in detoxification of ammonia in cultured mouse neurons and astrocytes. *Neurochem. Int.* 50, 1042–1051. doi: 10.1016/j.neuint.2007.01.009

Jubin, T., Kadam, A., Jariwala, M., Bhatt, S., Sutariya, S., Gani, A. R., et al. (2016). The PARP family: insights into functional aspects of poly (ADP-ribose) polymerase-1 in cell growth and survival. *Cell Prolif.* 49, 421–437. doi: 10.1111/cpr.12268

Kaplan, J. L., Marshall, M. A., C, C. M., Harmon, D. B., Garmey, J. C., Oldham, S. N., et al. (2015). Adipocyte progenitor cells initiate monocyte chemoattractant protein-1-mediated macrophage accumulation in visceral adipose tissue. *Mol. Metab.* 4, 779–794. doi: 10.1016/j.molmet.2015.07.010

Keiding, S., and Pavese, N. (2013). Brain metabolism in patients with hepatic encephalopathy studied by PET and MR. *Arch. Biochem. Biophys.* 536, 131–142. doi: 10.1016/j.abb.2013.05.006

Kieling, C. O., Backes, A. N., Maurer, R. L., Cruz, C. U., Osvaldt, A. B., Silveira, T. R., et al. (2012). The effects of anesthetic regimen in 90% hepatectomy in rats. *Acta Cir. Bras.* 27, 702–706. doi: 10.1590/s0102-86502012001000006

Kimura, N., Kumamoto, T., Hanaoka, T., Nakamura, K., Hazama, Y., and Arakawa, R. (2008). Portal-systemic shunt encephalopathy presenting with diffuse cerebral white matter lesion: an autopsy case. *Neuropathology* 28, 627–632. doi: 10.1111/j.1440-1789.2008.00898.x

Kitto, G. B., Stolzenbach, F. E., and Kaplan, N. O. (1970). Mitochondrial malate dehydrogenase: further studies on multiple electrophoretic forms.

Biochem. Biophys. Res. Commun. 38, 31–39. doi: 10.1016/0006-291x(70)91079-x

Kosenko, E., Kaminsky, Y., Grau, E., Minana, M. D., Marcaida, G., Grisolia, S., et al. (1994). Brain ATP depletion induced by acute ammonia intoxication in rats is mediated by activation of the NMDA receptor and Na^+, K^+-ATPase. *J. Neurochem.* 63, 2172–2178. doi: 10.1046/j.1471-4159.1994.63062172.x

Kosenko, E., Kaminsky, Y., Kaminsky, A., Valencia, M., Lee, L., Hermenegildo, C., et al. (1997). Superoxide production and antioxidant enzymes in ammonia intoxication in rats. *Free Radic. Res.* 27, 637–644. doi: 10.3109/10715769709097867

Kosenko, E., Kaminsky, Y., Lopata, O., Muravyov, N., Kaminsky, A., Hermenegildo, C., et al. (1998). Nitroarginine, an inhibitor of nitric oxide synthase, prevents changes in superoxide radical and antioxidant enzymes induced by ammonia intoxication. *Metab. Brain Dis.* 13, 29–41. doi: 10.1023/a:1020626928259

Lai, J. C., and Cooper, A. J. (1986). Brain α-ketoglutarate dehydrogenase complex: kinetic properties, regional distribution, and effects of inhibitors. *J. Neurochem.* 47, 1376–1386. doi: 10.1111/j.1471-4159.1986.tb00768.x

Langelier, M. F., Planck, J. L., Roy, S., and Pascal, J. M. (2011). Crystal structures of poly(ADP-ribose) polymerase-1 (PARP-1) zinc fingers bound to DNA: structural and functional insights into DNA-dependent PARP-1 activity. *J. Biol. Chem.* 286, 10690–10701. doi: 10.1074/jbc.M110.202507

Larsen, F. S., and Wendon, J. (2008). Prevention and management of brain edema in patients with acute liver failure. *Liver Transpl.* 14, S90–S96. doi: 10.1002/lt.21643

LeBel, C. P., Ischiropoulos, H., and Bondy, S. C. (1992). Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chem. Res. Toxicol.* 5, 227–231. doi: 10.1021/tx00026a012

Lin, S., and Raabe, W. (1985). Ammonia intoxication: effects on cerebral cortex and spinal cord. *J. Neurochem.* 44, 1252–1258. doi: 10.1111/j.1471-4159.1985.tb08751.x

Madrahimov, N., Dirsch, O., Broelsch, C., and Dahmen, U. (2006). Marginal hepatectomy in the rat: from anatomy to surgery. *Ann. Surg.* 244, 89–98. doi: 10.1097/01.sla.0000218093.12408.0f

Mans, A. M., DeJoseph, M. R., and Hawkins, R. A. (1994). Metabolic abnormalities and grade of encephalopathy in acute hepatic failure. *J. Neurochem.* 63, 1829–1838. doi: 10.1046/j.1471-4159.1994.63051829.x

Martinez-Hernandez, A., Bell, K. P., and Norenberg, M. D. (1977). Glutamine synthetase: glial localization in brain. *Science* 195, 1356–1358. doi: 10.1126/science.14400

McPhail, M. J., Bajaj, J. S., Thomas, H. C., and Taylor-Robinson, S. D. (2010). Pathogenesis and diagnosis of hepatic encephalopathy. *Expert Rev. Gastroenterol. Hepatol.* 4, 365–378. doi: 10.1586/egh.10.32

Mirandola, S. R., Melo, D. R., Schuck, P. F., Ferreira, G. C., Wajner, M., and Castilho, R. F. (2008). Methylmalonate inhibits succinate-supported oxygen consumption by interfering with mitochondrial succinate uptake. *J. Inherit. Metab. Dis.* 31, 44–54. doi: 10.1007/s10545-007-0798-1

Montana, V., Verkhratsky, A., and Parpura, V. (2014). Pathological role for exocytotic glutamate release from astrocytes in hepatic encephalopathy. *Curr. Neuropharmacol.* 12, 324–333. doi: 10.2174/1570159x12666140903094700

Montes-Cortes, D. H., Olivares-Corichi, I. M., Rosas-Barrientos, J. V., Manuel-Apolinar, L., Martinez-Godinez, M. L. A., Hernandez-Lopez, J. C., et al. (2019). Characterization of oxidative stress and ammonia according to the different grades of hepatic encephalopathy. *Dig. Dis.* doi: 10.1159/000503097 [Epub ahead of print].

Müller, A. P., Longoni, A., Farina, M., da Silveira, C. K., Souza, D. O., Perry, M. L., et al. (2013). Propylthiouracil-induced hypothyroidism during lactation alters leucine and mannose metabolism in rat cerebellar slices. *Exp. Biol. Med.* 238, 31–36. doi: 10.1258/ebm.2012.012255

Murthy, C. R., Rama Rao, K. V., Bai, G., and Norenberg, M. D. (2001). Ammonia-induced production of free radicals in primary cultures of rat astrocytes. *J. Neurosci. Res.* 66, 282–288. doi: 10.1002/jnr.1222

Narciso, L., Parlanti, E., Racaniello, M., Simonelli, V., Cardinale, A., Merlo, D., et al. (2016). The response to oxidative DNA damage in neurons: mechanisms and disease. *Neural Plast.* 2016:3619274. doi: 10.1155/2016/3619274

Norenberg, M. D. (1996). Astrocytic-ammonia interactions in hepatic encephalopathy. *Semin. Liver Dis.* 16, 245–253. doi: 10.1055/-2007-1007237

Oja, S. S., Saransaari, P., and Korpi, E. R. (2017). Neurotoxicity of Ammonia. *Neurochem. Res.* 42, 713–720. doi: 10.1007/s11064-016-2014-x

Paniz, L. G., Calcagnotto, M. E., Pandolfo, P., Machado, D. G., Santos, G. F., Hansel, G., et al. (2014). Neuroprotective effects of guanosine administration on behavioral, brain activity, neurochemical and redox parameters in a rat model of chronic hepatic encephalopathy. *Metab. Brain Dis.* 29, 645–654. doi: 10.1007/s11011-014-9548-x

Pilbeam, C. M., Anderson, R. M., and Bhathal, P. S. (1983). The brain in experimental portal-systemic encephalopathy. I. Morphological changes in three animal models. *J. Pathol.* 140, 331–345. doi: 10.1002/path.1711400403

Rajaram, P., and Subramanian, R. (2018). Management of acute liver failure in the intensive care unit setting. *Clin. Liver Dis.* 22, 403–408. doi: 10.1016/j.cld.2018.01.013

Rama Rao, K. V., Jayakumar, A. R., and Norenberg, M. D. (2014). Brain edema in acute liver failure: mechanisms and concepts. *Metab. Brain Dis.* 29, 927–936. doi: 10.1007/s11011-014-9502-y

Rosenthal, R. E., Hamud, F., Fiskum, G., Varghese, P. J., and Sharpe, S. (1987). Cerebral ischemia and reperfusion: prevention of brain mitochondrial injury by lidoflazine. *J. Cereb. Blood Flow Metab.* 7, 752–758. doi: 10.1038/jcbfm.1987.130

Sathyasaikumar, K. V., Swapna, I., Reddy, P. V., Murthy Ch, R., Dutta Gupta, A., Senthilkumaran, B., et al. (2007). Fulminant hepatic failure in rats induces oxidative stress differentially in cerebral cortex, cerebellum and pons medulla. *Neurochem. Res.* 32, 517–524. doi: 10.1007/s11064-006-9265-x

Schousboe, A., Waagepetersen, H. S., Leke, R., and Bak, L. K. (2014). Effects of hyperammonemia on brain energy metabolism: controversial findings *in vivo* and *in vitro*. *Metab. Brain Dis.* 29, 913–917. doi: 10.1007/s11011-014-9513-8

Scott, T. R., Kronsten, V. T., Hughes, R. D., and Shawcross, D. L. (2013). Pathophysiology of cerebral oedema in acute liver failure. *World J. Gastroenterol.* 19, 9240–9255. doi: 10.3748/wjg.v19.i48.9240

Shepherd, D., and Garland, P. B. (1969). The kinetic properties of citrate synthase from rat liver mitochondria. *Biochem. J.* 114, 597–610. doi: 10.1042/bj1140597

Sibson, N. R., Mason, G. F., Shen, J., Cline, G. W., Herskovits, A. Z., Wall, J. E., et al. (2001). *In vivo* (13)C NMR measurement of neurotransmitter glutamate cycling, anaplerosis and TCA cycle flux in rat brain during. *J. Neurochem.* 76, 975–989. doi: 10.1046/j.1471-4159.2001.00074.x

Souza, D. G., Almeida, R. F., Souza, D. O., and Zimmer, E. R. (2019). The astrocyte biochemistry. *Semin. Cell Dev. Biol.* 95, 142–150. doi: 10.1016/j.semcdb.2019.04.002

Strauss, G. I., Møller, K., Larsen, F. S., Kondrup, J., and Knudsen, G. M. (2003). Cerebral glucose and oxygen metabolism in patients with fulminant hepatic failure. *Liver Transpl.* 9, 1244–1252. doi: 10.1016/j.lts.2003.09.020

Stravitz, R. T., and Larsen, F. S. (2009). Therapeutic hypothermia for acute liver failure. *Crit. Care Med.* 37, S258–S264. doi: 10.1097/CCM.0b013e3181aa5fb8

Tretter, L., and Adam-Vizi, V. (2004). Generation of reactive oxygen species in the reaction catalyzed by α-ketoglutarate dehydrogenase. *J. Neurosci.* 24, 7771–7778. doi: 10.1523/JNEUROSCI.1842-04.2004

Ulm, J. W., Perron, M., Sodroski, J., and R, C. M. (2007). Complex determinants within the Moloney murine leukemia virus capsid modulate susceptibility of the virus to Fv1 and Ref1-mediated restriction. *Virology* 363, 245–255. doi: 10.1016/j.virol.2006.09.048

Voelter, W., Zech, K., Arnold, P., and Ludwig, G. (1980). Determination of selected pyrimidines, purines and their metabolites in serum and urine by reversed-phase ion-pair chromatography. *J. Chromatogr.* 199, 345–354. doi: 10.1016/s0021-9673(01)91386-x

Walker, V. (2014). Ammonia metabolism and hyperammonemic disorders. *Adv. Clin. Chem.* 67, 73–150. doi: 10.1016/bs.acc.2014.09.002

Warskulat, U., Görg, B., Bidmon, H. J., Müller, H. W., Schliess, F., and Häussinger, D. (2002). Ammonia-induced heme oxygenase-1 expression in cultured rat astrocytes and rat brain *in vivo*. *Glia* 40, 324–336. doi: 10.1002/glia.10128

Wendel, A. (1981). Glutathione peroxidase. *Meth. Enzymol.* 77, 325–333. doi: 10.1016/s0076-6879(81)77046-0

Xue, Z., Li, B., Gu, L., Hu, X., Li, M., Butterworth, R. F., et al. (2010). Increased Na, K-ATPase $\alpha 2$ isoform gene expression by ammonia in astrocytes and in brain *in vivo*. *Neurochem. Int.* 57, 395–403. doi: 10.1016/j.neuint.2010.04.014

Zoratti, M., Szabò, I., and De Marchi, U. (2005). Mitochondrial permeability transitions: how many doors to the house? *Biochim. Biophys. Acta* 1706, 40–52. doi: 10.1016/j.bbabio.2004.10.006

Zwingmann, C., Chatauret, N., Leibfritz, D., and Butterworth, R. F. (2003). Selective increase of brain lactate synthesis in experimental acute liver failure: results of a [H-C] nuclear magnetic resonance study. *Hepatology* 37, 420–428. doi: 10.1053/jhep.2003.50052

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Capítulo II

Blood brain barrier permeability and increased levels of cerebrospinal fluid amino acids are associated with neurological grading and mortality in rats with acute liver failure

Guazzelli PA, Fachim FS, Travassos AS, Casagrande SC, Ferreira PCL, de Assis AM, Fontella FU, Souza DO

Manuscrito submetido à revista científica Amino Acids

06 de julho de 2020

https://www.springer.com/journal/726

Blood brain barrier permeability and increased levels of cerebrospinal fluid amino acids are associated with neurological grading and mortality in rats with acute liver failure

Guazzelli PA[12], Fachim FS[2], Travassos AS[2], Casagrande SC[2], Ferreira PCL[4], de Assis AM[3], Fontella FU[12], Souza DO[12]

[1] Post-graduate Program in Biological Sciences: Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul – UFRGS, Porto Alegre, RS 90035-003, Brazil;

[2] Department of Biochemistry, Universidade Federal do Rio Grande do Sul - UFRGS, Porto Alegre, RS 90035-003, Brazil;

[3] Postgraduate Program in Health and Behavior, Center of Health Science, Universidade Católica de Pelotas - UCPel, Pelotas, RS 96015-560, Brazil.

[4] Graduate Program in Pharmaceutical Sciencesvior, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, 90610-000 Porto Alegre – RS, Brazil

*Corresponding author:
Diogo Onofre Gomes de Souza, Ph.D.
Department of Biochemistry, Postgraduate Program in Biochemistry
ICBS, Universidade Federal do Rio Grande do Sul, UFRGS
Ramiro Barcelos St 2600 Annex Building, 90035-003, Porto Alegre, RS, Brazil.
Tel.: +55 51 3308 5559
Fax: +55 51 3308 5540
E-mail: diogo.bioq@gmail.com

Abstract

Background: Acute liver failure [ALF] is a life-threatening medical condition that leads to hepatic encephalopathy (HE). Hyperammonemia, astrocyte dysfunction and glutamatergic system imbalance are involved in the pathogenesis of HE. Elevated levels of glutamine and glutamate have been encountered in the cerebrospinal fluid (CSF) of animals and humans with HE, which may be associated with decline in neurological function. This study aims to evaluate the relationship of blood brain barrier (BBB) integrity and CSF amino acids levels with the neurological status of rats submitted to subtotal hepatectomy.

Methods: Adult Wistar rats were submitted to subtotal hepatectomy (removal of 92% of hepatic mass) and monitored for 72 hours to evaluate the development of HE. A neurological scale was adapted to evaluate animals' symptoms and classify the HE progression. Samples of CSF were harvested at 12 or 24 hours after surgery to measure albumin and amino acid levels.

Results: Animals with ALF presented severe neurological impairment and elevated mortality. Analysis performed 24 hours after subtotal hepatectomy demonstrated significant elevation in amino acids and albumin in the CSF, which were correlated with the neurological grades of HE. An increased penetration of Evans Blue into the brain tissue was also encountered at this time mark, indicating an impairment in the BBB permeability. Furthermore, by measuring CSF samples at 12 hours after subtotal hepatectomy, the mortality rate of the animals was correlated with the observed increase of amino acid levels.

Discussion/Conclusion: This manuscript suggests modifications to the existing neurological grading scale for evaluating the progression of HE in rats submitted to ALF in which increased levels of amino acids and albumin in the CSF were observed. The intensity of these events was correlated with the severity of HE and mortality. Additionally, there was an increase of Evans Blue penetration into the brain parenchyma. It is hypothesized that these alterations indicate that our HE model affected blood brain barrier permeability. Moreover, these data suggest that CSF amino acids (emphasizing glutamine) and albumin CSF levels may be useful for clinical monitoring and evaluation of HE.

Financial Support

This work was supported by Brazilian agencies and grants: CNPq, CAPES, FAPERGS, Brazilian Institute of Neuroscience/FINEP, INCT/Excitotoxicity and Neuroprotection (465671/2014-4). The authors declare no competing financial interests.

Keywords

Acute liver failure, Hepatic encephalopathy, Cerebrospinal Fluid, Amino Acids, Albumin, Blood brain barrier, Glutamine, Glutamate.

Introduction

Acute liver failure [ALF] consists of the rapid loss of hepatic function that leads to liver dysfunction and neurological impairment within days or weeks in a patient without history of liver disease (Bernal, Auzinger et al. 2010, Bunchorntavakul and Reddy 2017). Among other alterations, this condition is clinically expressed as coagulopathy and hepatic encephalopathy (HE). Patients with ALF present a high risk of developing several life-threatening conditions such as cerebral edema, infections, bleeding and multi-organ failure, thus requiring intensive care and specialized caregivers (Warrillow, Fisher et al. 2019, Jayalakshmi and Bernal 2020). Intracranial hypertension and cerebral edema are the major factors that lead to death in patients with ALF (Detry, De Roover et al. 2006, Larsen and Wendon 2008, Lee 2012). Indeed, the prognosis of patients with ALF is related to the grade of encephalopathy: whereas patients with mild HE (Grades I and II) have a good chance of spontaneous recovery (around 70%), advanced stages (Grades III and IV) of the disease present a much worse scenario with only around 20% recovery (O'Grady, Alexander et al. 1989, Larsen and Wendon 2008).

The clinical management of ALF has improved in the last decades, but it is still a difficult challenge faced by hepatologists and critical cares doctors. Indeed, despite the close monitoring of ALF complications and intensive supervision of clinical parameters, drastic interventions such as liver transplantation (LT) is often necessary in order to save the patient’s life (Lee 2012, Kodali and McGuire 2015, Seetharam 2019). To aid in the decision-making processes between

clinical management and transplantation, different clinical criteria have been proposed to identify the patients that could benefit from LT. Among these, the most used are the King's College Criteria, the Clichy Criteria and MELD (Bernuau, Goudeau et al. 1986, O'Grady, Alexander et al. 1989, Zaman, Hoti et al. 2006). These tools evaluate both clinical characteristics of the disease (e.g. patient's age, ALF etiology and onset of jaundice) as well as blood biomarkers (e.g. blood pH, prothrombin time and serum creatinine, sodium, bilirubin and factor V) to evaluate the severity of liver failure and necessity of more aggressive treatment strategies. Nonetheless, despite HE and brain edema being a central cause of coma and death in ALF, no brain or cerebrospinal fluid (CSF) biomarker is currently used to evaluate the progression or severity of the disease.

Hyperammonemia and liver failure are associated with increased levels of amino acids in in the blood and CSF. In fact, classic studies documented and discussed high levels of amino acids in serum and CSF in several experimental models of ALF and HE (Smith, Rossi-Fanelli et al. 1978, James, Ziparo et al. 1979, Zanchin, Rigotti et al. 1979, Zanchin, Rigotti et al. 1979, Therrien and Butterworth 1991). More recently, studies performed in humans have also demonstrated similar findings in patients with these conditions (Record, Buxton et al. 1976, Holecek 2015, Helling, Wahlin et al. 2016). A possible cause for the amino acid rise in the CSF may be due to altered permeability in the blood brain barrier (BBB) caused by ALF. Indeed, BBB alterations have been demonstrated in animal models of hyperammonemia and ALF (Nguyen, Yamamoto et al. 2006, Wright, Sharifi et al. 2010, Nguyen 2012, Skowronska and Albrecht 2012, Cui, Sun et al. 2013). Regarding glutamine, high levels of this molecule in CSF have been correlated with the degree of hepatic encephalopathy in patients with chronic liver disease (Whitehead and Whittaker 1955, Hourani, Hamlin et al. 1971, Fraser and Arieff 1985, Chavarria, Alonso et al. 2013, Cudalbu and Taylor-Robinson 2019). Moreover, high CSF glutamine levels have been associated with a decline in electroencephalogram and neurological criteria in patients in intensive care units (Cascino, Cangiano et al. 1982). Recent data suggest that elevated blood levels of glutamine may be correlated with more severe cases and worse outcomes in patients with liver failure (Helling, Wahlin et al. 2016). Accordingly, our group recently published an experimental model of ALF induced by subtotal hepatectomy (surgical removal of 92% of the hepatic mass) which presented marked alterations in the EEG associated with high CSF glutamine levels (Cittolin-Santos, Guazzelli et al. 2019).

Considering the above, it is important to establish animal models of ALF that allow further investigations of acute HE in order to identify possible biomarkers in the progression of the disease, such as glutamine or amino acids levels in the CSF. Previous studies have proposed neurological grading of rats with acute HE in different animal models of ALF (Mans, DeJoseph et al. 1994, Cauli, Rodrigo et al. 2008). However, we find the criteria utilized to identify each neurological state confusing and difficult to apply objectively. Thus, the aims of this study are i) to propose a practical and reproducible method for evaluating the severity of hepatic encephalopathy in rats with ALF induced by subtotal hepatectomy; ii) to evaluate the CSF levels of amino acids and albumin as markers of the progression of hepatic encephalopathy; iii) to hypothesize that BBB impairment is involved in the increase amino acids and albumin levels in CSF, as well as the entrance of Evans Blue into the brain tissue. Thus, we suggest that CSF levels of amino acids (especially glutamine) may be potential biomarkers for identifying subjects at higher risk of poor outcome and therefore aid in the medical management patients with ALF.

Methods

Surgical procedure

Subtotal hepatectomy was performed as previously described (Detry, Gaspar et al. 2013, Cittolin-Santos, Guazzelli et al. 2019, Guazzelli, Cittolin-Santos et al. 2019), with minor modifications. Anaesthesia was induced and maintained with 3% isoflurane in oxygen at a flow rate of 1 L/min during the whole procedure. After cleaning the abdominal wall with povidone-iodine, a median laparotomy was performed, and liver ligaments and adhesions were cut. The pedicle of the left anterior lobe was ligated and resected followed by right anterior lobe resection and then by the resection of both right lobes. After ligation of the lobe hilum, the ischemic mass was removed from the abdominal cavity in order to reduce local inflammation. Both omental lobes (about 8% of liver mass) remained fully functional (Madrahimov, Dirsch et al. 2006).

Lidocaine (0.8 ml) was administered intramuscularly in the wound borders after suturing the abdominal wall to reduce pain. The rats were then kept in a heated box for 30 minutes before being returned to their home cages. Animals had free access to drinking water supplemented with 20% glucose. Additionally, 2 ml/kg of the glucose solution was administered i.p. at 3 different times: 0, 6 and 12 hours after surgery. Sham operations were performed with the exact

same protocol, except pedicle ligation and liver resection. The rats were observed every 6 hours over a 96 hour period to evaluate the progression of HE and mortality rate.

We emphasize that this surgical model of ALF has been utilized by our group in recent studies. Data regarding blood biomarkers of liver dysfunction, brain energy metabolism as well as electroencephalographic and behaviour alterations can be found in Cittolin-Santos, et al, 2019 (Cittolin-Santos, Guazzelli et al. 2019) and Guazzelli, et al, 2019 (Guazzelli, Cittolin-Santos et al. 2019).

Neurological evaluation

Neurological evaluation was performed every 6 hours after the surgical procedure. At first, the cage grid was removed to stimulate animals' locomotion (time 0). If animals presented any spontaneous locomotion or exploratory activity (e.g. interact with other animals, food or cage walls) within the first 10 seconds they were considered as Grade Ø. The animals that remained immobile were tested for both righting and corneal reflexes. Righting reflex was assessed by lifting and trying to place the animal on its back on the cage floor. The normal response is for an animal to turn its body to land on its feet before reaching the ground or to immediately return to normal position once released by a researcher. Corneal reflex was tested by softly poking the cornea with a cotton swab, which should trigger a response of eyelid closure.

