Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

EFEITOS NEUROPROTETORES DA GUANOSINA E DA INOSINA FRENTE ÀS AÇÕES NEUROTÓXICAS DA ISQUEMIA CEREBRAL *IN VIVO*

TESE DE DOUTORADO

Gisele Hansel

Porto Alegre, maio de 2014

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(Orientador)

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"Qualquer tecnologia suficientemente avançada é

indistinguível da magia"

A. C. Clarke

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SUMÁRIO

APRESENTAÇÃO

Esta tese está organizada em tópicos: Introdução, Objetivos, Capítulos (1 a 3, referente aos artigos científicos), Discussão, Conclusões, Perspectivas e Bibliografia.

A Introdução apresenta o embasamento teórico, que nos levou a formular a proposta de trabalho. O objetivo geral e os objetivos específicos estão dispostos no corpo da tese, e, especificamente, dentro de cada capítulo. Os capítulos contêm os artigos científicos, os quais foram organizados como resposta aos objetivos propostos. Todos os trabalhos foram desenvolvidos no Departamento de Bioquímica – ICBS – UFRGS; Centro de Ciências da Vida e da Saúde – UCPel; Laboratório de Investigação Medica da Faculdade de Medicina – USP; e Laboratório de Análises Clínicas – LABMED, Santa Maria – RS.

A seção Discussão contém uma interpretação geral dos resultados obtidos nos diferentes trabalhos. Os tópicos seguintes, Conclusões e Perspectivas, abordam as conclusões gerais da tese, bem como, possibilidades de futuros trabalhos a partir dos resultados descritos.

A seção Bibliografia lista as referências citadas na Introdução e Discussão. As referências utilizadas nos diferentes artigos estão listadas ao final de cada trabalho. Os resultados desta tese de doutorado estão apresentados sob a forma de artigos científicos. As seções Materiais e Métodos, Resultados, Discussão e Referências bibliográficas encontram-se nos próprios artigos.

LISTA DE ABREVIATURAS

A_1 – receptor de adenosina A1

A_{2A} – receptor de adenosina A_{2A}

ACM– artéria cerebral média

ADP – adenosina difosfato

AKT/PKB – proteína cinase B

AMP – adenosina monofosfato

AMPA – α-amino-3-hidroxi-5-metil-4-isoxazol-propionato

AMPc – adenosina monofosfato cíclico

ATP – adenosina trifosfato

AVE – acidente vascular encefálico

BDNF – fator neurotrófico derivado do encéfalo

Ca^{2+} – cálcio

CAT – catalase

Cl^- – cloro

COX-2 – ciclooxigenase 2

DNA – ácido desoxiribonucléico

EAAT – transportador de aminoácidos excitatórios

EAAT1/GLAST – transportador de glutamato e aspartato

EAAT2/GLT1 – transportador de glutamato 1

EAAT3/EAAC1– transportador de aminoácido excitatório 1

ERN – espécies reativas de nitrogênio

ERO – espécies reativas de oxigênio

FJC – fluoro jade C

GDP – guanosina difosfato

GMP – guanosina monofosfato

GMPc – guanosina monofosfato cíclico

GPx – glutationa peroxidase

GS – glutamina sintetase

GSH – glutationa reduzida

GTP – guanosina trifosfato

iGLUR – receptores ionotrópicos glutamatérgicos

IL-1 – interleucina 1

IL-1 β – interleucina 1beta

IL-10 – interleucina 10

IL-6 – interleucina 6

IL-8 – interleucina 8

IMP – inosina monofosfato

INF-γ – interferon gama

IP – iodeto de propídio

K^+ – potássio

KA – ácido caínico

LCR – líquido cefalorraquidiano

MAPK – proteína cinase ativadora de mitose

mGLUR – receptores metabotrópicos glutamatérgicos

Na^+ – sódio

Na^+-K^+- ATPase – sódio-potássio adenosina trifosfatase

NGF – fator de crescimento do nuclear

NMDA – N-metil-D-aspartato

NSE – enolase neurônio sensível

OMS – Organização Mundial da Saúde

PI3K – fofoinositol-3 cinase

POG – privação de oxigênio e glicose

PTZ – pentilenotetrazol

RNA – ácido ribonucléico

SNC – sistema nervoso central

SOD – superóxido dismutase

TNF- α – fator de necrose tumoral

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PARTE I

RESUMO

A isquemia cerebral é uma doença grave, sendo a segunda causa mais comum de morte e a principal causa de incapacidade em todo o mundo. A redução repentina do fluxo sanguíneo cerebral leva à diminuição do fornecimento de oxigênio e de glicose, resultando em uma falha no metabolismo energético cerebral. Este desequilíbrio no metabolismo energético é claramente o elemento chave no processo isquêmico, resultando em danos celulares e comprometimento das funções neurológicas. A excitotoxicidade glutamatérgica, o estresse oxidativo e processo inflamatório desempenham papéis importantes na lesão cerebral isquêmica, levando a danos teciduais que comprometem a integridade do tecido durante a isquemia. A guanosina e a inosina são conhecidas por desempenhar um papel neuroproteção ao sistema nervoso central, agindo como um modulador negativo do sistema glutamatérgico e possuindo efeitos tróficos em células neurais. Desta forma, nesta tese, avaliaram-se diversos mecanismos que são modulados pela guanosina e inosina em modelos experimentais de isquemia cerebral *in vivo*. Inicialmente, demonstrou-se que a guanosina é efetiva na neuroproteção contra a isquemia cerebral focal em ratos, causando redução de danos neuronais e astrogliais, diminuindo a peroxidação lipídica e o volume de enfarte cerebral e, consequentemente, recuperando a função motora do membro anterior debilitado pela isquemia. Esta neuroproteção estaria envolvida na manutenção do ambiente redox celular, na modulação da resposta inflamatória e na modulação do sistema glutamatérgico, mecanismos ligados à lesão isquêmica. A isquemia cerebral causou um aumento do número total de células micróglias e também uma maior ativação destas células, efeito inibido pela administração de guanosina. Nesta tese, também investigamos os efeitos agudos relacionados à neuroproteção da administração de guanosina e de inosina como reposição volêmica em um modelo de choque hemorrágico (que, potencialmente, diminui a perfusão sanguínea cerebral) em suínos. A guanosina e a inosina foram capazes de diminuir os níveis de glutamato mais rapidamente do que o controle e de modular o ambiente de citocinas pró-inflamatórias, diminuindo os níveis de IL-1β e TNF-α (apenas inosina) após choque hemorrágico. Esta supressão pode estar associada com diminuição na morte neuronal tardia, o que implicaria em uma melhora no prejuízo cognitivo que ocorre choque hemorrágico. No geral, nosso trabalho representa uma importante contribuição para o conhecimento sobre os possíveis mecanismos neuroprotetores da guanosina e da inosina em modelos de isquemia cerebral.

ABSTRACT

Cerebral ischemia is a devastating disease, being the second most common cause of death and the major cause of disability worldwide. The sudden reduction in cerebral blood flow leads to decreased oxygen and glucose supplies, resulting in a failure of cellular bioenergetics. Disruption of brain energetics metabolism is clearly a key element in stroke, resulting in cellular damage and impairment of neurological functions. Glutamate excitotoxicity, oxidative stress and neuroinflammation play important roles in ischemic brain injury, with harmful impacts on ischemic cerebral tissue. As guanosine and inosine play an important neuroprotective role in the central nervous system, exerting glutamatergic system antagonism and trophic effects on neural cells, in this study, it was evaluated the neuroprotective effects of guanosine and inosine against *in vivo* cerebral ischemia models. Initially, we demonstrated that guanosine was neuroprotective against cerebral focal ischemia in rats causing reduction of neuronal and astroglial damage, decreasing lipid peroxidation and cerebral infarct volume, and consequently recovery in the function of impaired forelimb. These neuroprotection could be involved in the maintenance of the cellular redox environment, modulating the inflammatory response and the glutamatergic systems. Furthermore, ischemia increased the total number of microglial cells, and changed the morphological characteristics. These effects were inhibited by guanosine treatment. Additionally, it was also investigated the acute neuroprotective effects of guanosine and inosine as a fluid resuscitation in a model of hemorrhagic shock in swines. The treatment with guanosine or inosine was able to decrease glutamate levels faster than control group and also was able to modulate the proinflammatory cytokines environment, decreasing IL-1β and TNF-α (only inosine) levels after hemorrhagic shock. These effects could be associated with reduction delayed neuronal cell damage, improving cognitive impairment that occurs in hemorrhagic shock. Overall, our work represents an important contribution to the knowledge regarding the putative neuroprotective mechanisms of guanosine and inosine in cerebral ischemia models.

1. INTRODUÇÃO

1.1.Isquemia Cerebral

A organização mundial da Saúde (OMS) define como isquemia cerebral, a perturbação focal ou global da função cerebral, causada pela falta de suprimento sanguíneo a alguma região do tecido cerebral, tendo como origem nenhuma outra causa aparente que não seja a origem vascular (1988). O sistema nervoso central (SNC) necessita de um contínuo suprimento de oxigênio e glicose para o seu metabolismo, pois a bioenergética cerebral normal tem algumas características especiais, que incluem uma taxa metabólica alta, estoques de energia limitados e uma grande dependência do metabolismo aeróbio da glicose. Desta forma, um desequilíbrio tanto na taxa de fluxo sanguíneo como no conteúdo arterial de oxigênio pode afetar gravemente a função cerebral e ocasionar alterações bioquímicas e moleculares consideráveis, resultando em danos celulares e perda das funções neurológicas (Lipton 1999; Donnan, Fisher et al. 2008; Brouns and De Deyn 2009). As causas mais comuns de interrupção transitória ou permanente do fluxo sanguíneo são: trombose, embolia, hemorragia, ataque cardíaco, choque hemorrágico (Donnan, Fisher et al. 2008; Brouns and De Deyn 2009; Go, Mozaffarian et al. 2014).

No Brasil, segundo o Ministério da Saúde, a isquemia cerebral é a principal causa de morte, com 70.232 óbitos registrados em 2008. Em abril de 2012, o Ministério da Saúde lançou a Portaria n° 664 que regulariza o “Protocolo Clínico e Diretrizes Terapêuticas – Trombólise no Acidente Vascular Encefálico Isquêmico Agudo”, que objetiva aprimorar a assistência aos pacientes com essa doença. Sua intenção é orientar a conduta dos profissionais de saúde sobre diagnóstico e tratamento clínico, além de estabelecer procedimentos para a assistência aos pacientes nos hospitais (2012). Por causa desse enorme fardo socioeconômico absorvendo 6% de todos os orçamentos de saúde, e com o aumento da expectativa de vida no mundo, as doenças cerebrovasculares são consideradas questão de saúde pública com alto

impacto social (Durukan and Tatlisumak 2007; Go, Mozaffarian et al. 2014). Neste sentido, novas estratégias terapêuticas necessitam ser desenvolvidas e testadas na tentativa de reduzir custos, melhorar a eficácia dos tratamentos e diminuir os efeitos adversos.

1.1.1. Isquemia Cerebral Focal

O acidente vascular encefálico (AVE) é responsável por 9 % de todas as mortes, sendo a segunda causa mais comum de morte e a principal causa de incapacidade no mundo. A maior prevalência é do AVE isquêmico, com 87 %, que ocorre a interrupção do fluxo sanguíneo através da formação de trombos ou por processo embólico. No AVE hemorrágico, ocorre a ruptura do vaso sanguíneo ocorrendo o extravasamento sanguíneo, destes, 10 % ocorrem por hemorragias intracerebrais e apenas 3 % por hemorragias subaracnóideas (Donnan, Fisher et al. 2008; Go, Mozaffarian et al. 2014). Devido ao envelhecimento da população, a incidência de AVE tende a aumentar nos próximos 20 anos, especialmente nos países em desenvolvimento (Donnan, Fisher et al. 2008; Go, Mozaffarian et al. 2014). No AVE isquêmico, a artéria mais comumente ocluída é a artéria cerebral media (ACM) ou suas ramificações profundas (Stokes 2004). A lesão no tecido cerebral ocasionada pela isquemia depende de dois fatores: da intensidade e da duração da isquemia. O AVE possui duas regiões específicas, o núcleo isquêmico, também chamado de “core”, e a penumbra. O core é região central isquêmica, onde ocorre uma redução abrupta do fluxo sanguíneo (somente 10 a 15 % do fluxo sanguíneo normal). Essa região sofre uma carência bioenergética e um desequilíbrio iônico muito severo, levando as células a danos irreversíveis. A penumbra é a região que se encontra entre o tecido sadio e a região do core. Desta forma, a penumbra recebe um aporte sanguíneo colateral suficiente para suprir as atividades celulares vitais, mas devido ao decréscimo deste fluxo sanguíneo, possui sua funcionalidade prejudicada. A penumbra é uma região dinâmica e se não houver nenhuma intervenção endógena ou exógena a fim de atenuar

os efeitos da isquemia, suas células perdem a viabilidade, acarretando a expansão da região do core. Desta forma, a lesão isquêmica progride continuamente ao longo de horas ou até mesmo dias (Lipton 1999; Donnan, Fisher et al. 2008; Brouns and De Deyn 2009; Heiss 2010). Tendo como alvo a região da penumbra, é possível intervir e buscar alternativas, como novas drogas, e também estudar mecanismos de ação com o intuito de proteger o tecido cerebral após a isquemia.

Atualmente, o único procedimento terapêutico com benefício comprovado na clínica da doença é a administração via endovenosa do ativador do plasminogênio tecidual. Por ter uma ação trombolítica, este medicamento é indicado somente em casos de AVE isquêmico e sua janela terapêutica é de até 4,5 h após o evento isquêmico. Também como medida paliativa, é utilizada a cirurgia descompressiva em alguns casos de edema cerebral (Go, Mozaffarian et al. 2014). Neste sentido, novas estratégias terapêuticas e farmacológicas juntamente com estudos que desvendem os eventos moleculares associados à morte celular causada pela hipóxia-isquemia, são necessários na tentativa de melhorar a eficácia de procedimentos, aumentar o tempo da janela terapêutica, e reduzir os efeitos adversos ocasionados pela isquemia.

1.1.2. Isquemia Cerebral Global

A isquemia global diferentemente da isquemia focal, não possui duas regiões distintas, porque o fluxo sanguíneo é interrompido de forma geral e abrupta, afetando o SNC como um todo (Lipton 1999; Zemke, Smith et al. 2004). A isquemia cerebral global é secundária a outras condições clínicas, como a parada cardíaca, cirurgias de grande porte que bloqueiam a circulação sanguínea, asfixia e choques hemorrágicos provenientes de traumas (Salazar, Wityk et al. 2001; Nussmeier 2002; Allen and Buckberg 2012). Além de prejuízo aos outros órgãos devido à hipoperfusão sistêmica, as sequelas neurológicas da lesão cerebral são

variadas e constituem um amplo espectro que inclui coma, convulsões e disfunção cognitiva (Murkin 1999). Na isquemia global, a interrupção do fluxo sanguíneo leva a uma falha energética no metabolismo e a um desequilíbrio iônico do SNC que, se não for revertida, leva à morte celular. O neurônio é a célula que mais sofre com a falha energética na isquemia global, sendo que no SNC existem regiões mais sensíveis ao dano, como por exemplo, os neurônios piramidais de da região CA1 do hipocampo, neurônios do córtex e estriado, além de células de Purkinje do cerebelo. Essa sensibilidade é variada e depende da duração e da gravidade da isquemia (Lipton 1999).

1.2. Mecanismos envolvidos na isquemia cerebral

A lesão isquêmica leva a uma série de eventos complexos e interligados, os quais são denominados de "cascata isquêmica". A cascata isquêmica é uma sequência complexa de eventos patofisiológicos espaciais e temporais, que apresentam importantes interrelações, perdurando por horas ou dias (Durukan and Tatlisumak 2007; Brouns and De Deyn 2009).

O SNC possui um elevado consumo de oxigênio e de glicose e depende quase exclusivamente de fosforilação oxidativa, formando adenosina trifosfato (ATP) para a utilização de energia. A hipoperfusão ocasiona diminuição do aporte de oxigênio (hipóxia) e glicose durante a isquemia, leva a uma falha na bioenergética cerebral e um desequilíbrio iônico (Hertz 2008; Brouns and De Deyn 2009). A falta de glicose e oxigênio resulta no acúmulo de lactato formado através da glicólise anaeróbica, o que causa uma acidose metabólica, levando a possíveis danos secundários (Lipton 1999; Hertz 2008). Essa deficiência energética provoca a inibição da enzima Na^+-K^+-ATPase, causando grande perda dos gradientes iônicos, pois há a elevação dos níveis extracelulares de potássio (K^+) e intracelulares de sódio (Na^+), cálcio (Ca^{2+}) e cloreto (Cl^-), conduzindo assim ao edema celular (Phan, Wright et al. 2002; Hertz 2008), alteração do potencial de membrana e a consequente

despolarização dos neurônios e também de células gliais. A despolarização neuronal ocasiona a liberação de grande quantidade de neurotransmissores (Brouns and De Deyn 2009).

A despolarização neuronal acarreta o aumento de liberação de neurotransmissores, principalmente, o aminoácido excitatório glutamato (Choi and Rothman 1990; Fei, Zhang et al. 2007). O glutamato é o principal neurotransmissor excitatório no sistema nervoso central e está envolvido em várias funções do cérebro, tais como a aprendizagem e a memória (Segovia, Porras et al. 2001; Izquierdo, Bevilaqua et al. 2006; Stevens 2008). Contudo, o acúmulo excessivo de glutamato extracelular leva à morte neuronal e está envolvida na patofisiologia de danos cerebrais isquêmicos (Durukan and Tatlisumak 2007; Brouns and De Deyn 2009).

Como o glutamato não é metabolizado no ambiente extracelular, a manutenção da neurotransmissão glutamatérgica normal ocorre pela presença de transportadores de aminoácidos excitatórios (EAAT), tanto nas células gliais, como nos neurônios (Danbolt 2001). Cinco isoformas de transportadores de glutamato já foram identificados: EAAT1, também chamado de transportador de glutamato e aspartato (GLAST); EAAT2, chamado de transportador de glutamato-1 (GLT1) - ambos encontrados principalmente em astrócitos; EAAT3, conhecido também como carreados de aminoácido excitatório-1 (EAAC1); EAAT4, principalmente encontrado nos neurônios; e o EAAT5 (Danbolt 2001; Had-Aissouni 2012). Vários estudos têm demonstrado que os transportadores GLT1 e GLAST desempenham um papel importante na manutenção fisiológica dos níveis de glutamato extracelular, consistindo em elemento crucial na proteção de neurônios contra a excitotoxicidade (Rothstein, Dykes-Hoberg et al. 1996). Com a falha energética e o desequilíbrio iônico causados pela isquemia, a captação de glutamato é prejudicada, podendo acarretar em um fluxo reverso, chamado de transporte reverso, que causo um aumento ainda mais acentuado dos níveis de glutamato extracelular (Malarkey and Parpura 2008). Os níveis excessivos de glutamato na fenda

sináptica, causados tanto pela despolarização neuronal quanto pela falha na captação desse glutamato, conduz à hiperativação dos receptores de glutamato, principalmente dos receptores ionotrópicos NMDA e AMPA (Romera, Hurtado et al. 2004). Essa hiperativação leva a um aumento do influxo de Ca^{2+} e Na^{+}, acarretando a ativação de diversas vias de sinalização, de genes associados à morte celular, bem como uma excessiva produção de radicais livres (Durukan and Tatlisumak 2007; Brouns and De Deyn 2009; Zadori, Klivenyi et al. 2012).

O SNC possui um sistema de defesa antioxidante eficaz, composto por enzimas antioxidantes, incluindo superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx). Estas enzimas em conjunto com antioxidantes não enzimáticos, como, por exemplo, a glutationa reduzida (GSH) e a vitamina C e vitamina E, são responsáveis pela inativação de radicais livres, evitando assim o dano oxidativo (Coyle and Puttfarcken 1993; Crack and Taylor 2005). Durante um evento isquêmico o excesso de produção de espécies reativas de oxigênio (ERO) e espécies reativas de nitrogênio (ERN) causa uma ineficiência enzimática e um esgotamento de moléculas que agem na remoção dos radicais livres (Brown 2010; Chen, Yoshioka et al. 2011; May 2012). Esse desequilíbrio do sistema antioxidante desencadeia diversos eventos celulares e moleculares, incluindo a oxidação/nitrosylation/nitração de proteínas, peroxidação lipídica e dano ao DNA, resultando em danos às macromoléculas e consequente ativação de mecanismos de sinalização que levam à morte celular (Nanetti, Raffaelli et al. 2011; Olmez and Ozyurt 2012).

A resposta inflamatória também está relacionada com a lesão isquêmica. A isquemia ativa moléculas de sinalização (citocinas, quimiocinas), moléculas de adesão e moléculas de reguladores da transcrição, amplificando o sinal celular, que desencadeia um maior dano celular e também o rompimento da barreira hemato-encefálica, o que contribui para a progressão do dano (Kaushal and Schlichter 2008). O rompimento da barreira hemato-

encefálica conduz à infiltração de células inflamatórias periféricas no SNC como granulócitos, polimorfonucleares e monócitos/macrófagos na região isquêmica cerebral (Iadecola and Anrather 2011). A resposta inflamatória frente à isquemia também causa a ativação de astrócitos e de células microgliais (Iadecola and Anrather 2011). Em função de todas essas modificações e da ativação de enzimas que danificam a estrutura das membranas celulares, ocorre um desbalanço na homeostase celular e, finalmente, morte neuronal (Figura 1) (Lipton 1999; Brouns and De Deyn 2009; Lakhan, Kirchgessner et al. 2009; Wu and Grotta 2013).

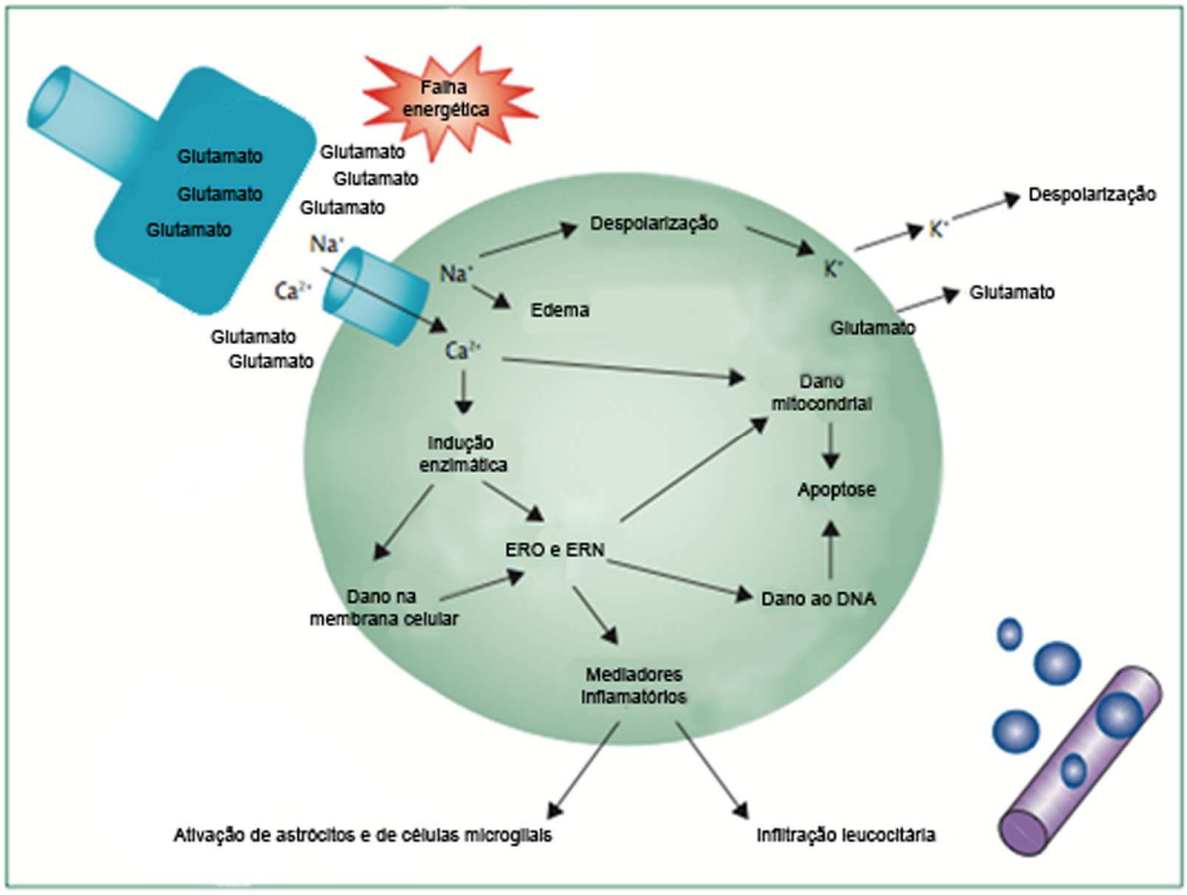

Figura 1. Mecanismos envolvidos na isquemia cerebral (Modificado de Wu and Grotta, 2013).

1.3. Modelos Experimentais de Isquemia Cerebral

Muitos modelos experimentais têm sido desenvolvidos para mimetizar a isquemia cerebral que ocorre em humanos, e servem como uma ferramenta indispensável no campo de pesquisa dessa doença. Estes modelos incluem a isquemia focal, a hipóxia-isquemia e a isquemia global (Taoufik and Probert 2008). Nesta Tese vamos abordar os modelos de isquemia focal e isquemia global.

1.3.1. Modelos experimentais de isquemia cerebral focal

Atualmente existem diversos modelos animais que mimetizam o AVE e suas utilizações são determinadas pelo objetivo experimental que se necessita alcançar. (Howells, Porritt et al. 2010). O primeiro grupo de métodos abordados são os que necessitam de craniotomia, permitindo o acesso direto às artérias cerebrais, principalmente ramificações distais dos vasos da ACM, principal artéria acometida durante a isquemia nos seres humanos. Estas técnicas podem envolver o ligamento (Crowell, Marcoux et al. 1981), o corte (Tamura, Graham et al. 1981), a formação de trombos (Markgraf, Kraydieh et al. 1993), eletrocoagulação (O'Brien and Waltz 1973) ou ainda a termocoagulação do vaso (Szele, Alexander et al. 1995). Embora a oclusão do vaso seja geralmente permanente, ligaduras podem ser liberadas permitindo uma oclusão transitória. Existem técnicas que utilizam drogas vasoconstritoras como a endotelina, que tem uma ação local constritora, levando a um bloqueio do fluxo sanguíneo de forma transitória (Agnati, Zoli et al. 1991). Embora esta técnica também exija a craniotomia, a abertura do crânio é pequena e a utilização de uma cânula permite a análise de uma região específica e pode ser utilizada sem a presença de anestesia.

