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Aline Alves Courtes

GUANOSINA MODULA A FUNCIONALIDADE E A BIOENERGÉTICA MITOCONDRIAL EM RATOS

Santa Maria, RS
2019

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS) como requisito parcial para obtenção do grau de **Doutora em Ciências Biológicas: Bioquímica Toxicológica**.

Orientador: Prof. Dr. Félix Alexandre Antunes Soares

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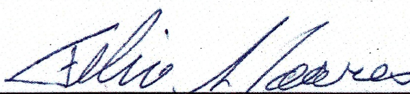

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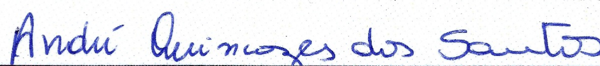

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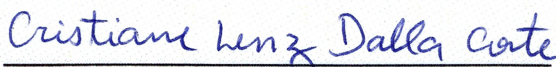

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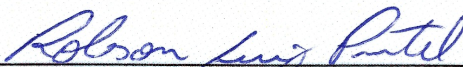

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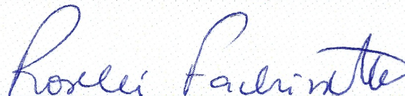

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DEDICATÓRIA

Dedico este trabalho aos meus primeiros educadores:
meus pais Tania e Fortunato, maiores incentivadores,
exemplo de luta, amor e dedicação!
A vocês toda minha gratidão!

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Ninguém é tão grande que não possa aprender,
nem tão pequeno que não possa ensinar.

Voltaire

APRESENTAÇÃO

No item INTRODUÇÃO consta uma revisão sucinta da literatura sobre os temas envolvidos nesta tese.

O DESENVOLVIMENTO da tese está apresentado sob a forma de dois artigos, os quais se encontram alocados no item ARTIGOS CIENTÍFICOS. As seções Materiais e Métodos, Resultados, Discussão dos Resultados, Conclusão e Referências Bibliográficas, encontram-se nos próprios artigos e representam a íntegra deste estudo.

O item DISCUSSÃO apresenta interpretações e comentários gerais sobre os trabalhos científicos aqui incluídos.

Os itens CONCLUSÕES e PERSPECTIVAS são encontrados no final desta tese e apresentam interpretações e comentários gerais sobre a investigação desenvolvida.

As REFERÊNCIAS BIBLIOGRÁFICAS referem-se somente às citações que aparecem no item INTRODUÇÃO e DISCUSSÃO, uma vez que o artigo científico contém as suas próprias referências.

RESUMO

GUANOSINA MODULA A FUNCIONALIDADE E A BIOENERGÉTICA MITOCONDRIAL EM RATOS

AUTOR: Aline Alves Courtes
ORIENTADOR: Félix Alexandre Antunes Soares

A disfunção mitocondrial é associada a uma grande variedade de doenças degenerativas e metabólicas, câncer e envelhecimento. Todas essas manifestações clínicas resultam de problemas na mitocôndria em desempenhar seu papel central da bioenergética na biologia celular. A bioenergética mitocondrial e o estado redox também são determinados pelos níveis intracelulares de cálcio (Ca^{2+}). O Ca^{2+} mitocondrial regula o metabolismo energético, entretanto em altas concentrações, estimula as vias de morte celular através das mitocôndrias. As mitocôndrias são sensíveis a alterações no estado fisiológico das células e parecem desempenhar um papel crítico na lesão secundária que ocorre após o traumatismo crânio encefálico (TCE). O estudo de agentes terapêuticos, com propriedades neuroprotetoras podem auxiliar na compreensão de desordens relacionadas a disfunções mitocondriais e permitir novas perspectivas para sua aplicação. Nesse contexto, o nucleosídeo guanosina, uma molécula endógena membro do sistema purinérgico, têm sido estudada em diferentes modelos experimentais, por demonstrar um efeito neuroprotetor devido à modulação do sistema glutamatérgico e manutenção do sistema redox. Sendo assim, a presente tese teve como objetivos avaliar os efeitos da guanosina sobre alterações na funcionalidade e bioenergética mitocondrial em ratos, através de um estudo *in vitro* frente a um dano induzido pelo cálcio, e *in vivo*, frente a um dano causado pelo TCE leve. Os resultados aqui apresentados do estudo *in vitro*, mostraram que a guanosina apresentou um efeito protetor contra a disfunção mitocondrial induzida pelo desequilíbrio de Ca^{2+}, uma vez que ela reduziu o inchaço mitocondrial na presença de Ca^{2+}, diminuiu os níveis de espécies reativas de oxigênio (EROs), peróxido de hidrogênio (H_2O_2), aumentou a atividade da enzima superóxido dismutase-Mn, fosforilação oxidativa e a atividade do ciclo do ácido tricarboxílico. Nossos achados do estudo *in vivo*, mostraram que uma única dose de guanosina injetada via intraperitoneal, 2 horas após um TCE leve em ratos, aumentou a fosforilação oxidativa, o sistema de transporte de elétrons na presença de um desacoplador e a razão do controle respiratório em córtex e hipocampo, avaliados através de respirometria de alta resolução. A guanosina também protegeu contra alterações locomotoras, exploratórias e déficits de memória em curto prazo induzidos pelo TCE 24 horas após a lesão. Dessa forma demonstramos aqui que a guanosina apresenta um efeito protetor em reduzir o dano mitocondrial induzido pelo Ca^{2+} e que esses efeitos não foram associados com suas propriedades antioxidantes diretas *per se* ou estabilização do potencial de membrana mitocondrial. Podendo ser considerada como uma estratégia para proteger contra danos neurológicos em patologias associadas ao sistema nervoso central bem como em perturbações a função mitocondrial, tornando essa molécula um atrativo terapêutico para o tratamento do TCE.

Palavras-chaves: purinas; cálcio; traumatismo crânio encefálico; respirometria.

ABSTRACT

GUANOSINE MODULATES RAT MITOCHONDRIAL BIOENERGETICS AND FUNCIONALITY

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ADVISOR: Félix Alexandre Antunes Soares

Mitochondrial dysfunction is associated with a wide variety of degenerative and metabolic diseases, cancer and aging. All these clinical manifestations result from problems in the mitochondria in playing their central role of bioenergetics in cellular biology. Mitochondrial bioenergetics and redox status are also determined by intracellular levels of calcium (Ca^{2+}). The Ca^{2+} mitochondrial regulates the energy metabolism, however in high concentrations, stimulates the cell death pathways through the mitochondria. Mitochondria are sensitive to changes in the physiological state of the cells and appear to play a critical role in secondary injury that occurs after traumatic brain injury (TBI). Therapeutic agents with neuroprotective properties may help in the understanding of disorders related to mitochondrial dysfunctions and allow new perspectives for their application. In this context, the nucleoside guanosine, an endogenous molecule member of the purinergic system, has been studied in different experimental models, since it demonstrates a neuroprotective effect due to modulation of the glutamatergic system and maintenance of the redox system. Thus, the present thesis aimed to evaluate guanosine effects on changes in mitochondrial bioenergetic functionality in rats, through an *in vitro* study against calcium-induced damage, and *in vivo*, against damage caused by mild TBI. The results presented in the *in vitro* study showed that guanosine presented a protective effect against mitochondrial dysfunction induced by Ca^{2+} imbalance, since it reduced mitochondrial swelling in the presence of Ca^{2+}, decreased levels of reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), increased the activity of the enzyme Mn-superoxide dismutase, oxidative phosphorylation and tricarboxylic acid cycle activity. Our findings from *in vivo* study showed that a single dose of guanosine injected intraperitoneally 2 hours after a mild TBI in rats increased oxidative phosphorylation, the electron transport system in the presence of a uncoupler and the ratio of respiratory control in the cortex and hippocampus, evaluated through high-resolution respirometry. Guanosine also protected against locomotor, exploratory and short-term memory deficits induced by the TBI 24 hours after the injury. Thus, we demonstrate that guanosine has a protective effect in reducing Ca^{2+}-induced mitochondrial damage and that these effects were not associated with its direct antioxidant properties *per se* or stabilization of the mitochondrial membrane potential. It can be considered as a strategy to protect against neurological damage in pathologies associated with the central nervous system as well as in mitochondrial disorders, making this molecule a therapeutic attraction for the treatment of TBI.

Key words: purines; calcium; traumatic brain injury; respirometry.

LISTA DE ABREVIATURAS E SIGLAS

ADP	Adenosina difosfato
ATP	Adenosina trifosfato
DNA	Ácido desoxirribonucleico
Ca^{2+}	Cálcio
BK	Canais de Potássio
MCU	Canal uniporter de Ca^{2+}
CAT	Catalase
UQ	Coenzima Q
UQH_2	Coenzima Q reduzida
Ecto-NTDase	Ecto-nucleotidases
EROs	Espécies reativas de oxigênio
FAD	Flavina adenosina dinucleotídeo
PI3K	Fosfatidilinositol 3-quinases
OXPHOS	Fosforilação oxidativa
FCCP	Fluorocarbonil-cianeto fenilhidrazona
GUA	Guanina
GUO	Guanosina
GDP	Guanosina difosfato
GMP	Guanosina monofosfato
GTP	Guanosina trifosfato
Glu	Glutamato
Gln	Glutamina
GS	Glutamina sintetase
GSSG	Glutationa oxidada
GPx	Glutationa peroxidase
GR	Glutationa redutase
HO-1	Heme oxigenase-1
RaM	Modo rápido de captação de Ca^{2+}
NAD	Nicotinamida adenina dinucleotídeo
NMDA	N-Metil-D-Aspartato
$NO^{\cdot}$	Óxido nítrico
MtNOS	Óxido nítrico sintase mitocondrial
ROX	Oxigênio residual
PGM	Piruvato, glutamato e malato
mPTP	Poro de transição de permeabilidade mitocondrial
$\Delta\Psi m$	Potencial de membrana mitocondrial
Akt	Proteína quinase B
MAPK/ERK	Proteíno-quinases ativadas por mitógenos
GBPs	Purinas à base de guanina
mRyR	Receptor de rianodina
ETS	Sistema de transporte de elétrons
SUIT	Substratos, inibidores e desacopladores
SOD	Superóxido dismutase
SOD-CuZn	Superóxido dismutase cobre zinco
SOD-Mn	Superóxido dismutase manganês
EAATs	Transportadores de aminoácidos excitatórios
TCE	Traumatismo crânio encefálico

TPx	Tioredoxina peroxidase
MPTP	1-metil-4-fenil-1,2,3,6-tetrahidropiridina

SUMÁRIO

1 INTRODUÇÃO 14
1.1 OBJETIVOS 15
1.1.1 Objetivo Geral 15
1.1.2 Objetivos Específicos 15
2 REVISÃO DE LITERATURA 16
2.1 FUNÇÃO MITOCONDRIAL, ESTRESSE OXIDATIVO 16
2.2 BIOENERGÉTICA MITOCONDRIAL E NÍVEIS INTRACELULARES DE CÁLCIO 20
2.3 CONSUMO DO OXIGÊNIO MITOCONDRIAL 22
2.4 HEPATOPROTEÇÃO MITOCONDRIAL 23
2.5 INSUFICIÊNCIA ENERGÉTICA 24
2.5.1 Traumatismo Crânio Encefálico 25
2.6 SISTEMA PURINÉRGICO 28
2.6.1 Guanosina 29
3 DESENVOLVIMENTO 32
3.1 ARTIGO CIENTÍFICO I 33
3.2 MANUSCRITO CIENTÍFICO I 43
4 DISCUSSÃO 73
5 CONCLUSÕES 78
5.1 CONCLUSÕES PARCIAIS 78
6 PERSPECTIVAS 79
REFERÊNCIAS BIBLIOGRÁFICAS 80
ANEXO – CARTA DE APROVAÇÃO 98

1 INTRODUÇÃO

A sobrevivência neuronal está intimamente ligada à homeostase mitocondrial. As mitocôndrias são importantes na bioenergética celular, sendo sensíveis às alterações no estado fisiológico das células e estão envolvidas na patogênese de muitas doenças associadas ao estresse oxidativo, tal como o acidente vascular encefálico (Dobrachinski *et al.*, 2014; Ramos, Denise Barbosa *et al.*, 2016; Garvin *et al.*, 2017), encefalopatia hepática (Paniz *et al.*, 2014), hipóxia (Stenvinkel e Haase, 2017), trauma (Gerbatin *et al.*, 2016; Dobrachinski *et al.*, 2017) e diabetes (Højlund *et al.*, 2008).

Vários mecanismos de defesa são utilizados para reduzir o estresse oxidativo induzido pela geração excessiva de radicais livres nas células. O sistema de defesa enzimático inclui a ação benéfica de várias enzimas antioxidantes, como a superóxido dismutase (SOD), a catalase (CAT), a glutationa redutase (GR) e a glutationa peroxidase (GPx) (Bhatti, J. S. *et al.*, 2017).

Disfunções nesta organela têm sido associadas à diminuição na transferência de elétrons e transdução de energia devido à sobrecarga de cálcio (Xiong *et al.*, 1997), aumento da produção de EROs, redução na produção de adenosina trifosfato (ATP), perturbações da homeostase (Azbill *et al.*, 1997; Matsushita e Xiong, 1997; Sullivan *et al.*, 1999; Bhatti, J. S. *et al.*, 2017) e morte celular (Robertson, 2004). Assim, moléculas potencialmente envolvidas na regulação da integridade mitocondrial são de considerável importância na biologia celular e em estudos farmacológicos (Shimura e Kunugita, 2016).

Os agentes farmacológicos que têm como alvo as mitocôndrias ou que evitem os desequilíbrios causados a esta organela têm demonstrado serem neuroprotetores. Várias intervenções terapêuticas que visam estabilizar as mitocôndrias têm mostrado resultados promissores (Sullivan *et al.*, 2004; Hino *et al.*, 2005; Xiong *et al.*, 2005; Clark *et al.*, 2007).

Nesse contexto, a guanosina, uma molécula endógena e membro do sistema purinérgico, possui importantes propriedades antioxidantes, provavelmente derivadas da sua atividade *scavenger* (Quincozes-Santos *et al.*, 2014) e tem sido usada como um composto neuroprotetor em diferentes modelos experimentais (Dobrachinski *et al.*, 2018; Courtes *et al.*, 2019; Marques *et al.*, 2019). No entanto, o mecanismo de ação específico da guanosina sobre parâmetros associados à bioenergética mitocondrial ainda permanecem incertos. Sendo assim, esse estudo pode auxiliar a uma melhor compreensão dos efeitos da guanosina sobre a funcionalidade mitocondrial e apresentar novas perspectivas de sua aplicação como uma molécula neuroprotetora em doenças relacionadas à mitocôndria.

1.1 OBJETIVOS

1.1.1 Objetivo geral

Avaliar os efeitos da guanosina sobre alterações na funcionalidade e bioenergética mitocondrial em ratos.

1.1.2 Objetivos específicos

- Investigar os efeitos da guanosina na função mitocondrial hepática, bem como frente ao dano induzido pelo cálcio (Ca^{2+}), através de um estudo *in vitro*;
- Investigar os efeitos da guanosina na bioenergética mitocondrial de córtex e hipocampo de ratos após um traumatismo crânio encefálico leve, assim como sobre os parâmetros comportamentais.

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2 REVISÃO DE LITERATURA

As mitocôndrias são organelas intracelulares com dupla membrana que desempenham papéis importantes no metabolismo energético celular, tem como função predominante a geração de ATP através da fosforilação oxidativa, sendo a fonte primária de compostos altamente energéticos na célula (Letts e Sazanov, 2017). Além disso, elas desempenham outras funções importantes como a biossíntese de aminoácidos e esteroides, a beta oxidação de ácidos graxos, produção e detoxificação de espécies reativas de oxigênio, regulação do cálcio intramitocondrial e citoplasmático e transdução de sinais nas vias de sinalização intracelular de apoptose (Milane *et al.*, 2015; Au - Djafarzadeh e Au - Jakob, 2017).

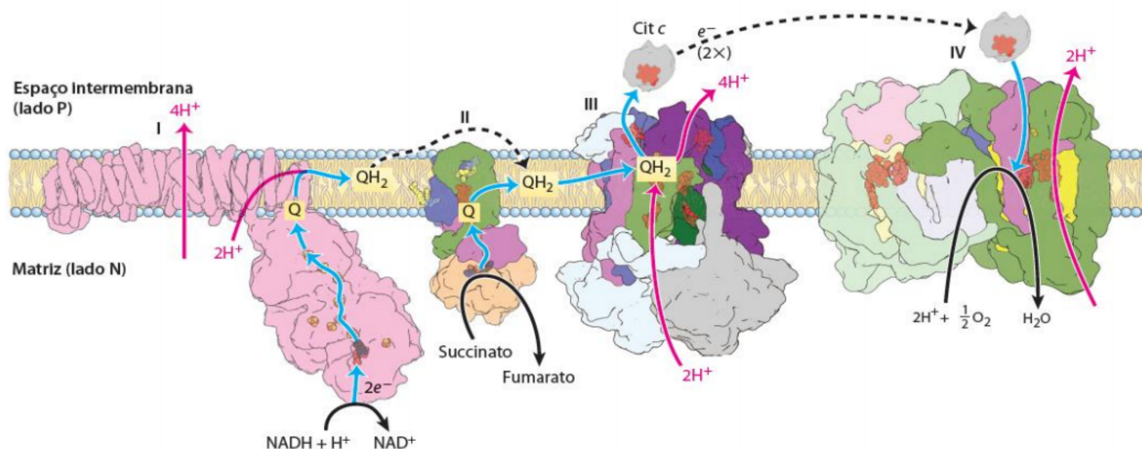
A fosforilação oxidativa utiliza a energia armazenada dos alimentos para gerar um gradiente de prótons através da membrana mitocondrial interna. Durante a respiração celular, os elétrons passam através da cadeia transportadora de elétrons para o oxigênio molecular, produzindo água (Papa *et al.*, 2012). A energia do gradiente de prótons conduz a síntese de ATP pela ATP-sintase. Ao longo dos anos, muitas desordens têm sido associadas a defeitos na fosforilação oxidativa (Arun *et al.*, 2016). Os tecidos requerem energia produzida pela fosforilação oxidativa e isso reflete sua dependência em relação à mitocôndria. Sendo assim, as células são bastante dependentes das mitocôndrias, possuindo uma alta densidade destas e, consequentemente, apresentam-se vulneráveis ao comprometimento do metabolismo energético e à disfunção mitocondrial (Bhatti, J. S. *et al.*, 2017; Molnar e Kovacs, 2018).