Animals that did not move in the first 10 seconds but presented no abnormal reflexes were considered to be Grade I. Animals that did not show spontaneous locomotion and also allowed themselves to be positioned on their backs, but quickly (<3 seconds) returned to normal position, were considered to be Grade II. Grade III consisted of animals with significant locomotor deficit, presenting no spontaneous locomotion or exploration and were unable to return to the upright position when placed on their backs. Grade III animals, however, still presented mild responses to pain stimuli (foot pinching) and presented normal corneal reflex. Animals in Grade IV were comatose and, therefore, did not present righting reflex, corneal reflex, or response to pain stimuli. Death time was registered as the last time-mark that the animal was seen alive.

During this study all sham operated animals remained in stage Ø.

Cerebrospinal fluid harvesting

CSF harvesting was performed according to previous reports from our group (Cittolin-Santos, de Assis et al. 2017, Cittolin-Santos, Guazzelli et al. 2019, Guazzelli, Cittolin-Santos et al. 2019). Animals were briefly anesthetized with 3% isoflurane and positioned in a stereotaxic frame. Samples of CSF (approximately 50 mcl) were collected by direct puncture of the fourth ventricle (cisterna magna) with insulin syringes (29G). CSF was immediately centrifuged at 1000g for 10 minutes and the supernatant was stored at -80ºC for posterior analysis.

CSF samples were collected 24 hours after surgery to evaluate amino acids and albumin levels and correlate them with the severity of HE. Also, whole brains were collected for evaluating Evans Blue color intensity. In a second cohort of animals, CSF samples were collected at 12 hours after the surgical procedure to evaluate the putative prognostic value of amino acid levels for mortality rate.

Amino acid CSF levels

Prior to analysis, CSF was filtered with a 0.22 μm filter and deproteinized (1:5) with methanol, centrifuged at 3000 g for 10 minutes, and after the supernatant was collected. The CSF levels of free amino acids (aspartate, glutamate, alanine, tryptophan, methionine, valine, phenylalanine, isoleucine, leucine, ornithine and lysine, as well as glutamine) were determined by high-performance liquid chromatography (HPLC) according to Joseph, et al, 1986 (Joseph 1986). The analysis was performed using a reverse phase column (Supelcosil LC-18, 250 mm × 4.6 mm × 5 μm, Supelco) in a Shimadzu Instruments liquid chromatograph (50μL loop valve injection, 40 uL injection volume) and fluorescent detection after pre-column derivatization with 100.5 μL OPA (5.4 mg OPA in 1 mL 0.2 M sodium borate pH 9.5) plus 25.5 μL 4% mercaptoethanol for 3 minutes. The mobile phase flowed at a rate of 1.4 mL/min and column temperature was 25 C. Buffer composition was: i) 0.04 mol/L sodium dihydrogen phosphate monohydrate buffer, pH 5.5, containing 80% of methanol; ii) 0.01 mol/L sodium dihydrogen phosphate monohydrate buffer, pH 5.5, containing 20% of methanol. The gradient profile was modified according to the content of buffer B in the mobile phase: 100% at 0.10 min, 90% at 15 min, 48% at 10 min, 100% at 60 min. Absorbance was read at 360 nm and 455 nm, excitation and emission respectively, in a Shimadzu fluorescence detector. Samples of 20 μL were used and concentration was expressed in μM (as mean ± SD). Amino acids were identified by their

retention time and were quantitatively determined by using their chromatographic peak area. A known amino acid standard mixture was used for calibration.

Albumin CSF level evaluation

High performance liquid chromatography coupled with fluorescence detector (HPLC-FLD) consisting of an LC Shimadzu system (Kyoto, Japan) equipped with a LC- 20AT pump, a DGU-14A degasser, thermostat set for CTO-10A column and fluorescence detector, RF 20A was used to quantify CSF albumin concentration. LC Solution software was used in data acquisition and processing. The FLD was set at 278 nm (excitation) and 335 nm (emission). Agilent reversed-phase ZORBAX SB-C18 column (5 μm particle size, 250 × 4.6 mm i.d.) was used. The method was performed using gradient condition, consisting of solvent A (H2O + 0.1% formic acid) and solvent B (acetonitrile (ACN)) as follows: A → 65% B → 35% (0–5.0 min), A → 70% B → 30% (5.0–10 min), A → 65% B → 35% (10.0–17.0 min). The flow rate was set at 0.7 mL/min. FDA bioanalytical guidance complemented by the EMA was used to perform method validation. Albumin stock solutions were prepared in water at 1 mg/mL and stored at − 20 ± 2 °C. For each day of analysis, standard solutions of albumin were prepared at 0.1, 0.5, 1, 10, 50 and 100 μg/mL. Sample preparation was performed by adding 10 μL liquor to the 40 μL of ACN and vortex mixed. Then, the solution was transferred to conical vials and 10 μL were injected. Procedures followed previous guidelines and recommendations (EMA 2012, FDA 2018) .

Evans Blue systemic administration and brain penetration

A small cohort of animals was selected for evaluation of albumin penetration in the central nervous system (CNS). Evans Blue (EB) staining was performed to indirectly access the penetration of albumin in the brain, as this molecule binds to serum albumin. In normal conditions, neither EB nor albumin are able to cross the BBB. However, in pathological conditions, albumin is able to enter the CNS carrying the EB stain (Yao, Xue et al. 2018). Animals had EB (2% dissolved in saline solution) administrated by tail vein puncture (3 mL/kg) at 24 hours after subtotal hepatectomy (Dal-Pizzol, Rojas et al. 2013). After 90 minutes, animals were anesthetized with isoflurane and euthanized by exsanguination. Brains were carefully dissected and kept on ice for image capturing.

Statistical analysis

Data are expressed as mean ± SD. All statistical tests were performed using GraphPad Prism v5 (La Jolla, CA, USA). The test utilized in each analysis is indicated in the corresponding figure legend. Differences were considered significant if *p* value was below 0.05.

Results

Neurological evaluation

Figure 1A summarizes the criteria utilized for establishment of neurological impairment due to HE. Figure 1B shows that animals presented a rapid impairment of brain function after subtotal hepatectomy. The mean onset of Grade I encephalopathy was at 8.66 hours and Grade II at 16.50 hours. The mean onset of severe stages of encephalopathy were 29.65 hours and 33.60 hours (Grades III and IV, respectively, Figure 1B). Mean time of death was 36 hours. A representation of the mean neurological stage over time is expressed in Figure 1C, demonstrating that after a progressive deterioration of neurological function animals that survived started to improve brain function from around 42 hours. It is noteworthy that observations were performed up to the 72 hours time mark, when the experiments were concluded.

Blood brain barrier permeability evaluation

CSF albumin levels and Evans Blue brain penetration

Figure 2A shows that animals submitted to hepatectomy presented an increase in albumin levels in CSF, which were correlated to the degree of neurological impairment: Sham 1.14 ± 0.29; Grade I 1.87 ± 0.26; Grade II 2.41 ± 0.43; Grade III 2.81 ± 0.03. It is noteworthy that no animal in this cohort reached Grade IV at the 24 hour time mark.

As expected, there was a diffuse and homogenous staining through the brain with Evans Blue, which correlated with the degree of neurological impairment (Grades I - III, Figure 2C).

CSF amino acid levels and HE

Figure 3 illustrates rats presenting HE had elevated levels of all analyzed amino acids in the CSF (except for Alanine), as compared to sham group.

CSF amino acid levels and HE severity

Figure 4 illustrates that the increase in CSF amino acid levels were correlated with the degree of neurological impairment (Grades I - IV). Values of specific individual amino acids (Mean ± SD) are in µM as Sham (Ø), Grade I, Grade II, Grade III and Grade IV, respectively: **Aspartate**: 0.46 ± 0.19; 0.53 ± 0.34; 0.71 ± 0.32; 2.00 ± 1.45 and 4.41 ± 0.28 **Glutamate**: 1.25 ± 0.39; 1.46 ± 0.39; 2.29 ± 0.57; 13.06 ± 5.71 and 19.92 ± 4.35 **Glutamine:** 386.9 ± 109.7; 1431.0 ± 476.1; 1959.0 ± 540.8; 2586.0 ± 834.9 and 3015.0 ± 429.6 **Alanine**: 53.05 ± 20.9; 51.27 ± 4.81; 49.40 ± 13.26; 69.83 ± 24.10 and 43.04 ± 14.32 **Tryptophan**: 2.00 ± 0.95; 3.94 ± 0.93; 2.94 ± 0.26; 6.70 ± 3.73 and 13.99 ± 6.34 **Methionine**: 3.82 ± 1.61; 14.91 ± 6.90; 15.74 ± 4.59; 32.55 ± 14.89 and 59.27 ± 19.57 **Valine**: 3.67 ± 1.83; 3.45 ± 2.79; 4.83 ± 2.13; 7.87 ± 3.25 and 12.23 ± 7.17 **Phenylalanine**: 4.43 ± 1.12; 17.54 ± 7.65; 18.14 ± 6.17; 53.42 ± 24.06 and 86.24 ± 8.72 **Isoleucine**: 1.77 ± 0.65; 2.79 ± 1.38; 2.89 ± 0.68; 5.49 ± 2.29 and 12.39 ± 0.86 **Leucine**: 3.45 ± 0.99; 3.89 ± 0.89; 5.10 ± 2.11; 8.12 ± 4.39 and 20.20 ± 5.66 **Ornithine**: 2.25 ± 1.04; 2.34 ± 0.77; 3.13 ± 0.66; 5.40 ± 2.24 and 13.58 ± 6.41 **Lysine**: 67.52 ± 19.89; 124.20 ± 41.75; 152.60 ± 45.87; 288.90 ± 108.50 and 592.50 ± 259.60.

Amino acid CSF levels and mortality rate

To evaluate the relationship between CSF amino acid levels and ALF induced mortality, CSF samples were collected at 12 hours after the surgery (Figure 5). Animals were then divided into 3 groups: i) Sham operated group; ii) Recovered, which consisted of animals that survived the 72 hour analysis; and iii) Deceased, which consisted of animals that died between 12 hours and 72 hours after surgery (conclusion of analysis). The results show that at 12 hours after surgery, the Recovered group did not present significant elevations in CSF levels for most amino acids, except for glutamine and methionine, which presented mild elevations. However, at the same time mark, animals in the Deceased group presented a significant increase in CSF levels for all amino acids (Figure 5). Values of specific individual amino acids (Mean ± SD) are expressed below in µM as Sham, Recovered and Deceased, respectively:

Aspartate: 0.46 ± 0.20; 0.47 ± 0.12 and 1.19 ± 0.76 **Glutamate**: 1.25 ± 0.40; 1.39 ± 0.37 and 4.28 ± 3.01 **Glutamine**: 386.90 ± 109.71; 1079.01 ± 149.97 and 1928.25 ± 414.21 **Alanine**: 53.05 ± 20.90; 50.95 ± 13.64 and 98.48 ± 57.82 **Tryptophan**: 2.00 ± 0.94; 3.72 ± 0.43 and 8.07

± 2.89 **Methionine**: 3.83 ± 1.61; 9.98 ± 2.45 and 23.08 ± 4.85 **Valine**: 3.68 ± 1.83; 5.70 ± 1.18 and 10.53 ± 5.94 **Phenylalanine**: 4.43 ± 1.12; 17.11 ± 2.96 and 40.84 ± 25.58 **Isoleucine**: 1.77 ± 0.65; 2.66 ± 0.26 and 4.92 ± 3.09 **Leucine**: 3.45 ± 0.99; 4.76 ± 0.76 and 10.76 ± 5.31 **Ornithine**: 2.25 ± 1.04; 2.78 ± 0.54 and 6.68 ± 3.86 **Lysine**: 67.52 ± 19.90; 137.51 ± 24.71 and 297.80 ± 130.23

Discussion

This study evaluates the correlation between the neurological progression of hepatic encephalopathy and the levels of amino acids and albumin in the CSF of rats with acute liver failure induced by subtotal hepatectomy. In this study, our group: i) proposes a scale for the reproducible neurological evaluation of rats with ALF; ii) correlates the increase in CSF amino acids and albumin levels with animals' neurological performance; and iii) correlates the increase in CSF amino acid levels with prognosis of death. Based upon these data, we hypothesize that the observed alterations are related to BBB impairment due to HE and, therefore, we propose that the increased levels of albumin and amino acids may be a potential biomarker for future medical evaluation and management of patients with ALF.

Acute liver failure is a life-threatening medical condition that inevitably entails significant brain impairment due to hepatic encephalopathy (Bunchorntavakul and Reddy 2017, Squires, McKiernan et al. 2018, Dong, Nanchal et al. 2020). The reduction in hepatic workload leads to the accumulation of ammonia and other neurotoxins, which interfere with brain metabolism (Montana, Verkhratsky et al. 2014, Butterworth 2016). Hepatic encephalopathy symptoms range from mild alterations such as loss of coordination, extremity tremor, mood and sleep disturbances and slurred speech up to severe neurological impairment, such as muscular rigidity, hyperreflexia, obtundation and even coma (Ferenci, Lockwood et al. 2002, Hadjihambi, Arias et al. 2018). Therefore, clinical classifications of encephalopathy degree have been established to understand and monitor the severity of the disease (Ferenci, Lockwood et al. 2002, Mouri, Tripon et al. 2015).

Animal models of liver disease have been studied for decades to understand the pathophysiology of hepatic encephalopathy, aiming to discover new therapeutic strategies for mitigating the impacts of this condition (Maes, Vinken et al. 2016). These experimental models

reproduce the various stages of the disease, thus allowing for the observation and classification necessary for identifying the progression of neurological impairment. Mans and colleagues described a neurological scale for rats with HE induced by portocaval shunt and hepatic artery ligation (Mans, DeJoseph et al. 1994). More recently, Cauli and colleagues described another neurological grading method for rats with ALF induced by galactosamine administration (Cauli, Rodrigo et al. 2008). These neurological scales, however, describe generic signs such as mild ataxia, mild lethargy, and poor gesture control to differentiate animals from stages 0, I and II. These criteria are susceptible to observational bias and may result in different interpretations. Hence, our study proposes a modified neurological evaluation utilizing simple and objective criteria with no additional costs or workload, which ensures more well-defined criteria for the purpose of sorting subjects into neurological grades for HE in animal models. Our criteria are described in detail in the section 'Methods – Neurological Evaluation'.

Elevated levels of free amino acids have been documented in both the serum and CSF of animals with HE (Holmin, Agardh et al. 1983, Therrien and Butterworth 1991, Swain, Butterworth et al. 1992, Mans, DeJoseph et al. 1994). These alterations seem to be consistent with elevated levels of glutamine and glutamate also found in patients with HE (Vergara, Plum et al. 1974, Record, Buxton et al. 1976, Oei, Kuys et al. 1979, Holecek 2015). In a similar way, our group previously described elevated levels of CSF glutamate and glutamine both in a model of hyperammonemia and of acute HE (Cittolin-Santos, de Assis et al. 2017, Cittolin-Santos, Guazzelli et al. 2019). In this present study, a significant increase in the CSF levels of all analyzed amino acids (except for alanine) was encountered in animals with ALF 24 hours after subtotal hepatectomy (Figure 3). This alteration was strongly correlated with the degree of hepatic encephalopathy (Figure 4). Indeed, animals with Grades I and II presented no alterations (or only mild elevations) in CSF amino acids, while animals in Grades III and IV presented large increases in the analyzed amino acids.

In the same way, CSF albumin levels were correlated with the neurological grades of HE, increasing significantly in late stages of the disease (Figure 2). Albumin is a molecule with a high molecular weight, and is unable to cross the BBB under normal conditions (Obermeier, Verma et al. 2016, Di Pardo, Castaldo et al. 2017). Thus, albumin CSF levels only increase under pathological conditions, such as HE (Chastre, Belanger et al. 2014), and therefore this is considered to be a marker of BBB permeability (Daneman and Prat 2015, Moretti, Pansiot et al.

2015, Tumani, Huss et al. 2017). Hyperammonemia has been shown to affect BBB permeability and permit amino acids to enter the brain (James, Ziparo et al. 1979, Skowronska and Albrecht 2012). On the other hand, previous studies in animal models of HE and ALF indicate mild alterations in BBB and do not demonstrate a clear breakdown of this brain system (Alexander, Li et al. 2000, Nguyen 2010, Wright, Sharifi et al. 2010, Nguyen 2012, Cui, Sun et al. 2013, Shaik, Miah et al. 2013). Similarly, a study of patients with chronic liver disease and overt HE did not encounter changes in BBB permeability using positron emission tomography (PET) scans (Keiding, Sorensen et al. 2006). In the present study, however, we found elevated levels of almost all amino acids alongside an in increase in albumin that were associated with the decline of neurological function due to HE. A diffuse penetration of various free amino acids in the CSF is likely caused by a single factor, such as altered BBB permeability, rather than specific disruptions in the metabolic pathway of each amino acid caused by ammonia accumulation. This hypothesis is reinforced by the diffuse and homogenous increased penetration of Evans Blue staining in the CNS of animals with HE, as shown in Figure 2B.

The amino acid profile and albumin evaluation discussed above were performed at the 24 hour time mark after subtotal hepatectomy, as were done in previous studies by our group (Cittolin-Santos, Guazzelli et al. 2019, Guazzelli, Cittolin-Santos et al. 2019). At 24 hours after surgery, most of the animals present significant neurological impairment and are not yet in the final stages of HE, where the mean time of death is at 36 hours (Figure 1B). Nonetheless, another group of animals was selected for a second experiment in order to harvest CSF at 12 hours after surgery. At this time mark, most animals presented HE Grade I, with few animals in Grade II (Figure 1B). In our experience, it is not possible at this point to differentiate via neurological parameters which animals are going to improve from those that are going to progress toward coma and death. CSF evaluation of amino acid levels allowed us to make a clear distinction between these two groups: the first group (Recovered group) presented a normal or mild elevation in amino acid levels and survived the 72 hour period of evaluation, while the second group (Deceased group) presented a marked elevation in CSF amino acid levels (Figure 5).

The glutamatergic system is believed to have an important role in ammonia detoxification in the brain, specifically via the production of glutamine (Montana, Verkhratsky et al. 2014, Cittolin-Santos, Guazzelli et al. 2019). However, it is also believed that the accumulation of glutamine in the brain may have dangerous effects, as it is implicated in the pathogenesis of

brain edema and may act as a Trojan Horse by releasing ammonium within the cell's mitochondria, thus disturbing brain bioenergetics (Albrecht and Norenberg 2006, Scott, Kronsten et al. 2013, Butterworth 2015). Elevated levels of glutamine in serum and CSF have been associated with neurological decline as evidenced by EEG and with poor clinical outcomes in patients with HE (Cascino, Cangiano et al. 1982, Helling, Wahlin et al. 2016). In this manuscript, the evaluation of CSF amino acid levels was of great value in differentiating not only healthy animals from those with HE, but also in differentiating animals with ALF and poor prognoses from those that spontaneously recovered (Figure 5). Indeed, elevated glutamine at the 12 hour time mark clearly differentiated rats with HE from sham operated animals. More importantly, glutamine levels allowed us to differentiate with great accuracy the animals with favorable prognoses from those that would die before the end of the experiment (Figure 5C). This was also true for methionine and phenylalanine (Figures 5F and 5H, respectively). It is important to emphasize that the sample collection was performed at 12 hours after surgery, a point at which it was not possible to predict the animals' outcome by neurological measures. To our knowledge, this is the first study that correlates the increase in CSF levels of amino acids to an important outcome such as mortality rate. Hence, we hypothesize that if the same pattern of CSF amino acid elevation were encountered in humans with ALF, elevated glutamine levels could potentially be a useful biomarker for evaluating these patients.

Conclusion

This study presents a neurological grading protocol for evaluating the progression of acute hepatic encephalopathy in rats with ALF and describes an increase of amino acids and albumin levels in the CSF of these animals. The CSF alterations are correlated to the severity of neurological symptoms and the mortality rate. Based upon the literature and our own results, we suggest that the alterations in CSF amino acids and albumin levels may be due to impaired BBB activity. To our knowledge, this is the first study that associates BBB permeability, amino acid levels and neurological evaluation under experimental ALF. We also believe that our data offers important information regarding the potential role of amino acids, especially glutamine, in detecting, even in early stages of HE, the subjects that will progress to coma and death.

Therefore, we hypothesize that these molecules have the potential to be useful biomarkers for monitoring and evaluating the progression of HE in humans.

Conflicts of interest

The authors have nothing to disclose.

Compliance with ethical standards

The experimental protocol was approved by the Ethics Committee for Animal Research of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, under the project number 29468, and followed the NIH Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1996). All efforts were made to minimize the number of animals used and their suffering.

References

Albrecht, J. and M. D. Norenberg (2006). "Glutamine: a Trojan horse in ammonia neurotoxicity." Hepatology **44**(4): 788-794.

Alexander, B., X. Li, I. S. Benjamin, M. B. Segal, R. Sherwood and J. E. Preston (2000). "A quantitative evaluation of the permeability of the blood brain barrier of portacaval shunted rats." Metab Brain Dis **15**(2): 93-103.

Bernal, W., G. Auzinger, A. Dhawan and J. Wendon (2010). "Acute liver failure." Lancet **376**(9736): 190-201.

Bernuau, J., A. Goudeau, T. Poynard, F. Dubois, G. Lesage, B. Yvonnet, C. Degott, A. Bezeaud, B. Rueff and J. P. Benhamou (1986). "Multivariate analysis of prognostic factors in fulminant hepatitis B." Hepatology **6**(4): 648-651.

Bunchorntavakul, C. and K. R. Reddy (2017). "Acute Liver Failure." Clin Liver Dis **21**(4): 769-792.

Butterworth, R. F. (2015). "Pathogenesis of hepatic encephalopathy and brain edema in acute liver failure." J Clin Exp Hepatol **5**(Suppl 1): S96-S103.

Butterworth, R. F. (2016). "Pathogenesis of hepatic encephalopathy in cirrhosis: the concept of synergism revisited." Metab Brain Dis **31**(6): 1211-1215.

Cascino, A., C. Cangiano, F. Fiaccadori, F. Ghinelli, M. Merli, G. Pelosi, O. Riggio, F. Rossi Fanelli, D. Sacchini, M. Stortoni and L. Capocaccia (1982). "Plasma and cerebrospinal fluid amino acid patterns in hepatic encephalopathy." Dig Dis Sci **27**(9): 828-832.

Cauli, O., R. Rodrigo, J. Boix, B. Piedrafita, A. Agusti and V. Felipo (2008). "Acute liver failure-induced death of rats is delayed or prevented by blocking NMDA receptors in brain." Am J Physiol Gastrointest Liver Physiol **295**(3): G503-511.

Chastre, A., M. Belanger, B. N. Nguyen and R. F. Butterworth (2014). "Lipopolysaccharide precipitates hepatic encephalopathy and increases blood-brain barrier permeability in mice with acute liver failure." Liver Int **34**(3): 353-361.