O segundo grupo de métodos abordados são os que não necessitam de craniotomia. O mais comumente usado é a oclusão da ACM por fio de sutura. Esses modelos têm sido

amplamente utilizados pela sua relevância, pois mimetizam condições clínicas do AVE. Um grande número de modelos de oclusão da ACM vem sendo utilizado tanto de natureza permanente, quanto transitória (Howells, Porritt et al. 2010). Embora este método tenha muitas variantes, notadamente no que diz respeito à construção da linha de oclusão e fecho para manipulação do fluxo de sangue colateral, a técnica básica descrita originalmente por Koizumi et al (1986) e modificado por Longa et al (1989) envolve a introdução de um fio de sutura no interior da artéria carótida interna (ACI) extracraniana, avançando até a sua ponta, que tapa a origem da MCA.

O modelo escolhido nessa tese foi o de isquemia cerebral focal permanente induzida por termocoagulação. Na técnica em questão, há a necessidade de uma craniotomia na região sensorial e motora do córtex. Nesta região especificada, há a aproximação de uma sonda, cujo calor, gerado na região próxima dos vasos da pia, resulta numa coagulação dos vasos e consequentemente uma indução isquêmica (Szele, Alexander et al. 1995). Esta técnica é de fácil desenvolvimento, possuindo uma lesão menor que a maioria das outras técnicas, e uma menor variabilidade, permitindo-nos utilizar um menor número de animais e realizar uma análise mais precisa na avaliação de eventuais drogas neuroprotetoras.

1.3.2. Modelos experimentais de isquemia cerebral global

A isquemia cerebral global é definida como uma diminuição do fluxo sanguíneo cerebral a um patamar crítico que propaga o dano para todo o cérebro. Os modelos experimentais que mimetizam a isquemia global são reproduzidos através de técnicas que ocluem os principais vasos que irrigam o cérebro (carótidas e vertebrais), por modelos que induzem a parada cardíaca, bem como por modelos que mimetizem o choque hemorrágico (Lipton 1999; Nussmeier 2002; Yu, Ono et al. 2002; Kalkan, Eser et al. 2006; Allen and Buckberg 2012).

Existem diversas variações nos modelos de isquemia cerebral por oclusão dos vasos, mas as duas mais utilizadas são a oclusão dos quatro vasos (4-VO), onde é realizada uma eletrocoagulação das artérias vertebrais, com uma oclusão transitória das artérias carótidas 24 h mais tarde (Pulsinelli and Brierley 1979). A técnica que oclui as duas artérias carótidas é realizada juntamente com uma hipoperfusão sistêmica, com uma pressão arterial média de 50 mmHg (Smith, Bendek et al. 1984).

Em modelos de choque hemorrágico, o sangue é retirado de forma constante até que a pressão arterial média seja reduzida a uma pressão crítica (menor que 40 mmHg). Essa hipoperfusão é mantida por um período prolongado, que normalmente é maior que 60 min. Após esse período inicia-se a reposição volêmica, que pode ser através de transfusão sanguínea ou ainda por soluções coloidais. Na reposição volêmica, a infusão da solução normalmente é 3 vezes maior que o volume sanguíneo retirado. Essa solução é infundida de forma contínua até que a pressão se estabilize nas condições basais observadas antes do choque hemorrágico (David, Spann et al. 2013; Fulop, Turoczi et al. 2013).

1.4. Sistema purinérgico

As purinas podem ser classificadas em derivados da adenina (ATP, ADP, AMP, adenosina) e derivados da guanina (GTP, GDP, GMP, guanosina). Ainda compõem o sistema purinérgico o nucleotídeo IMP, o nucleosídeo inosina, as bases purínicas adenina e guanina e os seus metabólitos diretos: hipoxantina, xantina e ácido úrico (Schmidt, Lara et al. 2007).

No SNC, as purinas estão envolvidas em importantes vias de sinalização celular e exercem seus efeitos através de diferentes mecanismos (Burnstock 2006). As purinas agem como importantes moduladoras da atividade sináptica no SNC, interagindo com vários sistemas, como glutamatérgico, dopaminérgico, serotoninérgico e colinérgico (Brundege and Dunwiddie 1997; Burnstock 2007). As purinas também possuem efeitos neuroprotetores

(Pettifer, Kleywegt et al. 2004; Pettifer, Jiang et al. 2007; Petronilho, Perico et al. 2012), anticonvulsivantes (Schmidt 2010; Ganzella, Faraco et al. 2011) e também antinociceptivos (Schmidt, Bohmer et al. 2010; Schmidt, Bohmer et al. 2010). Os derivados da guanina têm sido tradicionalmente estudados como moduladores de processos intracelulares. Entretanto, mais recentemente, os derivados da guanina, principalmente o nucleotídeo GMP e o nucleosídeo guanosina, têm demonstrado diversos efeitos biológicos extracelulares não relacionados a proteínas G, como efeitos tróficos em células neurais (Ciccarelli, Ballerini et al. 2001), modulador negativo do sistema glutamatérgico(Baron, Dudley et al. 1989; Malcon, Achaval et al. 1997; Burgos, Barat et al. 1998; Regner, Ramirez et al. 1998) e efeitos comportamentais (Schmidt, Lara et al. 2007).

1.4.1. Guanosina

O nucleosídeo guanosina tem ganhado atenção dos pesquisadores devido ao seu potencial efeito neuroprotetor em modelos experimentais de doenças cerebrais relacionadas com a excitotoxicidade glutamatérgica (Vinade, Schmidt et al. 2005; Schmidt, Lara et al. 2007; Moretto, Boff et al. 2009; Schmidt 2010; Dal-Cim, Martins et al. 2011), incluindo modelos animais de isquemia cerebral *in vivo* (Chang, Algird et al. 2008; Rathbone, Saleh et al. 2011; Connell, Di Iorio et al. 2013).

A guanosina no meio extracelular exerce uma série de efeitos tróficos nas células, estimulando a proliferação celular em cultura de células e aumentando a liberação de fatores tróficos tais como o fator de crescimento nuclear (NGF) e o fator neurotrófico derivado do encéfalo (BDNF) (Ciccarelli, Di Iorio et al. 2000; Giuliani, Romano et al. 2012). Em condições excitotóxicas, a guanosina parece estar envolvida no antagonismo da toxicidade do sistema glutamatérgico, principalmente modulando a captação de glutamato (Schmidt, Lara et

al. 2007; Thomazi, Boff et al. 2008; Schmidt, Paniz et al. 2010; Quincozes-Santos, Bobermin et al. 2013).

O primeiro trabalho que evidenciou uma relação da guanosina após a indução de um modelo de isquemia cerebral focal foi realizado no ano de 1991. Neste trabalho o aumento endógeno de guanosina ocorreu após 2 h da indução isquêmica e manteve-se elevada por 7 dias (Uemura, Miller et al. 1991). Esta constatação levou à investigações dos efeitos da administração exógena da guanosina em modelos isquêmicos tanto *in vitro* como *in vivo*. Os modelos *in vitro* de privação de oxigênio e glicose (POG) mostraram que a guanosina é neuroprotetora, aumentando a captação de glutamato e regulando as vias de sinalização inflamatórias (Oleskovicz, Martins et al. 2008; Thomazi, Boff et al. 2008; Dal-Cim, Martins et al. 2011; Dal-Cim, Ludka et al. 2013). Em modelos de hipóxia-isquemia, a guanosina foi capaz de aumentar a captação de glutamato em experimentos *ex vivo* (Moretto, Arteni et al. 2005; Moretto, Boff et al. 2009). Em modelos *in vivo*, a guanosina foi neuroprotetora na isquemia cerebral focal permanente e também transitória, reduzindo o tamanho da lesão ocasionada pela isquemia e também melhorando o comportamento motor dos animais (Chang, Algird et al. 2008; Rathbone, Saleh et al. 2011; Connell, Di Iorio et al. 2013).

Além disso, a guanosina apresenta atividade protetora contra do dano oxidativo ao DNA (Gudkov, Shtarkman et al. 2006; Gudkov, Shtarkman et al. 2006; Gudkov, Shtarkman et al. 2007), e modula o stress oxidativo e nitrosativo em modelos neurotóxicos (Roos, Puntel et al. 2009; Albrecht, Henke et al. 2013). Estudos apontam que o efeito neuroprotetor da guanosina é através de uma rota de sinalização dependente de proteínas G envolvendo nucleotídeos cíclicos e as rotas da p38 MAPK e da PI3K/Akt/PKB (Schmidt, Lara et al. 2007; Dal-Cim, Martins et al. 2011; Dal-Cim, Ludka et al. 2013).

1.4.2 Inosina

O nucleosídeo inosina é uma molécula pertencente ao sistema purinérgico, formada a partir da desaminação da adenosina ou também pela retirada de um fosfato inorgânico do IMP pela 5'nucleotidase (Schmidt, Lara et al. 2007). Foi evidenciado que há um aumento sistêmico da inosina quando ocorre um estresse metabólico (Hasko, Kuhel et al. 2000; Liaudet, Mabley et al. 2001) e que a infusão intravenosa de inosina é capaz de melhorar parâmetros hemodinâmicos e de sobrevida em modelos animais de choque hipovolêmico e tóxico, estimulando a bomba Na/K ATPase e controlando os níveis plasmáticos de K^+(Darlington and Gann 2005; Darlington and Gann 2005). A inosina é reconhecida como uma importante molécula endógena capaz de prevenir a lesão de tecidos isquêmicos, e os seus efeitos são, em parte, mediados pela inibição de citocinas inflamatórias e produção de radicais livres, aumentando a sobrevida em modelos animais e diminuindo o dano em diversos órgãos (Cain, Harken et al. 1999; Hasko, Kuhel et al. 2000; Liaudet, Mabley et al. 2001).

Comparado com a guanosina, a inosina possui poucos trabalhos na literatura científica referentes ao seu potencial efeito neuroprotetor no SNC. Dentre os estudos realizados, a inosina ou o seu análogo inosina 5` trifosfato reduziram respostas mediadas por receptores de glutamato em sinapses na região CA1 hipocampal (Macek, Schaffhauser et al. 1998) ou a neurotoxicidade mediada por NMDA em neurônios hipocampais em cultura (Ortinau, Laube et al. 2003). A inosina encontra-se aumentada no líquido cefalorraquidiano (LCR) em modelos de convulsão induzidos por PTZ (Oses, Leke et al. 2004) e possui ação neuroprotetora em modelos de convulsão induzidas por ácido quinolínico (Ganzella, Faraco et al. 2011). A inosina também se mostrou envolvida no mecanismo de transmissão de dor (Schmidt, Bohmer et al. 2010).

Estudos envolvendo modelos isquêmicos reportaram que a inosina reduziu a toxicidade causada pela POG em culturas de astrócitos de ratos (Haun, Segeleon et al. 1996), e preservou a viabilidade celular durante hipóxia química induzida por rotenona em cultura de células de medula (Litsky, Hohl et al. 1999). O efeito protetor da inosina também já foi demonstrado *in vivo*. A oclusão da ACM levou a liberação de inosina e seu metabólito hipoxantina do córtex isquêmico em modelos animais de AVE(Matsumoto, Graf et al. 1993). A inosina também foi capaz de estimular o crescimento axonal melhorando o perfil comportamental em animais que sofreram isquemia (Chen, Goldberg et al. 2002).

2. Objetivos

2.1. Objetivo geral

O objetivo geral deste trabalho é investigar o efeito neuroprotetor da guanosina e da inosina em modelos de isquemia cerebral. Para isso, o foco deste estudo é explorar parâmetros bioquímicos relacionados à excitotoxicidade glutamatérgica, alteração do sistema antioxidante e inflamatório celular, bem como avaliar dano e/ou alterações celulares em neurônios, astrócitos e microglia.

2.2. Objetivos específicos

2.2.1. Investigar os efeitos neuroprotetores da guanosina em um modelo *in vivo* de lesão isquêmica, utilizando o modelo de isquemia cerebral focal permanente induzido por termocoagulação em ratos.

- Verificar a curva dose-resposta da guanosina na lesão isquêmica.
- Verificar a efetividade da neuroproteção causada pela guanosina.
- Avaliar se a guanosina consegue reverter à lesão ocasionada pela isquemia, e se protege contra a morte neuronal.

- Avaliar se a neuroproteção da guanosina está relacionada com a modulação do sistema glutamatérgico.
- Avaliar se a neuroproteção da guanosina está relacionada com a modulação do ambiente redox, alterando parâmetros envolvidos no estresse oxidativo.
- Avaliar a modulação de fatores inflamatórios, através da análise de citocinas pro e antiinflamatórias e também a análise morfológica da microglia.

2.2.1. Investigar os efeitos da administração endovenosa de guanosina e inosina como reposição volêmica sobre parâmetros bioquímicos um modelo *in vivo* de choque hemorrágico em porcos.

- Avaliar se a guanosina e a inosina alteram parâmetros sistêmicos fisiológicos e sanguíneos durante e após a indução do choque hemorrágico
- Investigar sinais de toxicidade sistêmicas da guanosina e da inosina.
- Investigar os efeitos da guanosina e da inosina sobre a viabilidade celular e funcional do tecido nervoso.
- Investigar alterações no metabolismo energético cerebral no LCR.
- Avaliar a modulação de fatores inflamatórios no LCR.

PARTE II

3. Capítulo I

The Potential Therapeutic Effect of Guanosine after Cortical Focal Ischemia in Rats

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The Potential Therapeutic Effect of Guanosine after Cortical Focal Ischemia in Rats

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Abstract

Background and Purpose: Stroke is a devastating disease. Both excitotoxicity and oxidative stress play important roles in ischemic brain injury, along with harmful impacts on ischemic cerebral tissue. As guanosine plays an important neuroprotective role in the central nervous system, the purpose of this study was to evaluate the neuroprotective effects of guanosine and putative cerebral events following the onset of permanent focal cerebral ischemia.

Methods: Permanent focal cerebral ischemia was induced in rats by thermocoagulation. Guanosine was administered immediately, 1 h, 3 h and 6 h after surgery. Behavioral performance was evaluated by cylinder testing for a period of 15 days after surgery. Brain oxidative stress parameters, including levels of ROS/RNS, lipid peroxidation, antioxidant non-enzymatic levels (GSH, vitamin C) and enzymatic parameters (SOD expression and activity and CAT activity), as well as glutamatergic parameters (EAAC1, GLAST and GLT1, glutamine synthetase) were analyzed.

Results: After 24 h, ischemic injury resulted in impaired function of the forelimb, caused brain infarct and increased lipid peroxidation. Treatment with guanosine restored these parameters. Oxidative stress markers were affected by ischemic insult, demonstrated by increased ROS/RNS levels, increased SOD expression with reduced SOD activity and decreased non-enzymatic (GSH and vitamin C) antioxidant defenses. Guanosine prevented increased ROS/RNS levels, decreased SOD activity, further increased SOD expression, increased CAT activity and restored vitamin C levels. Ischemia also affected glutamatergic parameters, illustrated by increased EAAC1 levels and decreased GLT1 levels; guanosine reversed the decreased GLT1 levels and did not affect the EAAC1 levels.

Conclusion: The effects of brain ischemia were strongly attenuated by guanosine administration. The cellular mechanisms involved in redox and glutamatergic homeostasis, which were both affected by the ischemic insult, were also modulated by guanosine. These observations reveal that guanosine may represent a potential therapeutic agent in cerebral ischemia by preventing oxidative stress and excitotoxicity.

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Introduction

Ischemic stroke is the second most common cause of death and the major cause of disability worldwide [1–5]. According to the American Heart Association, someone has a stroke every 40 seconds, and stroke accounts for one of every 18 deaths in the United States [6]. The sudden reduction in blood flow leads to decreased oxygen and glucose supplies to the ischemic brain area, resulting in a failure of cellular bioenergetics. This condition triggers a series of events known as the ischemic cascade, during which the degree of damage is dependent on the affected area and length of blood flow blockage. Disruption of brain metabolism is clearly a key element in stroke, resulting in cellular damage and impairment of neurological functions [1,2,4,5].

Both excitotoxicity and oxidative damage are ischemic events related to cerebral energy failure [1,2,4]. Due to energy depletion, excitatory amino acid transporters (EAATs), EAAT1/glutamate-aspartate transporter (GLAST) and EAAT2/glutamate transporter-1 (GLT1) in astrocytes and EAAT3/excitatory amino acid carrier 1 (EAAC1) in neurons [7,8], responsible for glutamate uptake, are adversely affected, enabling high intracellular concentrations of glutamate to drive the transporters into reverse, releasing toxic amounts of the neurotransmitter into the synapse [7,9]. The excessive glutamate levels in the synaptic cleft leads to overstimulation of glutamate receptors. This overstimulation initiates several molecular events that trigger a massive generation of free radical species and extensive cellular damage [1,2,4,10].

Thus, the brain parenchyma undergoes dramatic changes in oxygen homeostasis, generating more free radical species that play important roles in ischemia and reperfusion injury [2,4,5,11–13]. The central nervous system (CNS) has an efficient antioxidant defense system, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), as well as scavenger molecules such as glutathione (GSH) and vitamin C. Despite the effectiveness of this system, the endogenous antioxidant capacity can be overwhelmed during cerebral ischemia, resulting in overproduction of free radicals including reactive oxygen species (ROS) and reactive nitrogen species (RNS), which have direct negative impacts on ischemic cerebral tissue [5,11,13,14]. ROS/RNS trigger many cellular and molecular events, including protein oxidation/nitrosylation/nitration, lipid peroxidation and DNA damage, resulting in damage to macromolecules and consequent activation of signaling mechanisms that lead to cell death [2,4,5,11,13]. Thus, molecules with antioxidant activities are anticipated to have beneficial effects on brain ischemia.

It has been demonstrated that guanosine (GUO), a guanine-based purine, plays important roles in the CNS [15–23]. Endogenous GUO levels increase after 2 h of focal stroke and remain higher for 7 days [24]. This finding led to the investigation of the effects of exogenously administered GUO on stroke models. The data from *in vitro* models suggests that GUO protects against oxygen and glucose deprivation (OGD) [25–29], increases glutamate uptake in hypoxia-ischemia models [30,31], and is neuroprotective against permanent and transient ischemic stroke [25,32,33]. In addition, GUO demonstrates antioxidant activity, protecting DNA from oxidative damage [34,35], and modulates oxidative and nitrosative stress in neurotoxic models [36,37]. Studies are pointing that GUO may exert its effects through modulation of mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K) signaling pathways [27–29], however, the mechanisms of the protective effects of GUO are not fully understood yet.

As previous experimental studies have demonstrated that GUO acts as a neuroprotective agent against stroke and is able to modulate oxidative response and glutamatergic parameters [33,35,36], the objectives of this study are to investigate the potential neuroprotective role of GUO using a model of permanent focal cerebral ischemia. For that, the focus of this study is directed to explore the neural intracellular biochemical parameters as well as underlying neuroprotective mechanisms.

Materials and Methods

Animals

Adult male Wistar rats (90–100 days old, weighing 300–350 g) were maintained under controlled light and environmental conditions (12 h light/12 h dark cycle at a temperature of 22±2°C) with water and commercial food *ad libitum*. All experimental procedures were performed according to the NIH Guide for Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for Animal Care and were approved by the ethical committee from Federal University of Rio Grande do Sul (Process number: 19283). All efforts were made to minimize the number of animals used and to prevent suffering.

Induction of Permanent Focal Ischemia

Ischemic lesion was induced by thermocoagulation of the blood in the pial vessels of the motor and sensorimotor cortices [38]. Briefly, the animals were anesthetized with ketamine hydrochloride (70 mg/kg, i.p.) and xylazine hydrochloride (10 mg/kg, i.p.) and placed in a stereotaxic apparatus. The skull was surgically exposed and a craniotomy was performed, exposing the left frontoparietal cortex (+2 to −6 mm A.P. and −2 to −4 mm M.L. from bregma), the motor and sensorimotor cortex regions [39]. Blood in the pial vessels was thermocoagulated transdurally by approximation of a hot probe to the Dura mater. The color of the blood vessels is normally light red, and the development of a dark red color was an indicator of complete thermocoagulation. After the procedure, the skin was sutured and body temperature was maintained at 37°C using a heating pad until recovery from the anesthesia.

Drug Treatment

The animals were divided into four groups: Sham Saline (SS), Sham GUO (SG), Ischemia Saline (IS) and Ischemia GUO (IG). GUO (60 mg/Kg in NaCl 0.9%) was purchased from Sigma (St. Louis, MO, USA). The GUO dose was chosen based on a dose-response curve test of the beneficial effect of GUO administration, as evaluated by the spontaneous exploratory behavior of rodents (cylinder test) for 15 days. First, a study was conducted with a GUO pretreatment 30 min before induction of ischemia, along with administration of GUO at 1 h, 3 h and 6 h after induction of ischemia (**Figure 1A**). After this initial dose-range finding experiment, a dose-response curve for GUO was obtained, but instead of pretreatment with GUO, GUO was administered immediately after induction of ischemia. Thus, all groups received a 1 mL/kg intraperitoneal (i.p.) administration (saline or GUO) immediately, 1 h, 3 h and 6 h after surgery (**Figure 1B**). Based upon these results, it was administrated GUO (60 mg/kg i.p.) immediately, 1 h, 3 h and 6 h after surgery.

Cylinder Test

This test evaluates the spontaneous exploratory behavior of rodents [40,41]. The cylinder test reveals forelimb preference when the animal rears to explore its environment by making forelimb contact with the cylinder walls. Animals were subjected to one trial on the pre-ischemic day. To prevent habituation to the cylinder, the number of movements recorded was limited to 20. The occurrences of sole use of the ipsilateral (to the lesion) or contralateral forelimb, or the simultaneous use of both forelimbs, were counted. The asymmetry score for each animal was calculated each day by the formula previously described [42]. For the dose-response curve experiments with GUO administration, animals were tested on 1, 3, 7 and 15 days post-ischemic injury. For all other experiments, the animals were tested 24 h after ischemia, before the biochemical experiments.

Measurement of Infarct Volume

Twenty-four hours after surgery, animals were sacrificed, and the brains were rapidly removed from the skull and sectioned in the coronal plane at 2 mm of thickness, using a rat brain matrix (Insight LTDA, Ribeirão Preto, SP, Brazil). The slices were immersed for 30 min into 2% of 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, USA) solution at 37°C, followed by overnight fixation in 4% paraformaldehyde (Sigma, USA). The infarct volume was calculated by the formula: *Infarct volume = [measured infarct area × slice thickness (2 mm)]+[area of contralateral corresponding structure × slice thickness]–[area of ipsilateral corresponding structure × slice thickness]* [43,44]. The brain slices were analyzed by Image J software (NIH, USA). The results are expressed as mm^3.

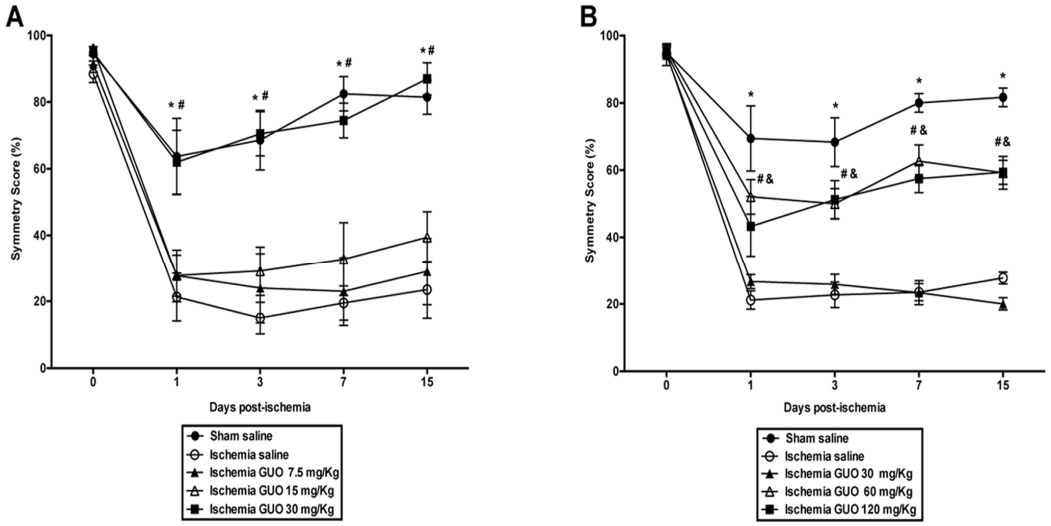

Figure 1. GUO dose-response curve. The beneficial effects of GUO were evaluated by a cylinder test. (**A**) Cylinder Test on 0, 1, 3, 7 and 15 days post-ischemia. GUO i.p. administration was performed 4 times (30 min before ischemia and 1 h, 3 h and 6 h after ischemia). *represents $P<0.001$ when comparing ischemia saline vs. sham saline and #represents $P<0.001$ when comparing ischemia saline vs. ischemia GUO 30 mg/Kg. n = 9–10 per group. (**B**) Cylinder Test on 0, 1, 3, 7 and 15 days post-ischemia. GUO i.p. administration was performed 4 times (immediately, 1 h, 3 h and 6 h after ischemia). *represents $P<0.001$ when comparing ischemia saline vs. sham saline, #represents $P<0.01$ when comparing ischemia saline vs. ischemia GUO 60 mg/Kg and &represents $P<0.01$ when comparing ischemia saline vs. Ischemia GUO 120 mg/Kg. n = 9–13 per group.
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Tissue Processing

All neurochemical parameters were evaluated 24 h after the ischemic insult. The animals were decapitated under deep anesthesia, and the brains were removed from the skull and maintained at 4°C. In ischemic animals, the cortical tissue surrounding the ischemic lesion, located between the lesion and the cerebral longitudinal fissure (a piece measuring approximately 8 mm×2 mm), was dissected. This region was chosen because previous studies using this model pointed that it has characteristics similar to the penumbra. In sham animals, the same region was dissected [42] (**Figure 2**).

For measurement of intracellular ROS levels, cortical slices (300 µm) were immediately incubated, and the experiment specimens were processed. For glutamine synthetase activity, the tissue was homogenized in a 150 mM KCl solution. For other oxidative stress assays, the tissue was homogenized in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. For Western Blot analysis, the tissue was homogenized using lysis solution [4% SDS, 2 mM EDTA, 50 mM Tris-HCl, pH 6.8], containing a protease and phosphatase inhibitors cocktail, and normalized with sample buffer [62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% β-mercaptoethanol, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue]. All homogenates were frozen (at −80°C) until the biochemical measurements were conducted.

Thiobarbituric Acid-reactive Substances (TBARS) Measurement

Lipid peroxidation can be evaluated by the TBARS assay [37], which evaluates the lipid damage via assay-based detection of malondialdehyde, the last product of lipid breakdown caused by oxidative stress. Briefly, homogenates (10 µL) were added to 20 µL of cold 10% trichloroacetic acid and 30 µL of 0.67% thiobarbituric acid in 7.1% sodium sulfate and boiled for 1 h. The mixture was cooled in water for 3 min. Afterwards, 40 µL of butyl alcohol were added, and then these samples were centrifuged at 5,000 *g* for 5 min. Pink-colored TBARS was determined in the resulting supernatants using a spectrophotometric microtiter plate reader set to read at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane. The data are expressed as nmol/mg of protein.