2.1 FUNÇÃO MITOCONDRIAL, ESTRESSE OXIDATIVO

Sabe-se que a maior parte da energia celular corporal (> 90%) é produzida pelas mitocôndrias na forma de ATP através do ciclo do ácido cítrico e da cadeia transportadora de elétrons (Bhatti, Jasvinder Singh *et al.*, 2017). A acetil-CoA formada pela oxidação de combustíveis orgânicos entra no ciclo do ácido cítrico e tem seus grupamentos acetil oxidados a dióxido de carbono (CO_2). A energia liberada no processo é armazenada pelas coenzimas derivadas respectivamente de nicotinamida e flavina, NADH e $FADH_2$. Ambas atuam como transportadores de elétrons em suas formas reduzidas até alcançarem a cadeia respiratória mitocondrial como demonstrado na Figura 1 (Milane *et al.*, 2015), a qual é composta por cinco complexos multienzimáticos: I, II, III, IV e V, localizados na membrana mitocondrial interna, e pelos transportadores de elétrons: coenzima Q e citocromo c e ainda as proteínas ferro-enxofre (Nicholls, 2002).

Na cadeia respiratória, elétrons provenientes de diferentes substratos são doados aos carreadores de elétrons tal como o NADH e transferidos para o átomo de ferro central da NADH desidrogenase. O complexo I então transfere elétrons para a forma oxidada da coenzima Q (UQ), levando a sua redução (UQH_2) (Formosa *et al.*, 2018). Elétrons originários do succinato são transferidos para a flavina adenina dinucleotídeo (FAD) e UQ pelo complexo II, resultando também em sua redução (Letts e Sazanov, 2017). A UQH_2 é então desprotonada, resultando na formação do ânion semiquinona ($UQH^{\cdot-}$), responsável pela doação de elétrons ao citocromo b-c_1 e posteriormente ao citocromo c (Wang e Hekimi, 2016). Existem dois *pools* distintos de $UQH^{\cdot-}$ um localizado na face citoplasmática da membrana mitocondrial interna, e outro na face matricial da membrana (Amigo *et al.*, 2016). As duas formas de $UQH^{\cdot-}$ são combinadas quando oxidadas, regenerando UQ e doando seus elétrons (Wang e Hekimi, 2016). O citocromo c então transporta elétrons para a citocromo c oxidase, responsável pela transferência de elétrons para o oxigênio, que resulta na formação de água. Esta passagem de elétrons se dá em quatro passos consecutivos de um elétron, devido à característica triplete do oxigênio (Nicholls, 2002). A passagem de elétrons pelas NADH desidrogenase, citocromo b-c_1 e citocromo c oxidase é acompanhada pelo bombeamento de prótons da matriz mitocondrial para o espaço intermembranas. O gradiente de prótons gerado favorece a re-entrada destes para a matriz mitocondrial através da ATP sintase, que utiliza a energia próton motriz para promover a síntese de ATP (Kowaltowski *et al.*, 2001; Papa *et al.*, 2012).

Figura 1: Movimentação dos elétrons através da cadeia respiratória



Fonte: Adaptado de (Nelson, 2014).

O transporte de elétrons na cadeia respiratória e a fosforilação oxidativa devem ser

regulados para a manutenção da funcionalidade e viabilidade celular. A falta de síntese mitocondrial de ATP pode levar a falência energética celular e morte necrótica, provocar acúmulo de EROs mitocondriais e, possivelmente, diminuir a longevidade (Kowaltowski *et al.*, 2001; Signes e Fernandez-Vizarra, 2018).

As mitocôndrias funcionam continuamente para metabolizar oxigênio e gerar EROs. Entretanto, o fluxo de elétrons através da cadeia respiratória não é um processo perfeito, pois, 0,4 a 4% do oxigênio consumido pela mitocôndria, não é reduzido e leva à produção de EROs, como por exemplo o ânion superóxido ($O_2^{\cdot -}$) designado como ERO "primária" (Debattisti *et al.*, 2017). A geração excessiva de ânion superóxido pode gerar EROs "secundárias" (Turrens, 1997). Além disso, a superprodução de EROs danifica as proteínas mitocondriais/enzimas, membranas e o ácido desoxirribonucleico (DNA), acarretando na interrupção da produção de ATP e outras funções essenciais nas mitocôndrias (Bhatti, Jasvinder Singh *et al.*, 2017).

O $O_2^{\cdot -}$ originado pela cadeia respiratória pode ser gerado pela NADH desidrogenase (complexo I), pela coenzima Q (complexo III) ou, possivelmente, por desidrogenases matriciais (Dröse e Brandt, 2012). A geração de $O_2^{\cdot -}$ ao nível do átomo central de ferro da NADH desidrogenase é de grande importância fisiopatológica, tendo sido correlacionada com danos celulares em distúrbios degenerativos como a doença de Parkinson (Ryan *et al.*, 2015). A presença de rotenona, um inibidor da transferência de elétrons entre o complexo I e a UQ, também estimula sensivelmente a geração de $O_2^{\cdot -}$ pela NADH desidrogenase (Wang e Hekimi, 2016). O tratamento de animais de experimentação com rotenona ou 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP), outro inibidor do complexo I, leva a uma neuropatia semelhante à doença de Parkinson (Milane *et al.*, 2015).

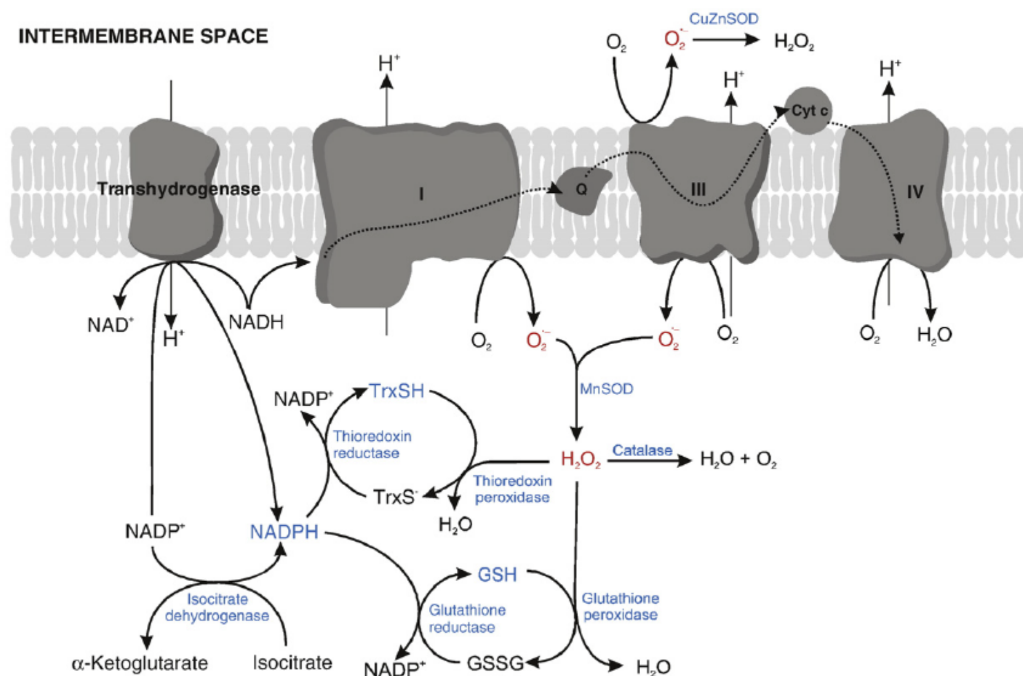
As células têm muitas maneiras de combater os efeitos do dano oxidativo induzido por EROs, seja diminuindo diretamente a geração de radicais livres ou capturando os radicais livres por uma série de antioxidantes, mecanismos enzimáticos e não enzimáticos como apresentado na Figura 2 (Bhatti, Jasvinder Singh *et al.*, 2017).

A SOD catalisa a conversão do radical ânion superóxido em peróxido de hidrogênio (H_2O_2) e oxigênio molecular (O_2). A isoforma da SOD presente na matriz mitocondrial é dependente de manganês (SOD-Mn), enquanto a isoforma da SOD presente no citosol e no espaço intermembrana é dependente de cobre e zinco (SOD-CuZn) (Xiong *et al.*, 2005; Cardoso *et al.*, 2017). O H_2O_2 formado, além de ser uma espécie reativa mais estável, é permeável pelas membranas e pode ser transportado por aquaporinas. Assim, o H_2O_2 difunde-se dentro da célula, podendo ser removido tanto por sistemas antioxidantes mitocondriais

quanto citosólicos, como por exemplo, através da enzima catalase (Buettner, 2011), tioredoxina peroxidase (TPx) e glutationa peroxidase (GPx). Esta última possibilita a conversão do H_2O_2 formado em água, ao mesmo tempo em que oxida a glutationa, que é prontamente reduzida pela ação da glutationa redutase (GR) (Apostolova e Victor, 2015).

A glutationa é importante também para manter os grupamentos sulfidril de proteínas em seu estado reduzido, impedindo de sofrerem os efeitos deletérios do estresse oxidativo (Marí *et al.*, 2009). Quando as espécies não são metabolizadas pelos sistemas antioxidantes, elas podem gerar o radical hidroxila ($OH^{\cdot}$), altamente reativo e citotóxico, por meio da reação de Fenton, na qual o H_2O_2 reage com íons cobre (Cu^+) ou ferro (Fe^{2+}). Não há nenhum sistema enzimático de defesa contra o $OH^{\cdot}$ (Venditti *et al.*, 2014). Assim, em condições em que ocorre um aumento da produção de $O_2^{\cdot -}$ ou uma falha no sistema antioxidante, pode ocorrer uma maior produção de $OH^{\cdot}$ com lesões oxidativas em biomoléculas (Prauchner, 2017). A mitocôndria é ainda uma possível fonte de espécies reativas de nitrogênio derivadas do óxido nítrico ($NO^{\cdot}$), produzido por uma óxido nítrico sintase mitocondrial (mtNOS) (Cardoso *et al.*, 2017).

Figura 2: Metabolismo mitocondrial de EROs. O radical ânion superóxido ($O_2^{\cdot -}$) é formado pela redução monoeletrônica de O_2, principalmente nos Complexos I e III da cadeia respiratória. $O_2^{\cdot -}$ é dismutado em H_2O_2 pela SOD-CuZn, no espaço intermembrana, e SOD-Mn na matriz. H_2O_2 pode ser removido pela catalase mitocondrial ou por peroxidases, tal como glutationa e tiorredoxina peroxidase, utilizando a glutationa reduzida (GSH) e tiorredoxina (TrxSH) como substratos, respectivamente. A glutationa oxidada (GSSG) e a tiorredoxina



Fonte: (Kowaltowski *et al.*, 2009).

($TrxS^-$) são reduzidas, utilizando o NADPH como fonte de elétrons. A nicotinamida adenina dinucleótido fosfato (NADP) pode ser mantido reduzido pela atividade da transidrogenase NAD / NADP, com transporte de prótons para a matriz, fornecendo uma ligação entre o potencial de membrana interna e a capacidade redox mitocondrial. Alternativamente, o $NADP^+$ é reduzido pela isocitrato desidrogenase.

2.2 BIOENERGÉTICA MITOCONDRIAL E NÍVEIS INTRACELULARES DE CÁLCIO

A bioenergética mitocondrial e o estado redox também são determinados pelos níveis intracelulares de cálcio (Ca^{2+}). Em condições normais, o Ca^{2+} mitocondrial regula o metabolismo energético, enquanto que, em altas concentrações, estimula as vias de morte celular mediadas por mitocôndrias (Bravo-Sagua *et al.*, 2017). A concentração de Ca^{2+} citosólico livre é estritamente controlada, e mudanças na faixa de 0,1-1 μM são sinais importantes em diversas vias, incluindo apoptóticas, sinalização neuronal e hormonal (Halestrap, 2006).

Em neurônios, a bioenergética mitocondrial e o estresse oxidativo, juntamente com o transporte mitocondrial de Ca^{2+} formam uma rede intimamente conectada. A geração deficiente de ATP na célula pode resultar em falha na atividade da bomba de Ca^{2+} da membrana plasmática e do retículo endoplasmático com sobrecarga de Ca^{2+}. Por sua vez, o estresse oxidativo pode restringir a capacidade da mitocôndria em gerar ATP (Javadov *et al.*, 2018).

As mitocôndrias afetam o metabolismo do Ca^{2+} citoplasmático de duas maneiras: indiretamente, via ATP, o qual é utilizado por ATPases dependentes de Ca^{2+} para bombear o Ca^{2+} para fora da célula; e diretamente, através do potencial de membrana mitocondrial ($\Delta\Psi m$) o qual impulsiona a absorção do Ca^{2+} liberado pelo retículo endoplasmático na mitocôndria através do canal uniporter de Ca^{2+} (Mnatsakanyan *et al.*, 2017). Os principais mecanismos de influxo de Ca^{2+} incluem: o canal uniporter de Ca^{2+} (MCU), modo rápido de captação de Ca^{2+} (RaM) e receptor de rianodina (mRyR) (Mammucari *et al.*, 2016).

A atividade do MCU é controlada pela proteína de absorção de Ca^{2+} mitocondrial 1 (MICU1), um regulador, que previne a sobrecarga de Ca^{2+} nas mitocôndrias. Estudos genéticos do MCU revelaram um papel importante no acúmulo de Ca^{2+}, sugerindo que o Ca^{2+} induz a abertura do poro de transição de permeabilidade mitocondrial (mPTP) devido à ativação do MCU (De Stefani *et al.*, 2015; Mammucari *et al.*, 2016).

Níveis aumentados de Ca^{2+} atuam como importantes ativadores da piruvato desidrogenase, de enzimas do ciclo do ácido cítrico e de componentes da cadeia respiratória

(Bravo-Sagua *et al.*, 2017). O Ca^{2+} também afeta a liberação de EROs mitocondriais. A absorção deste cátion pode levar a uma redução transitória no $\Delta\Psi m$ e aumentar o transporte de elétrons, o qual pode, sob algumas condições, reduzir a formação de EROs (Votyakova e Reynolds, 2005). Por outro lado, o acúmulo excessivo de Ca^{2+} está associado ao estresse oxidativo mitocondrial. O Ca^{2+} pode aumentar a formação de EROs mitocondriais através de vários mecanismos, incluindo aumento da atividade do ciclo do ácido cítrico e formação de NADH, ativando enzimas geradoras de EROs, como o glicerol fosfato e α-cetoglutarato desidrogenase (De Stefani *et al.*, 2015). Além disso, pode causar um aumento dos níveis de espécies reativas de nitrogênio derivadas do óxido nítrico ($NO^{\cdot}$) e consequente inibição respiratória, promovendo a perda do citocromo c devido à transição da permeabilidade mitocondrial, ocasionada pelo acúmulo excessivo de Ca^{2+}(Halestrap, 2006; Bravo-Sagua *et al.*, 2017).

A sobrecarga de Ca^{2+} aliada a altos níveis de EROs e fosfato inorgânico resultam em mudanças na permeabilidade da membrana mitocondrial e consequentemente acarretam na abertura do mPTP não seletivo e de alta condutância. A abertura do mPTP compromete ainda mais a função bioenergética e a integridade estrutural das mitocôndrias, acarretando na morte celular (Javadov *et al.*, 2018).

Notavelmente, o inchaço mitocondrial, o qual ocorre principalmente devido a abertura do mPTP, pode levar a morte celular através da apoptose ou necrose dependendo da disponibilidade de ATP (Javadov *et al.*, 2017). Ainda, ele está envolvido na patogênese de muitas doenças humanas associadas ao estresse oxidativo, como isquemia, hipóxia, inflamação entre outros (Belous *et al.*, 2006; Gouriou *et al.*, 2011). Para que as mitocôndrias possam manter sua estrutura funcional e morfológica, é necessário regular e/ou reduzir o volume da matriz mitocondrial como forma de aliviar o estresse, auxiliando na manutenção da vida celular (Bravo-Sagua *et al.*, 2017).

Como mencionado, a abertura do mPTP induz o edema mitocondrial. No entanto, nosso estudo demonstrou que a inibição da abertura do mPTP não impede o inchaço mitocondrial completamente (Courtes *et al.*, 2019), sugerindo que o mecanismo envolvido no inchaço mitocondrial pode ser independente do mPTP (Eliseev *et al.*, 2002; Javadov *et al.*, 2018). O exato mecanismo envolvido no inchaço mitocondrial não é claro, embora sabe-se que íons, pH e $\Delta\Psi m$ são os principais reguladores desse inchaço (Javadov *et al.*, 2017). Portanto, torna-se essencial elucidar os mecanismos envolvidos no inchaço mitocondrial para a compreensão da morte celular mediada por mitocôndrias e desenvolvimento de novas estratégias terapêuticas, visando à mitocôndria.