Chavarria, L., J. Alonso, R. Garcia-Martinez, M. Simon-Talero, M. Ventura-Cots, C. Ramirez, M. Torrens, V. Vargas, A. Rovira and J. Cordoba (2013). "Brain magnetic resonance spectroscopy in episodic hepatic encephalopathy." J Cereb Blood Flow Metab **33**(2): 272-277.
Cittolin-Santos, G. F., A. M. de Assis, P. A. Guazzelli, L. G. Paniz, J. S. da Silva, M. E. Calcagnotto, G. Hansel, K. C. Zenki, E. Kalinine, M. M. Duarte and D. O. Souza (2017). "Guanosine Exerts Neuroprotective Effect in an Experimental Model of Acute Ammonia Intoxication." Mol Neurobiol **54**(5): 3137-3148.
Cittolin-Santos, G. F., P. A. Guazzelli, Y. Nonose, R. F. Almeida, F. U. Fontella, M. V. Pasquetti, F. J. Ferreira-Lima, G. Lazzaroto, R. M. Berlezi, A. B. Osvaldt, M. E. Calcagnotto, A. M. de Assis and D. O. Souza (2019). "Behavioral, Neurochemical and Brain Oscillation Abnormalities in an Experimental Model of Acute Liver Failure." Neuroscience **401**: 117-129.
Cudalbu, C. and S. D. Taylor-Robinson (2019). "Brain Edema in Chronic Hepatic Encephalopathy." J Clin Exp Hepatol **9**(3): 362-382.
Cui, W., C. M. Sun and P. Liu (2013). "Alterations of blood-brain barrier and associated factors in acute liver failure." Gastroenterol Res Pract **2013**: 841707.
Dal-Pizzol, F., H. A. Rojas, E. M. dos Santos, F. Vuolo, L. Constantino, G. Feier, M. Pasquali, C. M. Comim, F. Petronilho, D. P. Gelain, J. Quevedo, J. C. Moreira and C. Ritter (2013). "Matrix metalloproteinase-2 and metalloproteinase-9 activities are associated with blood-brain barrier dysfunction in an animal model of severe sepsis." Mol Neurobiol **48**(1): 62-70.
Daneman, R. and A. Prat (2015). "The blood-brain barrier." Cold Spring Harb Perspect Biol **7**(1): a020412.
Detry, O., A. De Roover, P. Honore and M. Meurisse (2006). "Brain edema and intracranial hypertension in fulminant hepatic failure: pathophysiology and management." World J Gastroenterol **12**(46): 7405-7412.
Detry, O., Y. Gaspar, J. P. Cheramy-Bien, P. Drion, M. Meurisse and J. O. Defraigne (2013). "A modified surgical model of fulminant hepatic failure in the rat." J Surg Res **181**(1): 85-90.
Di Pardo, A., S. Castaldo, L. Capocci, E. Amico and M. Vittorio (2017). "Assessment of Blood-brain Barrier Permeability by Intravenous Infusion of FITC-labeled Albumin in a Mouse Model of Neurodegenerative Disease." J Vis Exp(129).
Dong, V., R. Nanchal and C. J. Karvellas (2020). "Pathophysiology of Acute Liver Failure." Nutr Clin Pract **35**(1): 24-29.
EMA (2012). European medicines agency. Guideline on bioanalytical method validation guideline on bioanalytical method validation. London, United Kingdom.
FDA (2018). Guidance for industry: bioanalytical method validation. U.S. Department of Health and Human Services**:** 1–44 22.
Ferenci, P., A. Lockwood, K. Mullen, R. Tarter, K. Weissenborn and A. T. Blei (2002). "Hepatic encephalopathy--definition, nomenclature, diagnosis, and quantification: final report of the working party at the 11th World Congresses of Gastroenterology, Vienna, 1998." Hepatology **35**(3): 716-721.
Fraser, C. L. and A. I. Arieff (1985). "Hepatic encephalopathy." N Engl J Med **313**(14): 865-873.
Guazzelli, P. A., G. F. Cittolin-Santos, L. A. Meira-Martins, M. Grings, Y. Nonose, G. S. Lazzarotto, D. Nogara, J. S. da Silva, F. U. Fontella, M. Wajner, G. Leipnitz, D. O. Souza and A. M. de Assis (2019). "Acute Liver Failure Induces Glial Reactivity, Oxidative Stress and Impairs Brain Energy Metabolism in Rats." Front Mol Neurosci **12**: 327.
Hadjihambi, A., N. Arias, M. Sheikh and R. Jalan (2018). "Hepatic encephalopathy: a critical current review." Hepatol Int **12**(Suppl 1): 135-147.
Helling, G., S. Wahlin, M. Smedberg, L. Pettersson, I. Tjader, A. Norberg, O. Rooyackers and J. Wernerman (2016). "Plasma Glutamine Concentrations in Liver Failure." PLoS One **11**(3): e0150440.
Holecek, M. (2015). "Ammonia and amino acid profiles in liver cirrhosis: effects of variables leading to hepatic encephalopathy." Nutrition **31**(1): 14-20.

Holmin, T., C. D. Agardh, G. Alinder, P. Herlin and B. Hultberg (1983). "The influence of total hepatectomy on cerebral energy state, ammonia-related amino acids of the brain and plasma amino acids in the rat." Eur J Clin Invest **13**(3): 215-220.
Hourani, B. T., E. M. Hamlin and T. B. Reynolds (1971). "Cerebrospinal fluid glutamine as a measure of hepatic encephalopathy." Arch Intern Med **127**(6): 1033-1036.
James, J. H., V. Ziparo, B. Jeppsson and J. E. Fischer (1979). "Hyperammonaemia, plasma aminoacid imbalance, and blood-brain aminoacid transport: a unified theory of portal-systemic encephalopathy." Lancet **2**(8146): 772-775.
Jayalakshmi, V. T. and W. Bernal (2020). "Update on the management of acute liver failure." Curr Opin Crit Care **26**(2): 163-170.
Joseph, M. A. (1986). Amino acids and sample peptides. Lim CK (ed) HPLC of Small Peptides. IRL Press, Oxford**:** pp 13-27.
Keiding, S., M. Sorensen, D. Bender, O. L. Munk, P. Ott and H. Vilstrup (2006). "Brain metabolism of 13N-ammonia during acute hepatic encephalopathy in cirrhosis measured by positron emission tomography." Hepatology **43**(1): 42-50.
Kodali, S. and B. M. McGuire (2015). "Diagnosis and Management of Hepatic Encephalopathy in Fulminant Hepatic Failure." Clin Liver Dis **19**(3): 565-576.
Larsen, F. S. and J. Wendon (2008). "Prevention and management of brain edema in patients with acute liver failure." Liver Transpl **14 Suppl 2**: S90-96.
Lee, W. M. (2012). "Acute liver failure." Semin Respir Crit Care Med **33**(1): 36-45.
Madrahimov, N., O. Dirsch, C. Broelsch and U. Dahmen (2006). "Marginal hepatectomy in the rat: from anatomy to surgery." Ann Surg **244**(1): 89-98.
Maes, M., M. Vinken and H. Jaeschke (2016). "Experimental models of hepatotoxicity related to acute liver failure." Toxicol Appl Pharmacol **290**: 86-97.
Mans, A. M., M. R. DeJoseph and R. A. Hawkins (1994). "Metabolic abnormalities and grade of encephalopathy in acute hepatic failure." J Neurochem **63**(5): 1829-1838.
Montana, V., A. Verkhratsky and V. Parpura (2014). "Pathological role for exocytotic glutamate release from astrocytes in hepatic encephalopathy." Curr Neuropharmacol **12**(4): 324-333.
Moretti, R., J. Pansiot, D. Bettati, N. Strazielle, J. F. Ghersi-Egea, G. Damante, B. Fleiss, L. Titomanlio and P. Gressens (2015). "Blood-brain barrier dysfunction in disorders of the developing brain." Front Neurosci **9**: 40.
Mouri, S., S. Tripon, M. Rudler, M. Mallet, J. Mayaux, D. Thabut and N. Weiss (2015). "FOUR score, a reliable score for assessing overt hepatic encephalopathy in cirrhotic patients." Neurocrit Care **22**(2): 251-257.
Nguyen, J. H. (2010). "Subtle BBB alterations in brain edema associated with acute liver failure." Neurochem Int **56**(2): 203-204; author reply 205-207.
Nguyen, J. H. (2012). "Blood-brain barrier in acute liver failure." Neurochem Int **60**(7): 676-683.
Nguyen, J. H., S. Yamamoto, J. Steers, D. Sevlever, W. Lin, N. Shimojima, M. Castanedes-Casey, P. Genco, T. Golde, E. Richelson, D. Dickson, M. McKinney and C. B. Eckman (2006). "Matrix metalloproteinase-9 contributes to brain extravasation and edema in fulminant hepatic failure mice." J Hepatol **44**(6): 1105-1114.
O'Grady, J. G., G. J. Alexander, K. M. Hayllar and R. Williams (1989). "Early indicators of prognosis in fulminant hepatic failure." Gastroenterology **97**(2): 439-445.
Obermeier, B., A. Verma and R. M. Ransohoff (2016). "The blood-brain barrier." Handb Clin Neurol **133**: 39-59.
Oei, L. T., J. Kuys, A. J. Lombarts, C. Goor and L. J. Endtz (1979). "Cerebrospinal fluid glutamine levels and EEG findings in patients with hepatic encephalopathy." Clin Neurol Neurosurg **81**(1): 59-63.
Record, C. O., B. Buxton, R. A. Chase, G. Curzon, I. M. Murray-Lyon and R. Williams (1976). "Plasma and brain amino acids in fulminant hepatic failure and their relationship to hepatic encephalopathy." Eur J Clin Invest **6**(5): 387-394.

Scott, T. R., V. T. Kronsten, R. D. Hughes and D. L. Shawcross (2013). "Pathophysiology of cerebral oedema in acute liver failure." World J Gastroenterol **19**(48): 9240-9255.
Seetharam, A. (2019). "Intensive Care Management of Acute Liver Failure: Considerations While Awaiting Liver Transplantation." J Clin Transl Hepatol **7**(4): 384-391.
Shaik, I. H., M. K. Miah, U. Bickel and R. Mehvar (2013). "Effects of short-term portacaval anastomosis on the peripheral and brain disposition of the blood-brain barrier permeability marker sodium fluorescein in rats." Brain Res **1531**: 84-93.
Skowronska, M. and J. Albrecht (2012). "Alterations of blood brain barrier function in hyperammonemia: an overview." Neurotox Res **21**(2): 236-244.
Smith, A. R., F. Rossi-Fanelli, V. Ziparo, J. H. James, B. A. Perelle and J. E. Fischer (1978). "Alterations in plasma and CSF amino acids, amines and metabolites in hepatic coma." Ann Surg **187**(3): 343-350.
Squires, J. E., P. McKiernan and R. H. Squires (2018). "Acute Liver Failure: An Update." Clin Liver Dis **22**(4): 773-805.
Swain, M., R. F. Butterworth and A. T. Blei (1992). "Ammonia and related amino acids in the pathogenesis of brain edema in acute ischemic liver failure in rats." Hepatology **15**(3): 449-453.
Therrien, G. and R. F. Butterworth (1991). "Cerebrospinal fluid amino acids in relation to neurological status in experimental portal-systemic encephalopathy." Metab Brain Dis **6**(2): 65-74.
Tumani, H., A. Huss and F. Bachhuber (2017). "The cerebrospinal fluid and barriers - anatomic and physiologic considerations." Handb Clin Neurol **146**: 21-32.
Vergara, F., F. Plum and T. E. Duffy (1974). "Alpha-ketoglutaramate: increased concentrations in the cerebrospinal fluid of patients in hepatic coma." Science **183**(4120): 81-83.
Warrillow, S., C. Fisher, H. Tibballs, M. Bailey, C. McArthur, P. Lawson-Smith, B. Prasad, M. Anstey, B. Venkatesh, G. Dashwood, J. Walsham, A. Holt, U. Wiersema, D. Gattas, M. Zoeller, M. Garcia Alvarez and R. Bellomo (2019). "Coagulation abnormalities, bleeding, thrombosis, and management of patients with acute liver failure in Australia and New Zealand." J Gastroenterol Hepatol.
Whitehead, T. P. and S. R. Whittaker (1955). "A method for the determination of glutamine in cerebrospinal fluid and the results in hepatic coma." J Clin Pathol **8**(1): 81-84.
Wright, G., Y. Sharifi and R. Jalan (2010). "Blood-brain barrier in liver failure: are cracks appearing in the wall?" Liver Int **30**(8): 1087-1090.
Yao, L., X. Xue, P. Yu, Y. Ni and F. Chen (2018). "Evans Blue Dye: A Revisit of Its Applications in Biomedicine." Contrast Media Mol Imaging **2018**: 7628037.
Zaman, M. B., E. Hoti, A. Qasim, D. Maguire, P. A. McCormick, J. E. Hegarty, J. G. Geoghegan and O. Traynor (2006). "MELD score as a prognostic model for listing acute liver failure patients for liver transplantation." Transplant Proc **38**(7): 2097-2098.
Zanchin, G., P. Rigotti, F. Bettineschi, P. Vassanelli and L. Battistin (1979). "Cerebral amino acid levels and uptake in rats after portocaval anastomosis: I. Regional studies in vitro." J Neurosci Res **4**(4): 291-299.
Zanchin, G., P. Rigotti, N. Dussini, P. Vassanelli and L. Battistin (1979). "Cerebral amino acid levels and uptake in rats after portocaval anastomosis: II. Regional studies in vivo." J Neurosci Res **4**(4): 301-310.

Figure legends

Figure 1: Neurological scale. (A) represents the criteria utilized for neurological classification of animals with hepatic encephalopathy. **(B)** represents the mean onset of each neurological stage after surgery. **(C)** represents the mean neurological grade over time after surgery – animals that

died before 72 hours were recorded and included until the last evaluation while still alive**.** Data is expressed as mean ± SD (hours). Experimental n = 39.

Figure 2: Albumin in CSF. (A) represents albumin concentration in CSF, measured at 24 h after surgery. The hepatectomy group is divided according to the neurological stage at the time of sample harvesting. **S**ham 1.14 ± 0.29 (n=9); Grade I 1.87 ± 0.26 (n=6); Grade II 2.41 ± 0.43 (n=5); Grade III 2.81 ± 0.03 (n=3). No animal of this cohort reached stage IV at the 24-hour time mark. **(C)** Representative images of the animals' brain 90 minutes after IV injection of Evans Blue. * represents difference from all the other groups ($p<0.001$) and # represents difference between stage I and III ($p<0.05$); ANOVA.

Figure 3: CSF amino acids levels. The table expresses the CSF concentration of each analyzed amino acid, measured at 24 hours after surgery. Data is expressed as mean ± SD (µM). Experimental n=14 (Sham) and n=19 (Hepatectomy). * and ** represent difference from the Sham group ($p<0.05$) and ($p<0.001$), respectively; Student's t test.

Figure 4: correlation between CSF amino acids and neurological grading. Data is expressed as mean ± SD (µM). Values are expressed as Sham (n=14), Grade I (n=6), Grade II (n=6), Grade III (n=4) and Grade IV (n=3). * represents difference from the other groups ($p<0.05$); ANOVA.

Figure 5: CSF amino acids as mortality predictors. Data represents CSF levels of amino acids, measured at 12 hours after subtotal hepatectomy of the following groups: Sham (n=14), Recovered (animals that survived during the 72 hours analysis after surgery) (n=8) and Deceased (animals that died from 12 hours to 72 hours after surgery) (n=9). Data is expressed as mean ± SD (µM). * represents difference from the other groups ($p<0.05$); ANOVA.

Figure 1

A

Neurological Grades	Modified Hepatic Encephalopathy Neurological Scale for rats
Ø	Freely moving and exploring the home cage. No reflex alterations.
I	No spontaneous exploratory activity. Righting reflex present. Researcher is not able to place the animal on it's back. Corneal reflex preserved.
II	No spontaneous exploratory activity. Researcher is able to place the animal on it's back, but the animal is able to return to normal position within 3 seconds. Corneal reflex preserved.
III	No spontaneous exploratory activity. Loss of righting reflex - animal is not able to return to normal position. Corneal reflex preserved.
IV	No spontaneous exploratory activity or locomotion. Loss of reflexes and no response to pain stimuli.

B

Mean onset (hours after surgery)	
Grade I	8.66 ± 5.43
Grade II	16.50 ± 7.56
Grade III	29.65 ± 13.66
Grade IV	33.60 ± 9.01
Death	36.00 ± 12.73

C

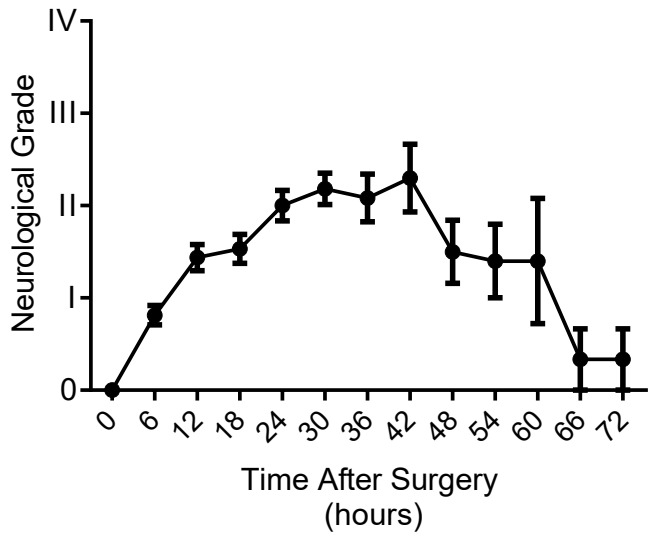


Figure 2
63

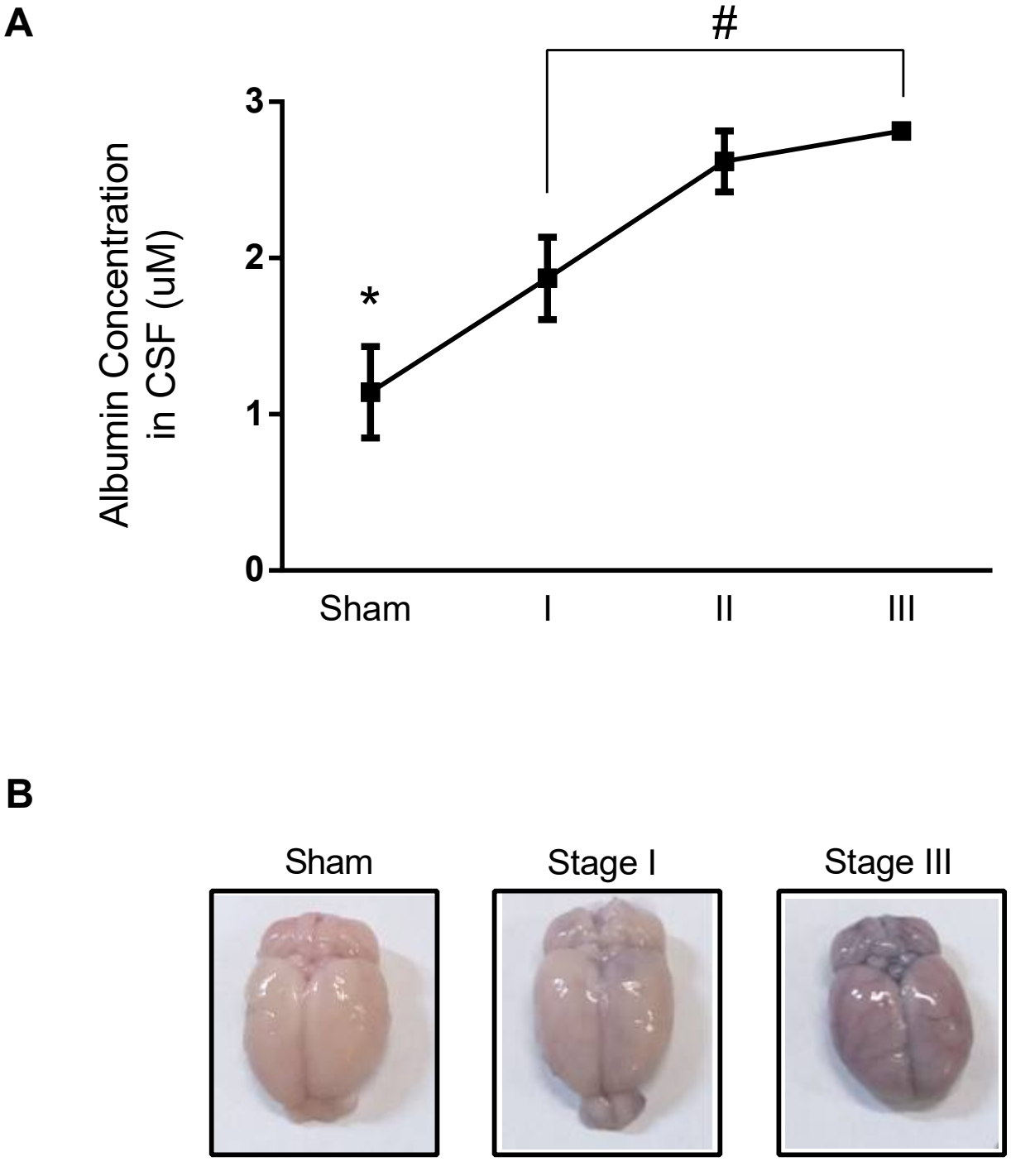
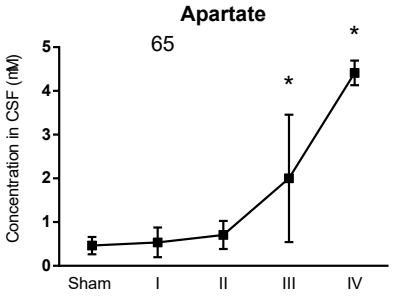
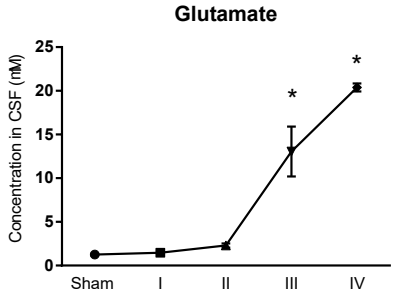

A
#
3
2
1
0
*
Albumin Concentration in CSF (uM)
Sham
I
II
III
B
Sham
Stage I
Stage III

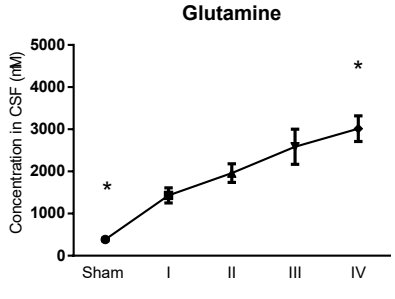
Figure 3

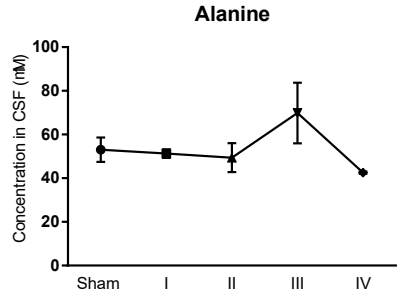
64

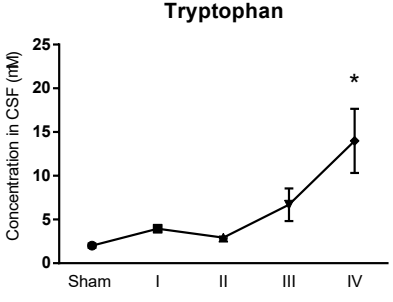
Amino Acid	Sham (μM)	Hepatectomy (μM)	
Aspartate	0.46 ± 0.05	1.30 ± 0.34	*
Glutamate	1.30 ± 0.11	5.61 ± 1.61	*
Glutamine	387.50 ± 29.65	2008.00 ± 178.06	**
Alanine	53.08 ± 5.62	54.73 ± 3.87	
Tryptophan	2.02 ± 0.25	5.81 ± 1.14	*
Methionine	3.81 ± 0.43	26.16 ± 4.43	*
Valine	3.78 ± 0.49	5.75 ± 0.93	*
Phenylalanine	4.46 ± 0.30	37.17 ± 7.24	*
Isoleucine	1.80 ± 0.17	4.92 ± 0.86	*
Leucine	3.57 ± 0.27	7.76 ± 1.55	*
Ornithine	2.31 ± 0.28	5.04 ± 1.14	*
Lysine	68.22 ± 5.33	242.00 ± 45.89	*

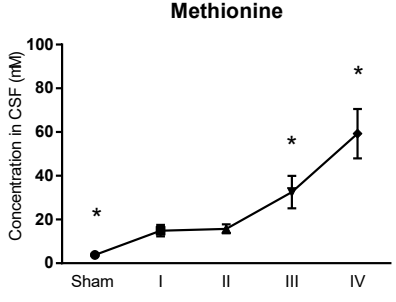

Apartate
Concentration in CSF (nM)
0
1
2
3
4
5
Sham
I
II
III
IV
*
*

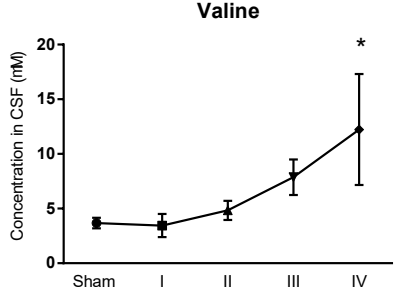

Glutamate
Concentration in CSF (nM)
0
5
10
15
20
25
Sham
I
II
III
IV
*
*

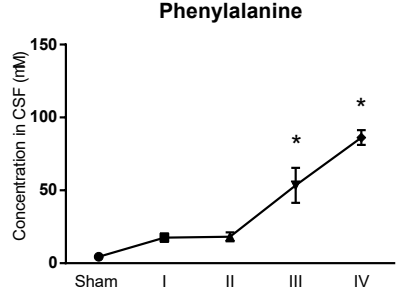

Glutamine
Concentration in CSF (nM)
0
1000
2000
3000
4000
5000
Sham
I
II
III
IV
*
*