Intracellular ROS Levels

DCFH oxidation was used to measure intracellular ROS production. DCFH-DA (2′-7′-dichlorofluorescein diacetate) is hydrolyzed by intracellular esterases to dichlorofluorescin (DCFH), which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) by the action of cellular oxidants. Cortical slices were treated with DCFH-DA (10 µM) for 30 min at 37°C. Following DCFH-DA exposure, the slices were placed into PBS with 0.2% Triton X-100. Fluorescence was measured in a plate reader (Spectra Max M5, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm [45]. The ROS production was calculated as fluorescence units per milligram protein (UF/mg) and then expressed as a percent of control.

Nitric Oxide (NO) Levels

NO was determined by measurement of nitrite (a stable oxidation product of NO), based on the Griess reaction [46]. Briefly, homogenates were mixed with 25% trichloroacetic acid and centrifuged at 1,800 *g* for 10 min. The supernatant was immediately neutralized to pH 7.0 with 2 M potassium bicarbonate. NO_3 was reduced to NO_2 by nitrate reductase. Total NO_2 was measured by a colorimetric assay at 540 nm. A standard curve was performed using sodium nitrate (0–80 µM). The results are expressed as µM of nitrite/mg of protein.

Vitamin C Levels

Ascorbic acid ($AscH^-$) was used to indicate vitamin C levels. Homogenates were centrifuged at 10,000 *g* for 2 min. Aliquots

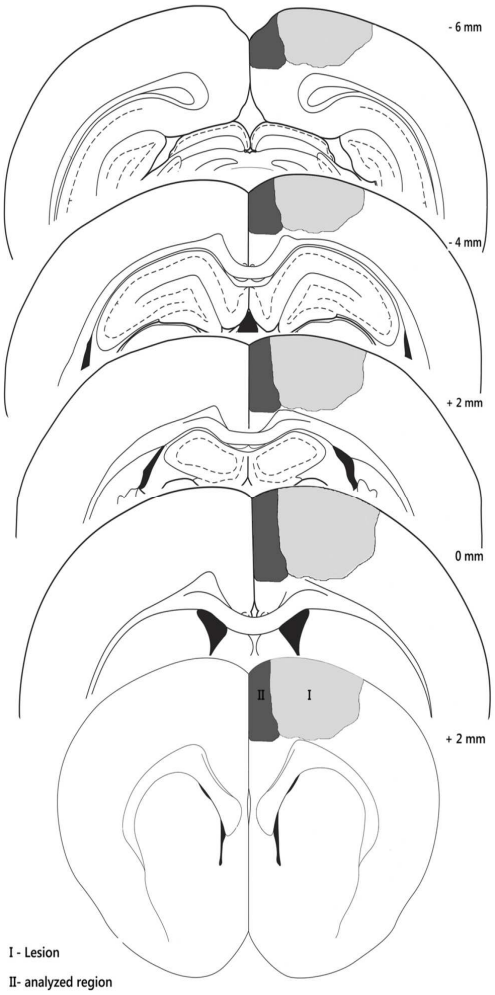

Figure 2. Schematic illustration of the ischemic lesion. Illustrations showing the region of the induced lesion (gray) and the analyzed region (dark gray) that were dissected for the experiments. The left frontoparietal cortex (+2 to −6 mm A.P. and −2 to −4 mm M.L. from bregma) was used.
doi:10.1371/journal.pone.0090693.g002

(50 µL) of the supernatant samples or $AscH^-$ standards were placed in a 96-well plate, and 50 µL of the 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (Tempol) stock solution (2.32 mM Tempol in acetate buffer) were added, and then these samples were incubated for 10 min at room temperature. While protecting the reaction from light, 21 µL of *o*-phenylenediamine (OPDA) solution (5.5 mM OPDA in acetate buffer) was added. Tempol promotes the oxidation of ascorbic acid to dehydroascorbic acid, which was measured by fluorescence detection (345 nm for excitation and a 425 nm for emission) in a Spectra Max GEMINI XPS plate reader (Molecular Devices, USA) [47]. The results were expressed as µM of $AscH^-$/mg of protein.

GSH Levels

GSH levels were assessed as previously described [48]. Briefly, homogenates were diluted in 10 volumes of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA, and the protein was precipitated with 1.7% meta-phosphoric acid. The supernatant was mixed with *o*-phthaldialdehyde (1 mg/mL methanol) and incubated at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0–500 µM). The GSH concentration was calculated as nmol/mg of protein.

SOD Activity

SOD activity was determined using the RANSOD kit from Randox (Autrim, United Kingdom). This method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and the superoxide radicals produced in the incubation medium from the xanthine and xanthine oxidase reaction system, which is assayed spectrophometrically at 505 nm. Inhibition of the produced chromogen is proportional to the activity of the SOD. The 50% inhibitory concentration is defined as one unit of SOD, and the specific activity is represented as U/mg of protein.

CAT Activity

CAT activity was assayed as previously described [49]. The absorbance was measured in homogenized tissue by measuring the absorbance decrease at 240 nm in a reaction medium containing: 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and 50 µg protein. One unit (U) of the enzyme is defined as 1 µmol of H_2O_2 consumed per minute. The results were expressed in U/mg of protein.

Glutamine Synthetase (GS) Activity

The enzymatic assay was performed as previously described [50]. Briefly, homogenates (0.1 mL) were added to 0.1 mL of the reaction mixture containing: 10 mM $MgCl_2$, 50 mM L-glutamate, 100 mM imidazole-HCl buffer (pH 7.4), 10 mM 2-mercaptoethanol, 50 mM hydroxylamine-HCl and 10 mM ATP, and incubated for 15 min at 37°C. The reaction was stopped by the addition of 0.4 mL of a solution containing 370 mM ferric chloride, 670 mM HCl, and 200 mM trichloroacetic acid. Samples were centrifuged at 1,000 *g* for 10 min, and the absorbance of the supernatant was measured at 530 nm and compared to absorbance generated by standard quantities of γ-glutamyl-hydroxamate treated with ferric chloride reagent. The results are expressed as µmol/h/mg of protein.

Western Blot Analysis

Samples (20 µg protein/well) were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were processed as follows: (1) blocking with 5% bovine serum albumin for 2 h; (2) incubation with primary antibody overnight [anti-SOD1, anti-EAAC1, anti-GLAST and anti-GLT1 (1:1000) from Alpha Diagnostic (St. Antonio, TX, USA) and anti-β-actin (1:5000) and anti-β-tubulin (1:10000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA)]; (3) incubation with peroxidase conjugated secondary antibody for 2 h; and, finally, (4) chemiluminescence (ECL kit) was detected using X-ray films. The films were scanned, and the bands were quantified using Image J software (NIH, USA). The results are expressed in percent of control levels.

Protein Assay

Protein content was measured using Pierce BCA protein kit (Thermo Scientific, USA) with bovine serum albumin as the standard. The results are expressed as mg of protein.

Statistical Analysis

The results are presented as the mean ± S.E.M. The cylinder test was analyzed with a repeated-measures analysis of variance (ANOVA), followed by Tukey's post-hoc test. Infarct volume was analyzed using Student's t-test. Oxidative stress assays and Western blots were statistically analyzed using two-way analysis of variance (ANOVA) followed by the Bonferroni's post-hoc test. Correlations were analyzed by Pearson's correlation. Probability values less than 0.05 were considered statistically significant. All analyses were performed using the Statistical Package for Social Sciences (SPSS) software version 15.0.

Results

GUO Treatment Partially Restored Function of the Impaired Forelimb, Reduced Brain Infarct Volume, and Prevented Lipid Damage in Brain Tissue

GUO treatment led to a partial recovery in the function of the impaired forelimb after 24 h of permanent focal cerebral ischemia, and the effect was maintained up to 15 days post-insult. No dysfunction of the forelimb was observed in the sham group (**Figure 3A**). Brain infarct volume analysis by TTC staining 24 h after ischemia showed tissue damage in the ischemic animals, visualized by pale stained-tissue (**Figure 3B**). GUO treatment significantly decreased the size of the area of tissue damage (**Figures 3B and 3C**). No tissue damage was observed in sham groups (**Figure 3B**). There was an increase in lipid damage in the ischemic group [ischemic effect: $F_{(1,32)} = 28.8$; $P<0.0001$], which was abolished by GUO treatment (**Figure 3D**).

GUO Treatment Decreased ROS/RNS Levels and Modulated Important Antioxidant Defenses Following Permanent Focal Cerebral Ischemia

Twenty-four hours after ischemic insult, ROS [ischemic effect: $F_{(1,32)} = 13.0$; $P = 0.0012$] and NO [ischemic effect: $F_{(1,32)} = 8.0$; $P = 0.008$] levels increased in the ischemic group, and these effects were prevented by GUO treatment (**Figures 4A and 4B**).

Analysis of the non-enzymatic antioxidant molecules in the CNS demonstrated that ischemic insult decreased GSH levels from 290.6±8.5 to 249.4±14.6 nmol/mg of protein [ischemic effect: $F_{(1,31)} = 16.1$; $P = 0.0003$] and that GUO treatment did not prevent this effect (**Figure 5A**). Additionally, vitamin C levels (**Figure 5B**) decreased significantly in the ischemic group [ischemic effect: $F_{(1,32)} = 130.0$; $P<0.0001$], and the GUO treatment partially restored these levels [treatment effect: $F_{(1,32)} = 11.5$; $P = 0.00018$].

Analysis of the antioxidant enzymes in the CNS suggests that GUO modulated the effects of ischemic injury. There was a large increase (5-fold) in SOD expression (**Figure 5C**) measured 24 h after the ischemic insult [ischemic effect: $F_{(1,24)} = 144.3$; $P<0.0001$] when compared to the sham group. Surprisingly, SOD activity did not demonstrate the same profile (**Figure 5D**); despite the presence of increased SOD expression, the SOD enzyme activity decreased in the ischemic group. GUO treatment following ischemia increased both SOD expression and activity.

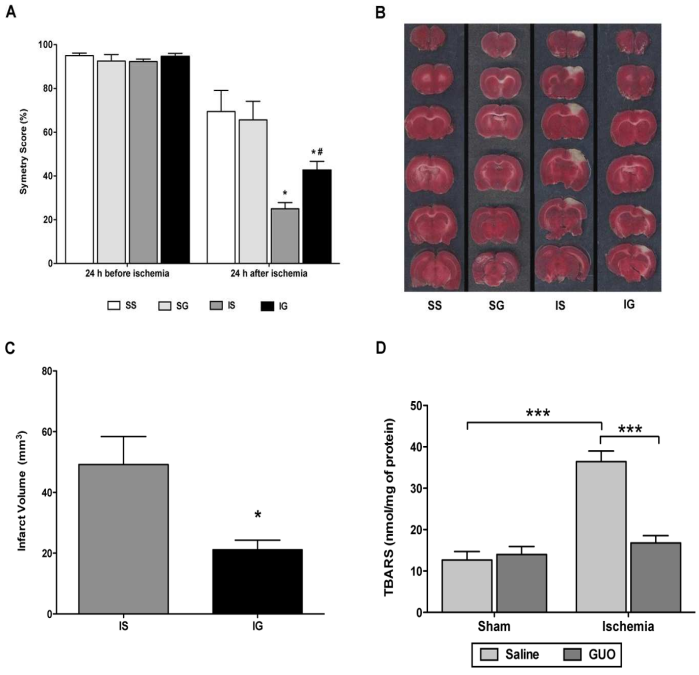

Figure 3. GUO treatment improved forelimb function and reduced cerebral damage caused by ischemic insult. (**A**) Cylinder Test 24 h before and 24 h after ischemia; *represents $P<0.001$ when comparing ischemia saline (IS) vs. sham saline (SS) and sham GUO (SG); #represents $P<0.001$ when comparing ischemia saline (IS) vs. ischemia GUO (IG); n = 10–15 per group. (**B**) Representative coronal brain sections (2 mm thick) stained with 2% TTC demonstrating infarction. Red-colored regions indicate a non-ischemic area, and pale-colored regions indicate the ischemic area. (**C**) Analysis of the cortical infarct volume measured by TTC staining, 24 h after cerebral ischemia; the data are expressed as mm^3. *represents $P = 0.012$ with n = 8 per group. (**D**) Lipid peroxidation was measured by the TBARS method 24 h after ischemia. The data are expressed as nmol/mg of protein. ***represents $P<0.001$ with n = 7–11 per group.
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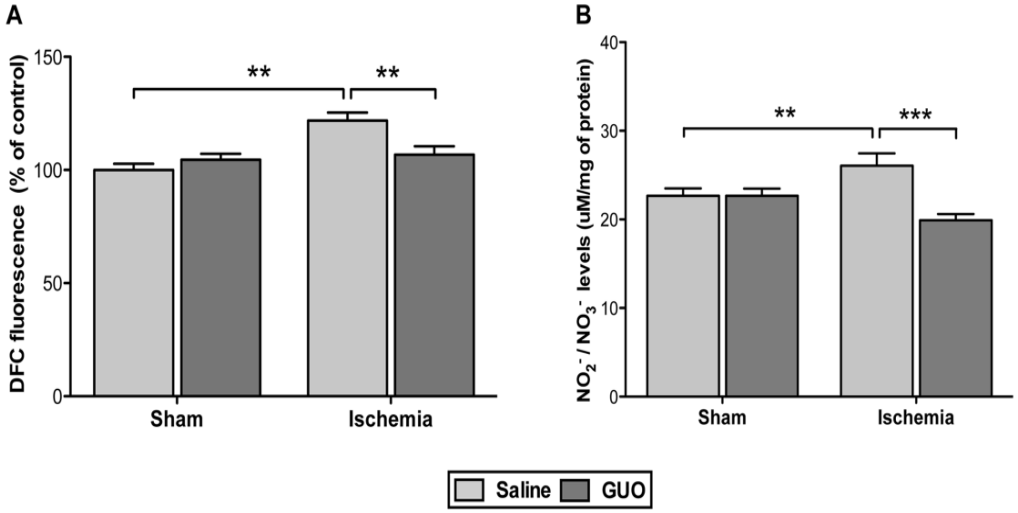

Figure 4. ROS/RNS levels increased 24 h after ischemia, and GUO treatment prevent this effect. (**A**) Effects on ROS levels, measured by DCF fluorescence as described in the Materials and methods section. The data are expressed as percent of control (n = 7–11 per group). (**B**) Effects on NO levels expressed as μM/mg of protein (n = 7–11 per group). The production of NO levels was indirectly measured by the formation of nitrite, as described in the Materials and methods section. The results expressed as the mean ± S.E.M. **represents $P<0.01$; ***represents $P<0.001$.
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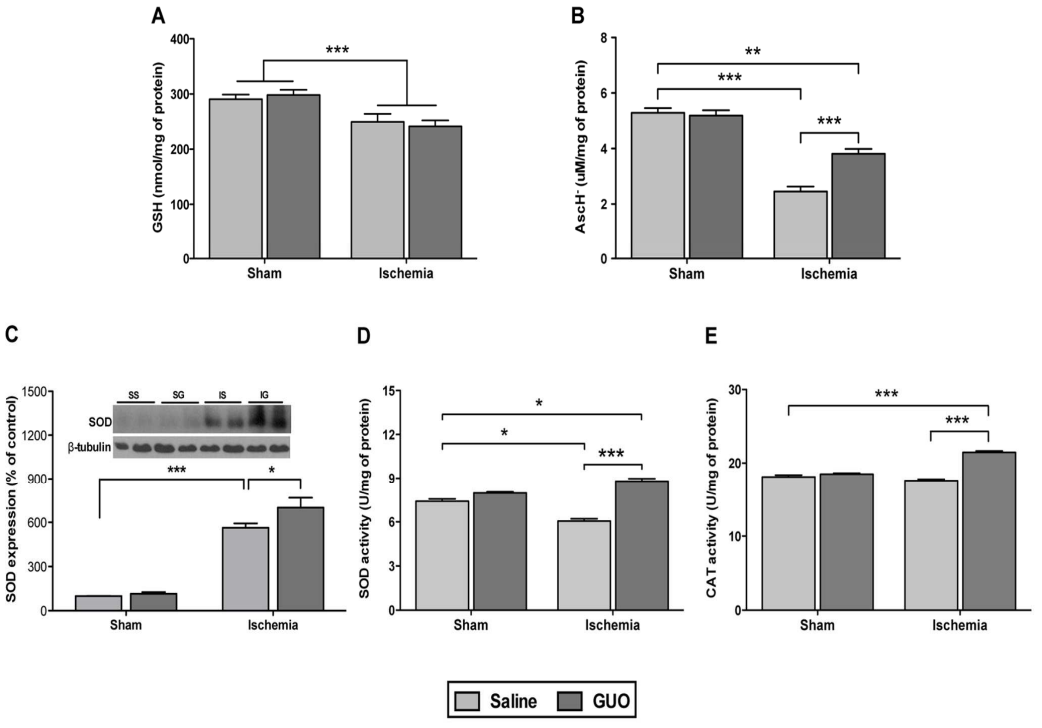

Figure 5. Analysis of non-enzymatic and enzymatic antioxidant defenses 24 h after the ischemia. (**A**) Effect on GSH levels, measured as described in the Materials and methods section. The data are expressed as nmol/mg of protein (n = 7–11 per group). (**B**) Effect on vitamin C levels measured as $AscH^-$, measured as described in the Materials and methods section. The data are expressed as μM/mg of protein (n = 7–11 per group). (**C**) Effect of SOD expression, measured by western blot. The data were normalized by β-tubulin and are expressed as a percent of control (n- 6–9 per group). Effect on enzymatic antioxidant activities: (**D**) SOD activity and (**E**) CAT activity. The data are expressed as U/mg of protein (n = 7–11 per group). *indicates $P<0.05$, **indicates $P<0.01$ and ***indicates $P<0.001$.
doi:10.1371/journal.pone.0090693.g005

Although the ischemic insult did not affect CAT activity (**Figure 5E**), ischemic animals treated with GUO presented an increased CAT activity [treatment effect: $F_{(1,32)} = 13,7$; $P = 0.008$].

GUO Modulated Glutamatergic Parameters Affected by the Ischemic Insult

When measured 24 h after ischemic injury, expression of the neuronal EAAC1 transporter (**Figure 6A**) increased in the ischemic group [ischemic effect: $F_{(1,26)} = 13.0$; $P = 0.0013$], and GUO had no effect on this increase. The analysis of glial glutamatergic transporters GLAST and GLT1 showed a different profile. The ischemic insult did not affect GLAST expression (**Figure 6B**). However, GLT1 expression (**Figure 6C**) following the ischemic insult was significantly diminished [ischemic effect: $F_{(1,24)} = 18.0$; $P = 0.0003$], and GUO treatment prevented this effect [treatment effect: $F_{(1,26)} = 5.5$; $P = 0.027$].

The activity and expression of GS, the enzyme responsible for conversion of glutamate to glutamine in astrocytes, were evaluated 24 h after ischemia. GS protein expression was similar in all groups (**Figure 7A**). Interestingly, ischemic insult did not affect GS activity [ischemic effect: $F_{(1,50)} = 3.6$; $P = 0.062$], but GUO treatment of the ischemic group resulted in increased GS activity (**Figure 7B**).

Correlation of Oxidative Stress Parameters and the Declining Function of the Impaired Forelimb

The data analysis was performed to investigate any potential correlation between the function of the impaired forelimb (symmetry score) and antioxidant scavenger (vitamin C) levels, reactive species (intracellular ROS) levels, and/or lipid damage (lipid peroxidation). There was a strong positive correlation of the symmetry score with vitamin C levels (**Figure 8A**; $R^2 = 0.72$, $P < 0.0001$), and there was a moderate negative correlation of the symmetry score with ROS levels (**Figure 8B**; $R^2 = 0.31$, $P = 0.008$) and with lipid peroxidation (**Figure 8C**; $R^2 = 0.41$, $P < 0.0001$).

Discussion

Acute ischemic stroke causes sudden impairment of blood circulation in a brain area, resulting in a failure of bioenergetics and cellular damage [1,2,4]. Despite considerable advances in the understanding of the pathophysiology of cerebral ischemia, therapeutic options for stroke are still limited. Previous studies have demonstrated beneficial effects of GUO against ischemic insult. GUO was able to recovery the sensorimotor function and reduce the cerebral infarct volume in both, permanent and transient Middle Cerebral Artery Occlusion (MCAO) [25,32,33]. Corroborating with these data, the present study found that GUO treatment caused a significant recovery in the function of impaired forelimb, and this effect was maintained up to 15 days post-insult (last measurement), and also significantly reduced the cerebral infarct volume. Moreover, GUO treatment significantly abolished the increase in lipid peroxidation caused by ischemia. Thus, GUO treatment was able to restore clinical sensorimotor function, decreased the associated morphological brain damage and abolished the neural cell membrane damage. These results demonstrate an effective neuroprotective role of GUO against ischemic insult to the brain.

The mechanisms of neuroprotective strategies against cerebral ischemia may target biochemical alterations involved in cellular damage and/or improve hemostatic and vascular systems involved in collateral blood flow. As the precise GUO neuroprotective mechanisms are unclear, this study aimed to search for putative

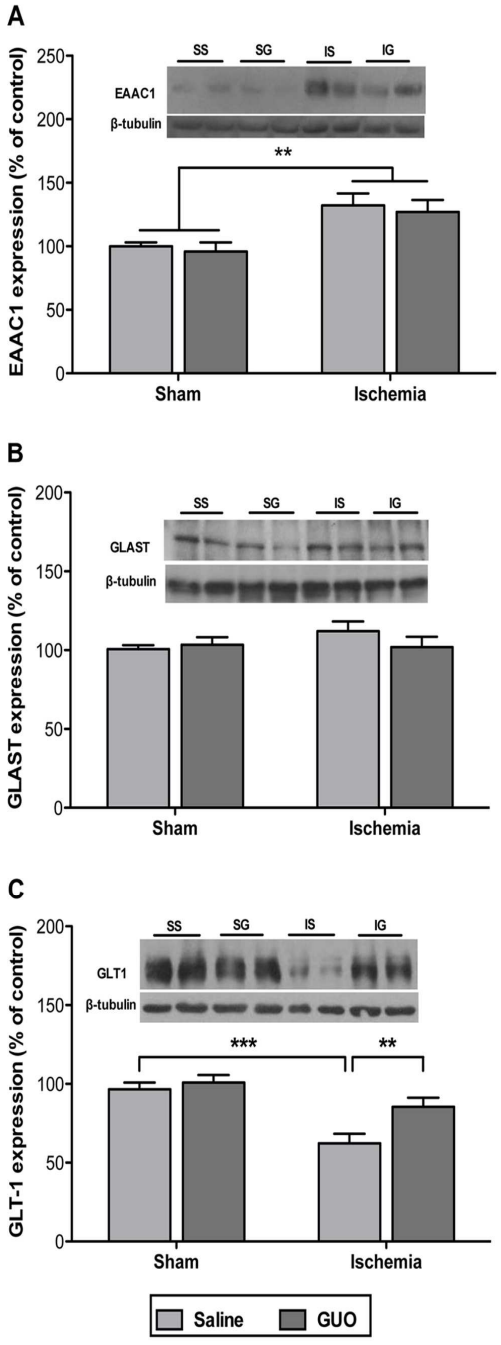

Figure 6. Analysis of glutamatergic transporters by Western blot 24 h after ischemic insult. (**A**) EAAC1 expression, (**B**) GLAST expression and (**C**) GLT1 expression measured by western blot. The data are expressed as a percent of control (n = 6–9 per group). The same membranes were blotted against β-tubulin to serve as a loading control. **indicates $P < 0.01$ and ***indicates $P < 0.001$.
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intracellular biochemical parameters in neural cells involved in this neuroprotection. Here, it was demonstrated for the first time that GUO treatment modulated important parameters related to both

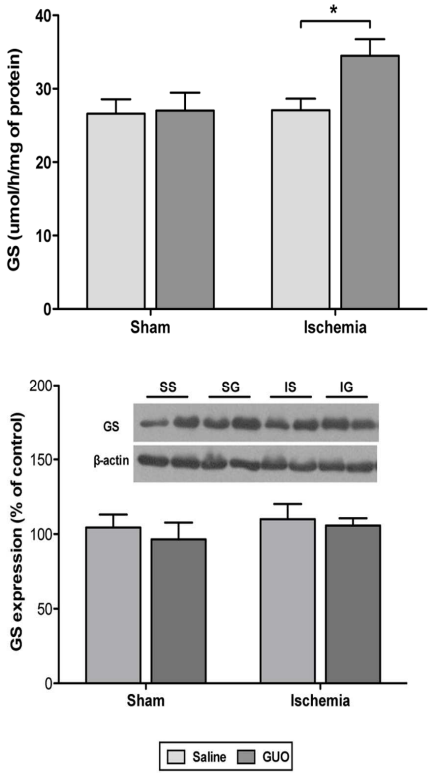

Figure 7. Effects of ischemia and GUO treatment on GS measured 24 h after ischemic insult. (**A**) GS activity, measured by colorimetric method that evaluate the enzyme activity metabolizing glutamate (substrate) into glutamine (product), as described in the Materials and methods section. The data are expressed as µmol/h/mg of protein (n = 11–16 per group). (**B**) GS expression, measured by western blot analysis. The data were normalized by β-actin and are expressed as a percent of control (n = 6–9 per group). *indicates $P < 0.05$.
doi:10.1371/journal.pone.0090693.g007

the oxidative stress response (redox homeostasis system) and the glutamatergic system after an *in vivo* ischemic event.