2.3 CONSUMO DO OXIGÊNIO MITOCONDRIAL

As medidas da respiração celular fornecem informações importantes sobre a capacidade respiratória mitocondrial específica dos complexos (I–IV), integridade mitocondrial e metabolismo energético (Formosa *et al.*, 2018). A fosforilação oxidativa pode ser avaliada *in vivo* e *in vitro* utilizando várias técnicas e vários estados da respiração mitocondrial (De Carvalho *et al.*, 2017; Signes e Fernandez-Vizarra, 2018).

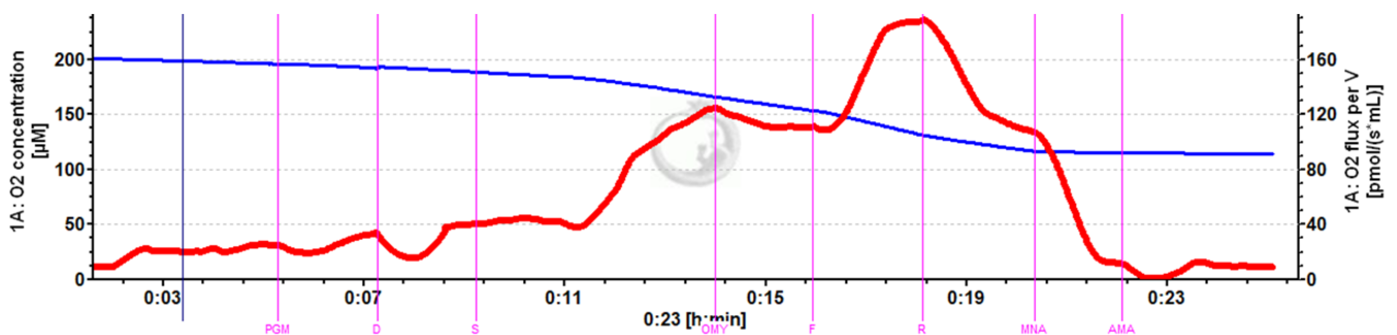
Um dos dispositivos que permite analisar o consumo do oxigênio mitocondrial com alta precisão, resolução e sensibilidade, é o oxígrafo de alta resolução (OROBOROS, Oxygraph-2k e DatLab software da OROBOROS Instruments, Innsbruck, Áustria) (Makrecka-Kuka *et al.*, 2015). O dispositivo de alta resolução contêm duas cubas com portas de injeção, cada cuba é equipada com um sensor de oxigênio polarográfico. Suspensões mitocondriais celulares ou isoladas são agitadas continuamente no respirômetro (Gnaiger, 2009). Para avaliar a função mitocondrial, podem ser adicionados substratos, desacopladores e inibidores em cada cuba, seguindo um protocolo padrão chamado de SUIT. Os quais podem ser titulados por injeções nas cubas do oxígrafo, e os níveis do consumo de oxigênio são calculados utilizando um software e seus resultados expressos como picomol por segundo por miligrama de tecido (Pesta e Gnaiger, 2012; Krumschnabel *et al.*, 2015).

Em homogenato de tecido ou mitocôndrias isoladas, os seguintes estados respiratórios podem ser avaliados como demonstrado na figura 3: i) respiração mitocondrial basal, ii) consumo de oxigênio após a adição de substratos específicos dos complexos da cadeia respiratória mitocondrial, iii) consumo máximo de oxigênio mitocondrial após a adição de concentrações saturantes de adenosina difosfato (ADP) (Gnaiger, 2009; Au - Djafarzadeh e Au - Jakob, 2017), iv) consumo do oxigênio na presença de oligomicina (um inibidor da ATP sintase), v) respiração desacoplada pelo fluorocarbonil-cianeto fenilhidrazona (FCCP). Protonóforos como o FCCP podem induzir um aumento na permeabilidade da membrana mitocondrial interna, permitindo o movimento passivo de prótons a fim de dissipar o gradiente quimiosmótico (De Carvalho *et al.*, 2017; Gonçalves *et al.*, 2019). Um aumento na permeabilidade de prótons desacopla a respiração oxidativa (sem produção de ATP) e induz a um aumento no consumo de oxigênio. Posteriormente, a rotenona, o malonato e a antimicina A são adicionados para inibir a respiração mitocondrial (Krumschnabel *et al.*, 2015).

A respirometria de alta resolução oferece várias vantagens em relação aos dispositivos de eletrodo de oxigênio polarográfico tradicionais e convencionais, incluindo maior sensibilidade e capacidade de trabalhar com um pequeno número de amostras biológicas (Au -

Djafarzadeh e Au - Jakob, 2017). Além disso, o fato do dispositivo conter duas cubas permite que as taxas respiratórias possam ser gravadas simultaneamente para comparações dos níveis de oxigênio. O oxígrafo de alta resolução também tem a vantagem de reduzir o vazamento de oxigênio das cubas do dispositivo em comparação com outros de eletrodo de oxigênio polarográfico tradicionais (Pesta e Gnaiger, 2012; Makrecka-Kuka *et al.*, 2015).

Figura 3: Representação da Respirometria de Alta Resolução. O protocolo consiste em uma adição sequencial de múltiplos substratos, desacopladores e inibidores (protocolo SUIT). Podendo avaliar a respiração mitocondrial basal (BASAL). Adição concomitante de piruvato, glutamato e malato (PGM) juntamente com a amostra, seguida por ADP (D) para medir a capacidade de fosforilação oxidativa do complexo I (CI_{OXPHOS}). Succinato (S) é adicionado para estimular a capacidade máxima da fosforilação oxidativa através dos complexos I e II (CI + CII_{OXPHOS}). A adição da oligomicina (OMY) demonstra a respiração mitocondrial independente da produção de ATP, comumente chamada de LEAK. A respiração máxima convergente do sistema de transporte de elétrons (ETS) foi avaliada através do FCCP (F) (CI + CII_{ETS}). A rotenona (R) inibe a respiração mitocondrial do complexo I, assim é possível avaliar a respiração individual do complexo II através do ETS (CII_{ETS}). O malonato (MNA) inibe a respiração mitocondrial do complexo II, sendo possível avaliar a capacidade do ETS através do complexo I. A antimicina-A (AMA), um inibidor do complexo III, foi adicionada para mensurar os níveis do oxigênio residual (ROX). Linha azul indica o consumo de oxigênio. Linha vermelha indica o fluxo de elétrons através dos complexos mitocondriais.



<table>
<tr><td>BASAL</td><td colspan="2">OXPHOS</td><td>LEAK</td><td colspan="2">ETS</td><td></td></tr>
<tr><td colspan="2">CI</td><td colspan="3">CI + CII</td><td>CII</td><td>ROX</td></tr>
</table>

Fonte: Autoria própria.

2.4 HEPATOPROTEÇÃO MITOCONDRIAL

O fígado é um dos mais importantes órgãos do corpo, pois desempenha um papel fundamental na regulação de diversos processos fisiológicos e a sua atividade está relacionada

a diferentes funções, tais como o metabolismo, secreção e armazenamento de vitaminas (Kandilis *et al.*, 2015). A lesão ou disfunção hepática é considerada um grave problema de saúde, pois os medicamentos sintéticos disponíveis para tratar doenças hepáticas são caros e podem causar efeitos adversos com uso prolongado (Kandilis *et al.*, 2015; Stefanello *et al.*, 2017).

Efetivamente, o fígado desempenha um papel importante na detoxificação de muitos fármacos, diferentes patologias produzem uma redução na sua função, diminuindo, por sua vez, a capacidade de regeneração dos hepatócitos (Liang *et al.*, 2004). Dado o seu importante papel no metabolismo celular, as mitocôndrias têm sido consideradas sensores de toxicidade e função de muitos tecidos e órgãos, incluindo o fígado (Gunter *et al.*, 2010). A disfunção dos complexos da cadeia respiratória tem um papel importante na patogênese de algumas doenças crônicas e na ocorrência de distúrbios metabólicos (Pecinova *et al.*, 2011) Também, leva à diminuição do mecanismo oxidativo de vários substratos, à diminuição da síntese do ATP e a tolerância dos hepatócitos perante o estresse oxidativo apresenta-se diminuída (Navarro e Boveris, 2004).

Assim sendo, vários mecanismos conducentes à disfunção mitocondrial nos hepatócitos têm sido descritos, incluindo a suscetibilidade à indução do poro de permeabilidade transitória (Cardoso *et al.*, 2017) e, consequentemente, ao esgotamento das reservas de ATP levando à necrose hepática ou a apoptose através da liberação de proteínas apoptóticas (Eliseev *et al.*, 2002), alterações deletérias na bioenergética mitocondrial hepática, e incremento do estresse e lesão oxidativa em consequência da elevada geração de EROS (Carvalho *et al.*, 2013).

2.5 INSUFICIÊNCIA ENERGÉTICA

A insuficiência energética celular causada pela incapacidade em utilizar o oxigênio a nível celular tem sido apontada como uma desordem que contribui para a falência múltipla de órgãos em pacientes acometidos por doenças críticas (Molnar e Kovacs, 2018). Estudos clínicos em pacientes sépticos internados em unidades de terapia intensiva (UTI) apresentaram redução nas subunidades de proteínas do complexo da cadeia respiratória mitocondrial evidenciada por biópsias musculares comparadas com pacientes saudáveis (Arulkumaran *et al.*, 2016). Os pacientes que sobreviveram tiveram um aumento nos fatores de transcrição para a biogênese mitocondrial, enquanto a falha em ativá-los acarretou na redução do conteúdo mitocondrial e insuficiência energética. Desta forma, as mitocôndrias

são fundamentais tanto no fracasso como na recuperação da função de células e órgãos (Arulkumaran *et al.*, 2016; Zhang *et al.*, 2018).

Inúmeras terapias são direcionadas às mitocôndrias, incluindo o fornecimento de substratos mitocondriais (Milane *et al.*, 2015), uso de antioxidantes (Apostolova e Victor, 2015), moléculas *scavengers* de EROs (Bhatti, J. S. *et al.*, 2017) e estabilizadores da membrana mitocondrial (Yu *et al.*, 2016). Tendo em vista que a disfunção mitocondrial tem sido apontada como um processo chave em doenças neurodegenerativas, seu estudo torna-se essencial (Arun *et al.*, 2016; Gao *et al.*, 2017).

A falência energética é um dos mecanismos responsáveis por danos cerebrais, logo após um traumatismo crânio encefálico (TCE) (Abramov e Duchen, 2008; Watson, W. D. *et al.*, 2013). As consequências da disfunção mitocondrial após TCE são numerosas, incluindo falhas energéticas e metabólicas, perda da homeostase celular do cálcio, estresse oxidativo e ativação de processos apoptóticos (Hiebert *et al.*, 2015; Li *et al.*, 2017).

2.5.1 Traumatismo Crânio Encefálico

O traumatismo crânio encefálico, também chamado de lesão cerebral, é gerado por um golpe ou choque na cabeça ou ainda por um objeto que penetra o crânio, perturbando o funcionamento normal do cérebro, produzindo assim, um estado alterado de consciência, o qual pode resultar em comprometimento das habilidades cognitivas e físicas (Lorente, 2015; Collins-Praino *et al.*, 2018). O TCE pode ser classificado em leve (75% dos casos), moderado ou grave de acordo com a escala de coma de Glasgow, a qual avalia o nível de consciência após a lesão (Kim *et al.*, 2017).

Estima-se que 40 milhões de pessoas sofrem de TCE, sendo uma das principais causas de morte e incapacidade em todo o mundo, além disso, constitui um grande problema socioeconômico e de saúde pública (Chien *et al.*, 2018). Nos Estados Unidos, 2,2 milhões de pessoas visitam o departamento de emergência devido a um TCE e 50.000 mortes ocorrem anualmente, de acordo com o Centro de Controle e Prevenção de Doenças (Taylor *et al.*, 2017). No Brasil, segundo dados do DATASUS, o TCE é responsável por 125.500 internações por ano e 9.700 mortes hospitalares, acometendo principalmente a população de adulto jovens na faixa etária dos 20 - 29 anos e idosos entre 70 - 79 anos (De Almeida *et al.*, 2016).

Comumente o TCE é causado por acidentes de trânsito, eventos relacionados a esportes, mergulho em águas rasas, agressões, quedas e projéteis de armas de fogo (Smith *et*

al., 2015). Pacientes com TCE têm um maior risco de hipotensão, hipoxemia e edema cerebral. Se essas sequelas não são prevenidas ou atenuadas adequadamente, podem agravar os danos cerebrais e aumentar o risco de morte (Rajesh *et al.*, 2017). Dois importantes processos fisiopatológicos que contribuem para a lesão cerebral após o trauma são a lesão primária, em que o dano é causado como resultado direto do impacto mecânico; e a lesão secundária, que é iniciada imediatamente após o trauma devido a novos danos celulares que partem dos efeitos das lesões primárias, e que continuam a se desenvolver ao longo de um período de horas ou dias após o insulto traumático inicial (Maas *et al.*, 2008; Patel *et al.*, 2016). Durante esta segunda fase, os principais mecanismos conhecidos da patogênese no dano celular são principalmente devido à liberação de neurotransmissores, sobrecarga de cálcio, danos causados por radicais livres, ativação de genes pró-apoptóticos, disfunção mitocondrial e respostas inflamatórias (Collins-Praino *et al.*, 2018; Rodriguez *et al.*, 2018).

As mitocôndrias são sensíveis a alterações no estado fisiológico das células e parecem desempenhar um papel crítico na lesão secundária que ocorre após o TCE (Finkel, 2001; Hunot e Flavell, 2001). Fisiologicamente ou patologicamente, as mitocôndrias são reguladas por uma complexa rede de proteínas, assim como por modificações pós-traducionais envolvidas no controle da fissão, fusão e autofagia mitocondrial. O processo contínuo de fissão e fusão faz parte da dinâmica da rede mitocondrial, causando alterações macroscópicas na morfologia dessas organelas (Fischer *et al.*, 2016). Sendo considerado um sistema de controle da qualidade mitocondrial para células eucarióticas. Em condições patológicas, a fusão é ativada para proteger as mitocôndrias que ainda apresentam-se funcionais, a fim de restaurar a função mitocondrial normal. Mitocôndrias persistentemente disfuncionais são prontamente separadas das funcionais por fissão e são removidas via autofagia (Cho *et al.*, 2013; Di Pietro *et al.*, 2017).

Um estudo mostrou que após um TCE leve houve a ativação da fusão mitocondrial acompanhada por uma inibição significativa da fissão e da mitofagia. Resultando em prevenção contra a via apoptótica, remodelação das cristas mitocondriais, com melhora da cadeia transportadora de elétrons acoplada a OXPHOS (Di Pietro *et al.*, 2017). Em contrapartida, após um TCE grave, as células cerebrais são mais propensas a ativar a fissão e a mitofagia, uma explicação válida para a falta de recuperação metabólica observada após esse tipo de trauma (Amorini *et al.*, 2016).

Em particular, as mitocôndrias acabam sofrendo danos estruturais e funcionais consideráveis após TCE. A disfunção mitocondrial após o TCE tem sido associada à diminuição na transferência de elétrons nas mitocôndrias cerebrais e transdução de energia

devido à sobrecarga de cálcio associada à mitocôndria, aumento da produção de EROs na mitocôndria, danos oxidativos, perturbações da homeostase e morte celular (Robertson, 2004; Di Deo *et al.*, 2016; Dobrachinski *et al.*, 2017). Em relação ao dano estrutural, as mitocôndrias apresentam-se inchadas, com as cristas fragmentadas, compartimento da matriz expandida e com ruptura da membrana externa, indicativo do início da perda de $\Delta\Psi m$ (Kim *et al.*, 2017). Em um modelo experimental de TCE, o inchaço mitocondrial foi observado 10 min após a lesão cerebral (Hiebert *et al.*, 2015). Alterações no tamponamento e captação do cálcio mitocondrial cortical também foram evidentes 3 horas após lesão (Harmon *et al.*, 2017).

A disfunção nesta organela tem sido demonstrada estar envolvida na neurotoxicidade induzida por aminoácidos excitatórios, principalmente vinculada ao glutamato. Na verdade, como o principal neurotransmissor excitatório, o glutamato desempenha um papel essencial em processos de plasticidade cerebral, tais como a aprendizagem/memória, desenvolvimento e envelhecimento (Dobrachinski *et al.*, 2017). Por outro lado, a hiperestimulação do sistema glutamatérgico pode levar a um processo conhecido como excitotoxicidade, que envolve aumento intracelular de íons, tais como sódio (Na^{+}), Ca^{2+}, depleção dos níveis de ATP e, finalmente, a danos neuronais (Nicholls, 2008). Consequentemente, estudos têm demonstrado que esta excitotoxicidade e outras vias causadas pelo TCE podem levar a alterações na capacidade exploratória, cognitiva, memória/aprendizado de animais com TCE (Cope *et al.*, 2012; Awwad, 2016; Nolan *et al.*, 2018), além disso, pode conduzir a insuficiência mitocondrial e consequentemente levar à perda irreversível da célula (Fischer *et al.*, 2016).