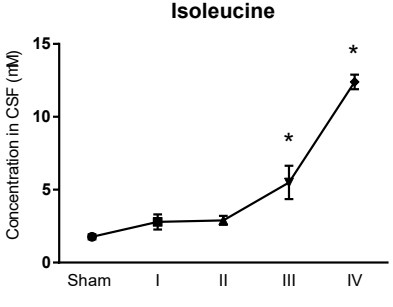

Alanine
Concentration in CSF (nM)
0
20
40
60
80
100
Sham
I
II
III
IV

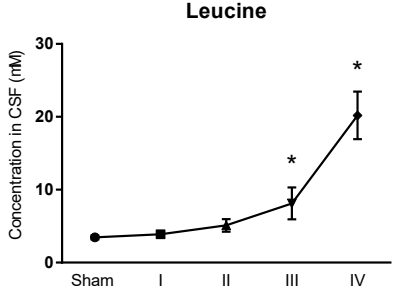

Tryptophan
Concentration in CSF (nM)
0
5
10
15
20
25
Sham
I
II
III
IV
*

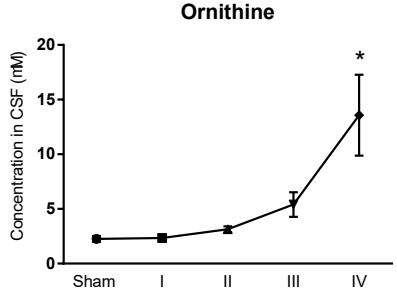

Methionine
Concentration in CSF (nM)
0
20
40
60
80
100
Sham
I
II
III
IV
*
*
*


Valine
Concentration in CSF (nM)
0
5
10
15
20
Sham
I
II
III
IV
*


Phenylalanine
Concentration in CSF (nM)
0
50
100
150
Sham
I
II
III
IV
*
*


Isoleucine
Concentration in CSF (nM)
0
5
10
15
Sham
I
II
III
IV
*
*


Leucine
Concentration in CSF (nM)
0
10
20
30
Sham
I
II
III
IV
*
*


Ornithine
Concentration in CSF (nM)
0
5
10
15
20
Sham
I
II
III
IV
*

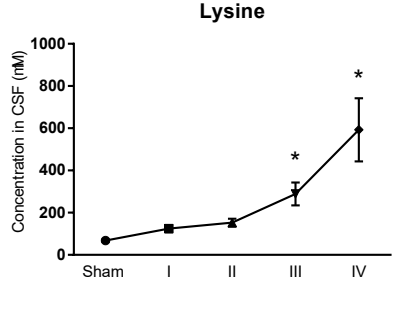
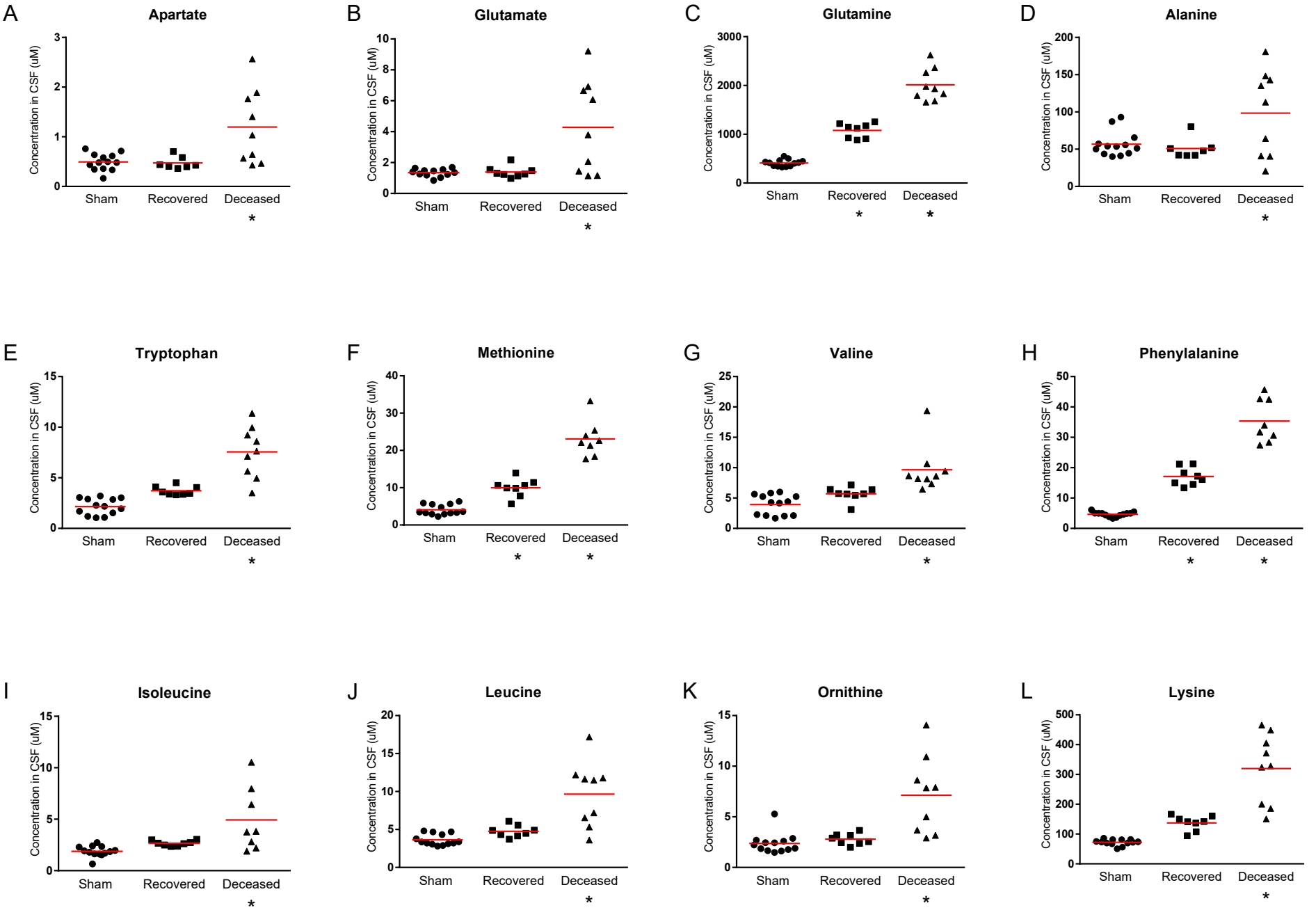

Lysine
Concentration in CSF (nM)
0
200
400
600
800
1000
Sham
I
II
III
IV
*
*

Figure 5


A
Apartate
Concentration in CSF (uM)
Sham
Recovered
Deceased
*
B
Glutamate
Concentration in CSF (uM)
Sham
Recovered
Deceased
*
C
Glutamine
Concentration in CSF (uM)
Sham
Recovered
Deceased
*
*
D
Alanine
Concentration in CSF (uM)
Sham
Recovered
Deceased
*
E
Tryptophan
Concentration in CSF (uM)
Sham
Recovered
Deceased
*
F
Methionine
Concentration in CSF (uM)
Sham
Recovered
Deceased
*
*
G
Valine
Concentration in CSF (uM)
Sham
Recovered
Deceased
*
H
Phenylalanine
Concentration in CSF (uM)
Sham
Recovered
Deceased
*
*
I
Isoleucine
Concentration in CSF (uM)
Sham
Recovered
Deceased
*
J
Leucine
Concentration in CSF (uM)
Sham
Recovered
Deceased
*
K
Ornithine
Concentration in CSF (uM)
Sham
Recovered
Deceased
*
L
Lysine
Concentration in CSF (uM)
Sham
Recovered
Deceased
*

Capítulo III

Guanosine administration reduces mortality and improves neurological and biochemical parameters of rats with hepatic encephalopathy induced by acute liver failure

Guazzelli PA, Fachim FS, Travassos AS, Nonose Y, Rocha A, Rohden F, Fontella FU, de Assis AM, Souza DO

Manuscrito em preparação para publicação

Guanosine administration reduces mortality and improves neurological and biochemical parameters of rats with hepatic encephalopathy induced by acute liver failure

Guazzelli PA[12], Fachim FS[2], Travassos AS[2], Nonose Y[12], Rocha A[12], Rohden F[12] Fontella FU[12,] de Assis AM[3], Souza DO[12]

[1] Post-graduate Program in Biological Sciences: Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul – UFRGS, Porto Alegre, RS 90035-003, Brazil;

[2] Department of Biochemistry, Universidade Federal do Rio Grande do Sul - UFRGS, Porto Alegre, RS 90035-003, Brazil;

[3] Postgraduate Program in Health and Behavior, Center of Health Science, Universidade Católica de Pelotas - UCPel, Pelotas, RS 96015-560, Brazil.

*Corresponding author:
Diogo Onofre Gomes de Souza, Ph.D.
Department of Biochemistry, Postgraduate Program in Biochemistry
ICBS, Universidade Federal do Rio Grande do Sul, UFRGS
Ramiro Barcelos St 2600 Annex Building, 90035-003, Porto Alegre, RS, Brazil.
Tel.: +55 51 3308 5559
Fax: +55 51 3308 5540
E-mail: diogo.bioq@gmail.com

Abstract

Introduction: Acute liver failure (ALF) is a medical condition that occurs in patients with rapid and extensive loss of hepatic function, presenting high mortality rates. It is characterized by the reduction of the liver's metabolic capacity and the development of Hepatic Encephalopathy (HE). HE consists of a spectrum of neurological symptoms caused by the accumulation of toxins in the blood and brains of patients with liver disease. HE's physiopathology is complex and multifactorial, but brain ammonia accumulation and glutamatergic excitotoxicity seem to play major roles in the development of the disease. The modulation of the glutamatergic system is being successfully used in animal models to treat this condition, including the use of the nucleoside Guanosine (GUO). This manuscript aims to study the potential neuroprotective effects of GUO in a rat model of ALF induced by hepatectomy.

Methods: To induce HE, our group used an animal model of ALF induced by subtotal hepatectomy (removal of 92% of the hepatic mass). Animals were treated with either GUO administration via intraperitoneal (i.p.) injection or saline solution during a 60-hour period. Neurological status, behavioral performance and overall mortality were observed for 72 hours. In a second cohort of animals, samples of cerebrospinal fluid (CSF) and cerebral cortex were harvested 24 hours after surgery for biochemical assays.

Results: animals with ALF presented reduced survival (11%), impaired neurological function and diminished exploratory activity on the Open Field Task (OFT). The administration of GUO was able to increase survival to 39%, to improve neurological function and improve behavioral parameters. The treatment with GUO also reduced astrocytic reactivity, evidenced by a morphological evaluation of astrocytes from cerebral cortexes stained for GFAP. Moreover, GUO improved biochemical parameters, such as the normalization of albumin levels in the CSF and increase in glutamate uptake by cerebral cortex slices.

Discussion: ALF is a rare but severe disease that involves acute ammonia rise and glutamatergic excitotoxicity, leading to neurological impairment, coma and even death. Subtotal hepatectomy in rats induced a high mortality rate, marked neurological deterioration and significant alterations in behavioral parameters, astrocytic morphology and biochemical parameters in the brain and CSF. The administration of GUO improved most of the evaluated biochemical and morphological parameters and presented a clear benefit by reducing mortality and improving the neurological function of treated animals. The results of this study bring new evidence that strengthen the potential neuroprotective effects of GUO under HE induced by ALF.

Introduction

Acute liver failure (ALF) is a clinical condition characterized by a marked loss of hepatic function in patients with previously healthy livers within days or weeks [1, 2]. The characteristic features of this syndrome include jaundice, coagulopathy, hepatic encephalopathy (HE) and, in severe cases, multi-organ failure [2]. ALF has a wide spectrum of etiologies including medication toxicity, viral infections, liver ischemia and autoimmune diseases [3, 4]. Most cases in Western countries result from acetaminophen overdose [3, 4], while viral infections account for a greater number of cases in underdeveloped countries, especially in regions where hepatitis E virus is endemic [5, 6]. Mortality rates of ALF have progressively declined over the last decades due to improved clinical and surgical management of these patients [7]. Nonetheless, this syndrome still represents a life-threatening condition as patients are at risk of developing cerebral edema, infections, excessive bleeding and multi-organ failure [8, 9].

The neurological impairment caused by liver failure, known as hepatic encephalopathy (HE), is associated with cerebral edema and brain stem herniation, representing the major cause of death in patients with ALF [10-12]. Early stages of HE may be identified by mild neurological or psychiatric symptoms such as mood changes, disorientation, sleep disorders and extremity tremor, but the disease may progress to severe alterations such as agitation, delirium, and, in critical stages, coma or death [1, 13]. While patients with mild HE (grades I and II) present good chances of spontaneous recovery (around 70%), those that reach grades III and IV of the disease present high lethality rates [12, 14, 15] and require aggressive treatment strategies such as liver transplantation [16, 17].

The physiopathology of HE has been studied for decades, demonstrating complex and multifactorial mechanisms that lead to neurological dysfunction [18, 19]. In this context, ammonia accumulation seems to play a major role in the development of HE [18, 20, 21]. Indeed, previous studies have shown that the arterial concentration of this molecule presents a positive correlation with intracranial hypertension in humans with decreased liver function [22, 23]. This effect is probably enhanced by the concomitant increase of other toxins under hepatic failure, as the sole administration of ammonia in healthy subjects causes only fatigue and no severe neurological alteration [24, 25]. Moreover, ammonia accumulation may serve as the trigger for other pathological

processes that have been associated with the development of HE such as glutamatergic excitotoxicity, neurotransmitter disorders, brain energy metabolism failure, oxidative stress, neuroinflammation and altered blood-brain barrier (BBB) permeability [19, 26].

Hyperammonemia seems to be particularly harmful to astrocytes, and HE is considered a gliopathy by some authors [27-29]. A typical altered astrocyte morphology, identified as Alzheimer type II astrocytes, have been described and studied in patients with liver disease for over a century [28-31]. This altered astrocyte morphology presents high sensitivity and specificity for chronic hepatic encephalopathy, although it may also be found in other brain disorders [32]. Nonetheless, data regarding astrocyte morphology under ALF is limited. In recent years, Belanger and colleagues described a reduction in glial fibrillary acid protein (GFAP) in animals with portocaval shunt followed by hepatic artery ligation [33]. On the other hand, our group described marked astrocytic reactivity with increased GFAP optical density due to augmented astrocytic volume and proliferation of cellular processes in the cerebral cortexes of animals with ALF induced by subtotal hepatectomy [34].

Regarding cellular function, astrocytes are responsible, among many other functions, for removing glutamate from the synaptic cleft, avoiding glutamatergic excitotoxicity, and are the only brain cells that express the enzyme glutamine synthetase (GS), a key factor for detoxification through the removal of excessive ammonia and glutamate using glutamine production [35]. Hyperammonemia and HE have been associated with a reduction in glutamate uptake by astrocytes [36, 37] as well as a reduction in the expression of membrane glutamate transporters GLT-1 and GLAST in these cells [38-41]. Evidence regarding the effects of ammonia on GS activity has also been described by our group and others [42, 43]. These data indicate a pathological interference of toxins in astrocytic activity and ammonia detoxification under HE, which compromises glial capacity to regulate the glutamatergic system's homeostasis.

Considering the above stated, the modulation of the glutamatergic system emerges as a potential therapeutic option for hyperammonemia and HE. Indeed, the inhibition of NMDA sub-type glutamatergic receptor levels/activity and glutamine synthetase, have independently been shown to reduce the mortality of animals with ALF [44-46]. Accordingly, our group has demonstrated that guanosine (GUO) was able to reduce mortality and improve several biochemical parameters in animals with acute ammonia intoxication [42]. Moreover, our group had also previously shown that GUO administration improved the behavioral performance and biochemical parameters of

animals with chronic HE induced by bile-duct ligation [47]. Guanosine is an endogenous purine nucleoside which modulates the glutamatergic system and has consistently presented neuroprotective effects in several animal models of neurological conditions related to glutamatergic excitotoxicity [48-53].

Therefore, this study aims to evaluate the potential neuroprotective effects of GUO in experimental HE induced by subtotal hepatectomy (resection of 92% of the liver mass). This is a reproducible animal model of ALF that induces hyperammonemia, neurological impairment and death by intracranial hypertension and brain herniation within one to three days after surgery [54-56]. Recently our group has successfully utilized this animal model for studying the pathophysiology of HE [34, 39, 57]. In this paper we aim to evaluate the effects of GUO administration in animals with ALF and provide data regarding: i) mortality rate; ii) neurological evaluation; iii) behavioral performance; iv) astrocytic morphology; v) glutamate uptake in the cerebral cortex; vi) cerebrospinal fluid (CSF) albumin levels.

Methods

Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glutamic acid, L-[^{14}C(U)] (#NEC290E250UC) and Optiphase 'Hisafe' 3 scintillation liquid was purchased from PerkinElmer (Boston, MA, USA). Protein quantification was performed with BCA Protein Assay kit from Thermo Fisher Scientific (#23227, Rockford, IL, USA), using bovine serum albumin as standard.

Animals

Experiments were performed on 90-day-old male Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. The animals were maintained in a colony room with 12 hours light/dark cycles (lights on 07:00–19:00) and with air-conditioned constant temperature (22 ± 1 °C). Animals had free access to water and standard commercial chow (SUPRA, Porto Alegre, RS, Brazil).

Surgical procedure

Subtotal hepatectomy was performed as described in previous studies of our group [34, 39, 57]. This animal model of ALF consists in the removal of 92% of the liver mass, preserving only the omental lobes functional (8% of liver mass), which is a small

modification of the surgical procedure described by Detry, et al, 2013 [55, 58]. Anaesthesia was induced and maintained with 3% isoflurane in oxygen at a flow rate of 1 L/min during the whole procedure. After cleaning the abdominal wall with povidone-iodine, a median laparotomy was performed, and liver ligaments and adhesions were cut. The pedicle of the left anterior lobe was ligated and resected followed by right anterior lobe resection and then by the resection of both right lobes. After ligation of the lobe hilum, the ischemic mass was removed from the abdominal cavity.

Lidocaine (0.8 ml) was administered intramuscularly in the wound borders after suturing the abdominal wall to reduce pain. The rats were then kept in a heated box for 30 minutes before being returned to their home cages. Animals had free access to drinking water supplemented with 20% glucose during the whole experiment. Additionally, 2 ml/kg of the same glucose solution was administered by intraperitoneal (i.p.) injections at 3 different times: 0, 6 and 12 hours after surgery. Sham operations were performed with the exact same protocol, except pedicle ligation and partial liver resection. The rats were observed every 6 hours over a 72-hour period to evaluate the progression of HE.

This surgical model of ALF has been recently utilized by our group, and data regarding blood and CSF biomarkers of liver dysfunction, brain energy metabolism as well as electroencephalographic, neurological and behaviour alterations can be found in Cittolin-Santos, et al, 2019 [39], Guazzelli, 2019 [34] and Guazzelli, 2020 [57].

After subtotal hepatectomy or sham operation, animals were divided into 2 different cohorts for the development of this study. The 1st cohort was utilized for neurological assessment and open field task performance. The 2nd cohort was euthanized at 24 hours after surgery for harvesting CSF and brain tissue samples to perform the immunohistochemical and biochemical assays.

Guanosine Administration

Guanosine was diluted to a concentration of 7.5 mg/ml in saline (NaCl 0.9%). A group of animals received i.p. injections of Guanosine 1 ml/kg of body weight in the following time marks: immediately after surgery, 3, 6, 12, 24, 36, 48 and 60 hours after surgery. Each injection of guanosine consisted of 7.5mg/kg of body weight. Another group received saline injections 1 ml/kg at the same time marks as the control group.

For the following presentation of results and the discussion of the data, this manuscript will address the experimental groups as **Naïve** (no intervention), **Sham + Saline** (sham operated animals that received saline), **Sham + GUO** (sham operated animals that received guanosine), **Hep. + Saline** (hepatectomized animals that received

saline) and **Hep. + GUO** (hepatectomized animals that received guanosine). A visual representation of the administration protocol is provided in Figure 1.

Neurological evaluation

Neurological evaluation was performed every 6 hours after the surgical procedure, in accordance to Guazzelli, 2020 [57]. The analysis was started by removing the cage grid (time 0). If animals presented any spontaneous locomotion or exploratory activity (e.g. interact with other animals, food, or cage walls) within the first 10 seconds they were considered as Grade Ø. If the animals were immobile for the first 10 seconds, they were then evaluated for both righting and corneal reflexes to assess neurological impairment. Righting reflex was assessed by lifting and positioning the animal on its back on the cage floor. Returning to upright position before reaching the ground or immediately after released by a researcher was considered as normal response. A delayed response or incapability to return to the upright position was considered as an altered response. Corneal reflex was evaluated by softly poking the cornea with a cotton swab, which must trigger a response of eyelid closure in normal animals.

Animals that did not move within the first 10 seconds but presented normal reflexes were considered to be Grade I. Animals that did not show spontaneous locomotion, and allowed to be positioned on their backs, but quickly (<3 seconds) returned to normal position were considered to be Grade II. Grade III consisted of animals with significant locomotor deficit, presenting no spontaneous locomotion or exploratory activity and that were unable to return to the upright position when placed on their backs. Grade I, II and III animals still presented normal corneal reflex. Animals in Grade IV were comatose and, therefore, did not present righting reflex, corneal reflex. Death time was registered as the last time-mark that the animal was seen alive.

All sham operated animals remained in stage Ø during the whole experiment.

Open Field task

The locomotor activity of the animals was evaluated as previously performed by our group [39, 59]. In each session, animals were individually placed in a black square arena (50 × 50 × 50 cm, length × width × height), where they could freely explore for 10 minutes. Each animal was submitted to 3 Open Field Task (OFT) sessions. The 1st session was performed immediately before the surgery to record the baseline activity of the animals (naïve group) and assure the homogeneity in the behavioral and locomotor activity presented by the animals before the surgical intervention. The 2nd session was performed 24 hours after the surgery and the 3rd session was performed 15 days after the

surgical procedure (animals that survived). Locomotor activity was measured by evaluating the total travelled distance and the time spent immobile during each session. Immobility was only considered after the animal remained 5 seconds immobile.

Between each task, the arena was cleaned with 70% alcohol. All sessions were recorded by a video camera positioned above the arena and the recordings were analyzed using ANY-Maze® dedicated software as a tracking system. Unfortunately, no animal of the Hep. + Saline group survived the 72-hour period; thus, the analysis of this group could not be performed at the 15 days task.

Immunohistochemistry and astrocyte morphological analysis

Immunohistochemistry for Glial Fibrillary Acidic Protein (GFAP) positive astrocyte was performed to evaluate cellular morphology. At 24 hours after surgery, animals were briefly anesthetized with 3% isoflurane and euthanized by decapitation. The brains were then harvested and fixed by immersion for 24 hours in 4% PFA diluted in phosphate buffer saline (PBS, pH 7.4), cryoprotected through immersion in sucrose solution (gradually, 15% to 30% until sank), and then frozen at -20ºC. Later, coronal brain slices of 30 µm, approximately ranging +2.20 mm rostrally from bregma were obtained using a cryostat (MEV, SLEE Medical GMBH, Mainz, Germany). The slices were incubated for 24h at 4ºC with polyclonal rabbit anti-GFAP (Z0334, 1:500 in PBS-Tx, Dako, Glostrup, Denmark), followed by 2 hours incubation with goat anti-rabbit AlexaFluor® 555 secondary antibody (1:1000 in PBS-Tx, Invitrogen, Carlsbad, CA, USA). Images were obtained in Olympus FV1000 confocal microscopy and acquired at 8-bit gray-scale (256 gray levels). All morphological analyses were performed at the center of microscopy and microanalysis of the Universidade Federal do Rio Grande do Sul (CMM-UFRGS) using the ImageJ software (NIH, Bethesda/USA).

Albumin levels in the CSF

CSF samples were collected 24 hours after surgery to evaluate albumin levels. CSF harvesting was performed according to previous reports from our group [34, 39, 42]. Animals were briefly anesthetized with 3% isoflurane and positioned in a stereotaxic frame. Samples of approximately 50 µl (29G). CSF was immediately centrifuged at 10,000g for 10 minutes and the supernatant was stored at -80ºC for posterior analysis.

Albumin measurement was performed with a high performance liquid chromatography coupled with fluorescence detector (HPLC-FLD) consisting of an LC Shimadzu system (Kyoto, Japan) equipped with a LC- 20AT pump, a DGU-14A degasser, thermostat set for CTO-10A column and fluorescence detector, RF 20A. LC

Solution software was used in data acquisition and processing. The FLD was set at 278 nm (excitation) and 335 nm (emission). Agilent reversed-phase ZORBAX SB-C18 column (5 µm particle size, 250 × 4.6 mm i.d.) was used. The method was performed using gradient condition, consisting of solvent A (H_2O + 0.1% formic acid) and solvent B (acetonitrile (ACN)) as follows: A → 65% B → 35% (0–5.0 min), A → 70% B → 30% (5.0–10 min), A → 65% B → 35% (10.0–17.0 min). The flow rate was set at 0.7 mL/min. Albumin stock solutions were prepared in water at 1 mg/mL and stored at − 20 ± 2 °C. For each day of analysis, standard solutions of albumin were prepared at 0.1, 0.5, 1, 10, 50 and 100 µg/ml. Sample preparation was performed by adding 10 µl of CSF to the 40 µl of ACN and vortex mixed. Then, the solution was transferred to conical vials and 10 µl were injected to the HPLC. Procedures followed FDA and EMA guidelines and recommendations [60, 61] .