Free radicals play an essential role in maintaining the physiological condition of the body. Because the CNS has a high oxidative metabolism rate, brain cells are especially vulnerable to free radical damage during ischemia [4,5,11]. Defense against free radicals is provided by a number of antioxidant enzymes, including SOD, CAT and GPx. SOD converts O_2^- to H_2O_2, whereas CAT and GPx convert H_2O_2 to H_2O, thus removing ROS. These enzymes are coupled with other non-enzymatic antioxidants, such as GSH and vitamin C, responsible for reducing both ROS and RNS levels [51–53]. During an ischemic event, there is a massive production of ROS (generated by the mitochondrial respiratory chain and NADPH oxidase) and RNS (generated by the combination of NOS and superoxide, forming the peroxynitrite) [54,55] that depletes intracellular brain GSH and vitamin C levels [55–57]. Despite increased expression of antioxidant enzymes during ischemic injury, there is an impairment of their activities, which implies a severe state of oxidative stress and enhanced lipid peroxidation rates [11,13]. Here, the ischemic insult increased SOD expression and decreased SOD activity; GUO treatment increased SOD expression and completely reestablished SOD activity. Studies have shown that

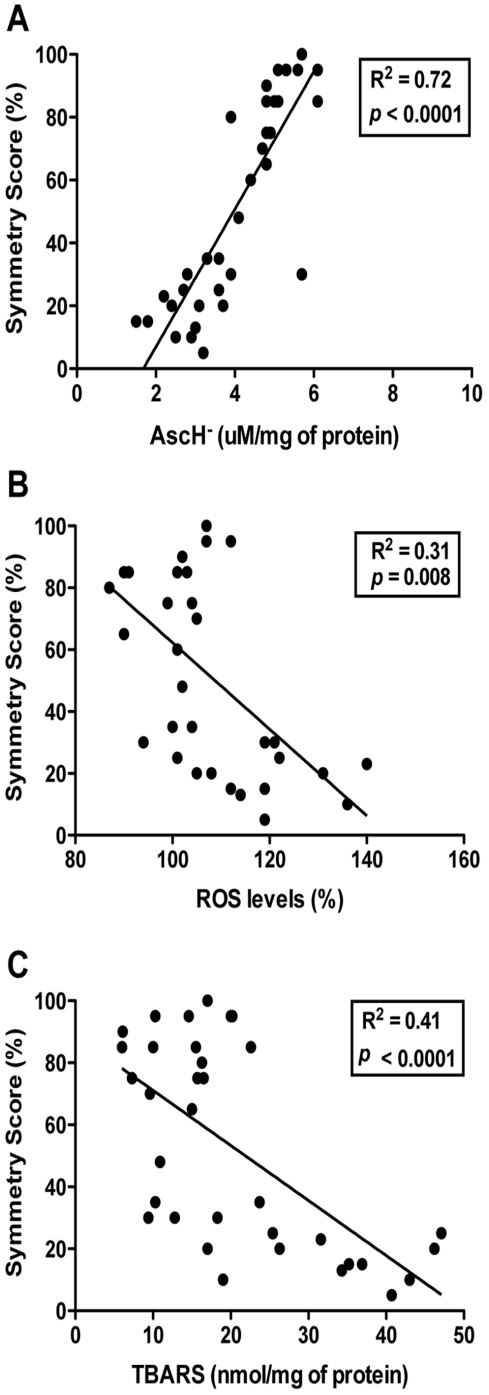

Figure 8. Correlation between oxidative stress parameters and forelimb function (symmetry score) measured 24 h after ischemia. (**A**) Positive correlation with vitamin C levels ($AscH^-$). (**B**) Negative correlation with ROS levels. (**C**) Negative correlation with lipid peroxidation.
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overexpression of SOD in transgenic mice resulted in a reduction of infarction volume and better neurological outcomes after

ischemia [51,58,59]. The increased CAT activity in the ischemic animals treated with GUO could be a beneficial response designed to remove H_2O_2. In this context, modulation of the expression and activity of SOD and the CAT activity by GUO may indicate that the neuroprotective effects of GUO are associated with attenuation of oxidative stress, consequently decreasing free radical levels [11,51,59].

Mounting evidence suggests that radical scavengers mediate protective effects following cerebral ischemia [60,61]. Studies have shown vitamin C is neuroprotective during ischemia, decreasing infarct volume, and this effect is likely related to scavenging for reactive species [56,61]. In the current study, ischemic insult decreased the levels of the non-enzymatic scavenger compounds GSH and vitamin C; although GUO treatment was not able to reverse the decreased GSH levels, GUO treatment did reverse the decreased vitamin C levels, increasing the presence of this non-enzymatic scavenger in the ischemic environment. Therefore, the neuroprotection of GUO in cerebral ischemia could be related to its enhancement of endogenous antioxidant capacity and inhibition of reactive species production, thereby mitigating the brain damage caused by reactive species production resulting from ischemia.

Glutamate excitotoxicity has long been recognized to play a key role in the pathophysiology of cerebral ischemia. Ischemia impairs glutamate uptake by EAATs, contributing to toxic amounts of the neurotransmitter into the synapse [7,9]. These events result in overstimulation of glutamatergic receptors and activation of intracellular pathways that lead to cell death [1,2,62]. Therefore, glutamate uptake activity is closely linked to ischemic events. GLAST and GLT1 are primarily expressed by astrocytes, which also express the enzyme GS to convert glutamate to glutamine, which is then recycled to glutamate into neurons. The connected activities of these proteins contribute to maintaining the extracellular glutamate concentration below toxic levels. EAAC1, on the other hand, is predominantly expressed in neurons [7]. The transport activities of EAAC1, GLAST and GLT1 are inhibited by oxidants via a direct action on the transporter proteins, reducing their activities [10,63]. Herein, ischemic insult decreased GLT1 expression (the major astrocytic EAAT), effect reversed by GUO, and increased the neuronal EAAC1 expression, measured 24 h after ischemia. Although ischemia did not modify GS expression, its activity increased with GUO treatment after the insult. Thus, in the ischemic group, GUO potentially increased both the glutamate uptake and its intracellular conversion to glutamine. These effects may have increased removal of glutamate from the synaptic cleft in the surrounding brain area subjected to the ischemic insult. The function of EAAC1 in the brain has not been fully established. EAAC1 is a neuronal glutamate and cysteine transporter, involved in the regulation of synaptic glutamate uptake and responsible for uptake of cysteine and glutamate, precursors of GSH [8,64,65]. In this study, EAAC1 expression significantly increased 24 h after ischemia; it could be hypothesized that this increase is an endogenous protective mechanism in response to ischemic insult. Importantly, GUO treatment increased EAAC1 expression.

The correlation between the functional recovery of animals and the capacity for administration of GUO to abolish the decreased vitamin C levels, the increased ROS and RNS levels, and the increase in lipid peroxidation, demonstrates that these parameters are active participants in the pathogenesis of ischemia and the neuroprotective effects of GUO. Additionally, the recovery of essential functions of the glutamatergic system (including glutamate uptake and its metabolism) following GUO administration suggests that this is another important factor in the attenuation the tissue damage. Thus, although the mechanisms by which GUO acts are not fully known, it was demonstrated that GUO modulated maintenance of the cellular redox environment and the glutamatergic system following ischemic injury in rodents. Overall, our work represents an important contribution to the knowledge regarding the putative neuroprotective mechanisms of GUO in cerebral ischemia models. However, due to limitations of our model of brain ischemia, further studies in other robust models, such as MCAO, are necessary to evaluate the global relevance of GUO neuroprotection against cerebral stroke.

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Author Contributions

Conceived and designed the experiments: GH DR CD AQ. Performed the experiments: GH DR CD D. Guerini Souza RA AQ. Analyzed the data: GH D. Guerini Souza LP AQ D. Onofre Souza. Contributed reagents/materials/analysis tools: LP D. Onofre Souza. Wrote the paper: GH D. Guerini Souza AQ D. Onofre Souza.

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4. Capítulo II

Guanosine protects against cortical focal ischemia. Involvement of inflammatory response.

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Main Points:

- Guanosine reduced neuronal and astrocytic damage after cerebral ischemia, restoring function of the impaired forelimb.
- Guanosine prevented a microglial increase and reduced pro-inflammatory cytokines after cerebral ischemia.

Abstract

Stroke is the major cause of death and the most frequent cause of disability in the adult population worldwide. Guanosine plays an important neuroprotective role in several cerebral ischemic models and is involved in the modulation of oxidative responses and glutamatergic parameters. Because the excessive reactive oxygen species produced during an ischemic event can trigger an inflammatory response, the purpose of this study was to evaluate the hypothesis that guanosine is neuroprotective against focal cerebral ischemia; inhibits microglia; and mediates an inflammatory response ameliorating the neural damage. Permanent focal cerebral ischemia was induced in rats, and guanosine was administered immediately, 1 h, 3 h, and 6 h after surgery. Twenty-four hours after ischemia, the asymmetry scores were evaluated with the cylinder test; neuronal damage was evaluated by Fluoro-Jade C (FJC) staining and propidium iodide (PI) incorporation; proliferation and activation of microglia was evaluated by flow cytometry and immunohistochemistry; and inflammatory parameters (IL-1, IL-6, IL-10, TNF-α, and INF-γ) were evaluated in the brain tissue and cerebrospinal fluid. Guanosine treatment reduced the infarct volume, PI incorporation and number of FJC positive cells and improved functional recovery. Immunohistochemistry revealed that activation and proliferation of microglia in the lesion periphery were increased, pro-inflammatory cytokines increased and IL-10 decreased with ischemia. Guanosine treatment decreased the production of pro-inflammatory cytokines; restored IL-10 levels (to sham levels) and decreased the proliferation of microglia. Thus, guanosine may be a promising therapeutic agent for the treatment of ischemic brain injury associated with microglial activation.

Keywords

Neuroprotection, neuroinflammation, microglia, cytokines.

Introduction

Stroke is currently a critical public health problem. Stroke is a major cause of death and the most frequent cause of long-term disability in the adult population worldwide. Ischemic stroke is more prevalent than hemorrhagic, representing approximately 87 % of all strokes (Brouns and De Deyn 2009; Doyle et al. 2008; Go et al. 2014). Acute ischemic stroke causes a sudden impairment of blood circulation within a brain area, which results in insufficient substrate delivery to support cellular homeostasis. This insufficient substrate delivery results in cellular bioenergetics failure and consequently a complex, multiple pathway event cascades, which results in cellular damage and loss of neurological functions (Brouns and De Deyn 2009; Donnan et al. 2008; Durukan and Tatlisumak 2007; Lipton 1999). Neuroinflammation plays an important role in the pathogenesis of ischemic stroke, inducing rapid neuronal death in the ischemic core, which gradually expands toward the penumbra area, and is crucial to brain damage (Brouns and De Deyn 2009). Microglia, the major immune cell in the central nervous system (CNS), is rapidly activated in response to ischemia and is typically converted from the resting cell type to an activated form (Jin et al. 2010; Kettenmann et al. 2011). During ischemic inflammation, cytokines are released, and the cytokines modulate the extent of damage in animal models. Peripheral cytokine levels are increased in ischemic patients (Jin et al. 2010). Tumor necrosis factors alpha (TNF-α) and Interleukin 1 (IL-1) are produced by microglia, intrathecal macrophages, and infiltrating, monocyte-derived macrophages. Interleukin-6 (IL-6) is produced by microglia and neurons, and interferon-gamma (INF-γ) is produced by $CD4^+$ and $CD8^+$ T cells (Lambertsen et al. 2012). The cytokines produced by immune and injured brain cells increase brain edema and/or promote the death of brain cells in the penumbra area, resulting in a secondary progression of the infarct lesion (Shichita et al. 2012). Interleukin 10 (IL-10) is an anti-inflammatory cytokine that is reduced during ischemic events. IL-10 may be neuroprotective

against ischemic brain injury (Ooboshi et al. 2006). Although the activation of microglia is a natural process and is necessary for defense against ischemia, microglial over-activation results in the progression of neuronal loss and brain injury following ischemic stroke. Thus, reducing cerebral microglial activation and inhibiting their release of pro-inflammatory cytokines is considered to be an important therapeutic strategy for ischemic stroke.

One potential neuroprotective agent is the nucleoside guanosine. Several neurodegenerative and neurotoxic models have demonstrated that guanosine plays an important role in the CNS (Giuliani et al. 2012; Petronilho et al. 2012; Pettifer et al. 2007; Quincozes-Santos et al. 2013; Schmidt et al. 2010a; Tarozzi et al. 2010; Tavares et al. 2008), exerting glutamatergic system antagonism and trophic effects on neural cells (Ciccarelli et al. 2000; Rathbone et al. 2008; Schmidt et al. 2007; Schmidt et al. 2010b; Schmidt AP 2010). Studies have shown that guanosine protects against several ischemic models, including oxygen and glucose deprivation (Chang et al. 2008; Dal-Cim et al. 2013; Oleskovicz et al. 2008), hypoxia-ischemia models (Moretto et al. 2009), and *in vivo* permanent or transient ischemic stroke (Chang et al. 2008; Connell et al. 2013; Rathbone et al. 2011). Researchers have claimed that guanosine is neuroprotective against oxidative and inflammatory processes induced by ischemia via MAPK/ERK pathway activation (Dal-Cim et al. 2013; Dal-Cim et al. 2011; Oleskovicz et al. 2008). Our laboratory has demonstrated that guanosine, by modulating the oxidative response and glutamatergic parameters, protects against damage provoked by permanent focal ischemia induced by thermocoagulation (Hansel et al. 2014). Excessive reactive oxygen species production during an ischemic event can trigger an inflammatory response through activation of the transcriptional nuclear factor kappa B (NF-κB) (Gloire et al. 2006), which promotes the expression of a large number of genes related to the pathology of cerebral ischemia, including those involved in the inflammatory response, such as IL-1 and TNF-α (Sethi et al. 2008).

Previous experimental studies have demonstrated that guanosine modulates the oxidative response during an ischemic event (Hansel et al. 2014), and this effect is linked to neuroinflammatory mechanisms (Gloire et al. 2006). Thus, in this study, we tested the hypothesis that guanosine attenuates cerebral ischemic injury by inhibiting the microglia-mediated inflammatory response and modulating the levels of anti- and pro-inflammatory cytokines in a model of permanent focal cerebral ischemia induced by thermocoagulation.

Materials and Methods

Animals

Wistar male adult rats (90 – 100 days old, weighing 300 – 350 g) were maintained under controlled light and environmental conditions (12 h light/12 h dark cycle at a constant temperature of 22 ± 2 ºC) with water and commercial food pellets available *ad libitum*. All experimental procedures were performed according to the NIH Guide for Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for Animal Care.

Induction of permanent focal ischemia

The ischemic lesion was induced by thermocoagulation of the blood in the pial vessels of the motor and sensorimotor cortices (Szele et al. 1995). Briefly, the animals were anesthetized with ketamine hydrochloride (70 mg/kg, i.p.) and xylazine hydrochloride (10 mg/kg, i.p.) and placed in a stereotaxic apparatus. The skull was surgically exposed, and a craniotomy was performed by exposing the left frontoparietal cortex (+ 2 to − 6 mm A.P. and – 2 to – 4 mm M.L. from bregma) (Paxinos and Watson 1986). Blood in the pial vessels was thermocoagulated transdurally by approximation of a hot probe close to the dura mater. The rostrocaudal extent of the surface of the frontal and parietal cortices was lesioned. After the

procedure, the skin was sutured, and the body temperature was maintained at 37 ºC using a heating pad until recovery from the anesthesia.

Drug treatment

The animals were divided into 4 groups: sham saline; sham guanosine; ischemia saline; ischemia guanosine. Guanosine (60 mg/kg in NaCl 0.9 %) was purchased from Sigma (St. Louis, MO, USA). All groups received 1 mL/kg i.p. administration of the drugs immediately, 1 h, 3 h, and 6 h after surgery.

Cylinder Test

This test is based on the spontaneous exploratory behavior of rodents and reveals forelimb preference when the animal rears to explore its environment by making forelimb contact with the cylinder walls (Macrae 2011; Schallert 2006). Animals were subjected to 1 trial, one day before ischemia (pre-ischemic day) and 24 h after ischemia (post-ischemic day). To prevent habituation to the cylinder, the number of movements recorded was limited to 20. The occurrences of ipsilateral (to the lesion) forelimb use, contralateral forelimb use, or simultaneous forelimb use were counted. The asymmetry score for each animal was calculated on each day by the formula previously described (de Vasconcelos Dos Santos et al. 2010).

Measurement of infarct volume

Twenty-four hours after surgery, the animals were sacrificed, and the brains were rapidly removed from the skull and sectioned in the coronal plane at a 2 mm thickness using a rat brain matrix. The slices were immersed for 30 min in a 2 % of 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO, USA) solution at 37 °C followed

by overnight fixation in 4 % paraformaldehyde (Sigma, St. Louis, MO, USA). The infarct volume was calculated by the formula: *Infarct volume = [measured infarct area x slice thickness (2 mm)] + [area of contralateral corresponding structure x slice thickness]–[area of ipsilateral corresponding structure x slice thickness]* (Liu et al. 2009; Swanson et al. 1990). The brain slices were analyzed with Image J software (NIH, Bethesda, Maryland, USA). The results are expressed as mm^3.

Cerebrospinal fluid and brain tissue processing

Cerebrospinal fluid (CSF) and tissue samples were collected 24 h after the ischemic insult and were frozen (-80 °C) until analysis. The animals were anesthetized with ketamine hydrochloride (70 mg/kg, i.p.) and xylazine hydrochloride (10 mg/kg, i.p.) and placed in a stereotaxic apparatus. The CSF was collected from the cisterna magna. The puncture was performed using an insulin syringe (27 gauge 9 1/200 length). The CSF was then centrifuged at 3000 g for 10 min at 4 °C to obtain a CSF cells-free supernatant. The animal was then submitted to transcardial perfusion with 0.9 % saline to eliminate blood from the cerebral tissue. For the flow cytometry assay, the brain was removed from the skull, maintained at 4 °C, and the cortical tissue was collected. In ischemic animals, the brain tissue adjacent to the ischemic lesion (located between the lesion and the cerebral longitudinal fissure, measuring approximately 8 mm x 2 mm) was considered the lesion periphery (Fig. 1). This region was chosen because previous studies (de Vasconcelos Dos Santos et al. 2010) of this model have determined that this region has characteristics that are similar to a penumbra area. The same region was dissected in sham brains and on the contralateral side of ischemic brains. For the immunohistochemistry assay, the animals were transcardially perfused with 0.9 % saline followed by 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and post-fixed in the same solution at room temperature for 4 h. For the

immunohistochemistry analysis, brains were incubated in a 30 % sucrose solution for 2 days. Coronal sections (50 μm) were obtained using a Vibratome (Leica Biosystems, Mannheim, Wetzlar, Germany). One section from six random series was collected for immunohistochemistry from the same region used for flow cytometry assay (Fig. 1). The images were captured, and a square region of interest was created.

Immunohistochemistry assay:

For the microglia assay, the sections were incubated for 24 h at 4 °C with polyclonal rabbit anti-Iba-1 (Wako, Tokyo, JP, 1:500) in Tris buffered saline (TBS), pH 7.4, containing 0.1 % of Triton-X 100 and 10 % of bovine serum. After being washed four times with TBS, the sections were incubated with 594 Alexa-Fluor conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories, Inc., PA, USA) for 2 h at room temperature. After washing, the sections were mounted on slides coated with 5 % gelatin with chromium and potassium sulfate using Vecta shield mounting medium (Vector Laboratories, São Paulo, Brazil). All sections were photographed with confocal microscopy (Olympus, Tokyo, JP). For immunohistochemical analyses, all lighting conditions and magnifications were kept constant.

The Fluoro-Jade C (FJC) stain was used to investigate neurodegeneration. Sections were subjected to FJC staining in accordance with the manufacturer's instructions (Gu et al. 2012). Briefly, the sections were immersed in a solution of 1 % sodium hydroxide in 80 % alcohol for 5 min; 70 % alcohol for 2 min; distilled water for 2 min; and 0.06 % potassium permanganate for 15 min. The sections were immersed in a solution of 0.0005 % FJC (Millipore Corporation, Billerica, MA, USA) in 0.1 % acetic acid vehicle for 30 min. The sections were then rinsed 3 times in distilled water and allowed to dry at 45 °C for 20 min before mounting with DPX medium (Electron Microscopy Sciences, Hatfield, PA, USA). FJC positive cell counting was performed as previously described (Giraldi-Guimaraes et al. 2009)

with modifications. Six sections located inside in the lesion periphery were analyzed. FJC positive cells were counted in each image (6 images per animal), and the area where the cells were included was measured using the Image J software. The final value for each animal was: Σ (cells counted per image) / Σ (area containing labeled cells per image, in μm^2).

Flow cytometry assay

The tissue samples (30 mg) were mechanically dissociated with 1 mL of phosphate-buffered saline (PBS), pH 7.4, containing 10 mg/mL of collagenase IV; were filtered with a 40 µm nylon mesh to remove large clumps of cells and debris; and were incubated with PBS/collagenase containing 10 µg/ml propidium iodide (PI). The cells were incubated at room temperature in the dark for 30 min, washed twice with PBS and centrifuged at 1000 g for 10 min at 4 °C to remove the supernatant containing free PI. Afterwards, the cells were permeabilized with 0.001 % PBS Triton X-100 and blocked for 15 min with bovine serum albumin 1 %. After blocking, the cells were incubated for 1 h in blocking solution containing the monoclonal antibodies anti-NeuN diluted 1:100 (Millipore Corporation, Billerica, MA, USA), anti-GFAP diluted 1:100 (Dako, California, USA) or anti-Iba-1 diluted 1:100 (Wako, Tokyo, JP). The cells were washed twice with PBS and were incubated for 1 h in blocking solution containing Alexa 488 anti-mouse IgG diluted 1:200 or Alexa 488-anti-rabbitIgG diluted 1:200 (Jackson ImmunoResearch Laboratories, Inc, PA, USA). The level of PI incorporation and number of positive NeuN, GFAP, or Iba-1 positive cells were determined by flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA). Alexa Fluor 488 and PI dyes were excited at 488 nm using an air-cooled argon laser. Negative controls (samples with the secondary antibody) were included for the machine voltage set-up. The emission of fluorochromes was recorded through specific band-pass fluorescence filters: green (FL-1; 530 nm/30) and red (FL-3; 670 nm long pass) using a CellQuest Pro software

(Becton Dickinson, Franklin Lakes, NJ, USA). Fluorescence emissions were collected by using logarithmic amplification. In brief, data from 20,000 events (intact cells) were acquired, and the mean relative fluorescence intensity was determined after exclusion of debris events from the data set. All flow cytometric analyses were performed using Flow Jo software 7.6.3 (Treestar, Ashland, OR). Flow cytometry data were analyzed and plotted by density as dot plots, which show the relative FL1 fluorescence on the x-axis and the relative FL3 fluorescence on the y-axis. The negative and positive quadrants were determined by using unstained samples. The number of cells in each quadrant was computed, and the proportion of cells stained with PI, NeuN, GFAP, and Iba-1 were expressed as percentage of control (Heimfarth et al. 2012).

Inflammatory cytokine measurements

The tissue samples (30 mg) were homogenized with PBS/Tris-HLC pH 7.4 and were centrifuged at 5,000 g for 10 min 4 °C to exclude debris. The supernatant was collected to analyze and measure protein content.

The inflammatory cytokine concentrations were measured by Enzyme Linked Immunosorbent Assay (ELISA) using commercial kits for rat TNF-α, IFN-γ, IL-1, IL-6, and IL-10 (eBIOSCIENCE, San Diego, USA) according to the manufacturer's instructions. Briefly, 96 well microplates were incubated with the primary antibody at 4 °C overnight, washed and blocked at room temperature for 1 hour. The cytokine standards, calibrators and samples (CSF or tissue) were added and incubated at room temperature for 2 hours. After washing, the secondary antibody conjugated with peroxidase was added and incubated at room temperature for 1 hour. After washing, a TMB chromogen (Tetramethylbenzidine) was added, and the reaction was allowed to proceed for 15 min. The enzyme reaction was stopped by adding Stop solution. The absorbance was measured at 450 nm. The presence and concentration of the

cytokines were determined by the color intensity measured by spectrometry in a micro ELISA reader. The results are expressed as pg/mL for CSF samples, and as pg/mg for tissue samples.

Protein assay

Protein content was measured using Pierce BCA protein kit (Thermo Scientific, Waltham, MA, USA) with bovine serum albumin as a standard. Results are expressed as mg of protein.

Statistical analysis

The results are presented as the mean ± S.E.M. The cylinder test was analyzed with a repeated measures analysis of variance (ANOVA) followed by Tukey's post-hoc test. FJC was analyzed using Student's t-test. Cytometry assays, cytokines, and cytokine ratios were statistically analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. Probability values less than 0.05 were considered to be significant. All analyses were performed using the Statistical Package for Social Sciences (SPSS) software version 18.0.

Results

Behavioral test and infarct volume

To evaluate whether the administration of guanosine after cortical ischemia leads to functional recovery, ischemic animals were treated with guanosine, and their sensorimotor performance was measured. Both day and group variables, visualized by the ''group x day'' interaction ($F_{(3,65)}$ = 23.9; P < 0.0001), were significantly different. The symmetry score decreased 24 h after ischemia. The guanosine treatment promoted significant recovery of the contralateral forelimb performance in support during vertical exploration (Fig. 2A).

In agreement with a previous study (Hansel et al. 2014), brain infarct volume analysis by TTC staining 24 h after ischemia demonstrated a large amount of tissue damage (visualized by pale staining). Guanosine treatment significantly decreased the extent of tissue damage (Fig. 2B). The sham procedure induced no damage (data not shown).

Cellular degeneration

As previously shown (Giraldi-Guimaraes et al. 2009), the procedure of thermocoagulation induced a large amount of neurodegeneration as revealed by FJC staining. The number of FJC positive cells observed in the lesion periphery 24 h after ischemia was significantly lower in ischemic animals treated with guanosine ($P < 0.05$) (Fig. 3A and 3B).

For assessing cell types and cell viability it was carried flow cytometry analysis. There was no difference among groups in the total number of neurons and astrocytes marked by NeuN and GFAP antibodies, in the contralateral and ipsilateral sites (Fig. 4A and 4C). In addition, the anti-NeuN and anti-GFAP antibodies co-stained with PI identified neuronal and astrocytic damage. PI incorporation in neurons in the ischemic group (in ipsilateral site) significantly increased more than 3-fold compared to the sham group. Guanosine treatment was able to abolish the increase in PI incorporation 24 h after ischemia (Fig. 4B). In the ischemic group (on the ipsilateral side), 24 h after ischemia, the number of anti-GFAP cells co-stained with PI increased 2-fold compared to the sham group. The ischemic group treated with guanosine had a partial decrease in PI incorporation compared to the ischemic group (Fig. 4D). Together, these findings indicate that ischemia increased neuronal and astrocytic damage in the lesion periphery, and guanosine treatment provided neuronal and astrocytic neuroprotection 24 h after ischemia.

Guanosine inhibited the ischemic microglial proliferation and activation

The Iba-1 antibody detects microglia and also resident and peripheral immune cells, such as monocytes/macrophages. To specifically mark microglia, the tissue was perfused with 0.9 % saline. Using flow cytometry analysis, there was a significant increase of microglia number in the ischemic group 24 h after ischemia. In contrast, the ischemic group treated with guanosine had a reduced number of microglia (Fig. 5).

Immunohistochemistry was carried out using Iba-1 for microglia labeling. The Iba-1 antibody allows visualization of microglia and their processes. No difference was detected on the contralateral side across all groups. In both sham groups, the microglia present in the lesion periphery had a typical resting morphology with cellular processes branching from the small soma with further distal arborization (Fig. 6). Twenty-four hours after insult, the ischemic group had several cells with enlarged somata, fewer and shorter processes, which is characteristic of the activated state (Fig. 6). The ischemic group treated with guanosine had an intermediary morphology. These groups had a smaller number of cells with activated characteristics and cells with intermediary processes branching from the somata in the lesion periphery (Fig. 6).

Guanosine attenuated changes in the levels of pro-inflammatory and anti-inflammatory cytokines

In this study, the levels of pro-inflammatory cytokines were measured in the CSF (Fig. 7) and in the lesion periphery (Fig. 8) 24 h after ischemia. The IL-1, IL-6, TNF-α and IFN-γ levels were significantly augmented in both the CSF and lesion periphery in the ischemic animals. Guanosine treatment significantly returned the levels of all these cytokines to sham levels. IL-10 is an anti-inflammatory cytokine. The levels of IL-10 decreased in the ischemic animals compared to the sham group, and guanosine treatment inhibited this reduction both in the CSF and lesion periphery. The IL1/IL10, IL6/IL10, TNFα/IL10 and IFN-γ/ IL-10 ratios

were calculated to verify the relationship between the pro- and anti-inflammatory cytokines. These ratios were increased in the ischemic animals in the CSF and lesion periphery, and this effect was minimized with guanosine treatment (Table 1).