Essas consequências, estimuladas pela lesão secundária são provavelmente um processo reversível, porque a morte celular neuronal tardia é um evento prolongado que pode ser regulado em muitos pontos da cascata de morte da célula (Hiebert *et al.*, 2015; Beauchamp *et al.*, 2018). No que diz respeito à importância da insuficiência mitocondrial e morte celular tardia, os agentes farmacológicos que têm como alvo as mitocôndrias ou que evitem os desequilíbrios causados a esta organela têm demonstrado serem neuroprotetores (Apostolova e Victor, 2015; Prauchner, 2017; Chien *et al.*, 2018). Várias intervenções terapêuticas que visam estabilizar as mitocôndrias têm mostrado resultados promissores, reduzindo danos gerais do tecido neuronal, bem como melhoras na evolução neurológica após o TCE (Bhatnagar *et al.*, 2016; Bhatti, Jasvinder Singh *et al.*, 2017). A guanosina (GUO) tem demonstrado atuar nesta cascata secundária, apresentando efeitos neuroproterores impedindo a excitotoxicidade, o dano mitocondrial e alterações comportamentais observados em modelos animais (Gerbatin *et al.*, 2016; Dobrachinski *et al.*, 2017; Dobrachinski *et al.*, 2018).

2.6 SISTEMA PURINÉRGICO

A sinalização purinérgica é uma importante via moduladora de variados processos fisiológicos, envolvida em muitos mecanismos neuronais e não neuronais e em eventos de curta e longa duração, incluindo respostas imunes, inflamação, proliferação, morte celular (Tasca *et al.*, 2018). As purinas são moléculas endógenas, constituintes estruturais dos ácidos nucleicos, atuam como segundos mensageiros nas vias de sinalização intracelular (Rathbone *et al.*, 1999).

As bases purinérgicas, como adenina e guanina, e seus nucleosídeos correspondentes, como adenosina e guanosina e seus produtos metabólicos apresentam importante papel como neurotransmissores e neuromoduladores no sistema nervoso central (SNC), sistema nervoso periférico e sistema nervoso entérico (Rathbone *et al.*, 1999). Os astrócitos são a principal fonte de purinas da adenina e da guanina e expressam receptores específicos para estes compostos (Ciccarelli *et al.*, 2000).

Os receptores melhor caracterizados são os da adenina, sendo o receptor do tipo P1 para os receptores de Adenina e os receptores P2 para ATP e ADP. A família de receptores P1 compreende os receptores de Adenosina A_1, A_2A, A_2B e A_3 sendo todos acoplados à proteína G. Os receptores P2 são de 2 tipos: P2X é da família de receptor ligado ao canal iônico e o P2Y é o receptor ligado à proteína G (Burnstock, 2018). A ativação de receptor A_1 inibe a adenilato ciclase, o que leva a diminuição dos níveis do segundo mensageiro AMPc. Resultando na inibição da liberação de neurotransmissores (Rathbone *et al.*, 1999). Já a ativação de receptor A_2A ativa a adenilato ciclase, facilitando a liberação de neurotransmissores. Os efeitos biológicos dos receptores A_2B e A_3 não são completamente conhecidos. A ativação de receptor de ATP pode estimular ou inibir a liberação de glutamato de neurônios hipocampais de ratos (Fields e Burnstock, 2006).

Entretanto, em oposição às múltiplas ações relatadas pelas purinas derivadas da adenina, a importância das purinas à base de guanina (GBPs) tem sido atribuída principalmente ao seu papel como reguladores da função da proteína G (Di Liberto *et al.*, 2016). A atividade da proteína G é, até o momento, modulada pelas interações com a guanosina difosfato (GDP) ou guanosina trifosfato (GTP) no estado basal e ativado, respectivamente. Outras GBPs, como guanosina monofosfato (GMP), guanosina ou guanina, não houve relatos de interação com proteínas G (Taylor, 1990).

Além disso, desempenham um importante papel extracelular, na modulação da atividade do sistema glutamatérgico, pois inibem a união do glutamato e análogos aos

receptores nas preparações da membrana celular sem envolvimento de proteínas G, e protegem contra a neurotoxicidade mediada por receptores glutamatérgicos (Tasca *et al.*, 1995; Tasca *et al.*, 1998).

Vários efeitos biológicos induzidos pela guanosina parecem ser dependentes dos receptores de adenosina, A_1 e A_2A (Dobrachinski *et al.*, 2018). Um estudo em fatias de hipocampo demonstrou que a inibição do receptor A_1 e ativação do receptor A_2A bloquearam o efeito da guanosina em reestabelecer a captação de glutamato alterada pela privação de glicose e oxigênio (Dal-Cim *et al.*, 2013). Desta forma, a guanosina parece depender de um receptor acoplado a proteína G e de uma interação antagônica entre os receptores de adenosina, A_1 e A_2A (Dal-Cim *et al.*, 2013).

Diversos efeitos benéficos relacionados às GBPs já foram descritos (efeitos neuroprotetores, neurotróficos, antidepressivos e analgésicos) em todo o SNC (Di Liberto *et al.*, 2016; Tasca *et al.*, 2018). Ultimamente pesquisas têm sido realizadas a fim de caracterizar e elucidar os mecanismos de ação ainda não descritos dessas moléculas (Lanznaster *et al.*, 2016). As mais diversas funções são desempenhadas pelos derivados da guanina, mas nessa tese abordaremos os efeitos protetores relacionados à guanosina.

2.6.1 Guanosina

No SNC a guanosina, o nucleosídeo de guanina endógeno, está disponível no meio extracelular através da liberação a partir de células da glia. Em culturas de células de astrócitos tem sido relatado que a guanosina pode ser liberada sob condições basais ou tóxicas (Ciccarelli *et al.*, 1999; Ciccarelli *et al.*, 2001). Alternativamente, os nucleotídeos, tais como GTP, GDP e GMP podem ser metabolizados por ecto-nucleotidases (Ecto-NTPDase) para produzir guanosina extracelular (Ciccarelli *et al.*, 2001). Ecto-NTPDase metaboliza GTP e GDP para produzir GMP. A GUO é hidrolisada pela purina nucleosídeo fosforilase (PNP) gerando a guanina (GUA) uma base. Pela ação de uma guanina deaminase, a guanina converte-se à xantina e sequencialmente ao ácido úrico pela ação de uma xantina oxidase (Fig.4).

Figura 4: Metabolismo dos derivados da guanina.

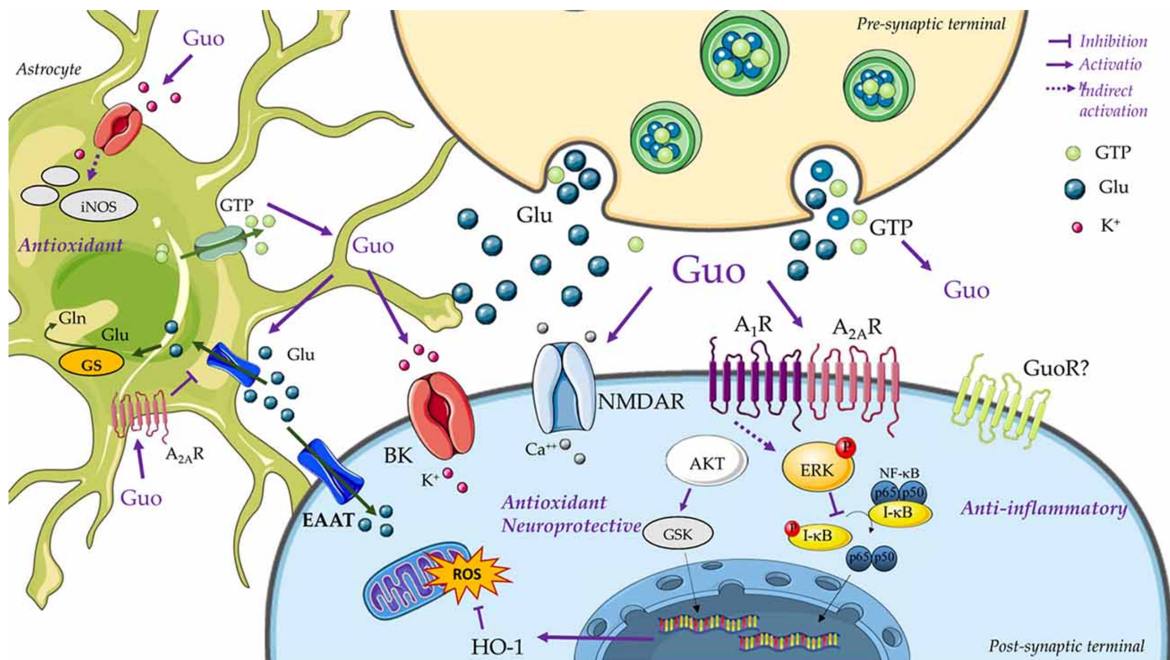
Fonte: Adaptado de (Lanznaster *et al.*, 2016).

Os nucleotídeos da guanina e a guanosina têm sido implicados na neuroproteção por exercerem efeitos tróficos (Ciccarelli *et al.*, 2001; Decker *et al.*, 2007), bem como por contrapor a excitotoxicidade glutamatérgica *in vitro* (Molz, Tharine*, et al.*, 2008; Oleskovicz, S. P. *et al.*, 2008) e *in vivo* (Dobrachinski *et al.*, 2017; Teixeira *et al.*, 2018). No que diz respeito aos seus efeitos sobre a atividade glutamatérgica, GBPs inibem a ligação de glutamato e análogos em seus receptores, demonstrados através de estudos *in vitro* (Burgos *et al.*, 1998), prevenindo respostas celulares para aminoácidos excitatórios (Rubin *et al.*, 1997) e aumentando a captação de glutamato pelos astrócitos (Frizzo *et al.*, 2001), o qual é um processo fisiológico envolvido na neuroproteção.

A guanosina apresenta efeitos antioxidantes como demonstrado na figura 5, através da redução da geração de EROs, prevenindo a expressão da óxido nítrico sintase indutível (iNOS) (Thomaz, Daniel T. *et al.*, 2016) e aumentando as defesas antioxidantes, como os níveis da heme oxigenase-1 (HO-1) (Quincozes-Santos *et al.*, 2014). A ativação de fosfatidilinositol 3-quinases (PI3K / Akt), proteína quinase C e proteíno-quinases ativadas por mitógenos (MAPK / ERK) pela guanosina (Molz *et al.*, 2011; Dal-Cim *et al.*, 2013) leva à estimulação da atividade dos transportadores de aminoácidos excitatórios (EAATs). GUO também aumenta a atividade da glutamina (Gln) sintetase (GS), reduzindo assim os níveis extracelulares de glutamato (Glu) e protegendo da excitotoxicidade do glutamato (Quincozes-Santos *et al.*, 2013; Cittolin-Santos *et al.*, 2017). Além disso, promove a neuroproteção, através de um efeito anti-inflamatório da inibição da ativação do fator nuclear kappa B (NF-κB) via MAPK / ERK (Dal-Cim *et al.*, 2013). Também há relatos de efeitos da GUO através de canais de potássio (BK) e de cálcio (Oleskovicz, S. P. B. *et al.*, 2008), receptores de adenosina (A_1R e $A_{2A}R$) (Dobrachinski *et al.*, 2018) e receptores glutamatérgicos de N-metil-D-aspartato (NMDAR) (Baron *et al.*, 1989; Schmidt *et al.*, 2010).

Estudos recentes demonstraram que a guanosina diminui a produção de EROs induzida pela privação de oxigênio e glicose em fatias hipocampais e previne a perda de potencial da membrana mitocondrial (Dal-Cim *et al.*, 2013). Em um protocolo de indução de estresse oxidativo através do bloqueio dos complexos I e V da cadeia respiratória em células SH-SY5Y, a guanosina induziu efeito protetor através da indução da HO-1(Dal-Cim *et al.*, 2012). A HO-1 também está envolvida na proteção induzida pela guanosina contra o estresse oxidativo e o aumento de citocinas pró-inflamatórias induzidos pelo tratamento com azida (um inibidor do complexo IV da cadeia respiratória) em células astrogliais C6 (Quincozes-Santos *et al.*, 2014).

Figura 5: Mecanismos envolvidos nos efeitos protetores da Guanosina.



Fonte: (Tasca *et al.*, 2018).

Através de um modelo *in vitro* para estudar os mecanismos de morte celular associado à doença de Parkinson foi avaliado usando MPP^+ (1-metil-4-fenilpiridínio), o metabólito ativo da neurotoxina MPTP que se acumula na mitocôndria e inibe a atividade do complexo I e, pode ocasionar a morte celular (Pettifer *et al.*, 2007). Observou-se que o tratamento com a guanosina reverteu o processo apoptótico, evitando a fragmentação do DNA e preveniu o aumento da atividade da caspase-3 induzida por MPP^+ em células de neuroblastoma SH-SY5Y (Pettifer *et al.*, 2007).

Nosso grupo mostrou que após um TCE em ratos, a guanosina apresentou um efeito neuroprotetor, evitando a perda neuronal através da modulação do sistema glutamatérgico, manutenção do sistema redox, diminuição dos níveis de citocinas, com consequente redução do desenvolvimento de edema cerebral (Gerbatin *et al.*, 2016; Dobrachinski *et al.*, 2017). Como demonstrado anteriormente, a guanosina exibe um papel protetor em diferentes modelos experimentais *in vitro* e *in vivo,* envolvendo o glutamato, estresse mitocondrial, modelos de isquemia, doença de Parkinson, TCE, entre outros (Li *et al.*, 2014; Ramos, D. B. *et al.*, 2016; Dobrachinski *et al.*, 2018; Tasca *et al.*, 2018). No entanto, pouco se sabe sobre o verdadeiro mecanismo de ação da guanosina, envolvido na proteção contra alterações na função mitocondrial.

3 DESENVOLVIMENTO

O desenvolvimento que faz parte desta tese está apresentado sob a forma de um artigo e um manuscrito científico. Os itens Introdução, Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos.

O artigo 1 foi publicado na revista *Biomedicine and Pharmacotherapy* e encontra-se no formato da mesma.

O manuscrito 1 encontra-se submetido na revista *Brain Research Bulletin* e está apresentado em forma de manuscrito, na formatação para publicação na revista.

3.1 ARTIGO CIENTÍFICO I

Guanosine protects against Ca^{2+}-induced mitochondrial dysfunction in rats

Aline Alves Courtes, Nelson Rodrigues de Carvalho, Débora Farina Gonçalves, Diane Duarte Hartmann, Pamela Carvalho da Rosa, Fernando Dobrachinski, Jeferson Luis Franco, Diogo Onofre Gomes de Souza, Félix Alexandre Antunes Soares.

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Guanosine protects against Ca^{2+}-induced mitochondrial dysfunction in rats

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ABSTRACT

Mitochondria play an important role in cell life and in the regulation of cell death. In addition, mitochondrial dysfunction contributes to a wide range of neuropathologies. The nucleoside Guanosine (GUO) is an endogenous molecule, presenting antioxidant properties, possibly due to its direct scavenging ability and/or from its capacity to activate the antioxidant defense system. GUO demonstrate a neuroprotective effect due to the modulation of the glutamatergic system and maintenance of the redox system. Thus, considering the few studies focused on the direct effects of GUO on mitochondrial bioenergetics, we designed a study to evaluate the *in vitro* effects of GUO on rat mitochondrial function, as well as against Ca^{2+}-induced impairment. Our results indicate that GUO prevented mitochondrial dysfunction induced by Ca^{2+} misbalance, once GUO was able to reduce mitochondrial swelling in the presence of Ca^{2+}, as well as ROS production and hydrogen peroxide levels, and to increase manganese superoxide dismutase activity, oxidative phosphorylation and tricarboxylic acid cycle activities. Our study indicates for the first time that GUO could direct prevent the mitochondrial damage induced by Ca^{2+} and that these effects were not related to its scavenging properties. Our data indicates that GUO could be included as a new pharmacological strategy for diseases linked to mitochondrial dysfunction.

1. Introduction

Mitochondria play an important role not only in cell life but also in the regulation of apoptotic or necrotic cell death [1,2]. Consistent with this role, several agents, such as calcium (Ca^{2+}), can regulate energy metabolism under normal conditions. High free cytosolic Ca^{2+} concentrations cause the mitochondrial permeability transition pore (mPTP) opening, leading to an excessive increase of mitochondrial swelling. This fact plays a central role in the induction of cell death through apoptosis or necrosis, depending on availability of adenosine triphosphate (ATP) [3,4].

Mitochondrial dysfunction is involved in the pathogenesis of many human diseases associated with oxidative stress, such as stroke [5–7], hepatic encephalopathy [8], hypoxia [9] and diabetes [10]. Besides, mitochondrial dysfunction has long been demonstrated as a common prominent early pathological feature of a variety of common neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and Huntington's disease [11]. Thus, molecules potentially involved in mitochondrial integrity regulation are of considerable importance in cell biology and in pharmacological studies [12].

Several studies have shown that the nucleoside guanosine (GUO), an endogenous molecule and member of the purinergic system, is known to participate in several intracellular processes [13–15]. In addition, GUO possesses important antioxidant properties, possibly derived from its direct scavenge [16]. GUO is released from astrocytes under basal and toxic conditions or is alternatively formed from the extracellular hydrolysis of guanine nucleotides [14]. GUO has also been used as a neuroprotective compound in experimental models of brain injury/diseases mainly by modulation of the glutamatergic system [13,15,17,18].