Glutamate uptake by cerebral cortex slices

Glutamate uptake by cerebral cortex slices was performed according to previous studies of our group [39, 42, 62]. 24 hours after surgery, the animals were briefly anesthetized with 3% isoflurane and euthanized by decapitation. The cerebral cortex was then immediately dissected on ice (4°C) and brain slices (0.2 mm thickness) of the frontoparietal cortex were rapidly obtained using a McIlwain Tissue Chopper. Samples were immersed in 4°C HBSS with the following composition (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. The slices were incubated with HBSS at 37°C for 15 min, followed by the addition of 0.33 µCi/mL of L-[3H] glutamate. Incubation was stopped after 7 minutes with 2 ice-cold washes using 1 ml of HBSS. After washing, the slices received 0.5 N NaOH and were then stored overnight. The incorporated radioactivity was measured using a liquid scintillation counter (Hidex 300 SL).

The Na^+-independent glutamate uptake was measured using a similar protocol as described above, but with changes in the incubation temperature (4ºC) and in the medium composition (N-methyl-D-glucamine instead of NaCl). Na^+-dependent uptake of glutamate was considered as the difference between the total uptake and the Na^+-independent uptake.

Results

Survival rate

Figure 2. As expected, subtotal hepatectomy presented a diminished survival rate of 11% (Figure 2). Nonetheless, GUO administration was able to improve the survival approximately 4-fold (39%). All deaths occurred between 18 and 66 hours after the surgery and the mean latency to death was 36 hours in the Hep. + Saline group and 38 hours in the Hep. + GUO Group, presenting no statistical difference.

Noteworthy, rats that died within the first 6 hours after surgery were excluded from the study because they most likely died from complications of the surgical procedure (e.g., excessive bleeding). Furthermore, no sham operated rats died during the experiment and all animals that endured the initial 72-hour period survived the next 6 months of follow-up (data not shown).

Neurological evaluation

Figure 3A summarizes the criteria utilized for classification of neurological impairment induced by ALF, as in Guazzelli, 2020 [57]. The mean neurological grade of each group over time is expressed in Figure 3B. Hepatectomized animals presented a deterioration of neurological function from around 6 hours after surgery that progressed up to 36 hours, when the mean neurological grading started to improve. As shown in Figure 3B, the administration of GUO markedly reduced the mean neurological grade, implying an improvement in overall neurological function, especially at the 12 and 24-hour time marks. The observations were performed up to the 72-hour time mark, when the experiments were concluded.

Behavioral analysis

Figure 4. Behavioral analysis performed 24 hours after the surgical procedure utilizing the OFT showed that animals submitted to subtotal hepatectomy presented marked decrease in the total travelled distance and marked increase in the time spent immobile (Figure 4A and 4B). Guanosine administration slightly diminished the effect on travelled distance (Figure 4A), with no effect on the immobility time (Figure 4B).

A second session of OFT was performed 15 days after the surgical procedure to evaluate long-term impact of hepatectomy. Animals from the Hep. + GUO group that survived the critical initial period of ALF partially recovered the locomotor activity, increasing the travelled distance and decreasing the time spent immobile, compared to the 24-hour session. As no animal of Hep. + Saline group survived the 72-hour period, we cannot say that the partial recuperation was due spontaneous recuperation or the GUO treatment. Values of each group (mean ± SD) are expressed below:

Total travelled distance at 24 hours after surgery (meters): **Naïve** 20.88 ± 8.33; **Sham + Saline** 19.46 ± 5.90; **Saline + GUO** 17.51± 3.60; **Hep. + Saline** 1.40 ± 1.99; **Hep. + GUO** 4.08 ± 2.79.

Time spent immobile at 24 hours after surgery (seconds): **Naïve** 153.3 ± 110.0; **Sham + Saline** 174.5 ± 90.0; **Saline + GUO** 195.7 ± 79.9; **Hep. + Saline** 541.3 ± 71.4; **Hep. + GUO** 488.4 ± 80.3.

Total travelled distance at 15 days after surgery (meters): **Naïve** 20.88 ± 8.33; **Sham + Saline** 21.59 ± 6.33; **Saline + GUO** 18.87± 6.14; **Hep. + Saline** Ø; **Hep. + GUO** 11.05 ± 5.47.

Time spent immobile at 15 days after surgery (seconds): **Naïve** 153.3 ± 110.0; **Sham + Saline** 173.5 ± 60.9; **Saline + GUO** 155.2 ± 67.3; **Hep. + Saline** Ø; **Hep. + GUO** 207.8 ± 102.1.

Astrocytic reactivity

Figure 5. Confocal images of frontoparietal cortex cells stained with GFAP showed that animals with acute liver failure presented alterations in astrocytes morphology when compared to control groups. Indeed, the Hep. + Saline group presented an increase in the number of both primary (Figure 5A) and secondary (Figure 5B) processes when compared to control groups, characterizing glial reactivity. This phenomenon was reversed with GUO treatment.

Biochemical analysis

Figure 6. Subtotal hepatectomy induced a marked increase in CSF levels of albumin that was reversed by guanosine treatment (Figure 6A). Data is expressed as mean ± SD (μg): **Sham + Saline** 1.08 ± 0.41; **Saline + GUO** 1.27 ± 0.39; **Hep. + Saline** 2.29 ± 0.46; **Hep. + GUO** 0.98 ± 0.47.

Guanosine administration increased glutamate uptake in cerebral slices of hepatectomized animals when compared to Hep. + Saline (Figure 6B). Data is expressed as mean ± SD (pmol/mg/min): **Sham + Saline** 1.43 ± 0.34; **Saline + GUO** 1.15 ± 0.56; **Hep. + Saline** 0.88 ± 0.41; and **Hep. + GUO** 1.79 ± 0.71.

Discussion

Acute liver failure (ALF) is a life-threatening medical condition that progresses with rapid and extensive loss of hepatic function and imposes high morbidity and

mortality rates [63, 64]. By definition, patients with ALF always present a decline in neurological function, which constitutes a syndrome know as hepatic encephalopathy (HE). Despite the advances in the management of patients with ALF over the last few decades, current treatment for ALF and HE consists of clinical support, strategies for reducing patients' ammonia absorption and, in most severe cases, liver transplantation [7, 65, 66]. To date there are no approved therapeutic strategies that directly affect neurological or glial function in order to avoid HE's progression and prevent critical medical conditions such as brain edema and intracranial hypertension [63, 66].

Astrocyte disfunction and glutamatergic excitotoxicity play important roles in the development of HE [27, 39, 42, 67]. For this reason, the modulation of the glutamatergic system is being studied in animal models of ALF and hyperammonemia in the search for brain-focused strategies for treating HE [42, 44-47, 68]. Guanosine (GUO) is a purine nucleoside that has been shown to present neuroprotective effects in different brain pathologies [53, 69]. Our group has demonstrated that GUO administration was able to improve biochemical and behavioral parameters in rats with chronic HE as well as reduce mortality and improve biochemical parameters in rats with acute hyperammonemia [42, 47]. Thus, this study was designed to test the potential neuroprotective effects of GUO in an animal model of HE induced by subtotal hepatectomy. This paper shows that guanosine administration in rats with ALF was able to: i) reduce the mortality rate; ii) improve neurological status; iii) improve locomotor activity in the Open Field Task; iv) prevent morphological alterations in astrocytes; v) normalize albumin levels in the CSF; and vi) enhance glutamate uptake in cerebral cortex slices.

Experimental treatments with guanosine have been successfully conducted with different doses and drug delivery strategies by our group and others [48, 70-73]. In the current study, the choice of administration (7.5 mg/kg/dose) of GUO via i.p. injection was based on the experience of the group with chronic HE induced by bile-duct ligation [47]. A higher dose of GUO (60mg/kg) was also initially tested in a small cohort of animals in the early stages of this study; however, it did not improve the effects of GUO when compared to lower doses. The time marks for the administration of GUO (represented in Figure 1) were chosen after pilot testing different administration protocols (data not shown). Overall, the described protocol achieved the best reduction in mortality: from an 89% mortality rate in control animals to 61% in treated animals, resulting in an approximately 4-fold increase in the number of animals that survived the experiments (Figure 2).

Moreover, similarly to Guazzelli, 2020 [57], animals with ALF presented a progressive impairment of neurological function following subtotal hepatectomy. GUO administration was able to improve the neurological status of the animals with ALF throughout the entire analysis by extending the early stages of the disease and without extending the duration in the final comatose stage. Indeed, by utilizing this neurological grading system for evaluating animals with ALF, our group demonstrated that a significant reduction in the mean neurological grade of animals treated with GUO was achieved throughout the experiment, and which started as soon as 12 hours after surgery (Figure 3B). These data were also accompanied by the improvement in exploratory activity of treated animals in the Open Field Task (represented in Figure 4) at 24 hours after surgery. Despite the marked reduction in locomotion encountered in both groups submitted to hepatectomy when compared to control groups, animals that received GUO presented a higher level of locomotion with 4.08 ± 2.79 meters, compared to only 1.40 ± 1.99 meters in the saline group. The time spent immobile, however, was not affected by the treatment.

Considering brain cellular function, astrocytes play an important role in the blood-brain barrier (BBB) as the astrocytic end foot envelops blood vessels in the brain and modulates the influx of substances into the central nervous system [74]. In the current paper, we found that animals with HE presented astrocytes with increased primary and secondary processes, which was consistent with previous studies conducted by our group [34]. This result was associated with elevated levels of CSF albumin (Figure 6A), which was also recently described by our group as associated with the rise of amino acid levels in ALF [57]. Elevated CSF albumin levels may be considered as a marker of BBB breakdown under pathological conditions because this molecule does not cross the BBB in healthy animals [75, 76]. Increased BBB permeability may be one of the events that contributes to HE's development, as impaired BBB facilitates the entrance of toxins into the brain, harming cellular function, and thus creating a vicious cycle that aggravates the disease. Alterations in the BBB of patients and animals with HE have been described before [74, 77-81]. Treatment with GUO was able to counteract the effects of liver failure regarding cellular morphology (Figure 5). Animals that received GUO also presented normal levels of albumin in the CSF (Figure 6A). These findings were associated with a significant improvement in survival rates and neurological performance, as described above. These data strengthen the support for the role of astrocyte disfunction and

increased BBB permeability in the development of HE, as well as reinforce the relation of the neuroprotective effects of GUO with astrocytic function.

Simultaneously, alterations in the glutamatergic neurotransmission are well described in hyperammonemia and HE [27, 67]. Ammonia accumulation leads to a hyperstimulation of NMDA receptors by excessively increasing glutamate levels in the synaptic cleft and by directly activating this glutamatergic receptor [67, 82, 83]. Astrocytes' diminished capacity for removing glutamate from the synaptic cleft is probably a major factor for glutamatergic excitotoxicity in hyperammonemia. In this sense, reduced glutamate uptake and the reduced expression of glutamate transporters have been reported in animals with ALF and/or hyperammonemia [36-41]. Recently, our group demonstrated that GUO administration was able to prevent a reduction in glutamate uptake in rats with acute ammonia intoxication, which was associated with reduced mortality [42]. Similarly, in the current paper, GUO administration enhanced the glutamate uptake in slices of cerebral cortex from animals with ALF (Figure 6B), and this was associated with a marked improvement in the overall survival of treated animals.

In conclusion, animals with HE induced by ALF and treated with GUO presented reduced mortality and improved neurological status, which were associated with improved astrocyte morphology, normal CSF levels of albumin and enhanced glutamate uptake. Hence, we hypothesize that GUO administration may prevent glutamatergic excitotoxicity and BBB malfunction by enhancing astrocytic activity and modulating the glutamatergic system. This study offers important evidence regarding the neuroprotective effects of GUO in animals with ALF. Moreover, this paper strengthens evidence regarding the beneficial effects of GUO in HE and hyperammonemia and reinforces the potential use of GUO as a future treatment for neurological impairment due to liver failure.

Financial Support

This work was supported by Brazilian agencies and grants: CNPq, CAPES, FAPERGS, UFRGS, Brazilian Institute of Neuroscience/FINEP, INCT/Excitotoxicity and Neuroprotection (465671/2014-4).

Keywords

Acute liver failure, Hepatic encephalopathy, Guanosine, Neuroprotection, Astrocytic reactivity, Immunohistochemistry, Albumin, Glutamate, Glutamate uptake, Open field test

Conflicts of interest

The authors have nothing to disclose.

Compliance with ethical standards

The experimental protocol was approved by the Ethics Committee for Animal Research of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, under the project number 29468, and followed the NIH Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1996). All efforts were made to minimize the number of animals used and their suffering.

References

1. Bernal, W., *Acute Liver Failure: Review and Update.* Int Anesthesiol Clin, 2017. **55**(2): p. 92-106.
2. Craig, D.G., et al., *Review article: the current management of acute liver failure.* Aliment Pharmacol Ther, 2010. **31**(3): p. 345-58.
3. Williams, R. and J. Wendon, *Indications for orthotopic liver transplantation in fulminant liver failure.* Hepatology, 1994. **20**(1 Pt 2): p. S5-10S.
4. Lee, W.M., et al., *Acute liver failure: Summary of a workshop.* Hepatology, 2008. **47**(4): p. 1401-15.
5. *Erratum: Nationwide Longitudinal Analysis of Acute Liver Failure in Taiwan: Erratum.* Medicine (Baltimore), 2017. **96**(44): p. e8577.
6. Thanapirom, K., et al., *The incidence, etiologies, outcomes, and predictors of mortality of acute liver failure in Thailand: a population-base study.* BMC Gastroenterol, 2019. **19**(1): p. 18.
7. Bernal, W., et al., *Acute liver failure: A curable disease by 2024?* J Hepatol, 2015. **62**(1 Suppl): p. S112-20.
8. Jayalakshmi, V.T. and W. Bernal, *Update on the management of acute liver failure.* Curr Opin Crit Care, 2020. **26**(2): p. 163-170.
9. Warrillow, S., et al., *Coagulation abnormalities, bleeding, thrombosis, and management of patients with acute liver failure in Australia and New Zealand.* J Gastroenterol Hepatol, 2019.
10. Detry, O., et al., *Brain edema and intracranial hypertension in fulminant hepatic failure: pathophysiology and management.* World J Gastroenterol, 2006. **12**(46): p. 7405-12.
11. Lee, W.M., *Acute liver failure.* Semin Respir Crit Care Med, 2012. **33**(1): p. 36-45.
12. Larsen, F.S. and J. Wendon, *Prevention and management of brain edema in patients with acute liver failure.* Liver Transpl, 2008. **14 Suppl 2**: p. S90-6.

13. Blei, A.T. and F.S. Larsen, *Pathophysiology of cerebral edema in fulminant hepatic failure.* J Hepatol, 1999. **31**(4): p. 771-6.
14. Stravitz, R.T. and F.S. Larsen, *Therapeutic hypothermia for acute liver failure.* Crit Care Med, 2009. **37**(7 Suppl): p. S258-64.
15. O'Grady, J.G., et al., *Early indicators of prognosis in fulminant hepatic failure.* Gastroenterology, 1989. **97**(2): p. 439-45.
16. Acharya, C. and J.S. Bajaj, *Current Management of Hepatic Encephalopathy.* Am J Gastroenterol, 2018.
17. Rajaram, P. and R. Subramanian, *Management of Acute Liver Failure in the Intensive Care Unit Setting.* Clin Liver Dis, 2018. **22**(2): p. 403-408.
18. Ott, P. and H. Vilstrup, *Cerebral effects of ammonia in liver disease: current hypotheses.* Metab Brain Dis, 2014. **29**(4): p. 901-11.
19. Ciecko-Michalska, I., et al., *Pathogenesis of hepatic encephalopathy.* Gastroenterol Res Pract, 2012. **2012**: p. 642108.
20. Felipo, V. and R.F. Butterworth, *Neurobiology of ammonia.* Prog Neurobiol, 2002. **67**(4): p. 259-79.
21. Fiati Kenston, S.S., et al., *Mechanistic insight, diagnosis, and treatment of ammonia-induced hepatic encephalopathy.* J Gastroenterol Hepatol, 2019. **34**(1): p. 31-39.
22. Bernal, W., et al., *Arterial ammonia and clinical risk factors for encephalopathy and intracranial hypertension in acute liver failure.* Hepatology, 2007. **46**(6): p. 1844-52.
23. Clemmesen, J.O., et al., *Cerebral herniation in patients with acute liver failure is correlated with arterial ammonia concentration.* Hepatology, 1999. **29**(3): p. 648-53.
24. Wilkinson, D.J., et al., *Absence of neuropsychological impairment in hyperammonaemia in healthy young adults; possible synergism in development of hepatic encephalopathy (HE) symptoms?* Metab Brain Dis, 2011. **26**(3): p. 203-12.
25. Butterworth, R.F., *Pathogenesis of hepatic encephalopathy in cirrhosis: the concept of synergism revisited.* Metab Brain Dis, 2016. **31**(6): p. 1211-1215.
26. Gonzalez-Regueiro, J.A., et al., *Pathophysiology of hepatic encephalopathy and future treatment options.* Rev Gastroenterol Mex, 2019. **84**(2): p. 195-203.
27. Montana, V., A. Verkhratsky, and V. Parpura, *Pathological role for exocytotic glutamate release from astrocytes in hepatic encephalopathy.* Curr Neuropharmacol, 2014. **12**(4): p. 324-33.
28. Norenberg, M.D., *The role of astrocytes in hepatic encephalopathy.* Neurochem Pathol, 1987. **6**(1-2): p. 13-33.
29. Butterworth, R.F., *Altered glial-neuronal crosstalk: cornerstone in the pathogenesis of hepatic encephalopathy.* Neurochem Int, 2010. **57**(4): p. 383-8.
30. Victor, M., R.D. Adams, and M. Cole, *The acquired (non-Wilsonian) type of chronic hepatocerebral degeneration.* Medicine (Baltimore), 1965. **44**(5): p. 345-96.
31. Kimura, N., et al., *Portal-systemic shunt encephalopathy presenting with diffuse cerebral white matter lesion: an autopsy case.* Neuropathology, 2008. **28**(6): p. 627-32.
32. Agarwal, A.N. and D.D. Mais, *Sensitivity and Specificity of Alzheimer Type II Astrocytes in Hepatic Encephalopathy.* Arch Pathol Lab Med, 2019. **143**(10): p. 1256-1258.
33. Belanger, M., et al., *Loss of expression of glial fibrillary acidic protein in acute hyperammonemia.* Neurochem Int, 2002. **41**(2-3): p. 155-60.
34. Guazzelli, P.A., et al., *Acute Liver Failure Induces Glial Reactivity, Oxidative Stress and Impairs Brain Energy Metabolism in Rats.* Front Mol Neurosci, 2019. **12**: p. 327.

35. Scott, T.R., et al., *Pathophysiology of cerebral oedema in acute liver failure.* World J Gastroenterol, 2013. **19**(48): p. 9240-55.
36. Monfort, P., et al., *Effects of hyperammonemia and liver failure on glutamatergic neurotransmission.* Metab Brain Dis, 2002. **17**(4): p. 237-50.
37. Rose, C., *Effect of ammonia on astrocytic glutamate uptake/release mechanisms.* J Neurochem, 2006. **97 Suppl 1**: p. 11-5.
38. Chan, H. and R.F. Butterworth, *Cell-selective effects of ammonia on glutamate transporter and receptor function in the mammalian brain.* Neurochem Int, 2003. **43**(4-5): p. 525-32.
39. Cittolin-Santos, G.F., et al., *Behavioral, Neurochemical and Brain Oscillation Abnormalities in an Experimental Model of Acute Liver Failure.* Neuroscience, 2019. **401**: p. 117-129.
40. Chan, H., et al., *Effects of ammonia on glutamate transporter (GLAST) protein and mRNA in cultured rat cortical astrocytes.* Neurochem Int, 2000. **37**(2-3): p. 243-8.
41. Knecht, K., et al., *Decreased glutamate transporter (GLT-1) expression in frontal cortex of rats with acute liver failure.* Neurosci Lett, 1997. **229**(3): p. 201-3.
42. Cittolin-Santos, G.F., et al., *Guanosine Exerts Neuroprotective Effect in an Experimental Model of Acute Ammonia Intoxication.* Mol Neurobiol, 2017. **54**(5): p. 3137-3148.
43. Fries, A.W., et al., *Effect of glutamine synthetase inhibition on brain and interorgan ammonia metabolism in bile duct ligated rats.* J Cereb Blood Flow Metab, 2014. **34**(3): p. 460-6.
44. Cauli, O., et al., *Acute liver failure-induced death of rats is delayed or prevented by blocking NMDA receptors in brain.* Am J Physiol Gastrointest Liver Physiol, 2008. **295**(3): p. G503-11.
45. Cauli, O., et al., *Blocking NMDA receptors delays death in rats with acute liver failure by dual protective mechanisms in kidney and brain.* Neuromolecular Med, 2014. **16**(2): p. 360-75.
46. Jambekar, A.A., et al., *A glutamine synthetase inhibitor increases survival and decreases cytokine response in a mouse model of acute liver failure.* Liver Int, 2011. **31**(8): p. 1209-21.
47. Paniz, L.G., et al., *Neuroprotective effects of guanosine administration on behavioral, brain activity, neurochemical and redox parameters in a rat model of chronic hepatic encephalopathy.* Metab Brain Dis, 2014. **29**(3): p. 645-54.
48. Schmidt, A.P., et al., *Guanosine prevents thermal hyperalgesia in a rat model of peripheral mononeuropathy.* J Pain, 2010. **11**(2): p. 131-41.
49. Schmidt, A.P., et al., *Guanosine and GMP prevent seizures induced by quinolinic acid in mice.* Brain Res, 2000. **864**(1): p. 40-3.
50. Chang, R., et al., *Neuroprotective effects of guanosine on stroke models in vitro and in vivo.* Neurosci Lett, 2008. **431**(2): p. 101-5.
51. Hansel, G., et al., *Guanosine Protects Against Cortical Focal Ischemia. Involvement of Inflammatory Response.* Mol Neurobiol, 2015. **52**(3): p. 1791-1803.
52. Su, C., et al., *Guanosine improves motor behavior, reduces apoptosis, and stimulates neurogenesis in rats with parkinsonism.* J Neurosci Res, 2009. **87**(3): p. 617-25.
53. Lanznaster, D., et al., *Guanosine: a Neuromodulator with Therapeutic Potential in Brain Disorders.* Aging Dis, 2016. **7**(5): p. 657-679.
54. Eguchi, S., et al., *Fulminant hepatic failure in rats: survival and effect on blood chemistry and liver regeneration.* Hepatology, 1996. **24**(6): p. 1452-9.
55. Detry, O., et al., *A modified surgical model of fulminant hepatic failure in the rat.* J Surg Res, 2013. **181**(1): p. 85-90.