Discussion

The neuroprotective effects of guanosine in ischemic stroke have been studied in several stroke models. The data from *in vitro* models suggest that guanosine protects against oxygen and glucose deprivation (Chang et al. 2008; Dal-Cim et al. 2013; Dal-Cim et al. 2011; Oleskovicz et al. 2008; Thomazi et al. 2008); increases glutamate uptake in hypoxia-ischemia models (Moretto et al. 2005; Moretto et al. 2009); and protects against glucose deprivation (Quincozes-Santos et al. 2013). *In vivo* guanosine is neuroprotective against permanent and transient ischemic stroke, promotes functional improvement, and reduces infarct volume (Chang et al. 2008; Connell et al. 2013; Rathbone et al. 2011). Our group recently demonstrated that guanosine neuroprotection during permanent focal ischemia involves the modulation and maintenance of the cellular redox environment and the glutamatergic system (Hansel et al. 2014). During ischemic events, the excessive reactive oxygen species production can trigger an inflammatory response and contribute to the expansion of brain injury and delayed neuronal death. Thus, the novel finding of this study is that treatment with guanosine early after permanent focal ischemia suppressed the activation and proliferation of microglia in the lesion periphery, reduced the levels of pro-inflammatory cytokines and increased the levels of anti-inflammatory cytokines in the brain and CSF. These effects lead to diminished neural damage and consequently functional recovery.

Neurons are extremely susceptible to changes in blood flow, and clinical studies have shown impaired neuronal function after only 10 min of ischemia (Oechmichen and Meissner 2006). In animal models of ischemic stroke, neural cells in the core rapidly become

committed to die. Therefore, therapeutic strategies aimed at rescuing these neurons have failed. As a consequence, attention has increasingly become focused on the penumbra, where neuronal death can be extended for days to weeks (Brouns and De Deyn 2009; Durukan and Tatlisumak 2007). FJC staining has a good affinity for entirely degenerating neurons (Gu et al. 2012). Here, FJC staining it was used to study the effect of guanosine treatment on ischemia-induced neurodegeneration in the lesion periphery. The number of degenerating neurons in the lesion periphery 24 h after ischemia significantly decreased in the ischemic group treated with guanosine. In addition, the flow cytometry analysis 24 h after ischemic insult indicated that although the total number of neurons and astrocytes were not decreased in the ipsilateral site in the ischemic animals, the number of PI positive in neurons and astrocytes was increased. The guanosine treatment totally reversed the increase in PI incorporation in neurons and partially restored this effect in astrocytes. These findings indicate that the beneficial effects of guanosine may be due to neuroprotection in the lesion periphery, the main presumptive site of restorative processes. Guanosine treatment diminishes the neurodegeneration and consequently promotes increased functional recovery.

Accumulating evidence demonstrates the relationship between inflammation and neuronal cell damage in cerebral ischemia (Kaushal and Schlichter 2008). Thus, the mechanisms of neuroprotective strategies against cerebral ischemia may target inflammatory alterations involved in cellular damage. Neuroinflammation is one of the key pathological events contributing to the progression of damage caused by ischemia (Lakhan et al. 2009). Post-ischemic inflammatory responses are characterized by the activation of astrocytes and microglia, as well as infiltration of polymorphonuclear granulocytes and monocytes/macrophages into the ischemic brain region (Iadecola and Anrather 2011). Resident microglia is the major cells involved in inflammatory processes in the brain and are the first cells to respond to brain injury. They are activated within minutes after the onset of

focal cerebral ischemia. This activation may last for several weeks and plays a critical role during ischemia mainly in the penumbra area (Jin et al. 2010; Madinier et al. 2009; Woodruff et al. 2011). In this study, the number of microglia in the lesion periphery, analyzed by flow cytometry, had substantially increased 24 h after ischemia, and these cells presented morphological transformation. The sham group had the characteristics of resting microglia with small somata and long, distal arborized processes. The ischemic group had characteristics of activated microglia. The microglia cells had reduced the complexity of their shape by retracting the branches of their processes (so that they are resorbed into the cell body), which increases the intensity stained near the soma. The ischemic group treated with guanosina had an intermediary morphology with some microglia having activated characteristics but still with branched processes.

Although microglial activation is necessary for the process of tissue damage in response to cerebral ischemia, the production of pro-inflammatory cytokine mediators contribute to the expansion of brain injury and the delayed neuronal death (Jin et al. 2010; Lakhan et al. 2009). Here, the activation and proliferation of microglia was accompanied by an increase of pro-inflammatory cytokines IL-1, IL-6, TNF-α and INF-γ and a decrease of the anti-inflammatory cytokine IL-10 in both the lesion periphery and CSF in the ischemic group. Increased production of pro-inflammatory cytokines has been observed in experimental models of brain ischemia and in patients with acute stroke and may be associated with large cerebral infarct volume and poor stroke outcome (Tuttolomondo et al. 2009). TNF-α, INF-γ and IL-1 are rapidly expressed in the ischemic brain after the disease onset, and their upregulation persists for days. Despite the unclear effects of IL-6, due to its anti-inflammatory properties to induce IL-1 receptor antagonist synthesis, studies have shown that the upregulation of TNF-α, INF-γ, IL-1, and IL-6 following ischemic events leads to the disruption of the blood brain barrier, increases the infarct volume and causes neuronal cell

death (Doyle et al. 2008; Lambertsen et al. 2012; Tuttolomondo et al. 2009). Some investigators have reported that IL-10 is the main down regulator of deleterious effects of pro-inflammatory cytokines. Clinical stroke studies have demonstrated that IL-10 decreases during ischemic events, and increases in IL-10 are associated with better patient outcomes (Nilupul Perera et al. 2006; Tuttolomondo et al. 2009). Interestingly, the guanosine treatment after ischemia decreased the production of pro-inflammatory cytokines and restored IL-10 to sham levels.

Studies have described the balance between pro and anti-inflammatory factors to be a more accurate reflection of the inflammatory environment (Gomes da Silva et al. 2013; Rawdin et al. 2013). Due to significant inter-individual differences in the concentrations of pro- or anti-inflammatory factors, here, we evaluated the ratio of pro- to anti-inflammatory factors, which provided a useful measure of the net immunological effect of the circulating cytokines and/or other factors associated with damage. It is important to note that the ratios of IL1/IL10, IL6/IL10, TNFα/IL10 and IFN-γ/ IL-10 were reduced in the ischemic animals treated with guanosine compared to ischemic group 24 h after ischemia in the lesion periphery and CSF. Taken together, these findings indicate a favorable effect of guanosine treatment, which altered the balance of pro- and anti-inflammatory cytokines.

Thus, the present data indicate that 24 h after insult, ischemic stroke results in microglial activation and proliferation in the lesion periphery. In addition, the expression of pro-inflammatory cytokines (TNF-α, INF-γ, IL-1 and IL-6) in this area increased, and anti-inflammatory cytokines (IL-10) decreased. These results are consistent with previous studies that demonstrated that cerebral ischemia activates microglia and increases expression of pro-inflammatory cytokines in the penumbra 24 h after middle cerebral artery occlusion and that the immunoreactivity for pro-inflammatory cytokines is co-localized with activated microglia (Wei et al. 2011). Furthermore, this study demonstrates that guanosine treatment decreased

the number of total microglia in the lesion periphery and was accompanied by a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines 24 h after ischemia. The suppression of some inflammatory components is associated with reduced neuronal cell death, reduced infarct volume and improved behavioral outcomes in stroke models (Doyle et al. 2008; Lambertsen et al. 2012; Nilupul Perera et al. 2006). Thus, the present study demonstrates for the first time that administration of guanosine after permanent focal cerebral ischemia reduced microglial proliferation and pro-inflammatory cytokine release and increased anti-inflammatory cytokines in the brain. The reduction of the inflammatory state may modulate oxidative parameters and reduce neuronal and astrocytic cell death, consequently improving the function of the impaired forelimb.

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Figure legends

Figure 1: Schematic illustration of the ischemic lesion. Illustrations showing the region of induced lesion (dark gray), and the analyzed region (gray) that were dissected for the experiments. The left (ipsilateral side) and right (contralateral side) frontoparietal cortices were used.

Figure 2. Recovery of the impaired forelimb in the cylinder tests 24 h before and after ischemia and infarct volume of the lesion 24 h after ischemia. (A) Graph showing the performance of sham saline (n = 8); sham guanosine (n = 8); ischemia saline (n = 10); and ischemia guanosine (n = 10) groups. Ischemia and ischemia guanosine groups were significantly different from the sham group 24 h after ischemia. The ischemia guanosine group partially recovered the use of the impaired forelimb. Data are expressed as percentage of symmetry score. * $P < 0.001$ comparing sham group vs. ischemia and ischemia guanosine groups; $^{\#}P < 0.01$ comparing ischemia and ischemia guanosine. (B) Analysis of cortex infarct volume 24 h after cerebral ischemia. Ischemia guanosine group had a significantly reduced infarct volume compared to the ischemia group. Data are expressed as mm^3. ** $P = 0.0014$, n = 8 per group. The representative TTC staining was evidenced in both ischemic and ischemic treated with guanosine groups. In sham animals, the TTC staining was analyzed, but no lesion was observed.

Figure 3. Neuronal neurodegeneration evaluation (FJC staining) 24 h after ischemia. Images of the lesion periphery were analyzed. The images were taken from representative coronal sections of ischemic animals (A) and ischemic animals treated with guanosine (B). Note that the number of FJC positive (degenerating) neurons was greater in ischemic animals compared to ischemic guanosine-treated animals. (C) Quantification of degenerating neurons. Ischemic

guanosine group had a significant reduction in the number of FJC positive neurons in the lesion periphery. Data are expressed as number of FJC positive cells. Symbol represent * P = 0.007, n = 5 per group.

Figure 4. Flow cytometry analysis 24 h after ischemia in the lesion periphery (contralateral and ipsilateral sides). (A) NeuN positive cells. (B) NeuN positive cells co-stained with propidium iodide (PI), * $P < 0.001$ comparing sham saline and ischemic guanosine vs. ischemic saline. (C) GFAP positive cells. (D) GFAP positive cells co-stained with PI, * $P < 0.001$ comparing sham saline vs. ischemic saline; $^{\#}P < 0.01$ comparing ischemia guanosine vs. ischemia saline; and $^{\&}P < 0.05$ comparing sham saline vs. ischemia guanosine. Cells stained only with the secondary antibody were used to set the negative region of the dot plot. Cells with fluorescence above the negative region were considered stained and were counted as neurons (NeuN positive) or astrocytes (GFAP positive). Data are reported as the means ± S.E.M. of 8 – 10 animals per group and are expressed as a percent of control. Statistical differences were determined by a two-way ANOVA followed by a Bonferroni post-test.

Figure 5. Flow cytometry analysis by Iba-1 24 h after ischemia in the lesion periphery (contralateral and ipsilateral sides). Cells stained only with the secondary antibody were used to set the negative region. Cells with fluorescence above the negative region were considered stained and were counted as microglia (Iba-1 positive). Data are reported as the means ± S.E.M. of 9 – 13 animals per group and are expressed as a percent of control. Statistical differences were determined by a two-way ANOVA followed by Bonferroni post-tests, * $P < 0.001$ comparing sham saline and ischemia guanosine vs. ischemia saline.

Figure 6. Iba-1 immunohistochemistry 24 h after permanent focal ischemia. Representative images (40X and 60X) of 5 animals per group. Six slices per animal were used. The lesion periphery in the ipsilateral and contralateral sides was analyzed.

Figure 7. CSF inflammatory cytokines levels: (A) TNF-α, (B) INF-γ, (C) IL-1, (D) IL-6, and (E) IL-10, 24 h after ischemia. Data are reported as the means ± S.E.M. of 8 – 16 animals per group and are expressed as pg/mL. Statistical differences were determined by a two-way ANOVA followed by Bonferroni post-tests. * $P < 0.001$ comparing sham saline and ischemia guanosine vs. ischemia saline in A, B, C, and E; * $P < 0.001$ comparing sham saline and vs. ischemia saline, and $^{\#}P < 0.01$ comparing ischemia guanosine vs. ischemia saline in D.

Figure 8. Inflammatory cytokines levels: (A) TNF-α, (B) INF-γ, (C) IL-1, (D) IL-6, and (E) IL-10 measured in the lesion periphery 24 h after ischemia. Data are reported as the means ± S.E.M. of 10 animals per group and are expressed as pg/mg of protein. Statistical differences were determined by a two-way ANOVA followed by Bonferroni post-tests. * $P < 0.001$ comparing sham saline and ischemia guanosine vs. ischemia saline in A, B and C; * $P < 0.001$ comparing sham saline and vs. ischemia saline; $^{\#}P < 0.01$ comparing ischemia guanosine vs. ischemia saline in D; * $P < 0.001$ comparing sham saline and vs. ischemia saline; $^{\#}P < 0.01$ comparing ischemia guanosine vs. ischemia saline; $^{\&}P < 0.05$ comparing sham saline vs. ischemia guanosine in E.

Figure 1:

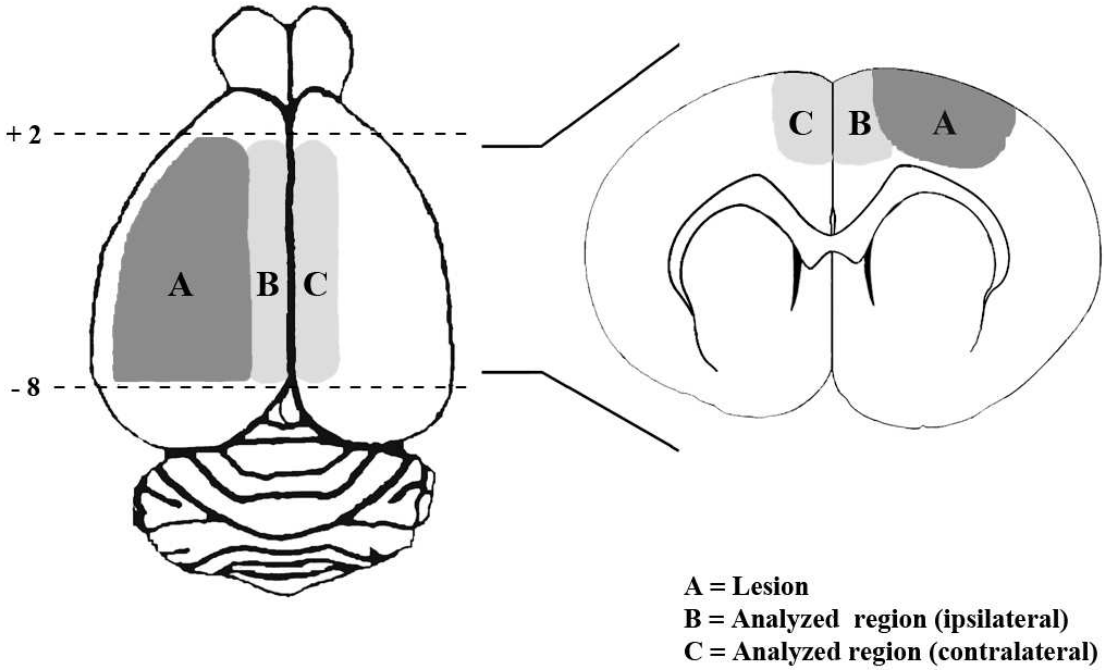
+ 2
- 8
A
B
C
C
B
A
A = Lesion
B = Analyzed region (ipsilateral)
C = Analyzed region (contralateral)

Figure 2:

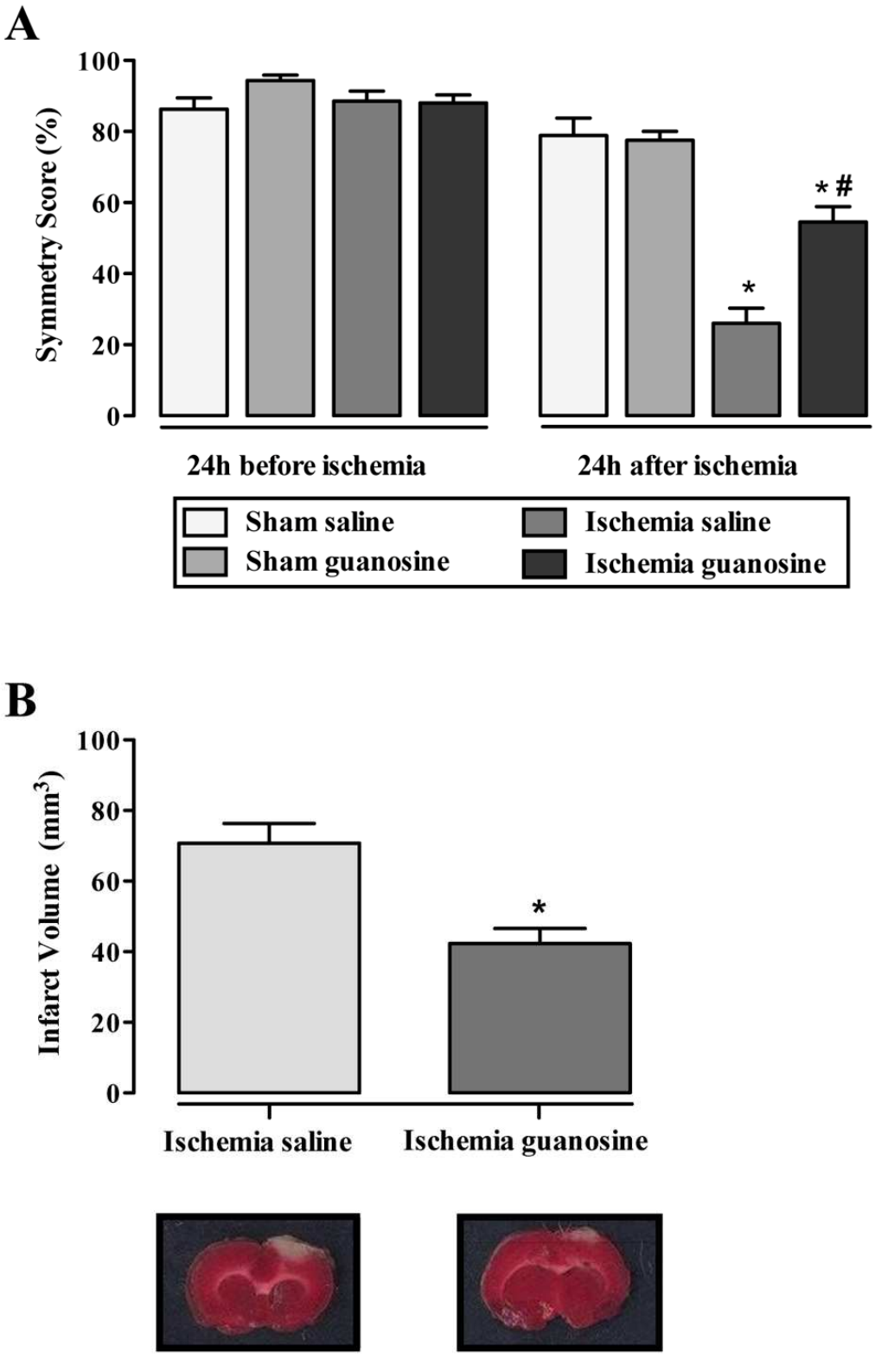
A
Symmetry Score (%)
100
80
60
40
20
0
*
*#
24h before ischemia
24h after ischemia
Sham saline
Sham guanosine
Ischemia saline
Ischemia guanosine
B
Infarct Volume (mm3)
100
80
60
40
20
0
*
Ischemia saline
Ischemia guanosine

Figure 3:

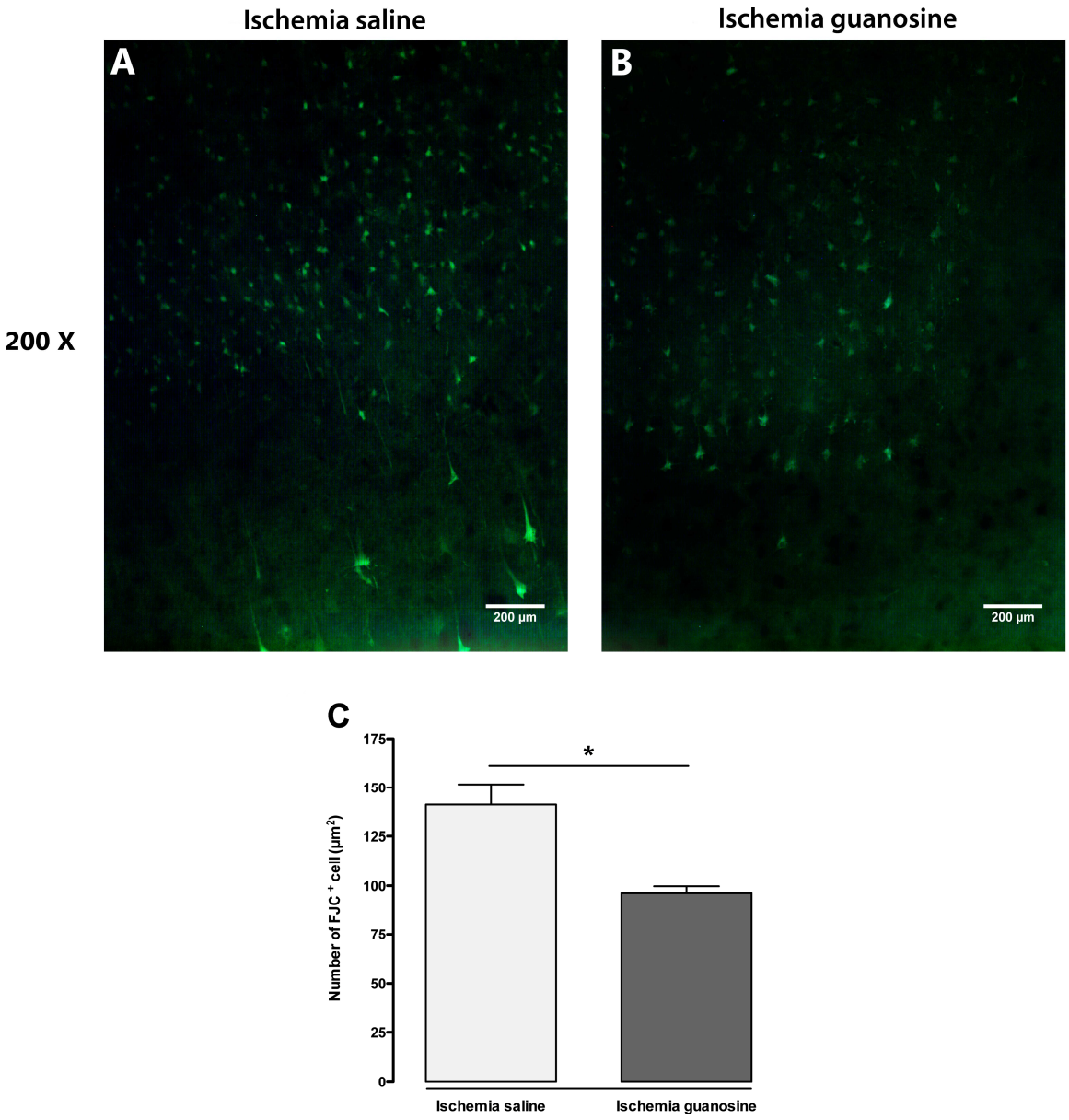
Ischemia saline
Ischemia guanosine
A
B
200 X
200 µm
200 µm
C
Number of FJC + cell (µm2)
175
150
125
100
75
50
25
0
*
Ischemia saline
Ischemia guanosine

Figure 4:

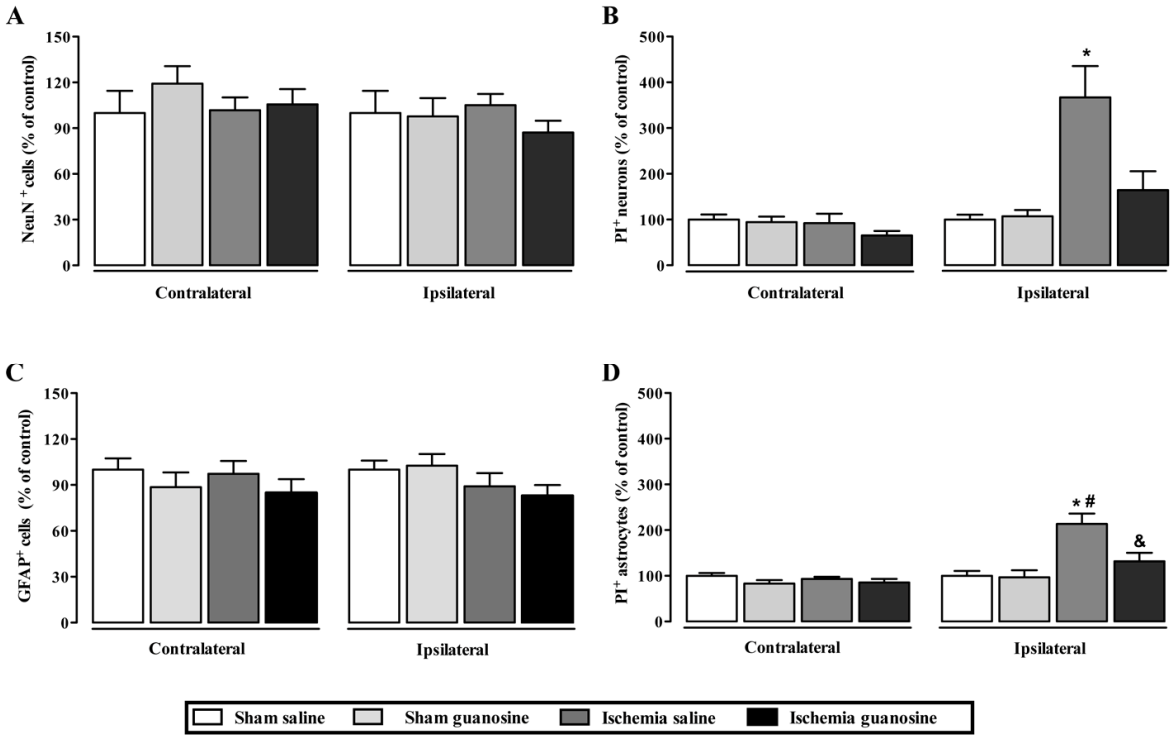
A
NeuN+ cells (% of control)
150
120
90
60
30
0
Contralateral
Ipsilateral
B
PI+ neurons (% of control)
500
400
300
200
100
0
*
Contralateral
Ipsilateral
C
GFAP+ cells (% of control)
150
120
90
60
30
0
Contralateral
Ipsilateral
D
PI+ astrocytes (% of control)
500
400
300
200
100
0
*#
&
Contralateral
Ipsilateral
Sham saline
Sham guanosine
Ischemia saline
Ischemia guanosine

Figure 5:

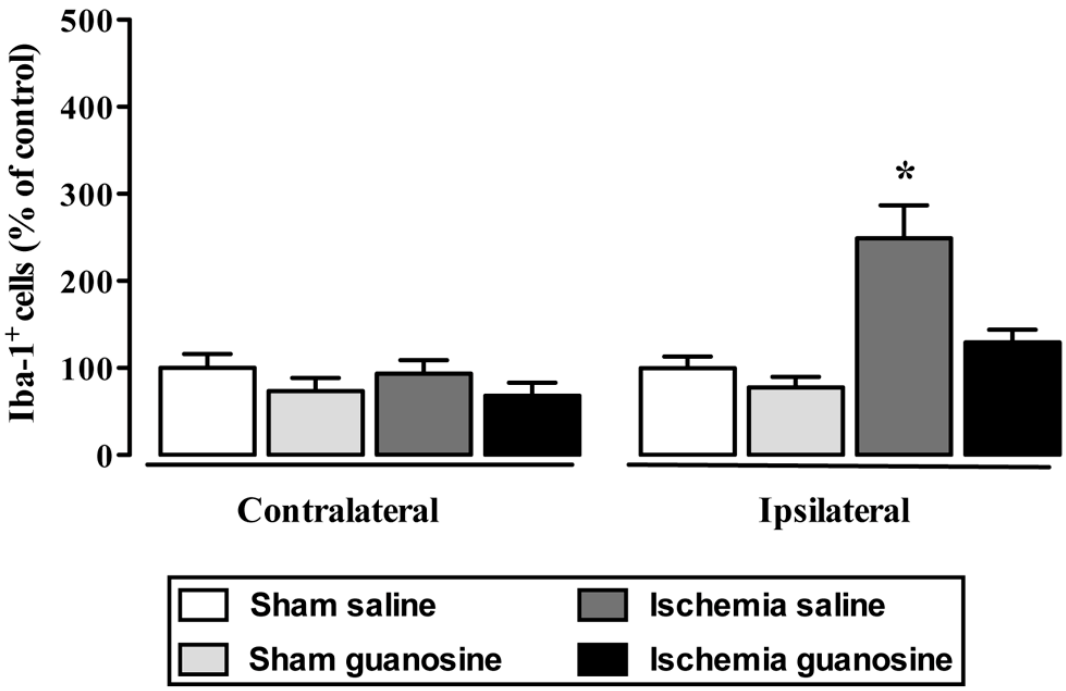
500
400
300
200
100
0
Iba-1+ cells (% of control)
*
Contralateral
Ipsilateral
Sham saline
Sham guanosine
Ischemia saline
Ischemia guanosine

Figure 6:

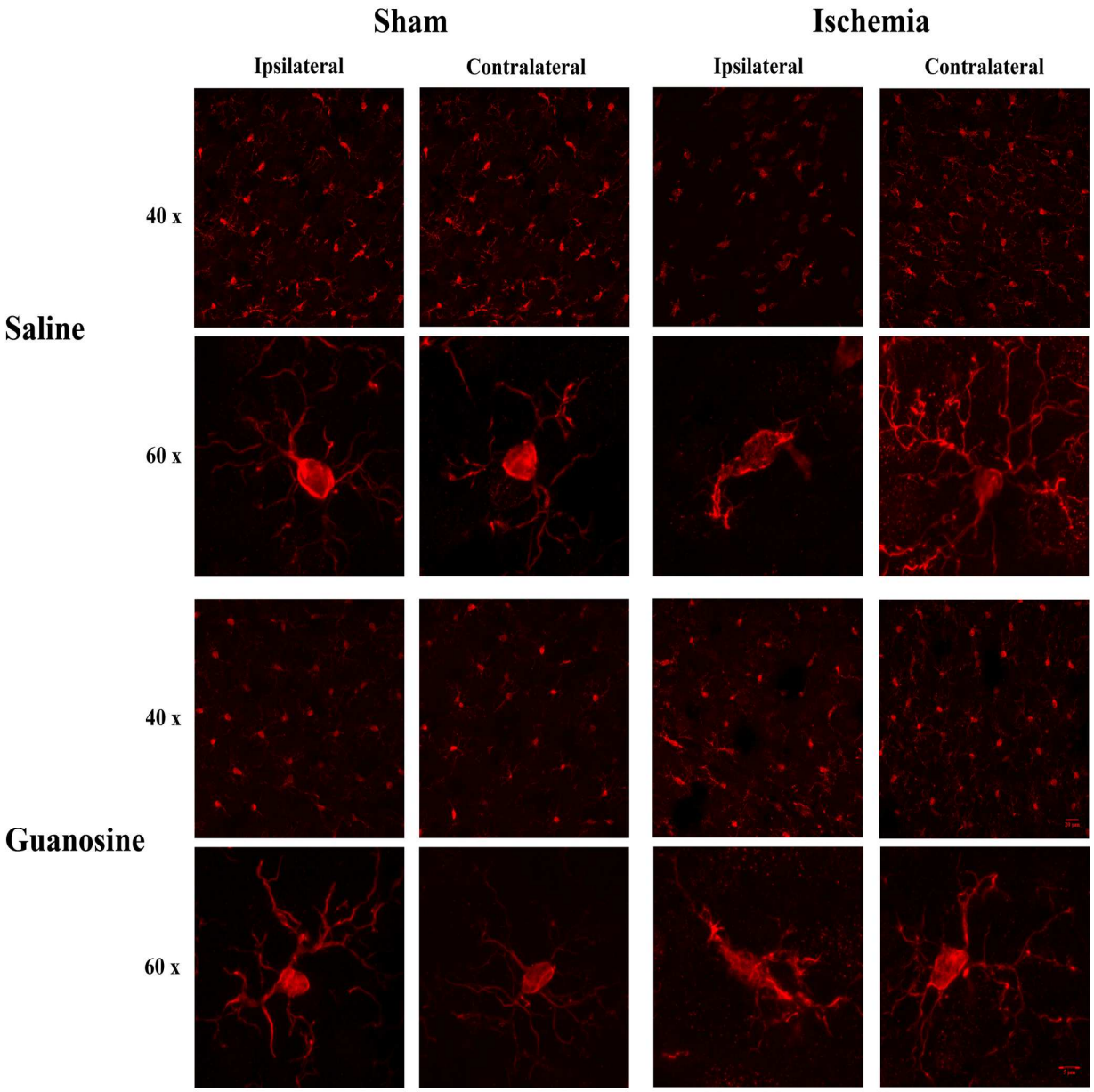
Sham
Ischemia
Ipsilateral
Contralateral
Ipsilateral
Contralateral
Saline
40 x
60 x
Guanosine
40 x
60 x

Figure 7:

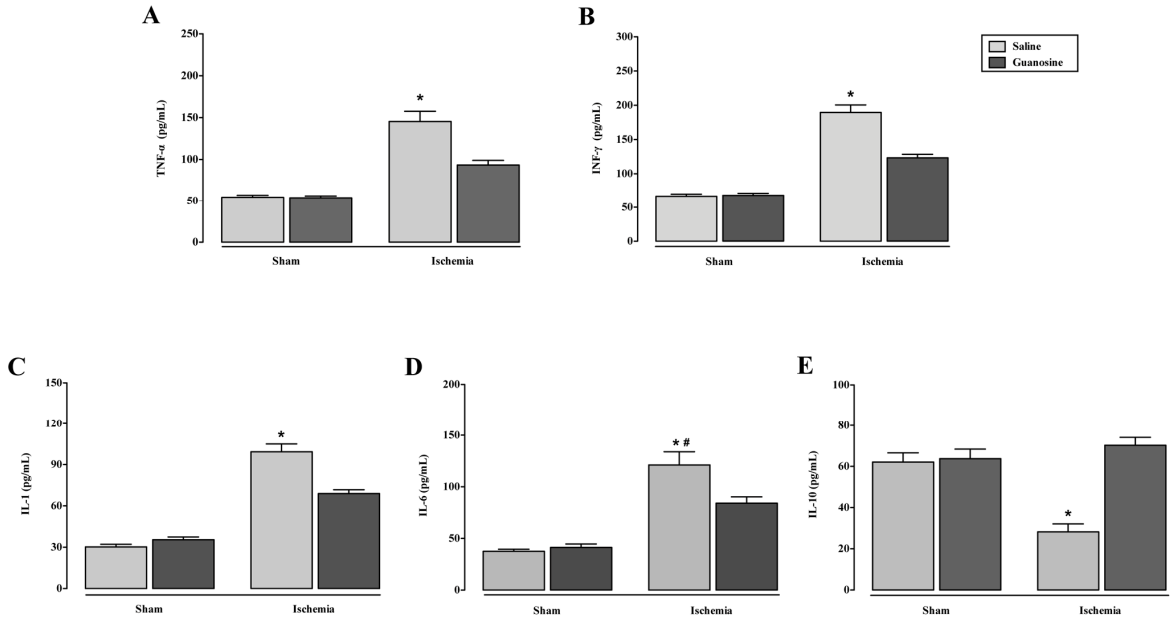
A
B
C
D
E
Saline
Guanosine
TNF-α (pg/mL)
INF-γ (pg/mL)
IL-1 (pg/mL)
IL-6 (pg/mL)
IL-10 (pg/mL)
Sham
Ischemia
*
* #

Figure 8:

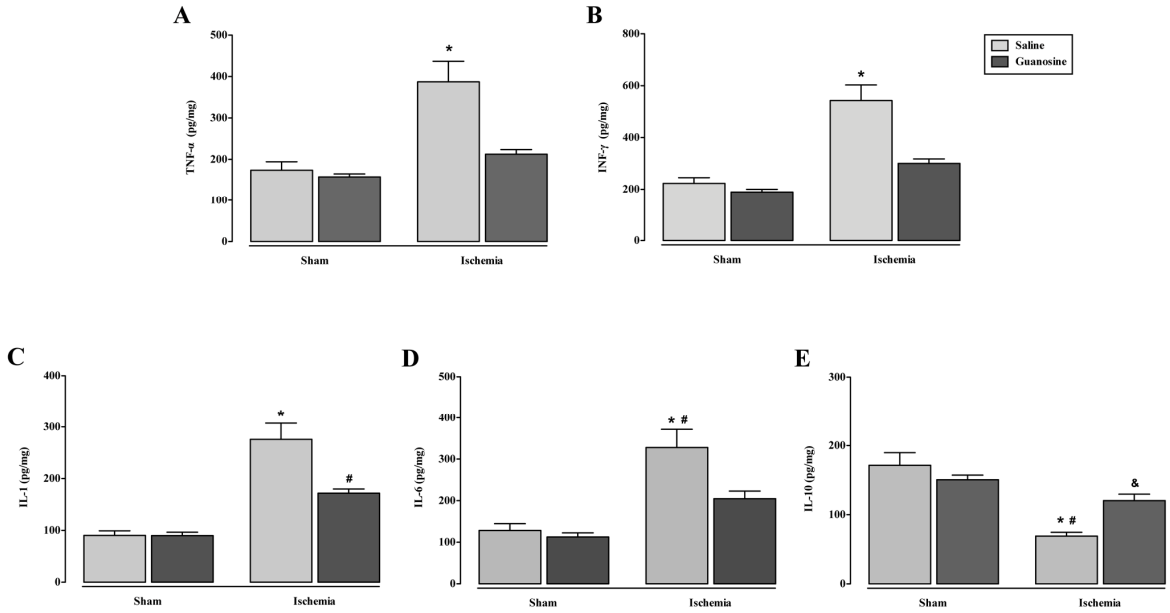
A
B
C
D
E
TNF-α (pg/mg)
INF-γ (pg/mg)
IL-1 (pg/mg)
IL-6 (pg/mg)
IL-10 (pg/mg)
Saline
Guanosine
Sham
Ischemia
*
& *#

TABLE 1. Proinflamatory/antiinflamatory ratio

Groups	IL-1/IL-10	IL-6/IL-10	TNF-α/IL-10	INF-γ/IL-10
CSF				
Sham saline	0.48 ± 002	0.62 ± 0.02	0.90 ± 0.03	1.09 ± 0.05
Sham guanosine	0.59 ± 0.05	0.68 ± 0.08	0.88 ± 0.06	1.09 ± 0.07
Ischemia saline	4.24 ± 0.76 *	5.36 ± 1.20 *	6.35 ± 1.31 *	8.07 ± 1.55 *
Ischemia guanosine	1.04 ± 0.08 †	1.27 ± 0.13 †	1.30 ± 0.12 †	1.94 ± 0.15 †
Cortical tissue				
Sham saline	0.52 ± 0.03	0.75 ± 0.06	1.01 ± 0.03	1.33 ± 0.06
Sham guanosine	0.60 ±0.06	0.75 ± 0.06	1.06 ± 0.05	1.27 ± 0.07
Ischemia saline	4.13 ± 0.53 *	5.00 ± 0.81 *	5.76 ± 0.90 *	8.12 ± 1.02 *
Ischemia guanosine	1.46 ± 0.09 †	1.74 ± 0.13 †	1.83 ± 0.15 †	2.57 ± 0.18 †

Sham saline vs. Ischemia saline, * $p < 0.001$; Ischemia saline vs. Ischemia guanosina, † $p < 0.001$. Data are shown as mean (SEM).

5. Capítulo III

Acute effects of guanosine and inosine in the central nervous system in swine model of hemorrhagic shock

Gisele Hansel, Clarissa Branco Hass, Jean Pierre Oses, Carolina David Wiener, Fernanda Pedrotti Moreira, Diogo Onofre Souza, André Prato Schmidt, Luís Valmor Portela

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Acute effects of guanosine and inosine in the central nervous systemin swine model of hemorrhagic shock

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Abstract

Hemorrhagic shock (HS) is defined as circulatory dysfunction causing whole-body hypoperfusion and decreased tissue oxygenation and accumulation of oxygen debt. This events lead to cardiovascular failure and impair several important organs including central nervous system (CNS). Purines, including guanosine and inosine, modulate the neurotransmitter system and have beneficial effects against neurodegenerative and neurotoxic experimental models, exerting various trophic effects on neural cells. In hemorrhagic and endotoxic shock, guanosine and inosine were able to stimulate the Na+/K+ ATPase, improve hemodynamic and prolong survival. This study aims to evaluate the acute effects of inosine and guanosine as fluid resuscitation. Thus, it was evaluated the effect of guanosine and inosine in brain energetic metabolism as well as acute neuroinflammation mechanisms in a swine model of HS. Twenty-seven pigs (25 - 30 kg) were submitted to a bleeding to cause hypotension (40 – 45 mmHg) for 60 min. After this period, the animals were randomized into three groups and received distinct fluid resuscitation (LR; LR + GUO; LR + INO) during 15 to 20 min. The animals were monitored continuously for 440 min with after the initial HS, and blood and CSF samples were collected in specific periods. Parameters involved in neural damage, energetic metabolism and inflammatory system were measured in the cerebrospinal fluid (CSF). The data of present study showed that HS modified brain energetic metabolism, increased glutamate levels and pro-inflammatory cytokines. Furthermore, guanosine and inosine were able to modulate these parameters, decreasing glutamate levels and proinflammatory cytokines levels after HS. This suppression of the activities of some inflammatory components could be associate with reduction delayed neuronal cell damage, improving cognitive impairment that occurs in HS.

Keywords

Hemorrhagic shock, guanosine, inosine, inflammation, energetic metabolism.

Introduction

Shock is a pathophysiology, which the circulatory system is unable to perfuse tissues adequately [1, 2]. The decrease in blood volume by bleeding leads to a hemorrhagic shock (HS). HS is cause of one third of deaths resulting from injury in the world, affects 90 % of shock cases of an emergency, and constitutes a leading cause of morbidity and mortality worldwide [3-5]. HS is defined as circulatory dysfunction causing whole-body hypoperfusion and decreased tissue oxygenation and accumulation of oxygen debt [4]. This process triggers hypoxia, poor distribution of nutrients to the cells, poor clearance of toxic substances, cellular transmembrane potential reduction, inadequate pH regulation, endothelial dysfunction, stimulation of pro-inflammatory and anti-inflammatory cascades [4, 6]. This events lead to cardiovascular failure and impair the several important organs including heart, liver, spleen, lung and kidney [3, 5]. HS is considered an imminent death situation that requires immediate treatment. The main strategies are the arrest of bleeding and the replacement of circulating volume by the fluid resuscitation [7]. Intravenous fluids appear to improve hemodynamic indices in the short term, being the predominant fluid resuscitation is Ringer's lactate (RL).

Most information about HS consequences has been obtained from studies in peripheral organs. However, even with rapid intervention procedures, HS causes suffering and damage of organs, thus affecting also the central nervous system (CNS), similarly global ischemia [8, 9]. CNS function can be maintained at low thresholds of blood pressure (60 - 70 mmHg). However, a decrease below this threshold triggers symptoms such as disorientation, cerebral edema, coma, and consequently may lead to death [8, 10]. Few studies have demonstrated the effects of HS on CNS. Studies in rat models showed cognitive impairment in behavior [11], glial cell swelling, disruption of the blood brain barrier and cell death by apoptosis and necrosis [10]. Thus, could be considered an important therapeutic strategy adding into the fluid resuscitation, neuroprotective molecules that may reduce CNS suffer during HS.

The purinergic system comprising adenine-based purines, guanine-based purines and finally, the metabolites inosine, xanthine, hypoxanthine, uric acid [12]. The purinergic system is widely known for exert several biological functions, including the pivotal role on energy metabolism and effects in several systems involved in HS event, such as cardiovascular, blood, respiratory and inflammatory response [12-15]. In the CNS, purines modulate the neurotransmitter system and have beneficial effects against neurodegenerative and neurotoxic experimental models [16-20], exerting various trophic effects on neural cells [12, 21, 22]. Particularly, clinical studies demonstrated that severe hypotension, hemorrhage or sepsis shock, and heart failure cause severe organ dysfunction associated with decreased transmembrane potential and cellular edema, an event related to decreased activity of Na/K ATPase [7]. In a model of sepsis systemic, guanosine was able to reduce oxidative stress parameters in the brain and recovering the memory impairment [23]. In animal models of hypovolemic and toxic shock, intravenous infusion of adenosine, inosine or guanosine was able to stimulate the Na^+/K^+ ATPase, improve hemodynamics and prolong survival [24, 25]. However, the infusion of adenosine caused secondary effects such as bradycardia, decreased cardiac output that are harmful to shock recovery, effect that not occur by the intravenous infusion of inosine and guanosine [6]. Purines are released into the extracellular space from cells upon metabolic stress, and are recognized as one of the most important endogenous molecules able to prevent tissue injury in ischemia, and its effects are partly mediated by the inhibition of deleterious immune-mediated processes, including the release of pro-inflammatory cytokines and free radicals [16, 26, 27].

As previous experimental studies have demonstrated that purines, mainly guanosine and inosine protect against several models of shock and that purines acts on energy metabolism and are neuroprotective in several neurotoxic models. This study aims to evaluate the acute effects of inosine and guanosine as fluid resuscitation. Thus, it was evaluated the effect of

guanosine and inosine in brain energetic metabolism as well as acute neuroinflammation mechanisms in a model of porcine HS.

Materials and Methods

Animals

Twenty-seven young, white, crossbreed male pigs (weighing 25 - 30 kg) from swine farms with high health, and temporarily housed in the Department of Reproduction, Veterinary Medicine and Animal Science School, University of São Paulo. All experimental procedures were performed according to the NIH Guide for Care and Use of Laboratory Animals and Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for Animal Care. All efforts were made to minimize the number of animals and their suffering.

Hemorrhagic shock and resuscitation protocol

Animals were fasted for 12 hours (h) and water for 1 h before the beginning of experiment. The animals received intramuscular administration of midazolam (0.25 mg/kg) and ketamine (5 mg/kg) as a premedication. After 15 minutes (min), an ear marginal vein was cannulated with a Teflon catheter (20 gauge), and general anesthesia was induced with propofol (5 mg/kg) and maintained using ketamine (80 μg/kg/min), fentanyl (10 μg/kg/h), and pancuronium bromide (0.3 mg/kg/h). After orotracheal intubation, the animals remained on mechanical ventilation (volume - controlled ventilation, Cicero; Dräger, Lubeck, Germany), a tidal volume between 6 and 8 mL/kg, a positive-end expiratory pressure of 5 cm H_2O, and an oxygen-inspired fraction of 40 %. To evaluate the ventilation, end-tidal carbon dioxide through capnography and peripheral hemoglobin oxygen saturation was continuously monitored. The partial pressure of carbon dioxide was maintained between 35 and 45 mm Hg. The temperature was maintained at around 38 °C with a warming blanket. When a deep

anesthesia level was achieved, the animals were positioned in the dorsal position. Both femoral arteries were catheterized, one for measuring the mean arterial pressure (MAP) and for arterial blood sampling, and the other for blood removal. Two large-bore venous catheters were inserted into the right femoral and jugular veins for fluid infusion. A pulmonary artery catheter (PAC) (CCO catheter; Edwards Lifesciences, Irvine, CA, U.S.A.) was introduced through the left femoral vein, positioned in the pulmonary artery, for the measurement of cardiac output (CO), mixed venous oxygen saturation (SvO_2), and central body temperature by means of a Vigilance Monitor (Edwards Life- sciences). Pressure transducers were connected to a multiparametric data collection system (Viridia CMS; Hewlett-Packard, Andover, MA, U.S.A.) for continuous monitoring of heart rate (HR) and atrial filling pressure. The bolus CO was determined by thermodilution using 10 mL of iced 5 % glucose, and the mean value of three measurements within ± 10 % variation of each other was recorded. The SvO_2 measured from the PAC catheter was obtained from the central venous blood collected from the distal extremity of the PAC catheter. The intermittent SvO_2 was obtained through blood gas analysis. The animals were monitored with transesophageal echocardiography, arterial blood gas analysis and continuous mixed venous, gastric tonometry, intracranial pressure (ICP) monitor systems for continuous assessment of systolic volume variation (SVV) and pulse pressure variation (deltaPP) and measure urine output.

After instrumentation, the animals were allowed to stabilize for 30 min, and then HS was induced by withdrawal of blood via femoral vein catheter. The blood is withdrawn using a pump with a constant withdrawal rate until the pressure is reduced to 40 - 45 mmHg. This hypotensive state will be maintained for 60 min. In the reperfusion, the animals were randomized into three groups (nine animals per group): LR, received infusion of LR; LR + GUO, received infusion of LR with guanosine (1 mmol/L); LR + INO, received infusion of LR with inosine (1 mmol/L). The animals received the infusion via femoral vein catheter

during 15 to 20 min, the infusion volume is three times the volume of blood withdrawn. The animals were monitored continuously for 8 h (440 min after the initial HS) (Figure 1).

Cerebrospinal fluid processing

Cerebrospinal fluid (CSF) were collected 8 times, after stabilization of animal (baseline); in the end of HS (60 min); immediately after infusion of fluid resuscitation (80 min); 30 min after infusion of fluid resuscitation (110 min); 60 min after infusion of fluid resuscitation (140 min); 120 min after infusion of fluid resuscitation (200 min); 240 min after infusion of fluid resuscitation (320 min); and 360 min after infusion of fluid resuscitation (440 min). The CSF was then centrifuged at 3000 x *g* for 10 min at 4 °C to obtain a CSF cells free supernatant and frozen (-80 °C) until analysis.

Measurement of NSE protein levels

An electrochemiluminescent assay kit was used to measure NSE and Insulin. It consists of a double sandwich assay that use an antibody anti-insulin or anti-NSE bound with ruthenium, which is the luminescent molecule. Reactions and quantification were performed by Elecsys-2010 (Roche Diagnostics Corporation) [28]. NSE was expressed as ng/ml (mean ± S.E.M.). The coefficient of variation was less than 5 % for samples and standard.

Measurements of glucose and lactate levels

Glucose and lactate were assayed in CSF using a glucose oxidase methodology manufactured by Vida Biotecnologica, MG, Brazil. All samples and standard were carried out in duplicate in the same experiment. The amount of glucose and lactate was determined by measuring the absorbance at 546 nm with a SpectraMax M5 spectrophotometer (Molecular Devices, USA). All samples and standards were measured in duplicate, and the coefficient of

variation was less than 5%. Calibration factors were determined using a standard of glucose (5.56 mmol/L) and lactate (4.44 mmol/L). The results were expressed as mmol/L.

Inflammatory cytokine levels

A standard capture sandwich assay was used to determine the levels of different cytokines in CSF. Each captured antibody was coupled to a different bead set (Invitrogen's Multiplex Bead Immunoassays). The system used a liquid suspension array of 7 sets of beads (Invitrogen, Swine Cytokine 7-plex) internally dyed with different ratios of two spectrally distinct fluorochromes to assign a unique spectral address. Each set of beads was combined with a monoclonal antibody raised against GM-CSF, IL-1β, IL-10, IFN-α, IFN-γ, TNF-α, IL-4, and IL-8. Beads were incubated first (2 h, at room temperature) with diluted standards (serial dilutions from 1.95 to 32 000 pg/ml) or the CSF sample, and then with biotinylated detector antibodies (30 min, at room temperature). They were washed twice in phosphate-buffered saline, and incubated for 30 min at room temperature with phycoerythrin- conjugated streptavidin. Cytokine levels were measured with a Luminex 200 TM system (Bio-Rad Laboratories). Each measurement was taken in duplicate. Standard curves were generated by using the reference cytokine concentrations supplied by the manufacturer. Raw data (mean fluorescent intensities) were analyzed by Bio-Plex Manager Software 4.1 version (Bio-Rad Laboratories) to obtain concentration as pg/mL.

HPLC procedure

High-performance liquid chromatography (HPLC) was performed with CSF cell-free supernatant aliquots to quantify glutamate levels [29]. Briefly, samples were derivatized with o-phthalaldehyde and separation was carried out with a reverse phase column (Supelcosil LC-18, 250 mm× 4.6 mm, Supelco) in a Shimadzu Instruments liquid chromatograph (200 μL loop

valve injection). The mobile phase flowed at a rate of 1.4 mL/min and column temperature was 24 °C. Buffer composition is A: 0.04 mol/L sodium dihydrogen phosphate monohydrate buffer, pH 5.5, containing 20 % of methanol; B: 0.01 mol/L sodium dihydrogen phosphate monohydrate buffer, pH 5.5, containing 80 % of methanol. The gradient profile was modified according to the content of buffer B in the mobile phase: 0 % at 0.00 min, 25 % at 13.75 min, 100 % at 15.00 – 20.00 min, 0 % at 20.01 – 25.00 min. Absorbance was read at 360 nm and 455 nm, excitation and emission respectively, in a Shimadzu fluorescence detector. Samples of 25 μL were used and concentration was expressed in μM.

Statistical analysis

Results are presented as mean ± S.EM. Statistical analysis was performed through Generalized Estimating Equation (GEE) plus Bonferroni's post hoc test. Area under curve was analyzed using one-way ANOVA. Probability values less than 0.05 were considered to be statistically significant. All analyses were performed using the Statistical Package for Social Sciences (SPSS) software version 18.0. The graphics were done using the Graphpad Prism 5.

Results

Physiologic parameters

All animals included in this study did not vary in the physiologic parameters at baseline. According to the experimental protocol, a target MAP of 40 mmHg was successfully achieved in all animals within 10 min and maintained for 60 min of the experiment. Mean body weight, the blood loss volume needed to induce HS, and total volume resuscitation infusion did not vary significantly among the study groups (Table 1).