It has been previously demonstrated [19] that GUO prevented the disruption of mitochondria membrane potential induced by oxygen-glucose deprivation (OGD) in hippocampal slices, as well as that it protected cells from oxidative damage induced by impairment of

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mitochondrial activity [20]. Additionally, our group demonstrates that GUO produced neuroprotective effect due modulation of the glutamatergic system, optimal maintenance of the redox system and inhibition of intracellular Ca^{2+} alterations in mitochondria in a traumatic brain injury rat model [21,15]. Some studies have focused on maintaining mitochondrial functionality and calcium homeostasis as one of the main targets of GUO in various pathologies [22,23]. However, the specific modulation of these parameters associated with mitochondrial bioenergetics still remains unclear.

Previous studies have demonstrated substantial mitochondrial heterogeneity among organs and within the central nervous system (CNS). In this tissue, distinct mitochondrial populations can be observed in different neuronal compartments (as synaptic vs. non-synaptic) [11]. Within the CNS, there are regional differences in mitochondrial populations, with regard to Ca^{2+}-induced mPTP threshold and reactive oxygen species (ROS) production [24]. In CNS, there is also regional and cellular heterogeneity concerning the composition, morphology, and trafficking of mitochondria [25]. This characteristic of CNS mitochondrial could cause some disruption in the data obtained when the study is not in a specific brain region.

Thus, considering the homogeneity of mitochondrial population that exists in the liver when compared to SNC [26], together with few studies focused on the direct effects of GUO on bioenergetics, this study was designed to evaluate the effects of GUO on hepatic mitochondrial function, as well as against Ca^{2+}-induced impairment, through *in vitro* study. This work may contribute to a better understanding of GUO mechanisms of action on mitochondrial functionality and bring some new perspectives for its application as a neuroprotective molecule for mitochondrial-related diseases.

2. Materials and methods

2.1. Chemical reagents

Guanosine (GUO), ruthenium red (RR), cyclosporine A (CsA), butylated hydroxytoluene (BHT), dithiothreitol (DTT), safranine-*O*, 2,4 dinitrophenol (DNP) and 2′ 7′ dichlorofluorescein diacetate (H_2-DCFDA), adenosine 5′- diphosphate sodium salt (ADP), taurine, pyruvic acid, glutamate, malate, antimycin A, rotenone, malonic acid and carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), acetyl coenzyme A (AcCoA), 5′,5′-dithiobis (2-nitrobenzoic acid), dichloroindophenol (DCIP) were obtained from Sigma Chemical Company (St Louis, MO). Amplex® Red (AmR) and horseradish peroxidase (HRP) were obtained from Molecular Probes. All other chemicals were of analytical grade and were obtained from standard commercial suppliers.

2.2. Animals

Adult male Wistar rats (250–350 g) from our own breeding colony were used in this study. The animals were housed in plastic cages with water and food ad libitum, at 22 ± 2 °C, 45–65% humidity, and 12-h light/dark cycle. The animals were used in accordance to guidelines of the Committee on Care and use of Experimental Animal Resources, Federal University of Santa Maria, Brazil, under the process number 153-2014.

2.3. Isolation of mitochondria

Rats liver mitochondria were isolated at 4 °C by differential centrifugation [61]. After removal, livers were immersed and homogenized in ice-cold isolation buffer I containing 320 mM sucrose, 1 mM EDTA, 1 mM EGTA and 10 mM TRIZMA-base (pH 7.4). The resulting suspension was centrifuged for 10 min at 2500 × *g* in a Hitachi CR 21E centrifuge. The supernatant was centrifuged at 10,000 × *g* for 10 min. The pellet was re-suspended in isolation buffer I with bovine serum albumin (BSA) containing 320 mM sucrose, 1 mM EDTA, 1 mM EGTA, 0.1% bovine serum albumin free fatty acid and 10 mM TRIZMA-base (pH 7.4). The resulting supernatant was decanted, and the final pellet was gently resuspended in isolation buffer II containing 250 mM sucrose, and 10 mM TRIZMA base (pH 7.4). The pellet was resuspended 3 times with isolation buffer II to get intact mitochondria (it has been suggested that after 3 resuspensions, the influence of contaminating microsomes on NAD(P)H oxidation, reactive oxygen species levels, and membrane permeabilization becomes negligible) [65].

2.4. Standard incubation procedure

Measurements of mitochondrial swelling, mitochondrial transmembrane electrical potential (Δψm), and estimation of reactive oxygen species were performed in a stirred cuvette mounted in a RF-5301 PC Shimadzu spectrofluorometer (Kyoto, Japan) at 30 °C. Liver mitochondria isolated (0.5 mg protein) were added to 3-ml standard incubation buffer containing 250 mM sucrose, 10 mM TRIZMA base buffer (pH 7.4), 100 μM adenosine diphosphate (ADP), 60 mM inorganic phosphate (Pi), 10 mM glutamate, and 5 mM succinate [27]. Other additions are indicated in the figure legends. The results shown are representative of a series of 3–5 independent experiments, using independently isolated mitochondrial preparations.

2.5. Mitochondrial swelling

Measurement of mitochondrial swelling was performed in RF-5301 Shimadzu spectrofluorometer at 600 nm and slit 1.5 nm for excitation and emission. Liver mitochondria isolated (0.5 mg protein) were incubated in buffer II (described earlier) in presence of 50, 100 or 300 μM guanosine (Votyakova and Reynolds 2005). Next, it was incubated with 100 μM Ca^{2+} plus 50, 100 or 300 μM guanosine to evaluated GUO effects on Ca^{2+}-induced swelling. Besides, the mitochondrial preparations were incubated with ruthenium red (RR; 1 μM), a specific inhibitor of mitochondrial Ca^{2+} transport [28,29], cyclosporin A (CsA; 1 μM), an inhibitor of mitochondrial permeability transition pores, butylated hydroxytoluene (BHT; 2.5 μM) or dithiothreitol (DTT; 10 μM) to evaluate antioxidant properties [30].

2.6. Mitochondrial transmembrane electrical potential (Δψm)

Mitochondrial Δψm was estimated by fluorescence changes in safranine-O [64]. Liver mitochondria isolated (0.5 mg of protein/mL) were incubated with isolation buffer II, 5 μM Ca^{2+} and/or 300 μM guanosine, 10 μM safranine-O, and the respiratory substrates 5 mM glutamate/malate and 5 mM succinate. In order to evaluate the maximal mitochondrial uncoupling, 2,4 dinitrophenol (DNP) (0.1 mM) was added to the medium at the end of assay period (5 min). The fluorescence analysis was performed at 495 nm for excitation and 586 nm for emission, with slit widths of 3 nm. The mitochondrial potential is presented as % of control.

2.7. Reactive oxygen species (ROS) levels

The liver mitochondrial isolated levels of ROS were spectrofluorimetrically determined, using the membrane permeable fluorescent dye dichlorofluorescein diacetate (H_2DCF-DA - 1 μM). The oxidation of H_2DCF-DA to 2′7′dichlorofluorescein (DCF) is used as an index of the peroxide production by cellular components [63]. Fluorescence was determined at 488 nm for excitation and 525 nm for emission, with slit widths of 3 nm. The values are expressed as arbitrary units of fluorescence (AFU).

2.8. Hydrogen peroxide (H_2O_2) production

H_2O_2 production was measured in the Oxygraph-2k (O2k,

OROBOROS Instruments, Innsbruck, Austria) using the Sensor Green of the O2k-Fluo LED2-Module for fluorescence while respiration was analyzed. The H_2O_2-sensitive probe Amplex® Red (Molecular Probes) was used to measure the peroxide flux [31]. Liver mitochondria isolated (1 mg/mL) were placed inside oroboros chamber containing MiR05, 10 µM Amplex® Red (AmR) and 1 U/mL horseradish peroxidase (HRP). The product of the reaction between AmR and H_2O_2, catalyzed by HRP, is fluorescent, and O2k-Fluo LED2-Module is sensitive to this fluorescence difference. Through this protocol was possible to evaluate peroxide production in different steps of the HRR with the addition of substrates and inhibitors. Experiments were performed with sequential additions of the following substrates and inhibitors: pyruvate (5 mM), malate (2.5 mM) and glutamate (10 mM); succinate (10 mM); rotenone (0.5 µM), oligomycin (2.5 µM), FCCP (0,25 µM), malonate (5 mM) and antimycin A (2.5 µM).

2.9. Manganese superoxide dismutase (MnSOD) activity

Aliquots (10 µL) of liver mitochondria isolated were added to a medium containing sodium bicarbonate-carbonate buffer (50 mM; pH 10.2), EDTA (2 mM), KCN (1 mM) and adrenaline (0.4 mM) [32]. The reactions were performed in a 96-well plate, and the results were read at 480 nm in SpectraMax i3×. The kinetic analysis of SOD was started after adrenaline addition, and the color reaction was measured spectrophotometrically for 5 min at 480 nm. The activities are expressed as units (U) SOD/mg of protein.

2.10. High-resolution respirometry (HRR)

For the respirometry determination, the samples were weighed and homogenized in 500 µL of cold buffer containing 5 mM Tris−HCl, 250 mM sucrose and 2 mM EGTA (pH 7.4) [33,34] and transferred into the Oxygraph-2 k (O2k, OROBOROS INSTRUMENTS, Innsbruck) 2 ml-chambers. Oxygen polarography was performed at 37 °C and the oxygen flux was recorded at real-time using DatLab software. In the present protocol, the homogenate (0.4 mg/mL) was added to the chamber containing the respiration medium-MIR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 0.1 mg/ml free fat acid BSA) and 300 µM guanosine. After stabilization of signal, the experimental protocol of high-resolution respirometry was performed by sequential addition of 10 mM glutamate, 2 mM malate, 2.5 mM ADP, 10 mM succinate, 0.5 µM rotenone and 2.5 µM antimycin A [33,35].

2.11. Citrate synthase enzyme (CS) activity

CS activity is frequently used as a biomarker of mitochondrial content, in liver mitochondria isolated was measured as previously described [36]. The reaction medium contained 1 M Tris−HCl (pH 8), 1 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), 10 mM acetyl coenzyme A (AcCoA), and 5 mg/ml mitochondrion protein. The medium was incubated for 5 min with continuous shaking at 37 °C, and the reaction was initiated by the addition of 10 mM oxaloacetate. The reactions were performed in a 96-well plate, and the results were read at 412 nm in SpectraMax i3×. The reduction of 5′,5′-dithiobis (2-nitrobenzoic acid) by CS was measured spectrophotometrically for 5 min at 412 nm (extinction coefficient = 13.6 mM/cm). The activities are expressed as % of control.

2.12. Mitochondrial complex II assay

The activity of complex II (succinate dehydrogenase) was determined by following the reduction of dichloroindophenol (DCIP) by succinate [27]. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM KCN, 0.05 mM DCIP, 16 mM succinate and 0.5 mg protein of mitochondrial isolated. Absorbance changes were followed at 600 nm, using an extinction coefficient of 19.1 $mM^{-1} cm^{-1}$ for dichloroindophenol.

2.13. Protein determination

Protein was determined by Bradford's test [62] using serum albumin as standard.

2.14. Statistical analysis

Statistical analysis was performed using unpaired Student's *t*-test and one-way analysis of variance (ANOVA), followed by Newman–Keuls test for *post-hoc* comparison. Data are expressed as means ± S.D. or S.E.M. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Effects of guanosine on mitochondrial swelling

In order to evaluate the influence of GUO *per se* in mitochondrial function, we induced mitochondrial swelling with different GUO concentrations (50, 100 and 300 µM). All GUO concentrations induced a significant increase of mitochondrial swelling ($F_{3,12} = 40.11$, $p < 0.05$) when compared to control group (Fig. 1A). The Ca^{2+}-induced mitochondrial swelling induced by 100 µM Ca^{2+} (Fig. 1B) was abolished by GUO (Fig. 1B) ($F_{4,16} = 15.70$, $p < 0.05$). Thus, 300 µM of GUO was chosen to be used in the further experiments [37]. The phosphorylated forms of GUO: guanosine monophosphate (GMP), guanosine diphosphate (GDP) and guanosine triphosphate (GTP) did not present any effect on mitochondrial parameters here evaluated (data not shown).

Aiming better understand this GUO effect on Ca^{2+}-induced mitochondrial swelling, we decided to use Ruthenium Red (RR) (inhibitor of the mitochondrial Ca^{2+} uniporter) (Fig. 1C). RR blocked the effects of Ca^{2+} and GUO on swelling ($F_{5,17} = 45.67$, $p < 0.05$). Moreover, it was performed swelling with cyclosporin A (CsA). As demonstrate in Fig. 1D, there was an inhibition in Ca^{2+} plus CsA, while GUO (300 µM) plus CsA was similar to control swelling ($F_{3,13} = 59.85$, $p < 0.05$).

As demonstrated in Fig. 1E, there was an increase in mitochondrial swelling in Ca^{2+} and GUO (300 µM) group alone, as well as in DTT and BHT plus Ca^{2+} group, when compared to control group. GUO was able to protect the Ca^{2+}-induced mitochondrial swelling ($F_{5,19} = 43.04$, $p < 0.05$).

3.2. Effects of guanosine on mitochondrial membrane potential (Δψm)

Fig. 2(A–B) illustrates the effect of Ca^{2+} and/or GUO (300 µM) on the mitochondrial membrane potential. It is observed a marked reduction of the safranine-O fluorescence (the increase in fluorescence is directly proportional to the increase of damage) in the mitochondria exposed to Ca^{2+}compared to the control group ($F_{3,18} = 56.02$, $p < 0.05$). GUO was not able to attenuate against depolarization of the mitochondrial transmembrane potential induced by Ca^{2+}.

3.3. Effects of GUO on ROS generation and H_2O_2 production

Initially, we performed a Ca^{2+} curve, to choose the better Ca^{2+}concentration (Fig. 3A). Fig. 3B shows that Ca^{2+} induced an increase in values of ROS levels ($F_{3,13} = 33.53$, $p < 0.05$). GUO (300 µM) has no effect in ROS formation when used alone but it caused a significant decrease in Ca^{2+}-induced ROS formation (Fig. 3B) ($F_{3,13} = 33.53$, $p < 0.05$). As demonstrated in Fig. 3C, Ca^{2+} induced an increase in levels of H_2O_2 production when compared to control group ($F_{3,15} = 10.42$, $p < 0.05$). GUO (300 µM) has no effect in H_2O_2 production when used alone but it caused a significant decrease in Ca^{2+}-

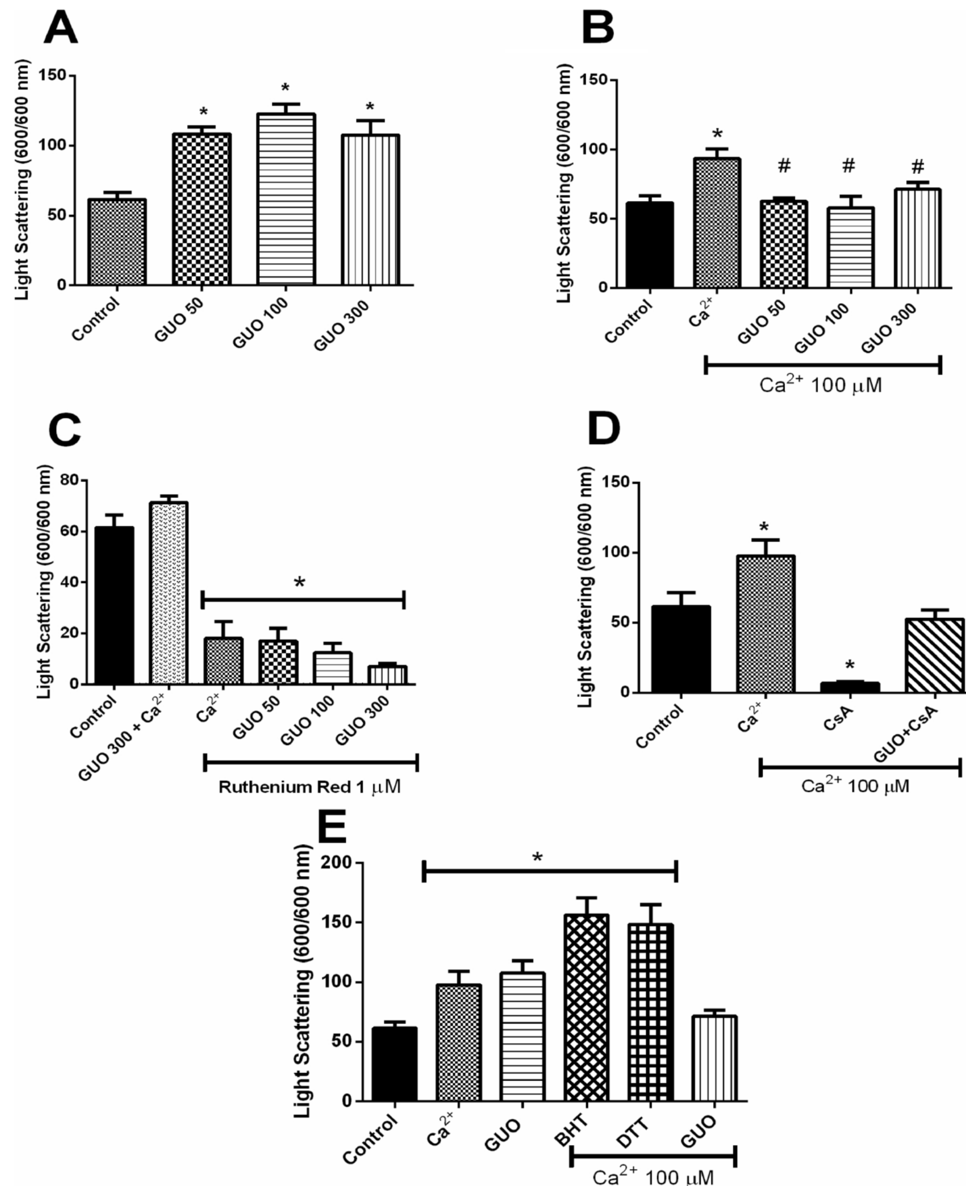

Fig. 1. Effects of 50, 100 and 300 μM of guanosine (GUO) (A), plus 100 μM of calcium (Ca^{2+}) (B), 1 μM of Ruthenium Red (RR) (C), 1 μM of cyclosporine A (CsA) (D), 2.5 μM of butylated hydroxytoluene (BHT) 10 μM of dithiothreitol (DTT) (E) on mitochondrial swelling in liver mitochondria isolated of rats. Each bar represents means ± S.E.M. (n = 4–5). (*) indicates statistic difference from control group by one-way ANOVA, followed by Newman Keuls's or Dunnet *post-hoc* test ($p < 0.05$). (#) indicates statistic difference from Ca^{2+} group by one-way ANOVA, followed by Newman Keuls's *post-hoc* test ($p < 0.05$).

induced H_2O_2 production (Fig. 3C) ($F_{3,15} = 10.42$, $p < 0.05$).