56. Cittolin-Santos, G.F., et al., *Behavioral, neurochemical and brain oscillation abnormalities in an experimental model of acute liver failure.* Neuroscience, 2019.
57. Guazzelli, P.A., *Blood brain barrier permeability and increased levels of cerebrospinal fluid amino acids are associated with neurological grading and mortality in rats with acute liver failure.* Amino Acids, 2020.
58. Madrahimov, N., et al., *Marginal hepatectomy in the rat: from anatomy to surgery.* Ann Surg, 2006. **244**(1): p. 89-98.
59. Almeida, R.F., et al., *Systemic administration of GMP induces anxiolytic-like behavior in rats.* Pharmacol Biochem Behav, 2010. **96**(3): p. 306-11.
60. FDA, *Guidance for industry: bioanalytical method validation.* 2018: U.S. Department of Health and Human Services. p. 1–44 22.
61. EMA, *European medicines agency. Guideline on bioanalytical method validation guideline on bioanalytical method validation*. 2012: London, United Kingdom.
62. Almeida, R.F., et al., *Effects of depressive-like behavior of rats on brain glutamate uptake.* Neurochem Res, 2010. **35**(8): p. 1164-71.
63. Bunchorntavakul, C. and K.R. Reddy, *Acute Liver Failure.* Clin Liver Dis, 2017. **21**(4): p. 769-792.
64. Bernal, W. and J. Wendon, *Acute liver failure.* N Engl J Med, 2013. **369**(26): p. 2525-34.
65. Grek, A. and L. Arasi, *Acute Liver Failure.* AACN Adv Crit Care, 2016. **27**(4): p. 420-429.
66. Seetharam, A., *Intensive Care Management of Acute Liver Failure: Considerations While Awaiting Liver Transplantation.* J Clin Transl Hepatol, 2019. **7**(4): p. 384-391.
67. Rodrigo, R., et al., *Role of NMDA receptors in acute liver failure and ammonia toxicity: therapeutical implications.* Neurochem Int, 2009. **55**(1-3): p. 113-8.
68. Hermenegildo, C., et al., *NMDA receptor antagonists prevent acute ammonia toxicity in mice.* Neurochem Res, 1996. **21**(10): p. 1237-44.
69. Bettio, L.E., J. Gil-Mohapel, and A.L. Rodrigues, *Guanosine and its role in neuropathologies.* Purinergic Signal, 2016. **12**(3): p. 411-26.
70. Teixeira, L.V., et al., *Neuroprotective Effects of Guanosine Administration on In Vivo Cortical Focal Ischemia in Female and Male Wistar Rats.* Neurochem Res, 2018. **43**(7): p. 1476-1489.
71. Lanznaster, D., et al., *Guanosine Prevents Anhedonic-Like Behavior and Impairment in Hippocampal Glutamate Transport Following Amyloid-beta1-40 Administration in Mice.* Mol Neurobiol, 2017. **54**(7): p. 5482-5496.
72. Almeida, R.F., et al., *Guanosine Anxiolytic-Like Effect Involves Adenosinergic and Glutamatergic Neurotransmitter Systems.* Mol Neurobiol, 2017. **54**(1): p. 423-436.
73. Ramos, D.B., et al., *Intranasal guanosine administration presents a wide therapeutic time window to reduce brain damage induced by permanent ischemia in rats.* Purinergic Signal, 2016. **12**(1): p. 149-59.
74. Cui, W., C.M. Sun, and P. Liu, *Alterations of blood-brain barrier and associated factors in acute liver failure.* Gastroenterol Res Pract, 2013. **2013**: p. 841707.
75. Obermeier, B., A. Verma, and R.M. Ransohoff, *The blood-brain barrier.* Handb Clin Neurol, 2016. **133**: p. 39-59.
76. Di Pardo, A., et al., *Assessment of Blood-brain Barrier Permeability by Intravenous Infusion of FITC-labeled Albumin in a Mouse Model of Neurodegenerative Disease.* J Vis Exp, 2017(129).
77. Alexander, B., et al., *A quantitative evaluation of the permeability of the blood brain barrier of portacaval shunted rats.* Metab Brain Dis, 2000. **15**(2): p. 93-103.

78. Shaik, I.H., et al., *Effects of short-term portacaval anastomosis on the peripheral and brain disposition of the blood-brain barrier permeability marker sodium fluorescein in rats.* Brain Res, 2013. **1531**: p. 84-93.
79. Nguyen, J.H., *Subtle BBB alterations in brain edema associated with acute liver failure.* Neurochem Int, 2010. **56**(2): p. 203-4; author reply 205-7.
80. Wright, G., Y. Sharifi, and R. Jalan, *Blood-brain barrier in liver failure: are cracks appearing in the wall?* Liver Int, 2010. **30**(8): p. 1087-90.
81. Nguyen, J.H., *Blood-brain barrier in acute liver failure.* Neurochem Int, 2012. **60**(7): p. 676-83.
82. Llansola, M., et al., *NMDA receptors in hyperammonemia and hepatic encephalopathy.* Metab Brain Dis, 2007. **22**(3-4): p. 321-35.
83. Hermenegildo, C., P. Monfort, and V. Felipo, *Activation of N-methyl-D-aspartate receptors in rat brain in vivo following acute ammonia intoxication: characterization by in vivo brain microdialysis.* Hepatology, 2000. **31**(3): p. 709-15.

Figure legends

Figure 1: Guanosine administration protocol. The figure represents the time marks for administration of either Guanosine (GUO)/Saline (S) and glucose (Glu) solution during the experiment.

Figure 2: Survival rate. The curve represents the survival rate over time (Kaplan-Meier estimator) of animals submitted to subtotal hepatectomy and treated with either saline solution or guanosine. * represents difference between the groups ($p < 0.05$), Gehan-Breslow-Wilcoxon test. Experimental n = 38 (Saline) and n = 41 (Guanosine).

Figure 3: Neurological scale. (A) represents the criteria utilized for neurological classification of animals with hepatic encephalopathy; **(B)** represents the mean neurological grade over time after surgery – animals that died before 72 hours were included until the last evaluation while still alive**.** Data is expressed as mean ± SD (hours). * represents difference between the groups ($p < 0.05$), Student's t test. Initial (time 0) experimental n = 38 (Saline) and n = 41 (Guanosine).

Figure 4: Open Field Task. (A) total travelled distance and **B)** time spent immobile during the 10 minutes of the OFT session 24 hours after surgery, respectively; **(C)** total travelled distance and **(D)** time spent immobile during the 10 minutes of the OFT session 15 days after surgery, respectively. No animal of the Hep. + Saline group survived over 72 hours of experiment. Data is expressed as mean ± SD (meters or seconds). *difference from all the other groups ($p<0.05$) and # represents difference from Naive and Sham operated groups ($p<0.05$); ANOVA. Experimental n = 18 (Naive), n = 12 (Sham + Saline), n = 12 (Sham + GUO), n = 15 (Hep. + Saline), n = 15 (Hep. + GUO) at 24 hours after surgery. Experimental n = 18 (Naive), n = 6 (Sham + Saline), n = 6 (Sham + GUO), n = 0 (Hep. + Saline), n = 3 (Hep. + GUO) at 15 days after surgery.

Figure 5. Immunofluorescence for GFAP. (**A)** represents the number of primary processes of astrocytes; **(B)** number of secondary processes of astrocytes. Data is expressed as mean ± SD. *represents difference from all the other groups ($p<0.05$), ANOVA; Experimental n = 6 animals (with 3 astrocytes analyzed per animal).

Figure 6. Biochemical evaluation 24 hours after the surgical procedures. **(A)** CSF levels of Albumin measured by HPLC (µg), n = 10 in all groups; **(B)** Na^+-dependent glutamate uptake (pmol/mg/min), n = 10 in all groups; Data is expressed as mean ± SD. *represents difference from all the other groups ($p<0.05$), ANOVA; and # represents difference between Hep. + Saline group and Hep. + GUO group ($p<0.05$), ANOVA.

Figure 1

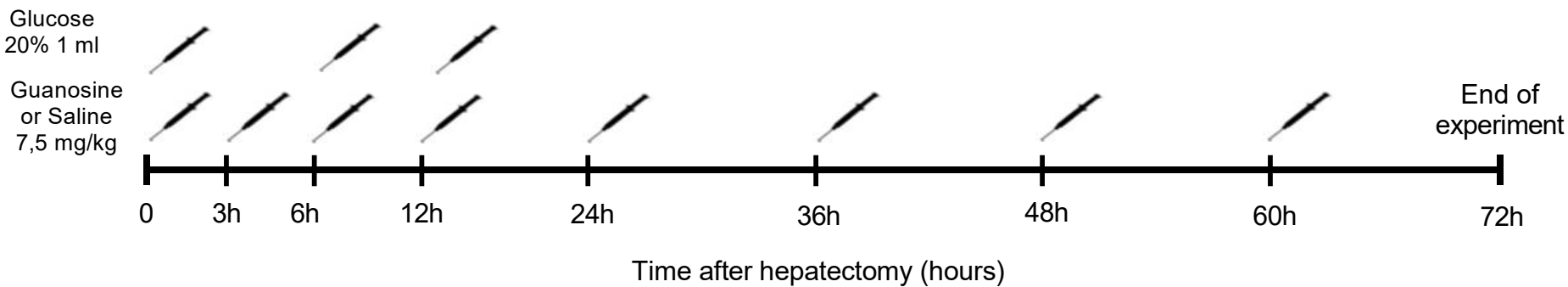

Glucose
20% 1 ml
Guanosine
or Saline
7,5 mg/kg
End of
experiment
0
3h
6h
12h
24h
36h
48h
60h
72h
Time after hepatectomy (hours)

Figure 2

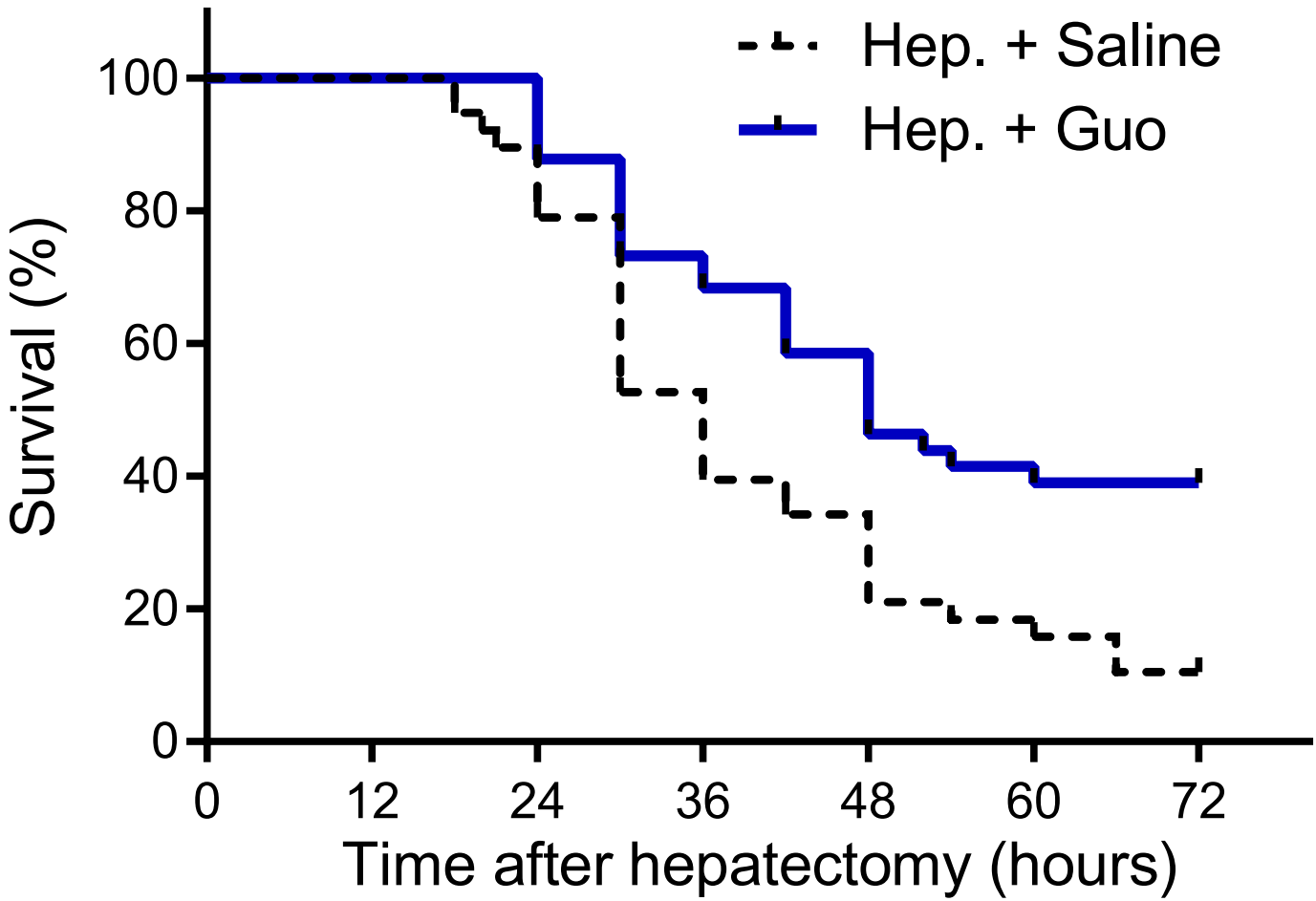

Hep. + Saline
Hep. + Guo
Survival (%)
100
80
60
40
20
0
0
12
24
36
48
60
72
Time after hepatectomy (hours)

Figure 3

A

Neurological Grade	Neurological tests and reflexe for accessing Hepatic Encephalopathy
Ø	Freely moving and exploring the home cage. No reflex alterations.
I	No spontaneous exploratory activity. Righting reflex present. Researcher is not able to place the animal on it's back. Corneal reflex preserved.
II	No spontaneous exploratory activity. Researcher is able to place the animal on it's back, but the animal is able to return to normal position within 3 seconds. Corneal reflex preserved.
III	No spontaneous exploratory activity. Loss of righting reflex - animal is not able to return to normal position. Corneal reflex preserved.
IV	No spontaneous exploratory activity or locomotion. Loss of both reflexes.

B

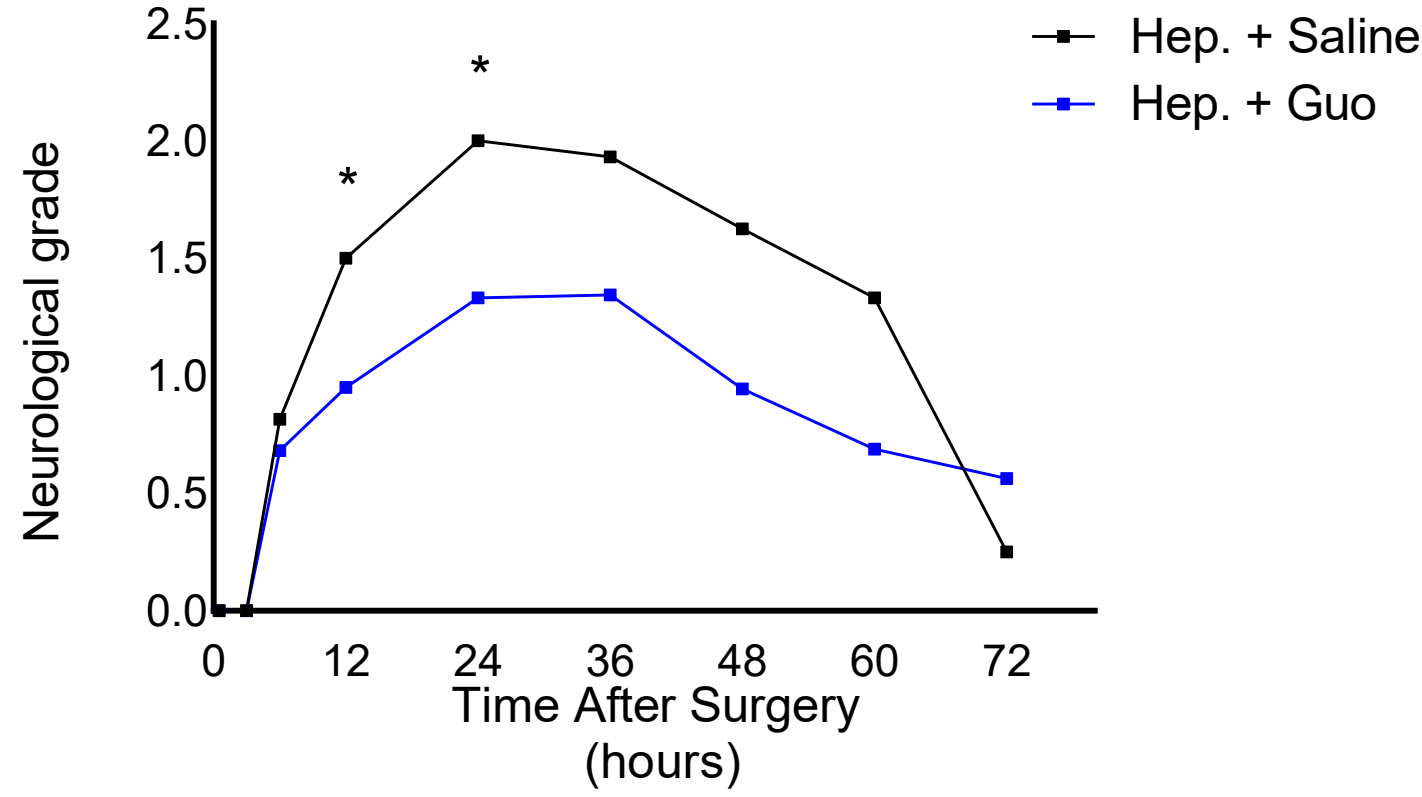


Figure 4

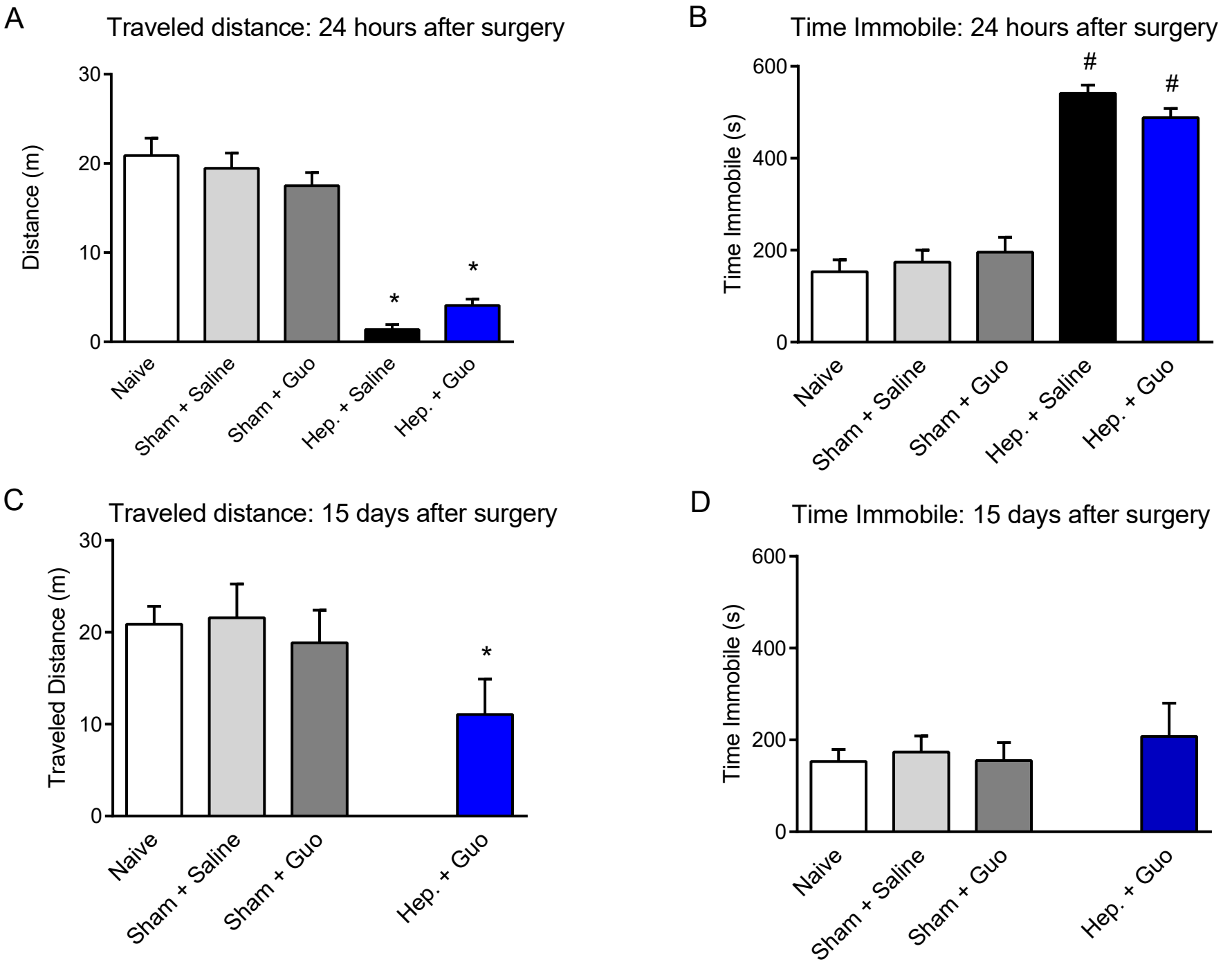

A
Traveled distance: 24 hours after surgery
Distance (m)
30
20
10
0
*
*
Naive
Sham + Saline
Sham + Guo
Hep. + Saline
Hep. + Guo
B
Time Immobile: 24 hours after surgery
Time Immobile (s)
600
400
200
0
#
#
Naive
Sham + Saline
Sham + Guo
Hep. + Saline
Hep. + Guo
C
Traveled distance: 15 days after surgery
Traveled Distance (m)
30
20
10
0
*
Naive
Sham + Saline
Sham + Guo
Hep. + Guo
D
Time Immobile: 15 days after surgery
Time Immobile (s)
600
400
200
0
Naive
Sham + Saline
Sham + Guo
Hep. + Guo

Figure 5

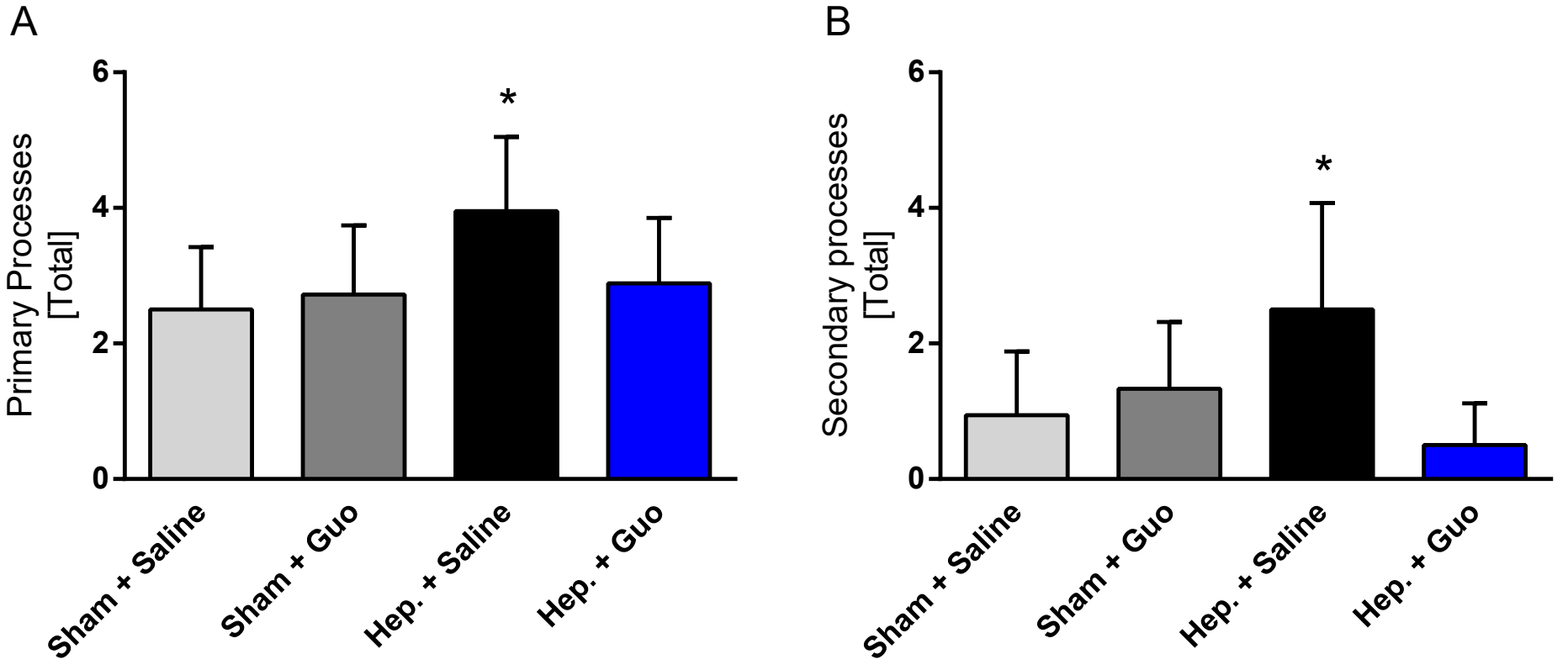
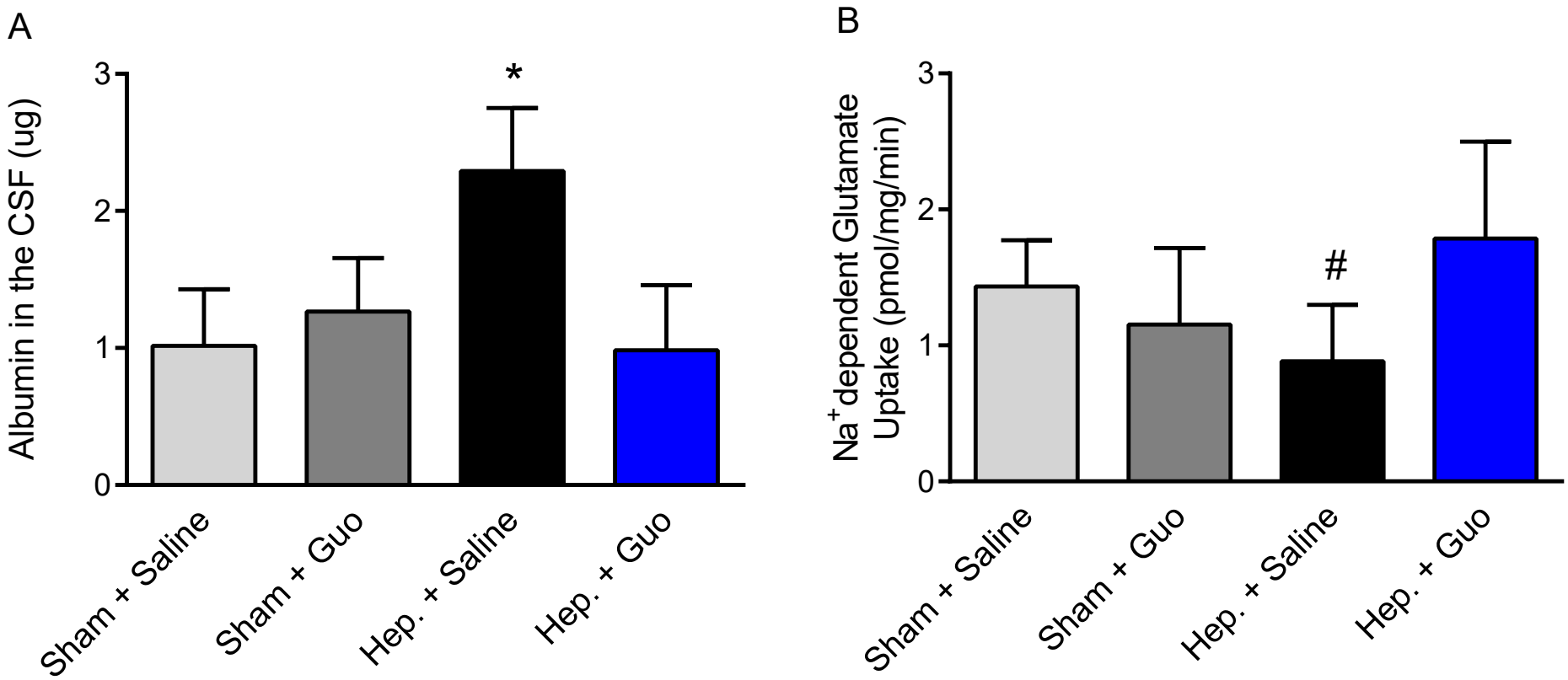