Hemodynamic data are shown in Table 2 and Figure 2. All groups displayed identical degree of shock following hemorrhage. During the shock, in an attempt to compensate the decrease in MAP, there was an increase in HR ($p \leq 0.05$). After resuscitation, HR decreased, but remained above baseline for all groups (Figure 2A). After reperfusion, MAP (Figure 2B) was significantly restored. MAP returned to decrease 320 min post-hock ($p \leq 0.05$). The ICP did not differ significantly compared baseline to shock ($p > 0.05$) (Figure 2C). After reperfusion ICP increased and remained high throughout all experiment. HR, MAP and ICP did not differ between the groups. After shock, massive metabolic acidosis was found in all groups. There was a large increase of blood lactate, increase of PaCO2, and decrease of PaO_2, resulting in a pH decreased ($p \leq 0.05$, Table 2). After reperfusion, there were $PaCO_2$ and PaO_2 establishments, however lactate levels continued to increase, maintained low pH. The lactate levels only restored 440 min post-shock. There were an increase of K^+ ($p \leq 0.05$), and a decrease of Na^+ ($p \leq 0.05$) at 440 min post-shock compared to baseline (Table 2).

Neuronal parameters

To determine whether HS caused alterations in the CNS, it was measured in the CSF the glutamate and NSE levels to analyze a potential neural suffering, and glucose and lactate levels to evaluate cerebral energetic metabolism (Figure 3). After shock, the glutamate levels increase dramatically ($p \leq 0.05$, Figure 3A) in all groups compared to baseline. Interestingly, the group that received guanosine and inosine were able to decrease the glutamate more rapid than the animals that received LR in the reperfusion ($p \leq 0.05$). Although the NSE levels had an apparent increase after 200 min of experiment, showing a potential neuronal damage, this data was not significant ($p = 0.09$, Figure 3B). The HS caused an increase in glucose level, remaining elevated until 200 min post-shock ($p \leq 0.05$, Figure 4A). The group that received guanosine in the reperfusion was able to decrease the glucose levels during reperfusion,

showing similar levels of baseline. Lactate levels did not differ in all groups (p = 0.08) (Figure 4B).

Markers of inflammation were measured in the CSF. IL-4 and IL-8 were not detectable in all periods of experiment, and INF-α had not changed during experiment (p = 0.95, data not shown). IL-1β increased significantly in the group that received LR after 200 min post-shock compared to baseline ($p \leq 0.05$, Figure 5A). 320 min post-shock, the both groups, LR + GUO and LR + INO were able to diminish this effect ($p < 0.01$ and $p < 0.05$, respectively). IL-8 increased abruptly in the shock ($p \leq 0.05$) and remained elevated during all times post-shock. IL-8 did not vary significantly among the study groups (p = 0.06, Figure 5B). INF- γ began increased 320 min post-shock compared to baseline ($p \leq 0.05$) and did not vary significantly among the study groups (p = 0.89,Figure 5C). TNF-α increased after 110 min post-shock compared to baseline ($p \leq 0.05$). After 200 min post-shock, TNF-α decreased continuously being similar to baseline at 320 min post-shock. The LR + INO group, although not statistically different at any point individually, the area under the curve decrease significantly compared to LR group ($p \leq 0.05$, Figure 5D).

Discussion

HS is one of the severe hypovolemic shocks induced by extensive blood loss or sudden and unexpected depletion of the circulating blood. HS is a major independent risk factor of the pathogenesis of trauma-related multiple organ failure and death [4, 8]. Stopping bleeding and providing adequate fluid resuscitation are the major keys to treating HS [30]. In this study, it was used LR as a reperfusion fluid (control group) and it was analyzed the acute effects in physiologic and neurologic parameters when added guanosine and inosine (1 mmol/L) in the fluid resuscitation. It was monitored several physiologic parameters (temperature, HR, MAP, ICP, pH, PaO_2, $PACO_2$, hematocrit, lactate, and electrolytes Na^+,

K^+), and there was no statistical difference in any of these variables between groups. Hemorrhagic and endotoxic shock in rats models demonstrated that purines, including guanosine and inosine, are able to prolong survival in the animals, suggesting a possible mechanism involving the Na/K ATPase, probably by reversing or overcoming the effects of an endogenous enzyme inhibitor [6, 24, 25]. In the present study, we did not have mortality outcome. The HS in rat's models mentioned above, the MAP is about 35 mmHg, more severe than our experiment that used 40 – 45 mmHg. It is possible that an HS more severe could lead death as its outcome, in LR group. This fact could imply in physiologic parameters changed, showing difference in the groups that received guanosine and inosine.

One of the most important organs affected by HS is the CNS. A decrease in cerebral blood flow HS-induced can lead to serious deleterious effects, similar to global ischemia [9], The reduced cerebral blood flow cause a decrease in oxygen supply and consequently leading to an increase in extracellular levels of glutamate [31, 32]. According with previous results, the HS led an increase dramatically the glutamate levels in the CSF. Interestingly, the groups that received guanosine and inosine were able to decrease the glutamate more rapid than the animals that received LR in the reperfusion. The excessive glutamate levels leads to overstimulation of glutamate receptors and will initiates several molecular events that trigger an extensive cellular damage [31, 33]. Guanosine and inosine play an important role in the CNS in several neurodegenerative and neurotoxic experimental models, exerting neuroprotective and trophic effects on neural cells [12, 16-18, 27, 34, 35]. Neuronal damage is evident 24 h after the ischemic insult, mainly in the hippocampus [31].Due to the fact our experiment approach be acute, neuronal damage, measured by NSE, was not observed. However, an increase of glutamate by HS could lead a delayed neural damage, and the guanosine and inosine were able to promote a decrease of glutamate levels diminishing a prolonged neurotoxicity event. Studies have shown that HS leads edema, apoptosis, and/or

necrosis of the brain cells, as well as modulation of the blood–brain barrier integrity [8, 9], and promotes cognitive dysfunction [11, 36]. Thus, the effect of decreased glutamate levels by guanosine and inosine could reduce delayed neural damage caused by HS.

The HS involves multiple interrelated factors including cellular ischemia, circulating or local inflammatory mediators, and energetic imbalance. In the ischemic tissues during HS, synthesis of ATP decreases because of negative effect on mitochondrial phosphorylation and increase of anaerobic glycolysis cycle. As the ATP synthesis decreases, plasma membrane sodium pump activity decreases, the cell swells and intracellular pH decreases [8, 33]. The HS caused an increase of glucose levels in CSF and this effect is maintained until 200 min post-shock. Guanosine was able to reduce glucose levels to basal levels before the LR group, with less variation in the glucose levels.

Accumulating evidence demonstrates the relationship between inflammation and neuronal cell damage in cerebral ischemic tissue [37]. Thus, the mechanisms of neuroprotective strategies against cerebral ischemia may target inflammatory alterations involved in cellular damage. Here, the HS did not change immediately the cytokines. However after infusion of reperfusion fluid, there were an immediately increase of IL-8 which remained high throughout the experiment; increased of TNF-α, IL-1β and INF- γ in distinct time points. Interestingly, guanosine and inosine inhibited the increase of IL-1β, and analyzing the area under the curve, inosine was able to decrease the total amount of TNF-α produced. The cytokines, IL-1β and TNF-α, are rapidly expressed in the ischemic brain and leads to the disruption of the blood brain barrier and causes neuronal cell death [38]. There are studies that demonstrate that guanosine has anti-inflammatory effect in oxygen and glucose deprivation conditions [27] and that inosine inhibits inflammatory cytokine production, protecting against endotoxic shock [15]. These data suggested that guanosine and inosine decrease proinflammatory mediators can contribute to the beneficial effects of HS.

Thus, the data of present study showed that HS modified brain energetic metabolism and increased glutamate levels and pro-inflammatory cytokines. The guanosine and inosine were able to modulate these parameters, decreasing glutamate levels and proinflammatory cytokines levels after HS. This suppression of the activities of some inflammatory components could be associated with reduction delayed neuronal cell damage, improving cognitive impairment in the HS, showed by studies that used this model [9,11,36].

Several limitations exist in this study and deserve to be mentioned. (i) Because of the lack of knowledge of clinical relevant values of the measured markers in pigs, it is difficult to conclude whether the observed differences are of clinical relevance as well as whether the response to the insult constitutes actual HS. (ii) The use of anesthetic agents may limit the generalizability compared to the findings of uncontrolled models. While anesthesia cannot be excluded for ethical reasons, the choice of catheter- based bleeding was made to ensure comparable and controlled levels of hypotension. The use of anesthesia and control parameters may have mitigated the deleterious effects of HS. (iii) The acute study (440 min post-shock) prevented us from analyzing effects that might have occurred in a delayed period such as death and cognitive impairment.

This approach has allowed us to gain important insight into the porcine response to injury, which will aid in designing future studies. For these reasons, this study should primarily be seen as hypothesis generating. The model was designed to replicate an injury scenario frequently observed in the emergency. The clinical relevance may, however, be limited by the constraints imposed by species differences as well as the laboratory environment and should be interpreted as such. However, these issues are inherent to all animal models. However, our findings present new insight to new approaches in the treatment of HS.

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Disclosures

None.

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Legends

Figure 1: Schematic representation of the experiment protocol. The HS was induced by withdrawal of blood via femoral vein catheter. The blood is withdrawn until the pressure is reduced to 40 - 45 mmHg, and maintained for 60 min. The fluid resuscitation was infused during 15 to 20 min, three times the volume of blood withdrawn. The animals were monitored continuously for 440 min after the initial HS.

Figure 2: (A) Heart rate (HR, beats per minute); (B) Mean arterial pressure (MAP, mmHg); (C) Intracranial pressure (ICP, mmHg), at baseline and during the evolution of the experiment. Data are reported as means ± S.E.M. of 9 animals per group. Statistically differences as determined by GEE followed by Bonferroni's posttest. Symbols represent * $p < 0.05$ comparing to baseline, and $^{\#}$ $p < 0.05$ comparing to Shock. The groups did not vary significantly during all periods of time.

Figure 3: (A) Glutamate levels (uM); (B) NSE levels (mg/mL), at baseline and during the evolution of the experiment. Data are reported as means ± S.E.M. of 9 animals per group. Statistically differences as determined by GEE followed by Bonferroni's posttest. Symbols represent * $p < 0.05$ comparing to baseline, and $^{\#}$ $p < 0.05$ comparing to shock, $^{\mathbf{a}}$ $p < 0.05$ comparing LR group to LR + GUO and LR + INO.

Figure 4: Energetic metabolites levels in CSF at baseline and during the evolution of the experiment. (A) Glucose levels (mmol/L); (B) Lactate levels (mmol/L). Data are reported as means ± S.E.M. of 9 animals per group. Statistically differences as determined by GEE followed by Bonferroni's posttest. Symbols represent * $p < 0.05$ comparing to baseline, and $^{\#}$ $p < 0.05$ comparing to shock, $^{\mathbf{a}}$ $p < 0.05$ comparing LR group to LR + GUO. Area under curve

(AUC) graphic, statistically differences as determined by one-way ANOVA. Symbols represent $^{@}$ $p < 0.05$ comparing LR + GUO vs. LR.

Figure 5: inflammatory cytokines levels in CSF at baseline and during the evolution of the experiment. (A) IL-1β (pg/mL); (B) IL-8 (pg/mL); (C) INF-γ(pg/mL); (D) TNF-α (pg/mL). Data are reported as means ± S.E.M. of 9 animals per group. Statistically differences as determined by GEE followed by Bonferroni's posttest. Symbols represent * $p < 0.05$ comparing to baseline; ^{a} $p < 0.05$ comparing LR group vs. LR + GUO and LR + INO. Area under curve (AUC) graphic, statistically differences as determined by one-way ANOVA. Symbols represent $^{@}$ $p < 0.05$ comparing LR vs. LR + GUO; $^{\#}$ $p < 0.05$ LR vs. LR + INO.

Table 1. Ressuscitation parameters

Groups	N	Total volume removed (mL)	Total volume infused (mL)	Body weight (Kg)	Total GUO or INO infused during resuscitation (mg/kg)
LR	9	1080.0 ± 43.6	3245.0 ± 132.2	26.9 ± 0.8	-
LR + GUO	9	1097.5 ± 51.2	3270.0 ± 132.8	26.5 ± 0.7	35.0 ± 1.2
LR + INO	9	1056.0 ± 32.3	3163.0 ± 98.3	25.8 ± 0.7	33.3 ± 0.6

Data are shown as mean ± S.E.M..

Table 2. Physiologic variables in the three groups of animals.

Groups	Baseline	Shock (60 min)	Inicial Ressuscitation (80 min)	Final ressuscitation (440 min)
Temperature (°C)				
LR	37.4 ± 0.3	38.6 ± 0.3 *	37.6 ± 0.3 †	39.1 ± 0.3 *‡
LR + GUO	37.3 ± 0.2	38.2 ± 0.3 *	37.2 ± 0.3 †	38.5 ± 0.2 *‡
LR + INO	37.3 ± 0.2	38.2 ± 0.3 *	37.1 ± 0.2 †	39.0 ± 0.3 *‡
pH				
LR	7.4 ± 0.02	7.2 ± 0.04 *	7.2 ± 0.03 *	7.3 ± 0.06
LR + GUO	7.4 ± 0.01	7.3 ± 0.02 *	7.3 ± 0.02 *	7.3 ± 0.08
LR + INO	7.4 ± 0.03	7.3 ± 0.03 *	7.3 ± 0.03 *	7.3 ± 0.06
$PaCO_2$ (mmHg)				
LR	49.9 ± 2.3	64.2 ± 3.2 *	51.7 ± 2.0	54.8 ± 4.2
LR + GUO	49.8 ± 1.4	58.1 ± 3.0 *	54.4 ± 1.7	54.7 ± 1.4
LR + INO	52.2 ± 3.3	59.0 ± 2.7 *	49.6 ± 1.8	52.7 ± 1.9
PaO_2 (mmHg)				
LR	47.1 ± 1.2	33.8 ± 1.6 *	46.9 ± 2.2 †	37.6 ± 1.4 *‡
LR + GUO	44.5 ± 1.6	38.0 ± 4.5 *	44.4 ± 1.8 †	35.6 ± 1.3 *‡
LR + INO	44.8 ± 0.7	35.7 ± 1.4 *	50.0 ± 3.8 †	39.0 ± 1.7 *‡
Hematocrit (%)				
LR	28.0 ± 0.7	26.8 ± 0.8	16.9 ± 0.8 *	18.7 ± 0.8 *
LR + GUO	25.4 ± 0.7	24.2 ± 1.3	15.8 ± 0.9 *	16.9 ± 0.9 *
LR + INO	25.9 ± 0.4	25.2 ± 0.5	15.7 ± 0.5 *	17.3 ± 0.5 *
Lactate (nmol/L)				
LR	1.7 ± 0.2	5.3 ± 1.3 *	8.9 ± 0.8 *†	1.63 ± 0.2
LR + GUO	1.5 ± 0.2	4.5 ± 0.8 *	7.2 ± 0.8 *†	1.84 ± 0.3
LR + INO	1.5 ± 0.2	4.7 ± 1.2 *	7.2 ± 0.9 *†	1.06 ± 0.1
Na (mEq/L)				
LR	140.3 ± 0.8	137.4 ± 0.7	137.4 ± 0.5	135.2 ± 0.6 *
LR + GUO	139.6 ± 0.9	137.4 ± 0.4	136.1 ± 0.7	133.0 ± 0.4 *
LR + INO	139.5 ± 0.9	137.4 ± 0.6	137.4 ± 0.8	134.4 ± 0.9 *
K (mEq/L)				
LR	3.9 ± 0.1	4.7 ± 0.1	4.2 ± 0.1	5.9 ± 0.5 *
LR + GUO	3.9 ± 0.1	4.8 ± 0.2	4.6 ± 0.2	6.1 ± 0.4 *
LR + INO	4.0 ± 0.1	4.7 ± 0.1	4.3 ± 0.1	5.3 ± 0.3 *

Data are shown as mean ± S.E.M..

* $p < 0.05$ compared to baseline; † $p < 0.05$ compared to shock; ‡ $p < 0.05$ compared to inicial ressuscitation

Figure 1:

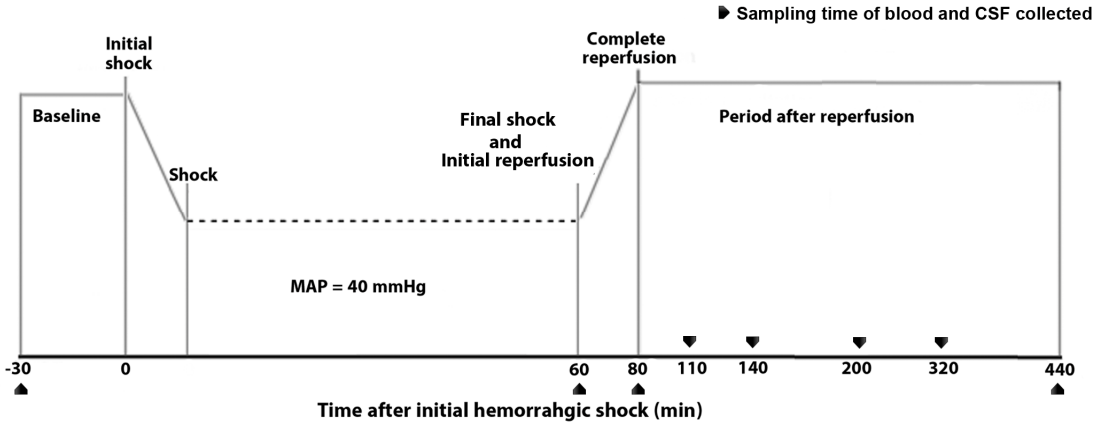

Sampling time of blood and CSF collected
Initial shock
Complete reperfusion
Baseline
Shock
Final shock and Initial reperfusion
Period after reperfusion
MAP = 40 mmHg
-30
0
60
80
110
140
200
320
440
Time after initial hemorrahgic shock (min)

Figure 2:

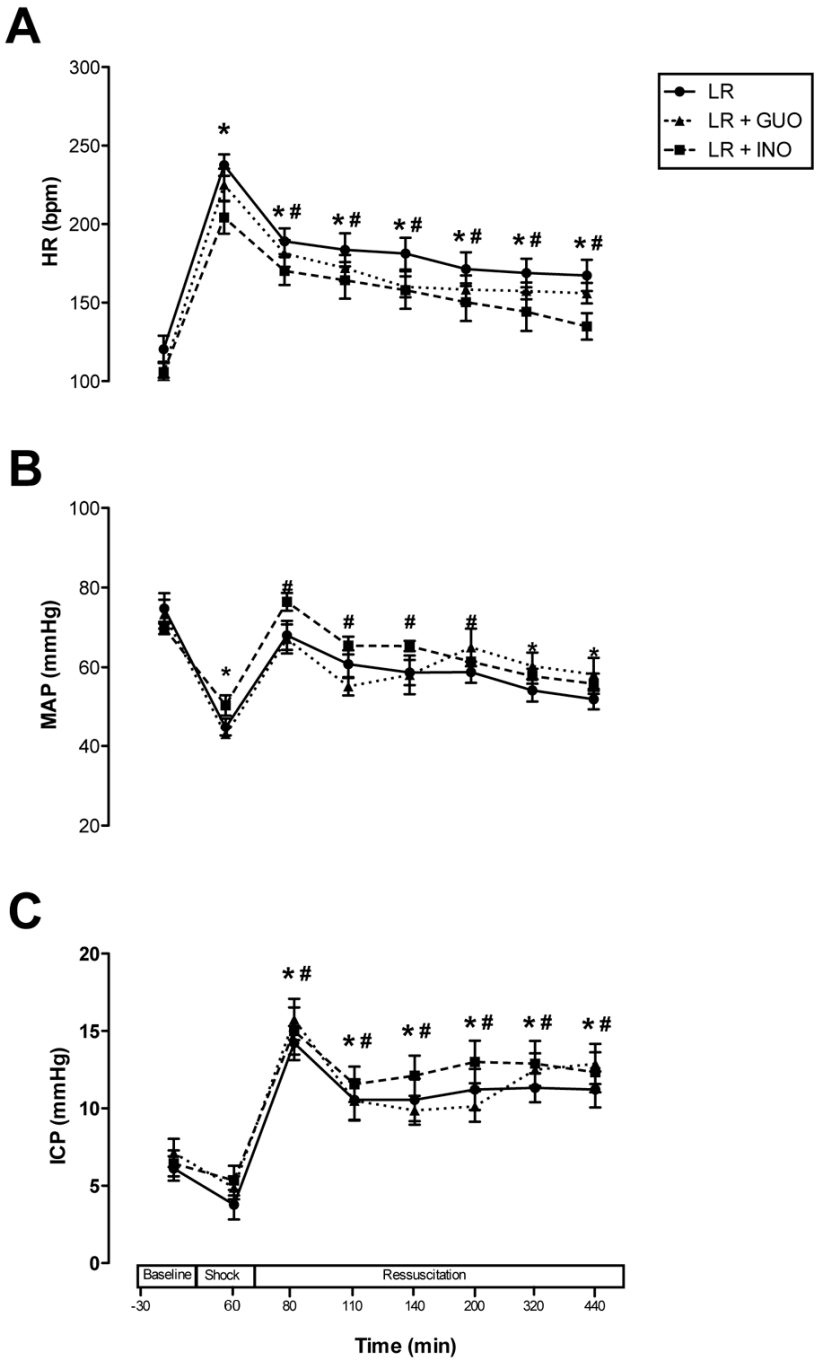

A
HR (bpm)
300
250
200
150
100
LR
LR + GUO
LR + INO
B
MAP (mmHg)
100
80
60
40
20
C
ICP (mmHg)
20
15
10
5
0
Baseline
Shock
Ressuscitation
-30
60
80
110
140
200
320
440
Time (min)

Figure 3:

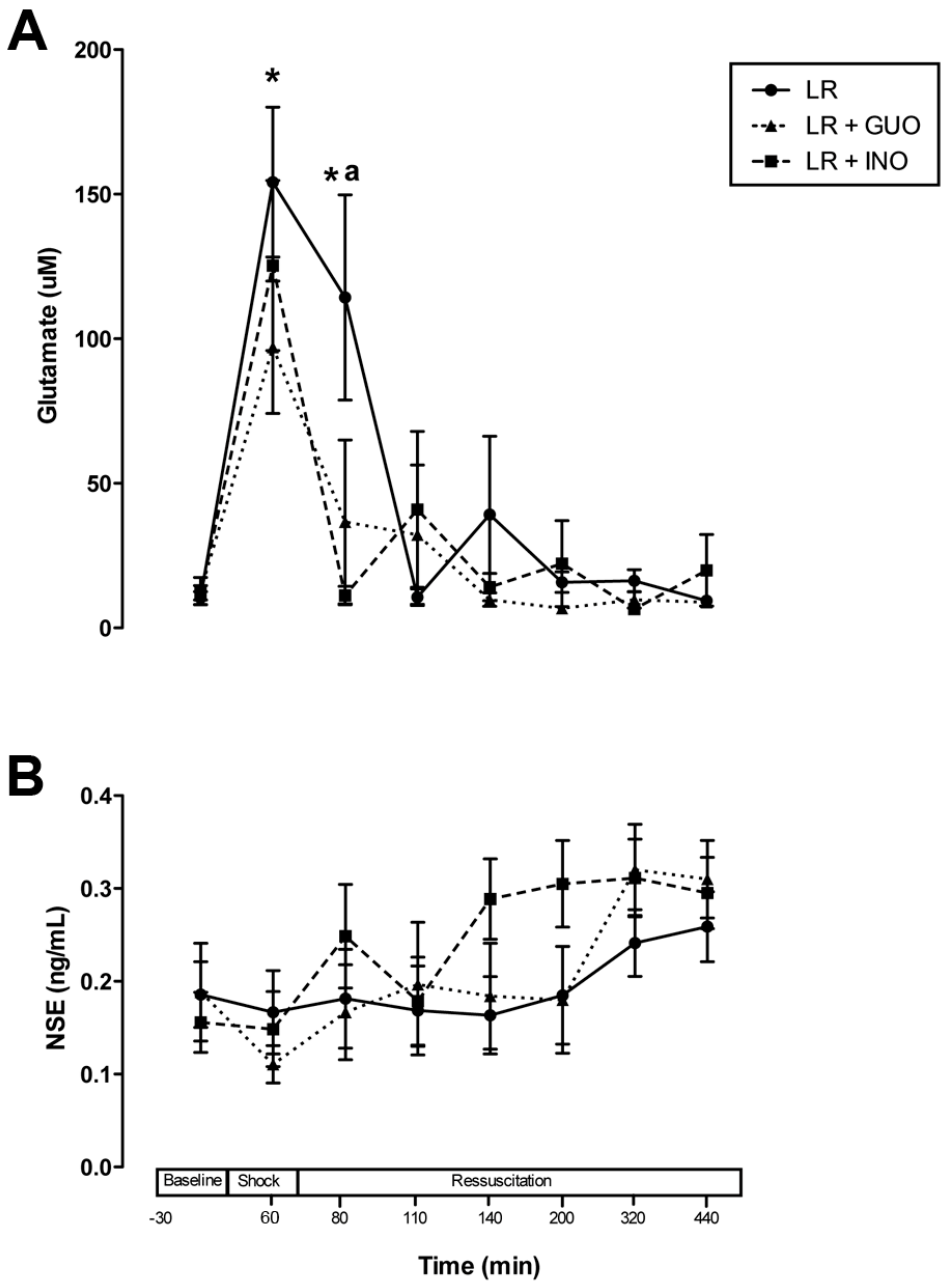

A
200
150
100
50
0
Glutamate (uM)
*
*a
LR
LR + GUO
LR + INO
B
0.4
0.3
0.2
0.1
0.0
NSE (ng/mL)
Baseline
Shock
Ressuscitation
-30
60
80
110
140
200
320
440
Time (min)

Figure 4:

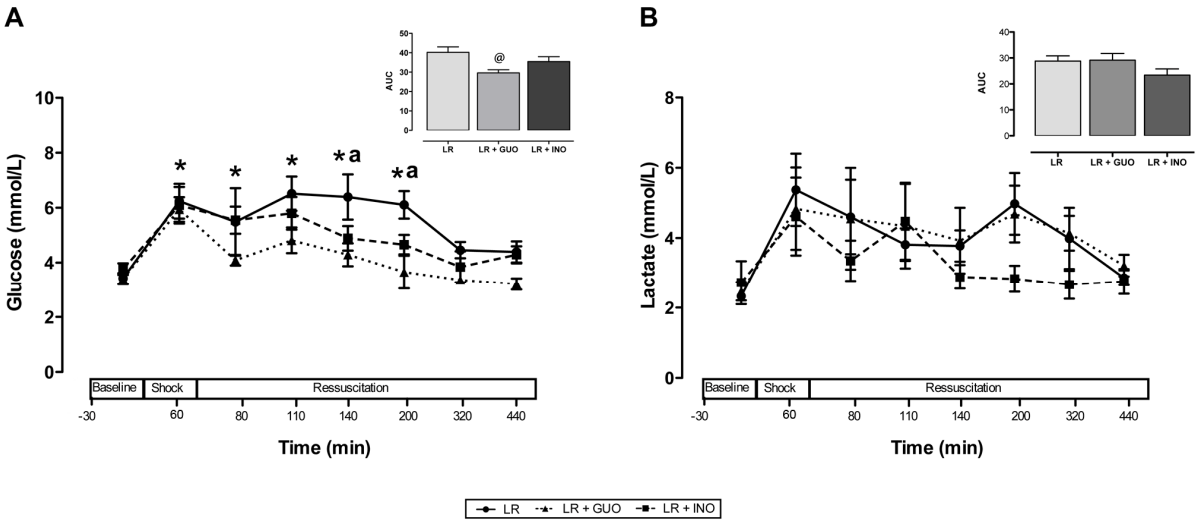
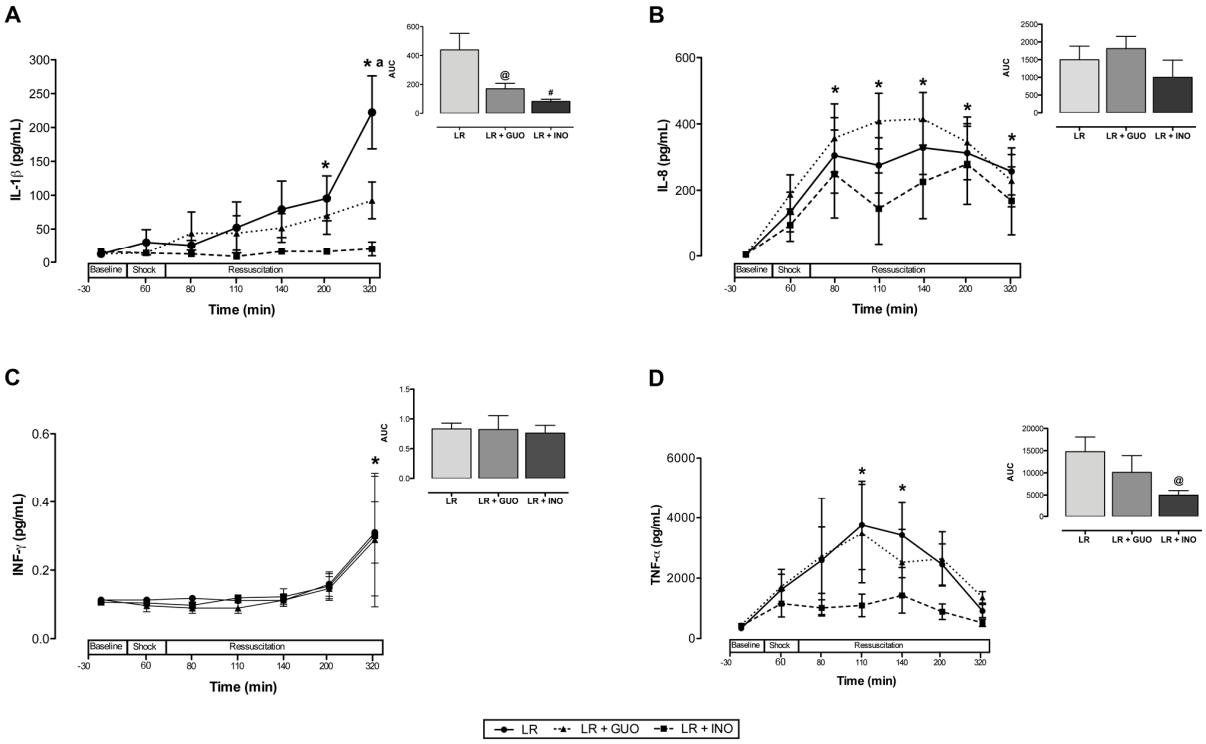

A
B
Glucose (mmol/L)
Lactate (mmol/L)
AUC
@
*
*a
Baseline
Shock
Ressuscitation
-30
60
80
110
140
200
320
440
Time (min)
LR
LR + GUO
LR + INO

Figure 5:


A
B
C
D
IL-1β (pg/mL)
IL-8 (pg/mL)
INF-γ (pg/mL)
TNF-α (pg/mL)
AUC
Baseline
Shock
Ressuscitation
Time (min)
LR
LR + GUO
LR + INO

PARTE III

6. Discussão

Durante as últimas décadas, evidências demonstram que as purinas, nesta tese exemplificada pela guanosina e pela inosina, desempenham um potente efeito neuroprotetor em diversos modelos neurotóxicos e neurodegenerativos (Chen, Goldberg et al. 2002; Schmidt, Lara et al. 2007; Ganzella, Faraco et al. 2011; Dal-Cim, Ludka et al. 2013).

Tendo em vista que a isquemia cerebral é um problema de grande relevância clínica, devido à sua alta incidência e morbidade, bem como aos elevados custos de manutenção de pacientes com sequelas, nossa proposta foi estudar os mecanismos envolvidos na neuroproteção promovida pela guanosina e pela inosina em modelos de isquemia cerebral, investigando parâmetros bioquímicos envolvidos nos sistemas: glutamatérgico, antioxidante e inflamatório, assim como marcadores de morte celular e alterações no metabolismo energético do SNC. Os resultados obtidos neste trabalho contribuem para avaliar os efeitos da guanosina e da inosina na isquemia cerebral experimental mais precisamente visando o melhor entendimento do seu mecanismo de ação.

Os dois primeiros capítulos desta tese buscaram aprofundar o conhecimento das propriedades neuroprotetoras do guanosina no modelo de isquemia cerebral focal permanente. Para a escolha da dose efetiva em nosso modelo experimental, foi realizada uma curva dose-resposta da guanosina baseada em estudos *in vivo* prévios de modelos de isquemia focal (Chang, Algird et al. 2008; Rathbone, Saleh et al. 2011; Connell, Di Iorio et al. 2013). A efetividade da dose foi analisada através do teste do cilindro, que avalia o comportamento exploratório espontâneo de roedores. O contato do animal com as paredes do cilindro revela a preferência do membro anterior utilizado na exploração do ambiente. Desta forma, é possível a avaliação da simetria do animal, que no processo isquêmico está alterada (Schallert 2006; Macrae 2011). No primeiro experimento, realizamos um pré-tratamento, em que administramos a guanosina 30 min antes da indução da isquemia, seguido de mais 3

administrações posteriores: 1 h, 3 h e 6 h após a lesão. A dose efetiva neste modelo de pré-tratamento foi 30 mg/kg. Como nosso objetivo foi a utilização da guanosina após o dano, realizamos um segundo experimento sem administração prévia, com 4 administrações sucessivas (0 h, 1 h, 3 h e 6 h) após o evento isquêmico. A dose efetiva neste modelo experimental com base no teste do cilindro, foi de 60 mg/kg, sendo esta dose utilizada em todos os demais experimentos bioquímicos realizados.

No capítulo 2, o tratamento com guanosina foi capaz de reduzir a incorporação de iodeto de propídio (IP), um marcador de integridade de membrana celular, tanto em neurônios como em astrócitos, e reduziu o número de neurônios marcados com Fluoro jade C (JFC), um marcador de degeneração neuronal. A guanosina foi capaz de reduzir significativamente o volume de infarto cerebral, levando a uma melhora clínica na atividade motora do membro anterior prejudicada pelo dano isquêmico. No capítulo 1, evidenciamos que a melhora clínica foi duradoura, sendo mantida por pelo menos 15 dias após a indução da isquemia (última vez avaliada). Estudos anteriores demonstraram efeitos semelhantes da guanosina em modelos de isquemia focal através da oclusão da ACM (tanto em modelos permanentes como em modelos transitórios) (Chang, Algird et al. 2008; Rathbone, Saleh et al. 2011; Connell, Di Iorio et al. 2013). Neste contexto, avaliando o efeito neuroprotetor da guanosina em outro modelo experimental de isquemia cerebral focal, nossos dados corroboraram e reforçaram os dados já existentes, causando um menor dano celular, um menor dano tecidual, e consequentemente uma visível melhora clínica.

Os resultados apresentados no primeiro capítulo demonstram que a guanosina foi neuroprotetora no modelo de isquemia cerebral focal em ratos, e que a neuroproteção está relacionada com a modulação da resposta ao estresse oxidativo ocasionado pelo dano isquêmico e também com a modulação do sistema glutamatérgico, grande responsável pela excitotoxicidade ocasionada durante isquemia (Durukan and Tatlisumak 2007; Brouns and De

Deyn 2009). Apesar da eficácia do sistema antioxidante, a elevada taxa de metabolismo oxidativo no CNS e a ativação de várias vias intracelulares durante o processo isquêmico leva a uma excessiva produção de radicas livres (ERO e ERN) e também a um prejuízo no funcionamento enzimático antioxidante (Chen, Yoshioka et al. 2011). Estes processos desencadeiam muitos eventos celulares e moleculares, resultando em danos às macromoléculas e consequente ativação de mecanismos de sinalização que levam à morte celular (Allen and Bayraktutan 2009; Nanetti, Raffaelli et al. 2011; Olmez and Ozyurt 2012). Estudos com outros modelos de toxicidade demonstraram o envolvimento da neuroproteção da guanosina em danos oxidativos (Roos, Puntel et al. 2009; Albrecht, Henke et al. 2013; Quincozes-Santos, Bobermin et al. 2013). Neste contexto, a guanosina foi efetiva na modulação do sistema antioxidante enzimático, aumentando a expressão e a atividade da enzima SOD, a atividade da enzima CAT e, consequentemente, proporcionando assim, uma redução de radicas superóxido e peróxido de hidrogênio. Apesar de não afetar os níveis de GSH, principal molécula antioxidante astrocitária (Aoyama, Watabe et al. 2008), durante o processo isquêmico, o tratamento com guanosina foi capaz de inibir o decréscimo de vitamina C no tecido. A vitamina C é considerada um dos principais antioxidantes neuronais e o decréscimo dos seus níveis está intimamente relacionado a um maior dano tecidual no processo isquêmico (Harrison and May 2009). Desta forma, Além de modular a atividade enzimática, a guanosina foi capaz de proporcionar uma maior disponibilidade deste antioxidante neuronal para potencial inativação dos radicais livres produzidos durante o processo isquêmico.Na lesão isquêmica a guanosina, 24 h após o dano, foi capaz de modular o sistema antioxidante, causando uma menor produção de ERO e de ERN, com a efetiva diminuição da peroxidação lipídica causada pela isquemia.

A excitotoxicidade glutamatérgica tem um papel chave na patofisiologia da isquemia cerebral, sendo a redução da captação de glutamato um dos grandes fatores que causam um

aumento da quantidade de glutamato na fenda sináptica (Danbolt 2001). Durante o processo isquêmico, a atividade dos transportadores GLAST e GLT1 é inibida devido à falha energética e também à oxidação proteica dos transportadores causada pelo excesso de ERO e ERN, reduzindo assim suas atividades (Trotti, Danbolt et al. 1998; Zadori, Klivenyi et al. 2012). A guanosina possui potencial efeito neuroprotetor em modelos experimentais de doenças cerebrais associadas à excitotoxicidade glutamatérgica (Schmidt, Lara et al. 2007; Schmidt 2010). O nosso grupo de pesquisa demonstrou que a guanosina é capaz de estimular a captação de glutamato em cultura de astrócitos e fatias de córtex cerebral (Frizzo, Lara et al. 2001; Frizzo, Lara et al. 2002; Frizzo, Schwalm et al. 2005). Em condições basais ou fisiológicas, os efeitos da guanosina sobre a captação de glutamato em fatias cerebrais parecem ser idade e estrutura dependentes, ocorrendo mais no córtex e em ratos jovens (Thomazi, Godinho et al. 2004). Porém, em condições excitotóxicas, a guanosina parece ser mais amplamente envolvida na modulação da captação de glutamato (Frizzo, Lara et al. 2001; Frizzo, Lara et al. 2002). Os modelos *in vitro* de POG (Oleskovicz, Martins et al. 2008; Thomazi, Boff et al. 2008; Dal-Cim, Martins et al. 2011) e modelos de hipóxia-isquemia (Moretto, Arteni et al. 2005; Moretto, Boff et al. 2009), mostraram que a guanosina é neuroprotetora, aumentando a captação de glutamato. No nosso estudo, 24 h após a isquemia, a guanosina foi capaz de manter elevados os níveis de EAAC1 (igualmente ao processo isquêmico), inibiu a redução da expressão de GLT1 (principal EAAT astrocitário) e também aumentou a atividade da glutamina sintetase (GS), enzima responsável pela conversão de glutamato à glutamina (Danbolt 2001). A função do EAAC1 no cérebro ainda não foi completamente estabelecida. EAAC1 é um transportador neuronal envolvido tanto na captação de glutamato, quanto na captação de cisteína, precursores de GSH (Tzingounis and Wadiche 2007; Had-Aissouni 2012). Neste estudo, a expressão de EAAC1, elevada tanto no grupo que sofreu a isquemia quando no grupo que recebeu guanosina, pode indicar um

mecanismo de proteção endógena em resposta ao dano isquêmico, na tentativa de elevar os níveis de GSH, diminuídos durante o processo isquêmico. A inibição da queda dos níveis de GLT1 e o aumento da atividade de GS no grupo isquêmico tratado com guanosina nos possibilitam inferir que, a guanosina estimula a captação de glutamato através de GLT1 e auxilia na conversão de glutamato em glutamina nos astrócitos, assim, modulando parâmetros glutamatérgicos, reduzindo os efeitos excitotóxicos do glutamato.

Com a finalidade de ampliar a investigação sobre os mecanismos envolvidos no efeito neuroprotetor da guanosina, no segundo capítulo propusemos investigar os efeitos neuroprotetores da guanosina no modelo de isquemia cerebral focal, avaliando a inibição da resposta inflamatória. A inflamação é uma resposta orquestrada que envolve a regulação e a rápida ativação de diversas proteínas como citocinas, quimiocinas e também e fatores de transcrição gênica (Lakhan, Kirchgessner et al. 2009). Esse processo dinâmico é crucial no progresso de dano causado pela isquemia (Durukan and Tatlisumak 2007; Lakhan, Kirchgessner et al. 2009; Jin, Yang et al. 2010; Shichita, Sakaguchi et al. 2012). Após a isquemia, há a ativação de astrócitos e células microgliais, que secretam moléculas que induzem a excitotoxicidade, o estresse oxidativo e a inflamação. Dentre estas moléculas, pode-se citar o glutamato, citocinas pro-inflamatórias (TNF-α, IL-1β, IL-6 e IL-8), e anti-inflamatórias (IL-10), prostaglandinas e o radical superóxido (Ooboshi, Ibayashi et al. 2006; Block, Zecca et al. 2007; Lakhan, Kirchgessner et al. 2009; Lambertsen, Biber et al. 2012). A microglia é considerada a célula inflamatória residente do CNS e é a primeira célula a responder a uma lesão cerebral, sendo ativada em minutos após a lesão isquêmica. Sua ativação pode perdurar por várias semanas, desempenhando um papel crucial durante o dano, principalmente, na área de penumbra (Madinier, Bertrand et al. 2009; Jin, Yang et al. 2010; Woodruff, Thundyil et al. 2011). Embora a ativação da microglia seja um processo natural e necessário no processo isquêmico, uma hiper-ativação resulta na progressão na lesão cerebral.

Assim, reduzir a ativação da microglia e inibir a libertação de citocinas pró-inflamatórias são consideradas estratégias terapêuticas importante no AVE.

Os resultados descritos no capítulo 2 demonstraram que o tratamento com guanosina foi capaz de reduzir o número total de células microgliais na região que circunda a lesão isquêmica. Além disso, a Imunoistoquímica das células microgliais, na região periférica ao dano, mostrou que a isquemia possui características de microglia ativadas com redução da complexidade de forma. As células microgliais possuíam retrações nas ramificações dos seus processos, aumentando a intensidade corada perto do soma. O tratamento com guanosina apresentou uma morfologia intermediária com algumas células microgliais com características ativadas, mas ainda com processos ramificados. Embora a ativação das células microgliais seja necessária durante o processo de reparo tecidual na isquemia, a produção de mediadores inflamatórios e um aumento das citocinas pró-inflamatórios contribuem para o aumento do dano cerebral e para a morte neuronal tardia (Lakhan, Kirchgessner et al. 2009; Jin, Yang et al. 2010).

Neste trabalho, a ativação e o aumento do número de células microgliais, no grupo isquêmico, foram acompanhadas por um aumento das citocinas pró-inflamatórias de IL-1, IL-6 , TNF-α e INF-γ e um decréscimo da citocina anti-inflamatória (IL-10), tanto no tecido quanto no LCR. O tratamento com guanosina foi capaz de reduzir as citocinas pró-inflamatórias e de restaurar os níveis de IL-10 na periferia da lesão e no LCR. O aumento da produção de citocinas pró-inflamatórias é observado em modelos de isquemia cerebral e em pacientes com AVE, estando associado com o aumento na área de enfarte e com um pior prognóstico da doença (Tuttolomondo, Di Sciacca et al. 2009). Estudos têm descrito que também é importante avaliar o balanço entre os fatores pró e anti-inflamatórios, por demonstrar um ambiente inflamatório mais preciso durante o dano (Rawdin, Mellon et al. 2013). Outra vez, a guanosina se mostrou efetiva, indicando um efeito positivo no equilíbrio

entre moléculas pró-inflamatórias e anti-inflamatórias no processo isquêmico. Experimentos *in vitro* de POG mostraram que a guanosina é neuroprotetora, aumentando a captação de glutamato e reduzindo os processos oxidativo e inflamatórios na isquemia cerebral (Dal-Cim, Ludka et al. 2013). Os dados da literatura, juntamente com os dados apresentados neste capítulo, evidenciam que a guanosina é capaz de reduzir o processo inflamatório causado durante a isquemia, inclusive com a diminuição da proliferação e da ativação microglial, bem como da liberação de citocinas pro-inflamatórias, aumentando, por outro lado, a produção da citocina antiinflamatória IL-10. A redução do estado inflamatório pode reduzir a morte tanto neuronal quando astrocitária e, consequentemente, melhorar a função motora prejudicada.

No terceiro capítulo, investigamos o potencial efeito neuroprotetor da guanosina e da inosina como solução de reposição volêmica em um modelo *in vivo* de choque hemorrágico em porcos. O choque hemorrágico é classificado com um choque hipovolêmico induzido por uma extensa perda de sangue ou pela exaustão súbita e inesperada do sangue circulante, causando uma redução da perfusão tecidual sistêmica, levando a uma disfunção orgânica (Hirano, Mantovani et al. 2003; Rawdin, Mellon et al. 2013). O choque hemorrágico é causa de um terço das mortes resultantes de traumas, afetando 90% dos casos de choque de uma emergência hospitalar, e constitui uma das principais causas de morbidade e mortalidade em todo o mundo (Fulop, Turoczi et al. 2013; Gann and Drucker 2013). Este processo provoca hipóxia, má distribuição de nutrientes para as células, deficiente eliminação das substâncias tóxicas, redução do potencial de membrana celular, regulação inadequada do pH, disfunção endotelial, estimulação de processos inflamatórios e oxidativos, sendo considerado uma situação de morte iminente que requer tratamento imediato (Fulop, Turoczi et al. 2013; Rawdin, Mellon et al. 2013). As principais estratégias são a cessação do sangramento e a reposição volêmica (Rawdin, Mellon et al. 2013). No SNC, a hipoperfusão causada pelo choque hemorrágico desencadeia prejuízos semelhantes à isquemia global (Lin, Koustova et

al. 2005). Estudos em modelos de choque hemorrágico em ratos demostram comprometimento cognitivo (Plaschke 2009), edema de células gliais, rompimento da barreira hemato-encefálica e morte celular por apoptose e necrose (Lin, Koustova et al. 2005). Assim, a adição de moléculas neuroprotetoras em soluções de reposição volêmica seria uma boa estratégia para reduzir o sofrimento do SNC durante o choque hemorrágico. Neste estudo, avaliamos os efeitos agudos causados pela administração endovenosa da guanosina e da inosina em parâmetros de metabolismo energético e mecanismos inflamatórios do SNC, adicionando estes substratos à solução de Lactato Ringer (solução controle de reposição volêmica).

A diminuição do fluxo sanguíneo cerebral leva a uma falha energética e consequentemente conduz a um aumento de glutamato extracelular, provocando não apenas uma estimulação excessiva dos receptores de glutamato, como também vários eventos moleculares que desencadeiam o dano celular (Lipton 1999; Zemke, Smith et al. 2004; Durukan and Tatlisumak 2007). Neste trabalho, os níveis de glutamato no LCR aumentaram drasticamente após o choque hemorrágico e se mantiveram altos no primeiro tempo após a reposição volêmica. Os grupos que receberam tanto guanosina quanto inosina na reposição volêmica foram capazes de reduzir estes níveis de glutamato, retornando aos níveis basais mais rapidamente. A guanosina e a inosina desempenham um papel importante no SNC em vários modelos experimentais neurodegenerativos e neurotóxicos, exercendo efeitos neuroprotetores e tróficos em células neuronais (Oses, Leke et al. 2004; Schmidt, Lara et al. 2007; Ganzella, Faraco et al. 2011; Connell, Di Iorio et al. 2013; Dal-Cim, Ludka et al. 2013; Quincozes-Santos, Bobermin et al. 2013). Neste trabalho não observamos alteração nos níveis de NSE, usado para avaliar o dano neuronal do LCR. Experimentos que utilizam modelos de isquemia global evidenciam um dano neuronal a partir de 24 h (Lipton 1999; Simao, Matte et al. 2012), como nossa abordagem experimental foi aguda, avaliada somente até 440 min após

o choque hemorrágico, o efeito de dano neuronal não foi observado. No entanto, este aumento de glutamato extracelular, ocorrido de forma mais prolongada no grupo controle, pode promover um processo excitotóxico, levando a um dano neuronal tardio. Assim, a eficácia da guanosina e da inosina, reduzindo o glutamato liquórico mais rapidamente que o grupo controle, pode estar relacionado com uma redução dos danos tardios ocorridos no choque hemorrágico.

Semelhante à isquemia focal, a isquemia global também demonstra uma íntima relação entre a ativação de processos inflamatórios e o dano neuronal (Nilupul Perera, Ma et al. 2006; Elkind 2010). Neste trabalho, a infusão de LR aumentou as citocinas IL-8, TNF-α, IL-1β e INF-γ em períodos distintos. A reposição volêmica com guanosina e inosina foi capaz de inibir o aumento de IL-1β, e analisando a área sob a curva, a inosina foi capaz de diminuir a quantidade total de TNF-α produzido. As citocinas, IL-1β e TNF-α, são rapidamente expressas no dano isquêmico. Elas desencadeiam à ruptura da barreira hemato-encefálica, que possibilita a entrada de células imunes periféricas no SNC. Seus níveis aumentados também causam a ativação de diversas vias de sinalização envolvidas na morte células, causando uma progressão do dano isquêmico (Doyle, Simon et al. 2008). Estudos mostram que a guanosina é capaz de modular as vias inflamatórias, causando assim uma neuroproteção em condições de POG (Dal-Cim, Ludka et al. 2013), e que a inosina é capaz de inibir citocinas inflamatórias, protegendo contra choque endotóxico (Hasko, Kuhel et al. 2000). Os resultados obtidos neste capítulo descrevem os efeitos benéficos da guanosina e da inosina, diminuindo os níveis de glutamato e de citocinas pró-inflamatórias de forma aguda no choque hemorrágico. Estes efeitos agudos poderiam estar associados a uma redução do dano neuronal tardio ocasionado pelo processo isquêmico desencadeado pelo choque hemorrágico, melhorando assim o comprometimento cognitivo.

Assim, esta tese representou um importante avanço na contribuição para o potencial efeito neuroprotetor da guanosina em modelos de isquemia cerebral focal e global e da inosina em um modelo de isquemia cerebral global. Embora os mecanismos neuroprotetores destas moléculas frente à isquemia cerebral não estejam completamente elucidados, esta tese deu um importante passo demonstrando que a guanosina modulou parâmetros do sistema oxidativo e glutamatérgico e também inibiu o processo inflamatório durante a isquemia cerebral. No modelo de isquemia global, a utilização de guanosina e inosina como solução de reposição volêmica, proporcionou uma modulação imediata do sistema glutamatérgico e de fatores inflamatórios que pode causar uma melhora no sofrimento do SNC no processo de choque hemorrágico.

7. Conclusões

Os dados obtidos durante a realização deste trabalho permitem concluir que:

1. O tratamento com guanosina exerceu efeito neuroprotetor no modelo de isquemia cerebral focal permanente induzida por termocoagulação em ratos. Esse efeito foi mediado por um conjunto de atividades, dentre elas:

    - Modulação do sistema antioxidante, prevenindo o estresse oxidativo através da redução de espécies reativas de oxigênio e de nitrogênio, inibindo a peroxidação lipídica. Esses efeitos foram seguidos por um aumento da atividade e na expressão de enzimas antioxidantes bem como a inibição do decréscimo de agentes antioxidantes como, por exemplo, a vitamina C no tecido cerebral que circunda o dano isquêmico (Capítulo 1).

    - Modulação de parâmetros do sistema glutamatérgico, principalmente ligados à captação de glutamato: mantendo a expressão de EAAC1 elevada, inibindo a redução da expressão de GLT1 e aumentando a atividade de GS; inibindo assim os efeitos deletérios causados pela excitotoxicidade glutamatérgica (Capítulo 1).

    - Modulação de processos inflamatórios, diminuindo a proliferação e a ativação microglial, reduzindo os níveis de citocinas inflamatórias como IL-1 , IL-6, TNF-α e INF-γ, restaurando os níveis de IL-10 (antiinflamatória). Reduzindo assim o dano neuronal e astrocitário (Capítulo 2).

2. O tratamento com guanosina e inosina acarretou uma redução mais efetiva nos níveis de glutamato no LCR e também proporcionaram um menor perfil inflamatório reduzindo, IL-1β (guanosina e inosina) e TNF-α (inosina). A modulação de

parâmetros relacionados tanto com o sistema glutamatérgico como com o sistema inflamatório poderia estar associada a uma redução do dano neuronal tardio ocasionado pelo processo isquêmico desencadeado pelo choque hemorrágico, melhorando assim o comprometimento cognitivo.

8. Perspectivas

8.1. Perspectivas no modelo de isquemia cerebral focal permanente induzida por termocoagulação:

- Avaliar as alterações morfológicas em astrócitos causadas por esse modelo experimental, bem como avaliar o efeito da guanosina sobre o mesmo.
- Avaliar a modulação de outros parâmetros associados à inflamação através da ativação do NF-κB e da JNK, além da regulação de iNOS e COX-2.
- Avaliar os efeitos da guanosina bloqueando os receptores adenosinérgicos A_1 e A_{2A} neste modelo.

8.2. Perspectivas no modelo de choque hemorrágico:

- Avaliar no LCR e no sangue outros marcadores de dano celular como Lactato desidrogenase e proteína C reativa ultrassensível.
- Avaliar no LCR outros parâmetros envolvidos no metabolismo energético como o piruvato e corpos cetônicos.
- Avaliar no LCR outros parâmetros inflamatórios como IL-6 e IL-12.
- Avaliar os efeitos sobre os níveis de fatores tróficos neurais diversos (BDNF, GDNF, NGF) e S100 no LCR.

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