3.4. Effects of GUO on high-resolution respirometry

Fig. 4A demonstrates the effect of 300 μM GUO on mitochondrial oxygen consumption. GUO caused an increase in electron transfer in OXPHOS CI-linked, CII-linked and CI + CII-linked, compared to control ($p < 0.05$); GUO presented no effect in CI-leak (Fig. 4A). GUO increased the Ratio control respiratory ($p < 0.05$) (Fig. 4B). Besides, it was evaluated ATP-linked production through the difference between basal respiration and LEAK, where oxygen consumption related to ATP production. In the Fig. 4C is possible to observe that GUO increased the ATP-linked production ($p < 0.05$).

3.5. Effects of GUO on MnSOD activity

Analysis of MnSOD activity showed a significant decrease Ca^{2+} induced in relation to the control group (Fig. 4D) ($F_{3,17} = 17.42$,

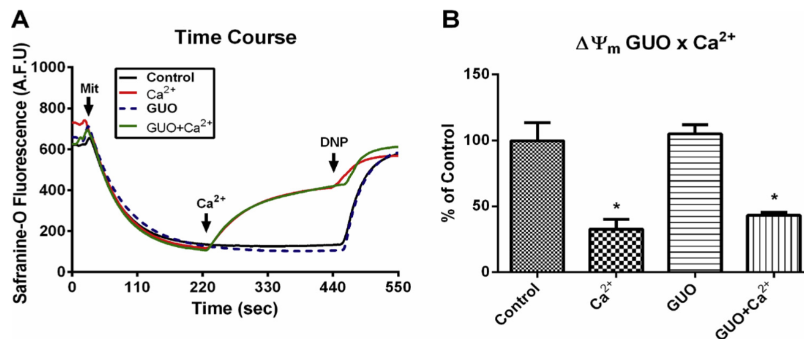

Fig. 2. Effects of 300 μM of guanosine (GUO) and 100 μM of calcium (Ca^{2+}) in the safranine-O fluorescence to evaluate mitochondrial membrane potential (Δψm) (Fig. 2A and 2B) in liver mitochondria isolated of rats. Each bar represents means ± S.E.M. (n = 5). (*) indicates statistic difference from control group by two-way ANOVA, followed by Newman Keuls's *post-hoc* test ($p < 0.05$).

$p < 0.05$). GUO (300 μM) treatment protected against this effect, showing increase activity MnSOD levels when compared to Ca^{2+} group (Fig. 4D) ($F_{3,17} = 17.42$, $p < 0.05$).

3.6. *Effects of GUO on citrate synthase (CS) enzyme activity and Mitochondrial complex II*

Different GUO concentrations increased the activity of CS (Fig. 5A) ($F_{3,12} = 25.89$, $p < 0.05$) and the complex II (succinate dehydrogenase) (Fig. 5B) ($F_{3,19} = 23.30$, $p < 0.05$).

4. Discussion

The data presented here clearly indicates that GUO could promote a protective effect against Ca^{2+}-induced mitochondrial dysfunction, through reduction of mitochondrial swelling, decrease in reactive oxygen species and hydrogen peroxide levels and increase on Mn-superoxide dismutase, oxidative phosphorylation, with a consequent increase in the tricarboxylic acid cycle activity. The nucleoside Guanosine has been studied in a variety of experimental neuropathological contexts associated with mitochondrial dysfunction [15,21,38]. In this case, the balance of mitochondrial respiration essential for cell survival is impaired, resulting in excessive production of reactive oxygen species, oxidative damage, and bioenergetics collapse. These events contribute to failure in astrocytic glutamate transporters (essential for avoiding glutamatergic excitotoxicity) and inhibition in the activity of the enzymes Na^{+}/K^{+}-ATPase and glutamine synthetase, accompanied by extensive neuronal loss [15,21].

It is well described that excessive matrix swelling associated with the sustained opening of mPTP and other independent mechanisms compromises mitochondrial function and integrity leading to cell death [39]. Changes in the mitochondrial matrix volume in the physiological range have a stimulatory effect on the electron transfer chain and oxidative phosphorylation to satisfy cell metabolic requirements [40]. Mitochondrial swelling is involved in the pathogenesis of many human diseases such as neurodegenerative and cardiovascular diseases

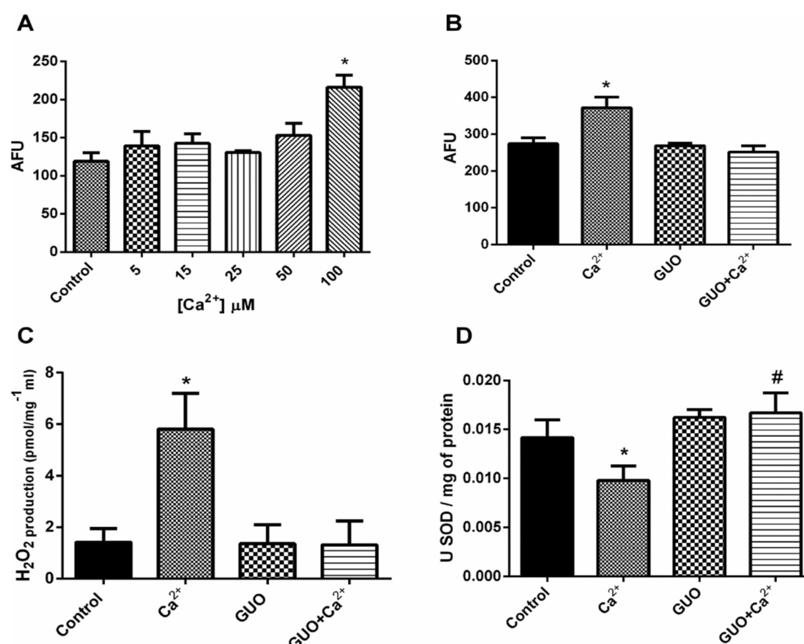

Fig. 3. Effects of different concentrations of calcium (Ca^{2+}) (A) and 300 μM of guanosine (GUO) and 100 μM of calcium (Ca^{2+}) (B) on DCF oxidation on arbitrary units of fluorescence (AFU) on H_2O_2 production (C) and MnSOD activity (D) in liver mitochondria isolated of rats. Each bar represents means ± S.E.M. (n = 5). (*) indicates statistic difference from control group by one-way ANOVA, followed by Newman Keuls's *post-hoc* test ($p < 0.05$). (#) indicates statistic difference from Ca^{2+} group by one-way ANOVA, followed by Newman Keuls's *post-hoc* test ($p < 0.05$).

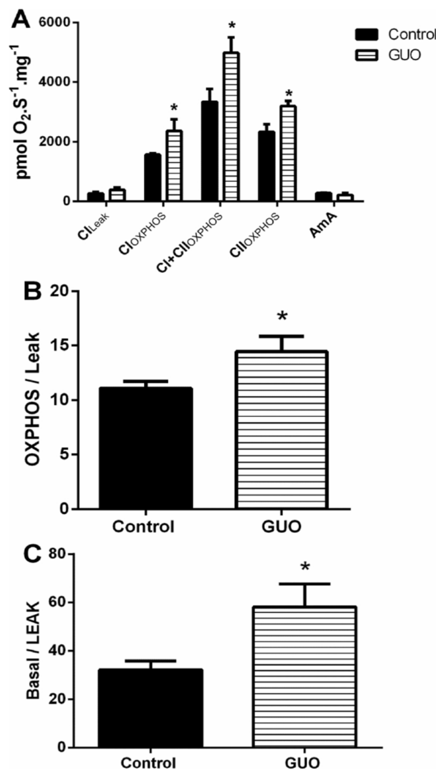

Fig. 4. Effects of 300 μM of guanosine (GUO) on HRR in mitochondria of rats (A), Ratio control respiratory (RCR) (B) and indirect ATP production (C). LEAK is demonstrated by the inactive state of phosphorylation system (without ADP), but in presence of CI (glutamate and malate). OXPHOS is the oxygen consumption in oxidative phosphorylation with saturated concentrations of substrates and ADP. OXPHOS was measured by sequential addition of glutamate, malate, ADP (CI-linked), succinate (CI&CII-linked) and rotenone (CII-linked). Ratio control respiratory (RCR) is the ratio between OXPHOS values (using the different substrates) and CI-leak values. ATP-linked production (C) is represented through the difference between Basal Respiration and LEAK. Each bar represents means ± S.E.M. (n = 5). (*) indicates statistic difference from control group by unpaired Student's *t*-test ($p < 0.05$).

[41,42]. The mechanisms of transition from reversible (physiological) to irreversible (pathological) swelling of mitochondria remain unclear. Our results showed that GUO itself could modulate mitochondrial swelling. These findings are in line with a study that demonstrated the caffeine, a purine receptor antagonist, effects, on heart mitochondria isolated from Wistar rats, which induced swelling mitochondrial by stimulate the opening of the mPTP and depicted a depletion in others mitochondrial respiratory parameters [43]. However, when different concentrations of GUO were used before Ca^{2+}, it was possible to observe an effect protective of GUO against Ca^{2+}-induced swelling. This result led us to postulate that GUO could act modulating the mPTP opening and protecting against excitotoxicity and the mitochondrial damage when the cell is exposed to high Ca^{2+} concentrations. In line with this, a previous work of our group demonstrated that GUO is involved in the maintenance of the redox system in a traumatic brain injury rat model [15]. In addition, other results depicted that GUO could prevent the mitochondrial alteration induced by a Parkinson's disease model [38] as well as oxygen and glucose deprivation (OGD) in hippocampal slices [20].

In this direction, we investigate if this protective effect of GUO on swelling could be dependent upon the sequestration of Ca^{2+} by mitochondria, which sequester and release Ca^{2+} through the mitochondrial membrane [29]. In order to study the involvement of Ca^{2+} on GUO effects, we observed (Fig. 1C) that Ruthenium Red blocked the mitochondrial swelling when used in combination with GUO, we did not detect any additive/synergistic effect in this association. This result reinforces that GUO modulates effects that are associated with calcium, once Ruthenium Red blocked this effects. When we used CsA, an inhibitor of mPTP opening, we demonstrated that the blockade caused by GUO may not be dependent on the opening of the classical mPTP pore, since there was no additive/synergistic effect between CsA and GUO (Fig. 6).

In previous studies, GUO demonstrates its protection of mitochondria function by direct radical scavenging activity and also by modulating signaling pathways that control antioxidant defense. The antioxidant effect of GUO has been demonstrated by its modulation of the homeostasis of GSH, the major non-enzymatic antioxidant in the CNS [44]. Besides, for demonstrating if the GUO effects on mitochondrial function were associated with homeostasis of GSH and/or direct antioxidant activity, we carried out experiments in the presence of the reducing agent DTT (effect in GSH/GSSG homeostasis) and the protective effects of BHT (a classic antioxidant used in vitro). Nevertheless, we demonstrated that the protective effect of GUO on mitochondrial swelling when exposed to high Ca^{2+} concentrations is not associated with GSH homeostasis and/or direct antioxidant activity. According to Dal-Cim and colleagues, GUO cannot reduce the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. GUO presented lower scavenge capacity of nitric oxide (NO) molecules, besides preventing the increase in NO production [18].

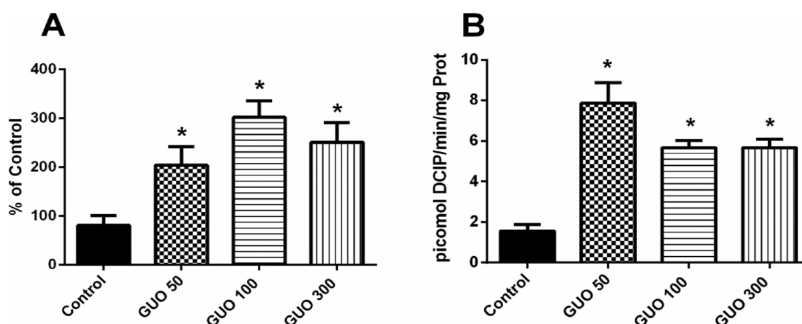

Fig. 5. Effects of 50, 100 and 300 μM of guanosine (GUO) on citrate synthase activity (A) and mitochondrial complex II (succinate dehydrogenase) (B) in liver mitochondria isolated of rats. Each bar represents means ± S.E.M. (n = 4). (*) indicates statistic difference from control group by one-way ANOVA, followed by Newman Keuls's *post-hoc* test ($p < 0.05$).

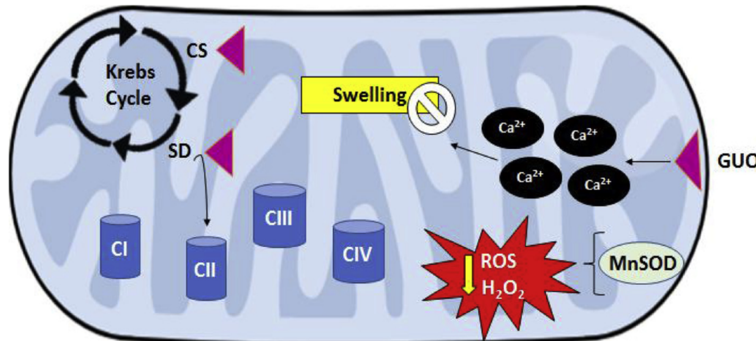

Fig. 6. Schematic illustration of the protective effect of guanosine (GUO) against liver mitochondrial impairment induced by calcium (Ca^{2+}). Ca^{2+} induced an increase of mitochondrial swelling, as well as reactive oxygen species (ROS), hydrogen peroxide levels (H_2O_2). We showed that GUO increased citrate synthase (CS), succinate dehydrogenase (SD) (Complex II activity) and manganese superoxide dismutase activities (MnSOD) corroborating with the increase in oxidative phosphorylation in the electron transport chair (Complex I (CI), Complex II (CII), Complex III and Complex IV (CIV)) as a compensatory mechanism to maintain mitochondrial energy homeostasis.

It is well demonstrated that an increase of intra-mitochondrial Ca^{2+} levels results in dysfunction, for example causing loss of mitochondria membrane potential and production of ROS with subsequent oxidative damage, disturbance of energy production and of redox homeostasis [45,46]. In fact, there is evidence that the mitochondrial ROS generation is strictly dependent upon the $\Delta\psi m$ [47]. In our study, we observed that Ca^{2+} severely impaired mitochondrial function through $\Delta\psi m$ loss, evidenced by increase safranine-O fluorescence and this effect was not prevented *in vitro* by GUO, as expected considering our results about GUO and CsA interaction in mitochondrial swelling (Fig. 1D). It seems that the initial loss of potential is not triggered by mPTP opening but rather by an energetic failure driven by a combination of increased energy demand. Besides, the potential can be restored by restoring electron flow through the respiratory chain with substrates [48].

In addition, Ca^{2+} increased ROS such as H_2O_2 levels, but here GUO prevented the increase, maintaining the ROS and H_2O_2 levels similar to the control group. It was demonstrated that ROS decrease mitochondrial motility, independently of mitochondrial membrane potential or mPTP opening, known effectors of oxidative stress, indicating that ROS likely target the adaptor complex with the involvement of p38 to control motility of mitochondria [49]. Another study also demonstrated that GUO considerably reduced the production of both H_2O_2 and hydroxyl radical, increasing survival of mice after a lethal dose of radiation [50]. This excessive ROS production can be neutralized by different antioxidant enzymes. Our study evaluated MnSOD and found that the activity of this enzyme was reduced by Ca^{2+}, GUO caused a higher increase in the activity of this enzyme as compared with Ca^{2+}. In fact, it is reported that the protective effect of GUO is mediated by HO-1 [20]. Considering that overexpression of SOD in several tissues is associated with increased HO-1 levels [51].

Inhibition of mitochondrial respiratory complexes and decreased electron flow in the mitochondrial electron transport system (mETS) has been studied in various models, in order to study the role of mitochondria in several diseases [52,53]. Our results depicted that GUO caused a significant increase in electron transfer in OXPHOS CI-linked, CII-linked and CI + CII-linked parameters when compared to control. It is possible to observe that GUO did not present changes in CI-leak when compared to control group, however, there was a significant increase on RCR in GUO group when compared to control group, demonstrating that GUO evokes a clear improvement in mitochondrial health. GUO-enhancing effect on OXPHOS suggesting possible regulatory actions ah the mitochondrial levels, mainly driven by GUO effects on complex I and II. These results corroborate findings showing an increase on OXPHOS of isolated mitochondria in different experimental models which it was used an antioxidant compound with neuroprotective properties in ischemia and Alzheimer's disease [54,55].