A
Primary Processes [Total]
6
4
2
0
*
Sham + Saline
Sham + Guo
Hep. + Saline
Hep. + Guo
B
Secondary processes [Total]
6
4
2
0
*
Sham + Saline
Sham + Guo
Hep. + Saline
Hep. + Guo

Figure 6


A
Albumin in the CSF (ug)
3
2
1
0
*
Sham + Saline
Sham + Guo
Hep. + Saline
Hep. + Guo
B
Na+dependent Glutamate Uptake (pmol/mg/min)
3
2
1
0
#
Sham + Saline
Sham + Guo
Hep. + Saline
Hep. + Guo

Parte 3

7. Discussão

A insuficiência hepática aguda (IHA) é uma condição médica infrequente, porém de grande importância devido ao impacto severo (potencialmente fatal) na saúde de indivíduos que sofrem danos súbitos ao funcionamento do fígado [1-3]. Por definição, todos os quadros de IHA evoluem com comprometimento do sistema nervoso central (SNC), compondo uma síndrome conhecida como encefalopatia hepática (EH) [21, 23, 24]. É sabido que a amônia tem um papel central no desenvolvimento desta condição [31, 36, 104] e que os níveis elevados de amônia na corrente sanguínea estão relacionados com o desenvolvimento de hipertensão intracraniana [105, 106]. A hiperamonemia também interfere em diversos processos fisiológicos do SNC, proporcionando o surgimento de distúrbios como o estresse oxidativo, aumento de permeabilidade da barreira hematoencefálica, alterações bioenergéticas, assim como o mal funcionamento de sistemas de neurotransmissão [31, 37, 104].Tratamentos que visam diminuir os níveis séricos de amônia e que são classicamente utilizados para o manejo da EH em pacientes com doença hepática crônica (como a lactulose e a rifaximina) tem seu benefício questionado em quadros de IHA, não sendo utilizados de maneira rotineira nos diferentes centros hospitalares [107]. Até a presente data, as equipes médicas não dispõem de tratamentos farmacológicos que comprovadamente consigam controlar ou reverter as alterações cerebrais secundárias a perda aguda de função hepática.

Considerando os efeitos patológicos ocasionados pela hiperamonemia no sistema glutamatérgico, a modulação desse sistema de neurotransmissão vem sendo estudada como possibilidade terapêutica para a EH [100-102]. Na mesma linha, nosso grupo de pesquisa publicou recentemente dados demonstrando efeitos neuroprotetores da guanosina (GUO) em modelos animais de hiperamonemia e encefalopatia hepática crônica [48, 103]. Tendo em vista o exposto acima, esta tese se propôs a investigar aspectos fisiopatológicos da EH no contexto da IHA, assim como a testar o uso de guanosina como possível medida terapêutica para esta condição. Nos estudos aqui apresentados, encontramos que animais submetidos à hepatectomia subtotal exibiram: i) reatividade astrocitária; ii) aumento de consumo de oxigênio mitocondrial e aumento de atividade enzimática, com consequente elevação dos níveis de ATP;

iii) aumento da oxidação de glutamato e diminuição da oxidação de glicose e lactato; iv) aumento da produção de ROS, com diminuição da atividade enzimática antioxidante; v) aumento dos níveis extracelulares de aminoácidos e albumina. Em conjunto, o tratamento com guanosina foi capaz de vi) aumentar a sobrevida dos animais; vii) melhorar parâmetros neurológicos e comportamentais; viii) normalizar a morfologia astrocitária; ix) normalizar os níveis de albumina liquórica e x) potencializar a capacidade de recaptação de glutamato por fatias cerebrais.

Em relação aos mecanismo celulares do desenvolvimento da EH, um fenômeno importante evidenciado nesta tese foi a presença de astrócitos reativos em fatias de córtex cerebral dos animais hepatectomizados. O estudo das células da gliais é realizado há décadas no contexto da doença hepática, sendo descrita a conexão entre hepatopatia crônica e astrócitos com anormalidades morfológicas (conhecidos como astrócitos tipo II de Alzheimer) desde 1953 [108]. O achado destas células de morfologia anômala se correlaciona com a gravidade da encefalopatia, apresentando boa sensibilidade e especificidade para o diagnóstico de EH em pacientes com insuficiência hepática crônica [109, 110]. No entanto, poucos relatos existem na literatura científica em relação à morfologia astrocitária em quadros agudos de EH (secundários à IHA), sendo o edema celular o principal dado citado [111]. No Capítulo I desta tese descrevemos um considerável aumento da densidade óptica e do volume astrocitário decorrentes da proliferação de processos celulares [112], o qual se confirmou no Capítulo III. Estes achados sugerem que a proliferação de processos astrocitários contribui com a expansão do volume celular e, provavelmente, estão relacionados com o aumento de pressão intracraniana em indivíduos com IHA. Ademais, é importante enfatizar que as alterações morfológicas encontradas nos astrócitos de animais com EH foram revertidas pelo uso da GUO, como demonstrado na Figura 4 do Capítulo III. De fato, o aumento no número de processos primários e secundários evidenciados nos animais que receberam salina foi encontrado após a administração de GUO, a qual resultou, paralelamente, em melhora neurológica e aumento de sobrevida no grupo tratado.

As alterações morfológicas descritas acima também foram associadas com mudanças significativas do perfil bioenergético do SNC dos animais

hepatectomizados. Embora estudos do metabolismo energético cerebral em pacientes com hepatopatia crônica sugiram uma redução da atividade cerebral e consumo de oxigênio [53, 54, 113, 114], estudos com modelos agudos e de hiperamonemia não descrevem um déficit significativo de energia nos tecidos cerebrais secundários aos efeitos da amônia [55-57, 59, 115]. No mesmo sentido, nesta tese encontramos que os animais submetidos à hepatectomia subtotal apresentaram aumento do consumo de oxigênio, aumento da atividade enzimática associada ao ciclo de Krebs e consequente aumento dos níveis de ATP (Capítulo I, Figuras 3 e 4). Esses animais apresentaram diminuição da oxidação de glicose e lactato à CO_2, porém, em contrapartida, a oxidação de glutamato à CO_2 se apresentou significativamente elevada (Capítulo I, Figura 5). Este mecanismo compensatório pode, em parte, explicar a manutenção de um estado hiper metabólico e sem déficit energético significativo nos estágios iniciais de evolução da encefalopatia no quadros de IHA.

Simultaneamente, o aumento do consumo de oxigênio e produção de ATP resultaram em importante incremento da produção de espécies reativas de oxigênio (ROS) (Capítulo I, Figura 6). Este evento se deve, também, a atividade reduzida das enzimas SOD e GSH-Px, as quais são fundamentais para a manutenção do equilíbrio redox intracelular. Este achado é, provavelmente, consequência direta da toxicidade da amônia no SNC, estando de acordo com outros estudos na literatura científica acerca do tema [48, 116-122]. O aumento da produção de ROS foi associado ao aumento da atividade da enzima PARP-1, a qual é ativada para proporcionar a reparação de dano ao DNA e está envolvida nos processos de morte celular [123, 124]. Assim sendo, concluímos que a EH induz reatividade astrocitária que demanda uma hiperatividade bioenergética cerebral e, como efeito colateral, resulta em estresse oxidativo por aumento de ROS e redução da atividade das enzimas SOD e GSH-Px, danificando o DNA e prejudicando o correto funcionamento celular.

Outro aspecto fundamental da atividade astrocitária é a composição da barreira hematoencefálica (BHE) através dos seus processos celulares, os quais revestem as estruturas vasculares cerebrais [61, 125]. De fato, a atividade da BHE na modulação do trânsito de substâncias no SNC é essencial para o adequado funcionamento neurológico. Em condições normais, a BHE impede a

entrada de grandes moléculas e proteínas como a albumina no líquido cefalorraquidiano (LCR) [126]. Nos capítulos II e III, demonstramos que animais submetidos à hepatectomia apresentaram um aumento significativo dos níveis de albumina no LCR (24 horas após o procedimento), sugerindo uma provável falha na atividade da BHE dos animais hepatectomizados. Este achado reforça o conjunto de evidências acerca do aumento de permeabilidade da BHE no contexto de EH [64-68]. Curiosamente, o uso de GUO foi capaz de reverter por completo esta elevação de albumina no LCR (Capítulo III, Figura 6).

Em conjunto, o grupo também avaliou os níveis de amino ácidos no LCR dos animais hepatectomizados no tempo de 24 horas após a cirurgia, sendo identificado um aumento generalizado dessas moléculas (Capítulo II, Figura 3). O aumento dos níveis do glutamato e glutamina já foram previamente estudados em pacientes com EH, pois estão intimamente ligados ao processo de detoxificação da amônia [127-130]. No entanto, encontramos evidências de que todos os outros amino ácidos (com exceção da alanina) também apresentaram elevação significativa no LCR. Este achado torna mais provável a hipótese de que o aumento dos amino ácidos no LCR (incluindo glutamato e glutamina) se deve ao mal funcionamento da BHE e não à interferência direta da amônia em uma rota metabólica específica. Além disso, a elevação dos níveis de amino ácidos no LCR apresentou correlação com a gravidade do dano neurológico, como demonstrado no Capítulo II, Figura 4. De fato, animais em estágios avançados de EH (estágios III e IV) apresentaram níveis de amino ácidos significativamente mais elevados que os animais em estágios iniciais e que os animais do grupo controle. Esta correlação neurológica/bioquímica também foi verificada em relação aos níveis de albumina no LCR, demonstrada no Capítulo II Figura 2, o que reforça a hipótese do aumento de permeabilidade da BHE conforme a progressão da EH.

Paralelamente, o grupo realizou a análise dos níveis de amino ácidos no LCR em outro grupo de animais, no tempo de 12 horas após o procedimento cirúrgico. Nesta coorte, o aumento de amino ácidos foi capaz de diferenciar com clareza o grupo de animais que sobreviveu (níveis normais ou discretamente elevados de amino ácidos) do grupo de animais que progrediu para morte, o qual apresentou níveis elevados de amino ácidos (Capítulo II, Figura 5). É importante reforçar que, neste ponto da evolução da EH, não há critérios neurológicos ou

comportamentais que diferenciem os animais com bom prognóstico dos que evoluem para a morte. Assim sendo, sugere-se que os níveis de amino ácidos (especialmente a glutamina) possam, futuramente, ser avaliados como biomarcadores da evolução da EH, do mesmo modo que estas moléculas vêm sendo propostas como biomarcadores de doenças neurodegenerativas [131]. De fato, a relação dos níveis liquóricos de glutamina com o grau de encefalopatia já fora descrita previamente em pacientes com hepatopatia crônica [132-136]. Ademais, recentemente os níveis elevados de glutamina no sangue de pacientes com IHA foram associados a piores desfechos clínicos [137]. Deste modo, levantamos a hipótese de que a detecção precoce de mau prognóstico em pacientes com IHA através do nível de glutamina no LCR poderia ser de grande utilidade para a indicação precoce de estratégias terapêuticas agressivas como o transplante hepático.

Por fim, outro ponto de destaque nos achados apresentados nesta tese se refere aos efeitos da guanosina sobre a mortalidade dos animais e sobre os parâmetros neurológicos e comportamentais aqui avaliados. De fato, de acordo com os trabalhos prévios do grupo, a hepatectomia subtotal apresentou uma elevada letalidade no período de 24 a 72 horas após a cirurgia (Capítulos I a III), [45]. A administração de guanosina foi capaz de diminuir a mortalidade destes animais de 89% para 61% (Capítulo III, Figura 1). Embora um número elevado de animais ainda apresente desfechos desfavoráveis, estes dados representam um aumento de aproximadamente quatro vezes no número de indivíduos vivos no grupo que recebeu o tratamento. Assim sendo, se eventuais estudos clínicos encontrarem um efeito similar da GUO no tratamento de pacientes com IHA, estaríamos, sem dúvidas, diante de uma mudança de paradigma no tratamento desta doença. Ademais, como demonstrado no Capítulo III, os efeitos neuroprotetores da GUO não se restringiram apenas à redução da mortalidade. Com efeito, os animais tratados apresentaram são só melhora dos parâmetros neurológicos durante todo o período de análise, como maior atividade locomotora nos testes comportamentais. Isto sugere que o aumento de sobrevida proporcionado pela administração de GUO se deva a uma melhora global do funcionamento cerebral com repercussões neurológicas significativas e não apenas a um prolongamento dos estágios terminais da doença.

Um dos possíveis mecanismos de ação da GUO para explicar esse efeito neuroprotetor é a sua capacidade de melhorar a recaptação de glutamato excessivo da fenda sináptica. Conforme discutido anteriormente, para o correto funcionamento do sistema glutamatérgico é necessário que, após a ativação dos receptores pós sinápticos, as moléculas de glutamato sejam retiradas da fenda sináptica pelos astrócitos através do processo conhecido como recaptação de glutamato [33, 138, 139]. Modelos animais de EH descrevem que essa patologia cursa com uma diminuição da expressão dos transportadores de glutamato (GLAST e GLT-1), assim como redução da recaptação de glutamato astrocitária [38, 45, 48, 140]. Recentemente, nosso grupo demonstrou que animais intoxicados por acetato de amônia não só apresentavam uma perda de aproximadamente 30% da recaptação de glutamato por fatias cerebrais, como demonstrou que o uso da GUO foi capaz de prevenir este efeito, resultando em aumento de sobrevida dos animais tratados [48]. Efeitos benéficos da GUO sobre a recaptação de glutamato também já foram demonstrados em outros modelos animais e culturas celulares [141-144]. No Capítulo III, demonstramos que os animais submetidos a hepatectomia subtotal que receberam GUO apresentaram um aumento da sua capacidade cerebral de recaptação de glutamato (Capítulo III, Figura 6), reforçando, assim, o papel da GUO na modulação do sistema glutamatérgico.

8. Conclusão

Os resultados expostos nesta tese nos permitem concluir que os animais submetidos à hepatectomia subtotal apresentaram sinais de comprometimento cerebral compatíveis com encefalopatia hepática, os quais foram mensurados por análises neurológicas, bioquímicas e testes comportamentais. O grupo levanta a hipótese de que o rápido incremento nos níveis de amônia em animais hepatectomizados induz reatividade astrocitária, a qual demanda um aumento da atividade energética cerebral, levando ao maior consumo de oxigênio, produção elevada de ATP e maior atividade enzimática. Consequentemente, há o aumento indesejado na produção de espécies reativas de oxigênio, a qual está classicamente ligada a danos nos funcionamento celular. Ademais, hipotetizamos que estas alterações astrocitárias provavelmente estão relacionadas à diminuição da integridade da barreira hematoencefálica, proporcionando a entrada de aminoácidos e albumina no SNC. Os níveis elevados de aminoácidos no líquor desses animais estão associados com a progressão dos estágios de encefalopatia, assim como são possíveis preditores de prognóstico de mortalidade mesmo em estágios iniciais da doença.

Por fim, demonstramos que a administração de guanosina em animais com IHA foi capaz de reduzir a mortalidade, melhorar parâmetros neurológicos e comportamentais, assim como normalizar alterações celulares e bioquímicas nos animais tratados. Embora os trabalhos aqui realizados não esclareçam os exatos mecanismos pelos quais a GUO proporcione esta melhora neurológica, acreditamos que seus efeitos neuroprotetores se devam ao aprimoramento da função astrocitária, com consequente melhora da modulação glutamatérgica e da função da barreira hematoencefálica. Estes dados reforçam o papel da modulação do sistema glutamatérgico como uma possível estratégia terapêutica para indivíduos com IHA e/ou hiperamonemia e, em especial, robustecem as evidências acerca dos efeitos neuroprotetores da guanosina.

9. Perspectivas

Os achados dos trabalhos realizados ao longo dos últimos 5 anos esclarecem questionamentos acerca do metabolismo energético, metabolismo

de amino ácidos e o papel neuroprotetor da guanosina no contexto de insuficiência hepática aguda e hiperamonemia. Estes resultados também geram diversas outras hipóteses e questionamentos, como é característico do processo científico. Acreditamos que dentre os dados trazidos nesta tese, duas vertentes principais surgem para guiar os estudos futuros do grupo e pesquisadores da área:

Glutamina: o aprofundamento do estudo da relação dos níveis liquóricos de aminoácidos, em especial da glutamina, em animais e pacientes com IHA. A ênfase deste trabalho deve ser o estudo da glutamina como biomarcador da progressão da encefalopatia hepática e preditor de mortalidade. De fato, os dados aqui apresentados demonstram um padrão de elevação dos aminoácidos no LCR capazes de prever o desfecho dos animais em estágios iniciais da doença, discriminando o grupo com recuperação espontânea do grupo que evoluiu para morte. Se este padrão de elevação precoce de aminoácidos for reprodutível em humanos com IHA, a dosagem de aminoácidos pode trazer grande impacto no desenvolvimento de novos *scores* clínicos para a avaliação de prognóstico destes pacientes e, por conseguinte, auxiliar nas tomadas de decisão acerca do transplante hepático.

Guanosina: este é o terceiro estudo do grupo demonstrando efeitos neuroprotetores da guanosina em modelos de hiperamonemia e encefalopatia hepática. Estes dados se somam a outros trabalhos que demonstraram efeitos benéficos na modulação do sistema glutamatérgico no contexto de EH. Ademais, a guanosina vem demonstrando resultados positivos em modelos animais de outras patologias neurológicas, como depressão, isquemia e Doença de Alzheimer. Em nenhum destes estudos foram observados efeitos adversos importantes ou limitações significativas ao uso do medicamento. Acreditamos que ainda há avanços importantes a serem feitos em estudos experimentais sobre o uso da guanosina, em especial no entendimento de seu mecanismo de ação. Não obstante, tendo em vista o baixo risco do uso deste medicamento somado ao seu potencial de auxiliar no manejo de uma condição clínica severa como a insuficiência hepática aguda, acreditamos estar próximos da proposição de estudos translacionais para testar a segurança e eficiência deste medicamento na prática clínica.

Parte 4

10. Lista de Abreviaturas

ALF = *Acute Liver Failure*
AMPA = (receptor) alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico
ATP = Adenosina trifosfato
BBB = *Blood-Brain Barrier*
BCAA = *Branched-chain Amino Acids ou* Aminoácidos de Cadeia Ramificada
BDL = *Bile Duct Ligation*
BHE = Barreira Hematoencefálica
CSF = *Cerebrospinal Fluid*
DNA = *Deoxyribonucleic Acid*
EEG = Eletroencefalograma
EH = Encefalopatia Hepática
EUA = Estados Unidos da América
GLAST = *glutamate-aspartate transporter*
GLT-1 = *glutamate transporter-1*
GS = Glutamina Sintase
GUO = Guanosina / Guanosine
HAV = Hepatite A vírus
HBV = Hepatite B vírus
HE = *Hepatic Encephalopathy*
LCR = Líquido cefalorraquidiano
IHA = insuficiência hepática aguda
INR = *International Normalized Ratio*
i.p. = intraperitoneal
NMDA = (receptor) N-metil D-Aspartato
SG = Sistema Glutamatérgico
SNC = Sistema Nervoso Central
TCA = *Tricarboxylic acid cycle* ou Ciclo de Krebs ou ciclo do ácido tri carboxílico
UTI = Unidade de Terapia Intensiva

11. Palavras chave

Insuficiência Hepática Aguda; Encefalopatia Hepática; Hiperamonemia; Glutamato; Glutamina; Aminoácidos; Excitotoxicidade Glutamatérgica; Estresse oxidativo; Bioenergética; Metabolismo Energético Cerebral; Mitocôndria; Reatividade Astrocitária; Barreira Hematoencefálica; Albumina; Recaptação de Glutamato; Guanosina; Neuroproteção;

12. Referências

1. Bunchorntavakul, C. and K.R. Reddy, *Acute Liver Failure.* Clin Liver Dis, 2017. **21**(4): p. 769-792.
2. Bernal, W., et al., *Acute liver failure.* Lancet, 2010. **376**(9736): p. 190-201.
3. Bernal, W. and J. Wendon, *Acute liver failure.* N Engl J Med, 2013. **369**(26): p. 2525-34.
4. O'Grady, J.G., S.W. Schalm, and R. Williams, *Acute liver failure: redefining the syndromes.* Lancet, 1993. **342**(8866): p. 273-5.
5. Gotthardt, D., et al., *Fulminant hepatic failure: etiology and indications for liver transplantation.* Nephrol Dial Transplant, 2007. **22 Suppl 8**: p. viii5-viii8.
6. Ichai, P. and D. Samuel, *Epidemiology of liver failure.* Clin Res Hepatol Gastroenterol, 2011. **35**(10): p. 610-7.
7. Williams, R. and J. Wendon, *Indications for orthotopic liver transplantation in fulminant liver failure.* Hepatology, 1994. **20**(1 Pt 2): p. S5-10S.
8. Lee, W.M., et al., *Acute liver failure: Summary of a workshop.* Hepatology, 2008. **47**(4): p. 1401-15.
9. Brandsaeter, B., et al., *Fulminant hepatic failure: outcome after listing for highly urgent liver transplantation-12 years experience in the nordic countries.* Liver Transpl, 2002. **8**(11): p. 1055-62.
10. Kasem, A., et al., *Epidemiology of hepatitis E virus infection.* Epidemiol Mikrobiol Imunol, 2019. **68**(4): p. 176-182.
11. Weiler, N., et al., *The Epidemiology of Acute Liver Failure.* Dtsch Arztebl Int, 2020. **117**(4): p. 43-50.
12. Bower, W.A., et al., *Population-based surveillance for acute liver failure.* Am J Gastroenterol, 2007. **102**(11): p. 2459-63.
13. *Erratum: Nationwide Longitudinal Analysis of Acute Liver Failure in Taiwan: Erratum.* Medicine (Baltimore), 2017. **96**(44): p. e8577.
14. Thanapirom, K., et al., *The incidence, etiologies, outcomes, and predictors of mortality of acute liver failure in Thailand: a population-base study.* BMC Gastroenterol, 2019. **19**(1): p. 18.
15. Shalimar and S.K. Acharya, *Management in acute liver failure.* J Clin Exp Hepatol, 2015. **5**(Suppl 1): p. S104-15.
16. Seetharam, A., *Intensive Care Management of Acute Liver Failure: Considerations While Awaiting Liver Transplantation.* J Clin Transl Hepatol, 2019. **7**(4): p. 384-391.
17. Mendizabal, M. and M.O. Silva, *Liver transplantation in acute liver failure: A challenging scenario.* World J Gastroenterol, 2016. **22**(4): p. 1523-31.
18. Adam, R., et al., *Evolution of indications and results of liver transplantation in Europe. A report from the European Liver Transplant Registry (ELTR).* J Hepatol, 2012. **57**(3): p. 675-88.
19. Mishra, A. and V. Rustgi, *Prognostic Models in Acute Liver Failure.* Clin Liver Dis, 2018. **22**(2): p. 375-388.
20. Bernal, W., et al., *Acute liver failure: A curable disease by 2024?* J Hepatol, 2015. **62**(1 Suppl): p. S112-20.
21. American Association for the Study of Liver, D. and L. European Association for the Study of the, *Hepatic encephalopathy in chronic liver disease: 2014 practice guideline by the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases.* J Hepatol, 2014. **61**(3): p. 642-59.
22. Stinton, L.M. and S. Jayakumar, *Minimal hepatic encephalopathy.* Can J Gastroenterol, 2013. **27**(10): p. 572-4.