Our group previously demonstrates a decrease in mitochondrial viability accompanied by inhibition of complex I activity in animals submitted to brain traumatic injury, and GUO treatment fully recovery these effects in the hippocampus and partially restored in the cortex [15]. Notably, the increase in oxidative phosphorylation caused by GUO maybe is not related to the energetic overproduction through the tricarboxylic acid cycle, as evidenced by an increase in citrate synthase activity (Fig. 5A). The GUO effect seems to be related with an interaction with the enzyme directly and not related with other factors that could modulate the citrate synthase and succinate dehydrogenase (complex II) in all GUO concentrations (data not shown). In line with this, several studies have demonstrated the protective effects and possible mechanisms of action of GUO when the mitochondria suffer an insult in some pathological condition. Most these neuroprotective effects are partly mediated through the stabilization of mitochondrial membrane potential via the modulation of the expression levels of intrinsic apoptotic proteins involved in the mitochondrial apoptotic pathway [20,38]. Unlike the others, our *in vitro* study demonstrates that GUO decreases ROS independently of the opening of the mPTP and that this effect is not related to its antioxidant properties directly.

Our data indicate that GUO improved mitochondrial function, alleviating mitochondria swelling induced by Ca^{2+} and protecting the mitochondria against excessive ROS generation, such as H_2O_2. Thus, the presence of mitochondrial calcium uniporter (MCU), a protein of the inner mitochondrial membrane responsible to regulate Ca^{2+} uptake [56] may be the site by which GUO induced a protective effect against mitochondrial dysfunction exerted by Ca^{2+}, an intracellular feedback loop which allows a cell to alter mitochondrial ATP production based on needs. The different MCU complex components expression levels have been analyzed in various tissues and cell lines. In some cases, the relative expression of MCU and of its interactors is in line with the predicted mitochondrial Ca^{2+} uptake behavior. So, our data concerning RR and GUO association could indicate that GUO acting by binding in mitochondrial calcium uniporter (MCU), which regulate Ca^{2+} uptake and modulate the mitochondrial swelling and ROS as demonstrated here. However, especially in breast carcinoma cell lines MDA-MB-231, this paradigm is not always confirmed, indicating that other regulatory systems contribute to MCU activity [57].

Our study involved GUO effects in mitochondrial preparation from liver essentially due to the mitochondrial homogeneity in this organ. However, considering the previous demonstrations of GUO neuroprotective effects in experimental models of brain diseases, we could postulate that the observed GUO beneficial effects on liver mitochondrial could open novel perspectives for using brain mitochondrial parameters as a target for GUO neuroprotection. Evidence in CNS and present data in liver mitochondria are compared as they could be directly cross-transferred assuming that liver mitochondria and brain could react in the same way in response to GUO.

Notably, GUO is accumulated under physiological conditions as well as in response to injury [58]. Here, we showed that this nucleoside may

be a protective molecule in liver mitochondria pathological situations. Despite the fact that, to date, several studies have indicated that this nucleoside can be modulating the mitochondria through its ability to directly scavenge oxidative species and/or from the activation of pathways involved in antioxidant defenses [59,60] or prevented the loss of mitochondrial membrane potential. In contrast, we demonstrate through in vitro study GUO effects in liver mitochondria were not associated with direct antioxidant properties or stabilization of mitochondrial membrane potential [19], it seems that GUO increases OXPHOS as a compensatory mechanism to maintain mitochondrial energy homeostasis.

In conclusion, our study indicates that GUO prevented *in vitro* Ca^{2+}-induced mitochondrial dysfunction, evidenced by the reduction of mitochondrial swelling, ROS levels, and maintenance of mitochondria bioenergetics activities (increase in CS activity). Our findings could support future investigations of direct interactions between GUO and MCU, as well as evaluations for the use of GUO in research efforts against mitochondrial dysfunction diseases.

Conflicts of interest

All authors report no conflict of interest.

Acknowledgments

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3.2 MANUSCRITO CIENTÍFICO I

Guanosine Protects Against Behavioural and Mitochondrial Bioenergetic Alterations after Mild Traumatic Brain Injury

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Guanosine Protects Against Behavioural and Mitochondrial Bioenergetic Alterations after Mild Traumatic Brain Injury

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ABSTRACT

Traumatic brain injury (TBI) constitutes a heterogeneous cerebral insult induced by traumatic biomechanical forces. Mitochondria play a critical role in brain bioenergetics, and TBI induces several consequences related with oxidative stress and excitotoxicity clearly demonstrated in different experimental model involving TBI. Mitochondrial bioenergetics alterations can present several targets for therapeutics which could help reduce secondary brain lesions such as neuropsychiatric problems, including memory loss and motor impairment. Guanosine (GUO), an endogenous neuroprotective nucleoside, affords the long-term benefits of controlling brain neurodegeneration, mainly due to its capacity to activate the antioxidant defense system and maintenance of the redox system. However, little is known about the exact protective mechanism exerted by GUO on mitochondrial bioenergetics disruption induced by TBI. Thus, the aim of this study was to investigate the effects of GUO in brain cortical and hippocampal mitochondrial bioenergetics in the mild TBI model. Additionally, we aimed to assess whether mitochondrial damage induced by TBI may be related to behavioral alterations in rats. Our findings showed that 24 h post-TBI, GUO treatment promotes an adaptive response of mitochondrial respiratory chain increasing oxygen flux which it was able to protect against the uncoupling of oxidative phosphorylation (OXPHOS) induced by TBI, restored the respiratory electron transfer system (ETS) established with an uncoupler. Guanosine also increased respiratory control ratio (RCR) and bioenergetics efficiency. In addition, mitochondrial bioenergetics failure was closely related with locomotor, exploratory and memory impairments. The present results suggest GUO promotes further efficiency of mitochondrial OXPHOS coupling determinated by HRR reducing the bioenergetics failure in different brain regions. These findings may contribute to the development of future therapies with a target on failure energetic metabolism induced by TBI.

KEYWORDS: nucleoside; weight drop model; high-resolution respirometry; mitochondrial functionality.

1. INTRODUCTION

Traumatic brain injury (TBI) is defined as a form of acquired brain injury and it remains as one of the major causes of death and disability worldwide (Hiebert *et al.*, 2015). To date there is no specific treatment for TBI, especially mild injury, in addition evidences of excessive edema or bleeding remain unknown (Chen *et al.*, 2016). It is well established the main cause of TBI-associated brain damage is secondary injury, which is mainly from mitochondrial dysfunction (Rodriguez *et al.*, 2018).

Cellular energy is produced by mitochondrial function through the electron transport system (ETS) coupled with oxidative phosphorylation (OXPHOS), a combination of electron flow and proton-motive force driving mitochondrial ATP synthesis (De Carvalho *et al.*, 2017; Gonçalves *et al.*, 2019). Damage to this organelle during TBI induces a drastic impairment of cellular respiration and bioenergetics, triggering an increase of reactive oxygen species (ROS) generation, dissipation of the mitochondrial transmembrane potential and depletion in ATP levels, all these processes are closely related with mitochondrial dysfunction (Gerbatin *et al.*, 2016; Dobrachinski *et al.*, 2017).

Mitochondrial dysfunction is considered a hallmark of a large number of diseases impinge on cognitive function and cause brain impairments, such as neurodegenerative diseases, aging, trauma and ischemia (Khacho *et al.*, 2017). Most of the drugs and antioxidants undergoing preclinical trials in the mitochondria of rats submitted to a TBI event have not shown satisfactory results, due to their nonspecific cellular localization, poor transport properties across multiple biological barriers, and associated side effects (Di Deo *et al.*, 2016; Sharma *et al.*, 2018). Besides, the majority of studies involving TBI have as main focus reversing or preventing neuronal cell death (Loane e Faden, 2010; Scheff e Ansari, 2017). In this context, researches involving therapeutic agents that promote further efficiency

of mitochondrial OXPHOS coupling to maintaining the cell energy balance against an injury are extremely important.

Guanosine (GUO), an endogenous neuroprotective nucleoside, is an intercellular signaling molecule affecting multiple cellular processes (Bettio, Gil-Mohapel*, et al.*, 2016). Studies in different *in vitro* and *in vivo* experimental models demonstrated the neuroprotective effects of GUO through its ability to protect cells in oxidative stress conditions (Li *et al.*, 2014; Teixeira *et al.*, 2018; Marques *et al.*, 2019). It is effective in decreasing ROS levels (Dobrachinski *et al.*, 2017), reducing mitochondrial swelling induced by calcium (Courtes *et al.*, 2019), preventing the collapse of mitochondrial membrane potential in hippocampal slices subjected to oxygen glucose deprivation (Thomaz, D. T. *et al.*, 2016). GUO can promote cytoprotection through an intracellular biochemical pathway that implicates the activation of PI3K/Akt leading to inactivation of glycogen synthase kinase-3β (GSK-3β) (Dal-Cim *et al.*, 2012) and induction of the antioxidant enzyme heme oxygenase-1 (HO-1) (Quincozes-Santos *et al.*, 2014). Although the exact protective mechanism exerted by GUO on disruption electron transfer chain induced by TBI is still not completely understood.

Given these observations and the lack of efficacious trauma treatments, the aim of this study was to investigate the effects of GUO, post mild TBI, on mitochondrial bioenergetics function in brain cortex and hippocampus, using high-resolution respirometry (HRR) and to assess whether mitochondrial damage induced by TBI may be related to behavioral alterations in rats. Besides, we evaluated if the effect of GUO on mitochondrial bioenergetics after TBI could be the same in different brain regions.

2. MATERIALS AND METHODS

Animals, Ethics Statement, and Reagents

Male Wistar adult rats (200 - 220 g), supplied from our own breeding colony, were used in this study. The animals were housed in groups of three animals per cage (polypropylene, 41 × 34 × 16 cm L × W × H, 1394 cm^2) with the floor covered with autoclaved shavings under controlled conditions (12:12-h light-dark cycle, lights turned on at 07:00 a.m.; 24 ± 1 °C; 55% relative humidity) on a ventilated rack with access to water and food (Puro Lab 22 PB) *ad libitum*. All procedures with animals followed guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil (#153/2014). All reagents were purchased from Sigma (St. Louis, MO, USA).

Mild TBI method

Mild TBI was made using weight drop model, based on (Mannix *et al.*, 2014), with modifications. The animals received only one concussion. Before the concussion process, the animals received lidocaine topical on head (Mychasiuk *et al.*, 2014). Rats were anesthetized (Isoflurane, 1 % inhaled, Baxter), stayed on aluminum paper with small cuts along there. Below of aluminum paper we have added some small pieces of sponges to cushion the drop of animal. Either, a trigger fires an acrylic weight of 54 g (2.5 cm diameter) free fall until the animal head, on a height 100 cm (39.4 inches). A fishing line (0.30 mm, Mazzaferro) supported the weight.

Drug Treatment

GUO solution dissolved in 0.9 % saline was maintained at 37 °C before injections to prevent any drug precipitation. Dose used was selected based on our previous studies with a

dose-response curve (Soares *et al.*, 2004) and others (Tavares *et al.*, 2008; Gerbatin *et al.*, 2016), in which GUO (7.5 mg/kg) conferred neuroprotective action when intraperitoneally (i.p.) administered to rats in different experimental models.

Experimental design

TBI group was submitted to the weigh drop model, which together with the control group received a single injection of GUO or its vehicle 0.9 % saline i.p. 2 h after brain injury, such as demonstrated in the Figure 1. This procedure generated the following four groups: control-saline, control-Guanosine, TBI-saline, and TBI-Guanosine group. Control group was exposed to the same experimental conditions of TBI group except for brain injury. Finally, 24 h after TBI, the behavioral evaluation with tests of exploratory, locomotor behavior and memory, such as Open Field (OF), Training Object Recognition (TOR), Short-Term Memory (STM), Rotarod (RR) and Long-Term Memory (LTM) have been performed, as well as mitochondrial bioenergetics utilizing high-resolution respirometry (HRR) (Fig. 1).

High resolution respirometry (HRR)

For respirometry determination was used an Oxygraph-2k (O2k, Oroboros Instruments, Innsbruck, Austria. At 24 h post-TBI, animals were euthanized and the brain was removed, following dissection of the cortex and hippocampus. Tissues were gently homogenized on ice in isolation buffer (320 mM sucrose, 10 mM Trizma base, and 2 mM EGTA) using a 5 mL Potter–Elvehjem teflon-glass. Cerebral structures homogenates were added to the chamber containing the respiration medium - MiR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110

mM sucrose, 0.1 mg/mL fatty acid free BSA). A final concentration of tissue homogenate with MiR05 analyzed was 1 mg/ml at a constant 37 °C, and the rate of oxygen consumption was expressed as oxygen flux in pmols O_2/ [s * mg of tissue homogenate], (DatLab software all from OROBOROS Instruments). The oxygraph was calibrated daily, and oxygen concentration was automatically calculated from barometric pressure (De Carvalho *et al.*, 2017; Gonçalves *et al.*, 2019).

Mitochondrial respiration assays

Using titration, we assayed the abilities of a series of substrates and inhibitors to influence mitochondrial function as reflected in the difference in respiration states. Mitochondrial bioenergetics in cortex and hippocampus were carried out according to the literature (Gnaiger, 2009; Pesta e Gnaiger, 2012). Pyruvate + Glutamate + Malate and succinate were used as oxidizable substrates in all experiments. After signal stabilization, the basal respiration supported by endogenous substrates, the complex I- mediated LEAK (CI_{LEAK}) respiration was determined using 5 mM Pyruvate, 5 mM Glutamate and 5 mM Malate. CI-mediated OXPHOS (CI_{OXPHOS}) was determined using ADP (1 mM). The convergent electron flow during the maximal OXPHOS respiration (CI + CII_{OXPHOS}) was determined with substrates of CI and CII (10 mM Succinate). The ETS respiration represents the uncoupled respiration using Carbonyl Cyanide p-(trifluoromethoxy) Phenylhydrazone (FCCP); CI + CII-mediated ETS respiration (CI + CII_{ETS}) was determined using titrating the protonophore (optimum concentration reached between 0.5 and 1.5 µM). CII-mediated ETS respiration (CII_{ETS}) was determined with 0.5 µM rotenone. The addition of 2.5 µM antimycin A (AMA) inhibited complex III, resulting in nonmitochondrial respiration, the residual oxygen consumption (ROX) with small contributions from electron leak in the uncoupled

state. Respiratory control ratios (RCR = CI_{OXPHOS}/CI_{LEAK}) was used as mitochondrial quality control (Pesta e Gnaiger, 2012).

Behavioural tests

Open Field Test

Open-field was made of plywood and surrounded by walls 30 cm in height. The floor of the open-field, 45 cm in length and 45 cm in width, was divided by masking tape markers into 9 squares (3 rows of 3). Each animal was placed individually at the center of the apparatus and observed for 5 min to record the spontaneous locomotor (number of segments crossed with the four paws) and exploratory activities (expressed by the number of time rearing on the hind limbs) (Walsh e Cummins, 1976).

Object Recognition (OR) Memory Test

Training and testing in the OR task were carried out in an open-field arena built with black-painted wood (Ennaceur e Delacour, 1988). Rats were first habituated individually in the apparatus and left to freely explore it before the training session. In the training session, 24 h after mTBI, two different objects (A and B) were placed in the apparatus, and rats were allowed to freely explore for 5 min. The objects were made of metal, glass, or glazed ceramic. Exploration was defined as sniffing or touching the objects with the nose and/or forepaws. Sitting on or turning around the objects were not considered as exploratory behaviors. After 1.5 h and 24 h of the training session, in the STM and LTM test sessions, one of the objects was randomly exchanged for a novel object (C and D, respectively), and the rats were

reintroduced into the apparatus for 5 min. The time spent exploring the familiar and the novel object was recorded. To avoid confounding effects of lingering olfactory stimuli and preferences, the object and the arena were cleaned with 70% ethanol after testing each animal.

Rotarod Test

The Rotarod test (RR) (EFF 411, Insight, Brazil) included both forelimb and hindlimb motor coordination (Whishaw *et al.*, 2003). Before of concussion, the animals were habituated and trained to remain in the rotarod under a constant speed of 8 rpm for 300 s, the same criteria used for the following tests. Before mTBI, after habituation on the rotarod for 1 min, the animals who failed to stay on the RR were disqualified to avoid a false positive. Twenty four hours after mTBI, the RR test was repeated, and the results of latency to fall were measured and expressed in seconds (s). After each test, the equipment was cleaned with 70% ethanol solution.

Statistical Analysis

The normality of the data was analyzed using the D'Agostino and Pearson's omnibus normality test. Data were expressed as mean ± standard error of the mean (SEM) for five or eight animals per group and were analyzed by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test, except behavior test, which were analyzed by unpaired Sudent's t- test and Kruskal–Wallis test followed by Dunn's post hoc test, respectively. $p < 0.05$ was considered statistically significant.