23. Wijdicks, E.F., *Hepatic Encephalopathy.* N Engl J Med, 2016. **375**(17): p. 1660-1670.
24. Weissenborn, K., *Hepatic Encephalopathy: Definition, Clinical Grading and Diagnostic Principles.* Drugs, 2019. **79**(Suppl 1): p. 5-9.
25. Saunders, J.B., et al., *A 20-year prospective study of cirrhosis.* Br Med J (Clin Res Ed), 1981. **282**(6260): p. 263-6.
26. Jepsen, P., et al., *Clinical course of alcoholic liver cirrhosis: a Danish population-based cohort study.* Hepatology, 2010. **51**(5): p. 1675-82.
27. Amodio, P., et al., *Prevalence and prognostic value of quantified electroencephalogram (EEG) alterations in cirrhotic patients.* J Hepatol, 2001. **35**(1): p. 37-45.
28. Stepanova, M., et al., *In-hospital mortality and economic burden associated with hepatic encephalopathy in the United States from 2005 to 2009.* Clin Gastroenterol Hepatol, 2012. **10**(9): p. 1034-41 e1.
29. Groeneweg, M., et al., *Screening of subclinical hepatic encephalopathy.* J Hepatol, 2000. **32**(5): p. 748-53.
30. Sharma, P., et al., *Critical flicker frequency: diagnostic tool for minimal hepatic encephalopathy.* J Hepatol, 2007. **47**(1): p. 67-73.
31. Ciecko-Michalska, I., et al., *Pathogenesis of hepatic encephalopathy.* Gastroenterol Res Pract, 2012. **2012**: p. 642108.
32. Gonzalez-Regueiro, J.A., et al., *Pathophysiology of hepatic encephalopathy and future treatment options.* Rev Gastroenterol Mex, 2019. **84**(2): p. 195-203.
33. Rimmele, T.S. and P.A. Rosenberg, *GLT-1: The elusive presynaptic glutamate transporter.* Neurochem Int, 2016. **98**: p. 19-28.
34. Danbolt, N.C., *Glutamate uptake.* Prog Neurobiol, 2001. **65**(1): p. 1-105.
35. Norenberg, M.D. and A. Martinez-Hernandez, *Fine structural localization of glutamine synthetase in astrocytes of rat brain.* Brain Res, 1979. **161**(2): p. 303-10.
36. Ott, P. and H. Vilstrup, *Cerebral effects of ammonia in liver disease: current hypotheses.* Metab Brain Dis, 2014. **29**(4): p. 901-11.
37. Felipo, V. and R.F. Butterworth, *Neurobiology of ammonia.* Prog Neurobiol, 2002. **67**(4): p. 259-79.
38. Montana, V., A. Verkhratsky, and V. Parpura, *Pathological role for exocytotic glutamate release from astrocytes in hepatic encephalopathy.* Curr Neuropharmacol, 2014. **12**(4): p. 324-33.
39. Hermenegildo, C., P. Monfort, and V. Felipo, *Activation of N-methyl-D-aspartate receptors in rat brain in vivo following acute ammonia intoxication: characterization by in vivo brain microdialysis.* Hepatology, 2000. **31**(3): p. 709-15.
40. Rodrigo, R., et al., *Role of NMDA receptors in acute liver failure and ammonia toxicity: therapeutical implications.* Neurochem Int, 2009. **55**(1-3): p. 113-8.
41. Monfort, P., et al., *Molecular mechanism of acute ammonia toxicity: role of NMDA receptors.* Neurochem Int, 2002. **41**(2-3): p. 95-102.
42. Monfort, P., et al., *Effects of hyperammonemia and liver failure on glutamatergic neurotransmission.* Metab Brain Dis, 2002. **17**(4): p. 237-50.
43. Rose, C., *Effect of ammonia on astrocytic glutamate uptake/release mechanisms.* J Neurochem, 2006. **97 Suppl 1**: p. 11-5.
44. Chan, H. and R.F. Butterworth, *Cell-selective effects of ammonia on glutamate transporter and receptor function in the mammalian brain.* Neurochem Int, 2003. **43**(4-5): p. 525-32.
45. Cittolin-Santos, G.F., et al., *Behavioral, Neurochemical and Brain Oscillation Abnormalities in an Experimental Model of Acute Liver Failure.* Neuroscience, 2019. **401**: p. 117-129.
46. Chan, H., et al., *Effects of ammonia on glutamate transporter (GLAST) protein and mRNA in cultured rat cortical astrocytes.* Neurochem Int, 2000. **37**(2-3): p. 243-8.

47. Knecht, K., et al., *Decreased glutamate transporter (GLT-1) expression in frontal cortex of rats with acute liver failure.* Neurosci Lett, 1997. **229**(3): p. 201-3.
48. Cittolin-Santos, G.F., et al., *Guanosine Exerts Neuroprotective Effect in an Experimental Model of Acute Ammonia Intoxication.* Mol Neurobiol, 2017. **54**(5): p. 3137-3148.
49. Fries, A.W., et al., *Effect of glutamine synthetase inhibition on brain and interorgan ammonia metabolism in bile duct ligated rats.* J Cereb Blood Flow Metab, 2014. **34**(3): p. 460-6.
50. Scott, T.R., et al., *Pathophysiology of cerebral oedema in acute liver failure.* World J Gastroenterol, 2013. **19**(48): p. 9240-55.
51. Vaquero, J. and R.F. Butterworth, *The brain glutamate system in liver failure.* J Neurochem, 2006. **98**(3): p. 661-9.
52. Bessman, S.P. and A.N. Bessman, *The cerebral and peripheral uptake of ammonia in liver disease with an hypothesis for the mechanism of hepatic coma.* J Clin Invest, 1955. **34**(4): p. 622-8.
53. Iversen, P., et al., *Low cerebral oxygen consumption and blood flow in patients with cirrhosis and an acute episode of hepatic encephalopathy.* Gastroenterology, 2009. **136**(3): p. 863-71.
54. Dam, G., et al., *Hepatic encephalopathy is associated with decreased cerebral oxygen metabolism and blood flow, not increased ammonia uptake.* Hepatology, 2013. **57**(1): p. 258-65.
55. Hindfelt, B., F. Plum, and T.E. Duffy, *Effect of acute ammonia intoxication on cerebral metabolism in rats with portacaval shunts.* J Clin Invest, 1977. **59**(3): p. 386-96.
56. Hindfelt, B. and B.K. Siesjo, *Cerebral effects of acute ammonia intoxication. II. The effect upon energy metabolism.* Scand J Clin Lab Invest, 1971. **28**(3): p. 365-74.
57. Mans, A.M., M.R. DeJoseph, and R.A. Hawkins, *Metabolic abnormalities and grade of encephalopathy in acute hepatic failure.* J Neurochem, 1994. **63**(5): p. 1829-38.
58. Lin, S. and W. Raabe, *Ammonia intoxication: effects on cerebral cortex and spinal cord.* J Neurochem, 1985. **44**(4): p. 1252-8.
59. Fitzpatrick, S.M., et al., *Effects of acute hyperammonemia on cerebral amino acid metabolism and pHi in vivo, measured by 1H and 31P nuclear magnetic resonance.* J Neurochem, 1989. **52**(3): p. 741-9.
60. Schousboe, A., et al., *Effects of hyperammonemia on brain energy metabolism: controversial findings in vivo and in vitro.* Metab Brain Dis, 2014. **29**(4): p. 913-7.
61. Obermeier, B., A. Verma, and R.M. Ransohoff, *The blood-brain barrier.* Handb Clin Neurol, 2016. **133**: p. 39-59.
62. Moretti, R., et al., *Blood-brain barrier dysfunction in disorders of the developing brain.* Front Neurosci, 2015. **9**: p. 40.
63. Liebner, S., et al., *Functional morphology of the blood-brain barrier in health and disease.* Acta Neuropathol, 2018. **135**(3): p. 311-336.
64. Skowronska, M. and J. Albrecht, *Alterations of blood brain barrier function in hyperammonemia: an overview.* Neurotox Res, 2012. **21**(2): p. 236-44.
65. Nguyen, J.H., et al., *Matrix metalloproteinase-9 contributes to brain extravasation and edema in fulminant hepatic failure mice.* J Hepatol, 2006. **44**(6): p. 1105-14.
66. Nguyen, J.H., *Blood-brain barrier in acute liver failure.* Neurochem Int, 2012. **60**(7): p. 676-83.
67. Wright, G., Y. Sharifi, and R. Jalan, *Blood-brain barrier in liver failure: are cracks appearing in the wall?* Liver Int, 2010. **30**(8): p. 1087-90.
68. Cui, W., C.M. Sun, and P. Liu, *Alterations of blood-brain barrier and associated factors in acute liver failure.* Gastroenterol Res Pract, 2013. **2013**: p. 841707.
69. Wilkinson, D.J., et al., *Absence of neuropsychological impairment in hyperammonaemia in healthy young adults; possible synergism in development of hepatic encephalopathy (HE) symptoms?* Metab Brain Dis, 2011. **26**(3): p. 203-12.

70. Butterworth, R.F., *Pathophysiology of hepatic encephalopathy: The concept of synergism.* Hepatol Res, 2008. **38 Suppl 1**: p. S116-21.
71. Butterworth, R.F., *Pathogenesis of hepatic encephalopathy in cirrhosis: the concept of synergism revisited.* Metab Brain Dis, 2016. **31**(6): p. 1211-1215.
72. Warrillow, S.J. and R. Bellomo, *Preventing cerebral oedema in acute liver failure: the case for quadruple-H therapy.* Anaesth Intensive Care, 2014. **42**(1): p. 78-88.
73. Bernal, W., et al., *Intensive care management of acute liver failure.* Semin Liver Dis, 2008. **28**(2): p. 188-200.
74. Zhu, Y., et al., *Protective effects of mild hypothermia against hepatic injury in rats with acute liver failure.* Ann Hepatol, 2019. **18**(5): p. 770-776.
75. Butterworth, R.F., *Mild hypothermia prevents cerebral edema in acute liver failure.* J Hepatobiliary Pancreat Surg, 2001. **8**(1): p. 16-9.
76. Rose, C., et al., *Mild hypothermia delays the onset of coma and prevents brain edema and extracellular brain glutamate accumulation in rats with acute liver failure.* Hepatology, 2000. **31**(4): p. 872-7.
77. Jalan, R., et al., *Moderate hypothermia in patients with acute liver failure and uncontrolled intracranial hypertension.* Gastroenterology, 2004. **127**(5): p. 1338-46.
78. Bernal, W., et al., *A multicentre randomized controlled trial of moderate hypothermia to prevent intracranial hypertension in acute liver failure.* J Hepatol, 2016. **65**(2): p. 273-9.
79. Romero-Gomez, M., S. Montagnese, and R. Jalan, *Hepatic encephalopathy in patients with acute decompensation of cirrhosis and acute-on-chronic liver failure.* J Hepatol, 2015. **62**(2): p. 437-47.
80. Said, V.J. and E. Garcia-Trujillo, *Beyond Lactulose: Treatment Options for Hepatic Encephalopathy.* Gastroenterol Nurs, 2019. **42**(3): p. 277-285.
81. Amodio, P., *Hepatic encephalopathy: Diagnosis and management.* Liver Int, 2018. **38**(6): p. 966-975.
82. Leise, M.D., et al., *Management of hepatic encephalopathy in the hospital.* Mayo Clin Proc, 2014. **89**(2): p. 241-53.
83. Gluud, L.L., H. Vilstrup, and M.Y. Morgan, *Nonabsorbable disaccharides for hepatic encephalopathy: A systematic review and meta-analysis.* Hepatology, 2016. **64**(3): p. 908-22.
84. Patidar, K.R. and J.S. Bajaj, *Antibiotics for the treatment of hepatic encephalopathy.* Metab Brain Dis, 2013. **28**(2): p. 307-12.
85. Sharma, B.C., et al., *A randomized, double-blind, controlled trial comparing rifaximin plus lactulose with lactulose alone in treatment of overt hepatic encephalopathy.* Am J Gastroenterol, 2013. **108**(9): p. 1458-63.
86. Zhu, G.Q., et al., *Systematic review with network meta-analysis: the comparative effectiveness and safety of interventions in patients with overt hepatic encephalopathy.* Aliment Pharmacol Ther, 2015. **41**(7): p. 624-35.
87. Gluud, L.L., et al., *Branched-chain amino acids for people with hepatic encephalopathy.* Cochrane Database Syst Rev, 2017. **5**: p. CD001939.
88. Sharma, P., et al., *An open-label randomized controlled trial of lactulose and probiotics in the treatment of minimal hepatic encephalopathy.* Eur J Gastroenterol Hepatol, 2008. **20**(6): p. 506-11.
89. Prasad, S., et al., *Lactulose improves cognitive functions and health-related quality of life in patients with cirrhosis who have minimal hepatic encephalopathy.* Hepatology, 2007. **45**(3): p. 549-59.
90. Sidhu, S.S., et al., *Rifaximin improves psychometric performance and health-related quality of life in patients with minimal hepatic encephalopathy (the RIME Trial).* Am J Gastroenterol, 2011. **106**(2): p. 307-16.

91. Bajaj, J.S., et al., *Rifaximin improves driving simulator performance in a randomized trial of patients with minimal hepatic encephalopathy.* Gastroenterology, 2011. **140**(2): p. 478-487 e1.
92. Nardone, R., et al., *Minimal hepatic encephalopathy: A review.* Neurosci Res, 2016. **111**: p. 1-12.
93. Vinade, E.R., et al., *Effects of chronic administered guanosine on behavioral parameters and brain glutamate uptake in rats.* J Neurosci Res, 2005. **79**(1-2): p. 248-53.
94. Schmidt, A.P., et al., *Guanosine prevents thermal hyperalgesia in a rat model of peripheral mononeuropathy.* J Pain, 2010. **11**(2): p. 131-41.
95. Schmidt, A.P., et al., *Guanosine and GMP prevent seizures induced by quinolinic acid in mice.* Brain Res, 2000. **864**(1): p. 40-3.
96. Chang, R., et al., *Neuroprotective effects of guanosine on stroke models in vitro and in vivo.* Neurosci Lett, 2008. **431**(2): p. 101-5.
97. Hansel, G., et al., *Guanosine Protects Against Cortical Focal Ischemia. Involvement of Inflammatory Response.* Mol Neurobiol, 2015. **52**(3): p. 1791-1803.
98. Su, C., et al., *Guanosine improves motor behavior, reduces apoptosis, and stimulates neurogenesis in rats with parkinsonism.* J Neurosci Res, 2009. **87**(3): p. 617-25.
99. Lanznaster, D., et al., *Guanosine: a Neuromodulator with Therapeutic Potential in Brain Disorders.* Aging Dis, 2016. **7**(5): p. 657-679.
100. Cauli, O., et al., *Acute liver failure-induced death of rats is delayed or prevented by blocking NMDA receptors in brain.* Am J Physiol Gastrointest Liver Physiol, 2008. **295**(3): p. G503-11.
101. Cauli, O., et al., *Blocking NMDA receptors delays death in rats with acute liver failure by dual protective mechanisms in kidney and brain.* Neuromolecular Med, 2014. **16**(2): p. 360-75.
102. Jambekar, A.A., et al., *A glutamine synthetase inhibitor increases survival and decreases cytokine response in a mouse model of acute liver failure.* Liver Int, 2011. **31**(8): p. 1209-21.
103. Paniz, L.G., et al., *Neuroprotective effects of guanosine administration on behavioral, brain activity, neurochemical and redox parameters in a rat model of chronic hepatic encephalopathy.* Metab Brain Dis, 2014. **29**(3): p. 645-54.
104. Fiati Kenston, S.S., et al., *Mechanistic insight, diagnosis, and treatment of ammonia-induced hepatic encephalopathy.* J Gastroenterol Hepatol, 2019. **34**(1): p. 31-39.
105. Stravitz, R.T. and F.S. Larsen, *Therapeutic hypothermia for acute liver failure.* Crit Care Med, 2009. **37**(7 Suppl): p. S258-64.
106. Larsen, F.S. and J. Wendon, *Prevention and management of brain edema in patients with acute liver failure.* Liver Transpl, 2008. **14 Suppl 2**: p. S90-6.
107. Rabinowich, L., et al., *Clinical management of acute liver failure: Results of an international multi-center survey.* World J Gastroenterol, 2016. **22**(33): p. 7595-603.
108. Norenberg, M.D., *The role of astrocytes in hepatic encephalopathy.* Neurochem Pathol, 1987. **6**(1-2): p. 13-33.
109. Butterworth, R.F., *Altered glial-neuronal crosstalk: cornerstone in the pathogenesis of hepatic encephalopathy.* Neurochem Int, 2010. **57**(4): p. 383-8.
110. Agarwal, A.N. and D.D. Mais, *Sensitivity and Specificity of Alzheimer Type II Astrocytes in Hepatic Encephalopathy.* Arch Pathol Lab Med, 2019. **143**(10): p. 1256-1258.
111. Jayakumar, A.R. and M.D. Norenberg, *Endothelial-astrocytic interactions in acute liver failure.* Metab Brain Dis, 2013. **28**(2): p. 183-6.
112. Guazzelli, P.A., et al., *Acute Liver Failure Induces Glial Reactivity, Oxidative Stress and Impairs Brain Energy Metabolism in Rats.* Front Mol Neurosci, 2019. **12**: p. 327.
113. Alman, R.W., et al., *Cerebral metabolism in hepatic insufficiency.* Am J Med, 1956. **21**(6): p. 843-9.

114. Strauss, G.I., et al., *Cerebral glucose and oxygen metabolism in patients with fulminant hepatic failure.* Liver Transpl, 2003. **9**(12): p. 1244-52.
115. Holmin, T., et al., *The influence of total hepatectomy on cerebral energy state, ammonia-related amino acids of the brain and plasma amino acids in the rat.* Eur J Clin Invest, 1983. **13**(3): p. 215-20.
116. Kosenko, E., et al., *Nitroarginine, an inhibitor of nitric oxide synthase, prevents changes in superoxide radical and antioxidant enzymes induced by ammonia intoxication.* Metab Brain Dis, 1998. **13**(1): p. 29-41.
117. Gorg, B., F. Schliess, and D. Haussinger, *Osmotic and oxidative/nitrosative stress in ammonia toxicity and hepatic encephalopathy.* Arch Biochem Biophys, 2013. **536**(2): p. 158-63.
118. Murthy, C.R., et al., *Ammonia-induced production of free radicals in primary cultures of rat astrocytes.* J Neurosci Res, 2001. **66**(2): p. 282-8.
119. Kosenko, E., et al., *Superoxide production and antioxidant enzymes in ammonia intoxication in rats.* Free Radic Res, 1997. **27**(6): p. 637-44.
120. Warskulat, U., et al., *Ammonia-induced heme oxygenase-1 expression in cultured rat astrocytes and rat brain in vivo.* Glia, 2002. **40**(3): p. 324-36.
121. Ulm, J.W., et al., *Complex determinants within the Moloney murine leukemia virus capsid modulate susceptibility of the virus to Fv1 and Ref1-mediated restriction.* Virology, 2007. **363**(2): p. 245-55.
122. Jayakumar, A.R., et al., *Glutamine in the mechanism of ammonia-induced astrocyte swelling.* Neurochem Int, 2006. **48**(6-7): p. 623-8.
123. Jubin, T., et al., *The PARP family: insights into functional aspects of poly (ADP-ribose) polymerase-1 in cell growth and survival.* Cell Prolif, 2016. **49**(4): p. 421-37.
124. Eustermann, S., et al., *The DNA-binding domain of human PARP-1 interacts with DNA single-strand breaks as a monomer through its second zinc finger.* J Mol Biol, 2011. **407**(1): p. 149-70.
125. Di Pardo, A., et al., *Assessment of Blood-brain Barrier Permeability by Intravenous Infusion of FITC-labeled Albumin in a Mouse Model of Neurodegenerative Disease.* J Vis Exp, 2017(129).
126. Chastre, A., et al., *Lipopolysaccharide precipitates hepatic encephalopathy and increases blood-brain barrier permeability in mice with acute liver failure.* Liver Int, 2014. **34**(3): p. 353-61.
127. Vergara, F., F. Plum, and T.E. Duffy, *Alpha-ketoglutaramate: increased concentrations in the cerebrospinal fluid of patients in hepatic coma.* Science, 1974. **183**(4120): p. 81-3.
128. Oei, L.T., et al., *Cerebrospinal fluid glutamine levels and EEG findings in patients with hepatic encephalopathy.* Clin Neurol Neurosurg, 1979. **81**(1): p. 59-63.
129. Record, C.O., et al., *Plasma and brain amino acids in fulminant hepatic failure and their relationship to hepatic encephalopathy.* Eur J Clin Invest, 1976. **6**(5): p. 387-94.
130. Holecek, M., *Ammonia and amino acid profiles in liver cirrhosis: effects of variables leading to hepatic encephalopathy.* Nutrition, 2015. **31**(1): p. 14-20.
131. Socha, E., M. Koba, and P. Koslinski, *Amino acid profiling as a method of discovering biomarkers for diagnosis of neurodegenerative diseases.* Amino Acids, 2019. **51**(3): p. 367-371.
132. Whitehead, T.P. and S.R. Whittaker, *A method for the determination of glutamine in cerebrospinal fluid and the results in hepatic coma.* J Clin Pathol, 1955. **8**(1): p. 81-4.
133. Hourani, B.T., E.M. Hamlin, and T.B. Reynolds, *Cerebrospinal fluid glutamine as a measure of hepatic encephalopathy.* Arch Intern Med, 1971. **127**(6): p. 1033-6.
134. Fraser, C.L. and A.I. Arieff, *Hepatic encephalopathy.* N Engl J Med, 1985. **313**(14): p. 865-73.

135. Cudalbu, C. and S.D. Taylor-Robinson, *Brain Edema in Chronic Hepatic Encephalopathy.* J Clin Exp Hepatol, 2019. **9**(3): p. 362-382.
136. Chavarria, L., et al., *Brain magnetic resonance spectroscopy in episodic hepatic encephalopathy.* J Cereb Blood Flow Metab, 2013. **33**(2): p. 272-7.
137. Helling, G., et al., *Plasma Glutamine Concentrations in Liver Failure.* PLoS One, 2016. **11**(3): p. e0150440.
138. Fonnum, F., *Glutamate: a neurotransmitter in mammalian brain.* J Neurochem, 1984. **42**(1): p. 1-11.
139. Ozkan, E.D. and T. Ueda, *Glutamate transport and storage in synaptic vesicles.* Jpn J Pharmacol, 1998. **77**(1): p. 1-10.
140. Trotti, D., N.C. Danbolt, and A. Volterra, *Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration?* Trends Pharmacol Sci, 1998. **19**(8): p. 328-34.
141. Moretto, M.B., et al., *Hypoxic-ischemic insult decreases glutamate uptake by hippocampal slices from neonatal rats: prevention by guanosine.* Exp Neurol, 2005. **195**(2): p. 400-6.
142. Frizzo, M.E., et al., *Guanosine enhances glutamate transport capacity in brain cortical slices.* Cell Mol Neurobiol, 2005. **25**(5): p. 913-21.
143. Dal-Cim, T., et al., *Guanosine prevents oxidative damage and glutamate uptake impairment induced by oxygen/glucose deprivation in cortical astrocyte cultures: involvement of A1 and A2A adenosine receptors and PI3K, MEK, and PKC pathways.* Purinergic Signal, 2019. **15**(4): p. 465-476.
144. Frizzo, M.E., et al., *Extracellular conversion of guanine-based purines to guanosine specifically enhances astrocyte glutamate uptake.* Brain Res, 2003. **972**(1-2): p. 84-9.