3. RESULTS

Mitochondrial O_2 flux consumption

To investigate the effects of GUO treatment post-TBI at mitochondrial levels, we evaluated the oxygen flux in the cortex and hippocampus using high-resolution respirometry (HRR). Little attention is given to the GUO effects on the mitochondrial bioenergetics function, so, at the first time, our research group have shown in cortex and hippocampus that GUO treatment promoted a meaningful increase of oxygen flux in Basal, CI_{Leak}, CI_{OXPHOS}, $CI+CII_{OXPHOS}$, $CI+CII_{ETS}$ and CII_{ETS} state during the GUO when compared to the control group. Basal respiration showed a decrease after TBI when compared with the control group, in the cortex and hippocampus ($p<0.05$) (Fig. 2 and 3). GUO treatment post-TBI restored the oxygen flux similar to the control group levels in the cortex (Fig. 2) and partially in hippocampus (Fig. 3). CI_{Leak} respiration showed no significant differences between control, TBI and TBI + GUO (Fig. 2 and 3). In contrast, the addition of saturating ADP concentration to induce CI_{OXPHOS} and succinate to induce the convergent pathway ($CI+CII_{OXPHOS}$) have indicated a critical bioenergetics collapse in mitochondrial respiration in cortex and hippocampus of animals submitted to the TBI when compared to control group and the GUO treatment post-TBI restored the normal function ($p<0.05$) (Fig. 2 and 3). Similarly, when the respiration was uncoupled by FCCP addition, there was a significant decrease on ETS at the $CI+CII_{ETS}$ and CII_{ETS} in the cortex and hippocampus of the animals' TBI group when compared with the control group. GUO treatment after TBI increased maximal respiration induced by FCCP addition in both cerebral structures compared to the TBI group ($p<0.05$) (Fig. 2). To evaluate the residual oxygen consumption (ROX), it was added antimycin A (AMA), we can observe a decrease in the oxygen flux post-TBI compared with the control

group ($p<0.05$) (Fig. 2 and 3). GUO treatment after TBI increased partially oxygen flux in the cortex and hippocampus ($p<0.05$).

Mitochondrial Bioenergetics Capacity

We evaluated the bioenergetics capacity by subtracting the ADP-induced CI_{OXPHOS} values from the CI_{Leak} values (Fig. 4). At both, cortex and hippocampus, TBI induced a reduction in the mitochondrial bioenergetics efficiency compared with the control group ($p<0.05$) (Fig. 4A and 4B). GUO treatment post-TBI showed an increase in the mitochondrial bioenergetics capacity in relation to the TBI group ($p<0.05$) (Fig. 4A and 4B). GUO alone induced a significant increase in the bioenergetics capacity when compared with control group, in the cortex and hippocampus ($p<0.05$) (Fig. 4A and 4B).

Respiratory Control Ratio (RCR)

Mitochondrial functionality was evaluated through of the RCR for complex I (CI_{OXPHOS}/CI_{Leak}), an indicator of the state of mitochondrial coupling (Table 1). TBI reduced the RCR in the cortex and hippocampus when compared with the control group ($p<0.05$), as demonstrated in the table 1. GUO treatment after TBI was able to increase the RCR in both brain structures compared with TBI group ($p<0.05$). No significant differences were observed between control group and GUO alone.

Motor and Exploratory Activity Alterations Induced by mTBI

Animals in the TBI group were characterized by a decrease in the number of crossings

($p<0.0002$) (Fig. 5A) and rearings ($p<0.0005$) (Fig. 5B) compared with those of the control group. In addition, GUO treatment post-TBI protected against a decrease in the number of crossings ($p<0.0002$) and rearings ($p<0.0005$), in TBI-treated rats.

mTBI-Induced Alterations in Object Recognition Memory

As expected, the rats explored each object (A and B) for a similar percentage of total time in the training session. The control group did not show deficits in short-term memory (STM) in object recognition testing (Fig. 6A) ($p<0.005$). Animals treated with GUO alone also did not show deficits in STM; they spent more time exploring a new object (object C) ($p<0.005$). Animals treated with mTBI presented deficits in STM; they spent more time exploring the familiar object than the new one. This kind of behaviour was not seen in animals treated with GUO post-TBI (Fig. 6A).

The control group showed no deficits in long-term memory (LTM) in object recognition testing. Animals treated with GUO alone also did not show deficits in LTM; they spent more time exploring the new object (object D) ($p<0.005$) (Fig. 6B). The TBI and GUO post-TBI groups did not present a significant difference in total exploration time between familiar and novel objects ($p<0.005$) (Fig. 6B).

Motor Coordination Damage Induced by mTBI

Figure 7 shows the latency to fall during the Rotarod test. Statistical analysis revealed a decrease in latency to fall induced by TBI ($p<0.0001$) compared with the control group, and GUO protected against damage to motor coordination in TBI-treated rats ($p<0.05$).

4. DISCUSSION

Previous studies highlight the importance of proper mitochondrial function in restoring behavior, cognition and memory after mild TBI (Alessandri *et al.*, 2002; Rhein *et al.*, 2010). In this context, the brain is highly vulnerable to changes in bioenergetics levels and oxidative damage, which are the main contributing factors in the etiology of neurological disorders and ageing (Gelfo *et al.*, 2018). Our results clearly demonstrate the crucial role played by GUO in the mitochondria from cortex and hippocampus against secondary injury TBI-induced and their effects on behavioral tasks.

Therefore, here we used a more sensitive oxygraph to measure picomolar changes in oxygen flux, which enabled analysis of more subtle changes in mitochondrial function (Gnaiger, 2009; Pesta e Gnaiger, 2012). We chose to analyze homogenates instead of isolated mitochondria to reveal possible short-acting factors affecting mitochondrial respiration. Another study reported in isolated mitochondria raises the possibility that defective mitochondria are removed to a higher extent in the isolation process, and the process may also affect mitochondrial structure and function (Pecinova *et al.*, 2011).

Bioenergetics failure, observed post-TBI, is a process associated not only with the loss of mitochondrial membrane potential ($\Delta\Psi m$) but also impairment of ETS and OXPHOS capacity (Kilbaugh *et al.*, 2015). Our HRR assays showed that TBI caused a reduction in basal respiration and OXPHOS in CI_{OXPHOS} and $CI{+}CII_{OXPHOS}$. Through this result, it was possible to observe an impairment of the mitochondrial OXPHOS support system, which may cause a compromise in mitochondrial ability to phosphorylate ADP at OXPHOS level. GUO treatment post-TBI restored the basal respiration and increased CI_{OXPHOS} and $CI{+}CII_{OXPHOS}$ respiration. Considering that other studies related that GUO possesses important antioxidant properties, possibly derived from its ability to directly scavenge oxidative species and/or from

the activation of pathways involved in antioxidant defenses (Quincozes-Santos *et al.*, 2013; Quincozes-Santos *et al.*, 2014). One may suppose that the neuroprotective effect of the GUO on OXPHOS is due to the increase in antioxidant defenses against reactive species resulted from mitochondrial dysfunction.

Electrons flow changes induced by TBI can promote an increase of ROS levels and consequently to impair the fluidity of the mitochondrial membrane, resulting in the leakage of radicals (Hiebert *et al.*, 2015). In agreement, we observed trough electron transfer system (ETS) in the presence of an uncoupler (FCCP) which represents the maximum oxygen consumption capacity and indicated the disruption of ETS in $CI+CII_{ETS}$ and CII_{ETS}. Treatment with GUO improves the electron flow in TBI-treated rat cortex and hippocampus. It was able to maintain electron flow through $CI+CII_{ETS}$ and CII_{ETS} at 24 h after mild TBI.

The complex III inhibitor antimycin-A was added in order to measure residual oxygen consumption. In this way, it is possible to observe a decrease in ROX in the cortex and hippocampus of TBI-treated animals when compared with the control group. Another study demonstrated that uncoupler-stimulated rates of oxygen consumption and membrane potential presented a significant decrease in 7-day post-TBI in cortical mitochondria. In contrast, hippocampal mitochondria at 7 days showed only non-significant decreases in rates of oxygen consumption and membrane potential (Watson, William D. *et al.*, 2013). Our results demonstrated that GUO treatment post-TBI partially increased ROX levels in the cortex and hippocampus; it is likely that GUO treatment induced an attempt of mitochondrial adaptation to stress in order to reduce post-TBI ROS levels.

Moreover, it was possible to observe a decrease in mitochondrial bioenergetics efficiency after TBI. In parallel with this result, TBI caused a reduction of RCR in the cortex and hippocampus, leading to an impairment of mitochondrial functionality, probably through damage in the outer and inner membranes. Treatment with GUO post-TBI demonstrated a

clear improvement of the mitochondrial functionality, evidenced by increasing RCR values, an indicator of the state of mitochondrial coupling.

A previous study demonstrated that 24 h after diffuse TBI in the immature brain, there were significant alterations in cerebral mitochondrial bioenergetics, with pronounced impairment in complex I of the cortex and hippocampus mitochondria (Kilbaugh *et al.*, 2016). This disruption can be due to NADH-dehydrogenase loss of its coenzyme – flavine mononucleotide (FMN) - a key link in the transfer of electrons from NADH to respiratory chain (Wirth *et al.*, 2016). As a result, there is an increase of the superoxide anion in cells leading to oxidative stress, which may be a cause of many pathological conditions (Nita e Grzybowski, 2016). Another study, demonstrated that Guanosine phosphates (GTP, GDP, GMP) as well as adenosine phosphate (ATP, ADP, AMP) effectively prevent the release of FMN from NADH-dehydrogenase of rats liver mitochondria (Frolova e Vekshin, 2014). Therefore, we postulate that the protective effect of GUO observed after TBI may be related with stabilization of the mitochondrial NADH-dehydrogenase. Additionally, other work of our group showed that the effect of GMP as an anticonvulsant seems to be related to its ability to generate GUO through the action of ecto-5′-nucleotidase (Soares *et al.*, 2004). The conversion of GMP to GUO plays a crucial role on its effects on glutamate uptake by astrocyte cultures, besides this conversion was also necessary to prevent convulsions in an *in vivo* model (Soares *et al.*, 2004). Thus, reinforcing our hypothesis, the effects observed on the stabilization of the NADH-dehydrogenase can be via GUO, since dephosphorylation is inhibited, GMP, GDP and GTP lose their effect.

We demonstrated that after mitochondrial failure induced by TBI, simultaneously, there were behavioural alterations related to motor function, cognitive deficits and memory impairment, indicating neurological function damage. Accordingly, TBI decreased motor coordination and exploratory parameters, and GUO protected against these alterations in TBI-

treated rats. In the same way, GUO ameliorated TBI-induced short-term memory impairments. However, there were no alterations in long-term memory in either of the groups. In our experiments, the weight drop model used here did not produce a long-term memory deficit at 48 h post-injury. In some previous studies, functional deficits following a single mild TBI, such as impaired cognition and memory, were most robustly reported at 1 month post-injury (Darwish *et al.*, 2012; Collins-Praino *et al.*, 2018). In this way, GUO showed a joint action in improving the balance of mitochondrial respiration, essential for cell survival, at the same time that it restored the behavioral alterations induced by mild TBI.

Our findings indicated that the administration of GUO after a mild TBI event promoted protective effects in mitochondrial bioenergetics, increasing oxygen flux through of the complexes, as well as OXPHOS, ETS, bioenergetics efficiency, respiratory control ratio, similarly in the cortex and hippocampus. Compounds that pass through biological membranes and accumulate within mitochondria, rendering them far more effective in protecting against mitochondrial oxidative damage are extremely important (Jin *et al.*, 2014).

GUO has been studied in several neuroprotection models; here we show that it can be used in complex pathologies involving mitochondrial dysfunction, behavioral and neurological changes. Brain structures are affected differently by TBI (Mcgee *et al.*, 2016) and in this study it can be observed that GUO similarly protected cortex and hippocampus after TBI, demonstrating that it has multiple action targets.

CONCLUSION

The present work indicates that in a mild TBI rat model, GUO treatment produced a neuroprotective effect and controlled neurological injury, which may provide a new therapeutic approach to control TBI-induced mitochondrial dysfunction in the cortex and

hippocampus of rats. Little attention is given to the GUO effects on the mitochondrial bioenergetics function, so, for the first time, our research group showed in cortex and hippocampus that GUO treatment promoted a meaningful increase of oxygen flux in Basal, CI_{Leak}, CI_{OXPHOS}, $CI+CII_{OXPHOS}$, $CI+CII_{ETS}$ and CII_{ETS} state using high-resolution respirometry. In addition, mitochondrial bioenergetics failure induced by TBI was closely related with locomotor, exploratory and memory impairments. Guanosine treatment improved mitochondrial function and restored behavioral alterations after mild TBI.

Our results suggest that GUO was able to maintain electrons flow through mitochondrial complexes, promoted further efficiency of mitochondrial OXPHOS coupling reducing the bioenergetics failure in different brain regions. This stabilization of mitochondrial function GUO-induced could explain its neuroprotective effects observed in several studies. Thus, endogenous compounds that protect mitochondrial function in pathological conditions can be considered as strategies to control neurological damage in early stages of mild TBI.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure 1

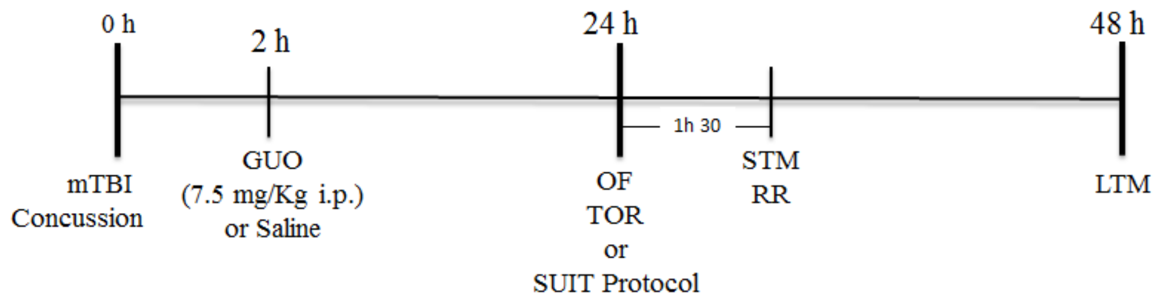

Figure 1: Experimental design, the animals were submitted to a single concussion, 2h after received guanosine (GUO) or saline solution intraperitoneally (i.p.). 24 h after TBI were performed the behavioral test (tests of exploratory, locomotor behavior and memory, such as Open Field (OF), Training Object Recognition (TOR), Short-Term Memory (STM), Rotarod (RR) and Long-Term Memory (LTM) was evaluated as well as mitochondrial bioenergetics utilizing high-resolution respirometry (SUIT Protocol).

Figure 2

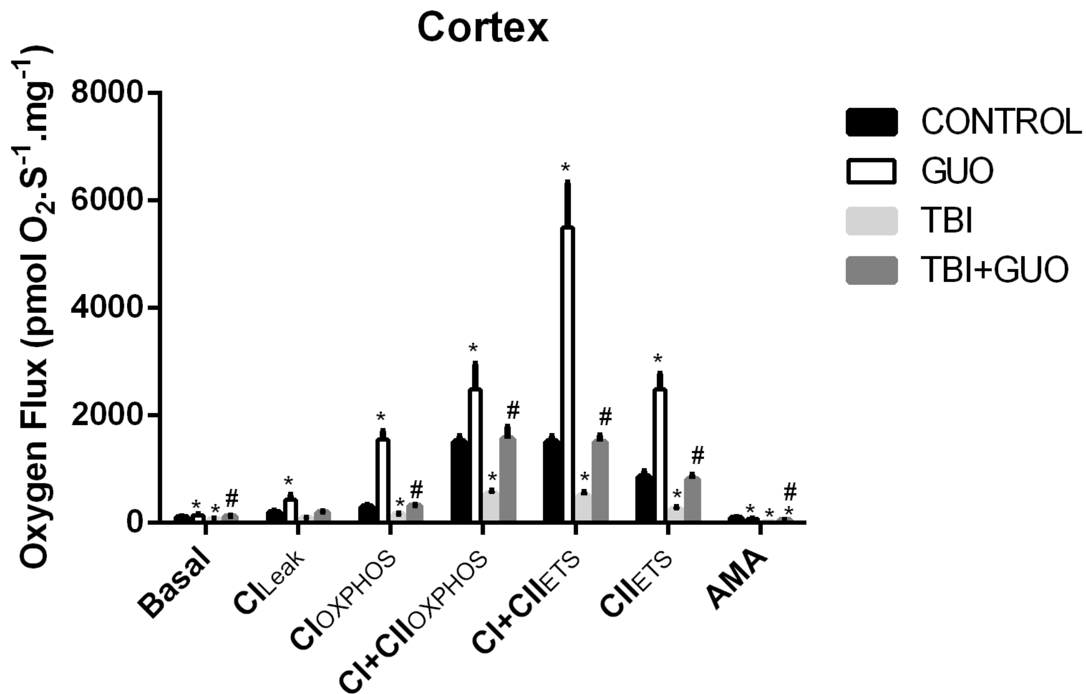

Figure 2: Effect of Guanosine (GUO) treatment on mitochondrial function (HRR) 24 h after mild traumatic brain injury (TBI). O_2 flux was measured in the mitochondria of cortex homogenate of the rats. Mitochondrial function is presented with the abbreviation(s) of the complex(es) involved followed by the state of respiration measured in the presence of Pyruvate + Glutamate + Malate (CI_{Leak}), + ADP (CI_{OXPHOS}), + Succinate ($CI + CII_{OXPHOS}$), + FCCP ($CI + CII_{ETS}$), + Rotenone (CII_{ETS}). Antimycin A (AMA) was used to correct for residual O_2 consumption. Results are expressed as the mean ± SEM for five animals per group. (*) indicates statistic difference from control group by one-way ANOVA, followed by Newman Keuls's post-hoc test ($p<0.05$). (#) indicates statistic difference between TBI and TBI + GUO group by one-way ANOVA, followed by Newman Keuls's post-hoc test ($p<0.05$).

Figure 3

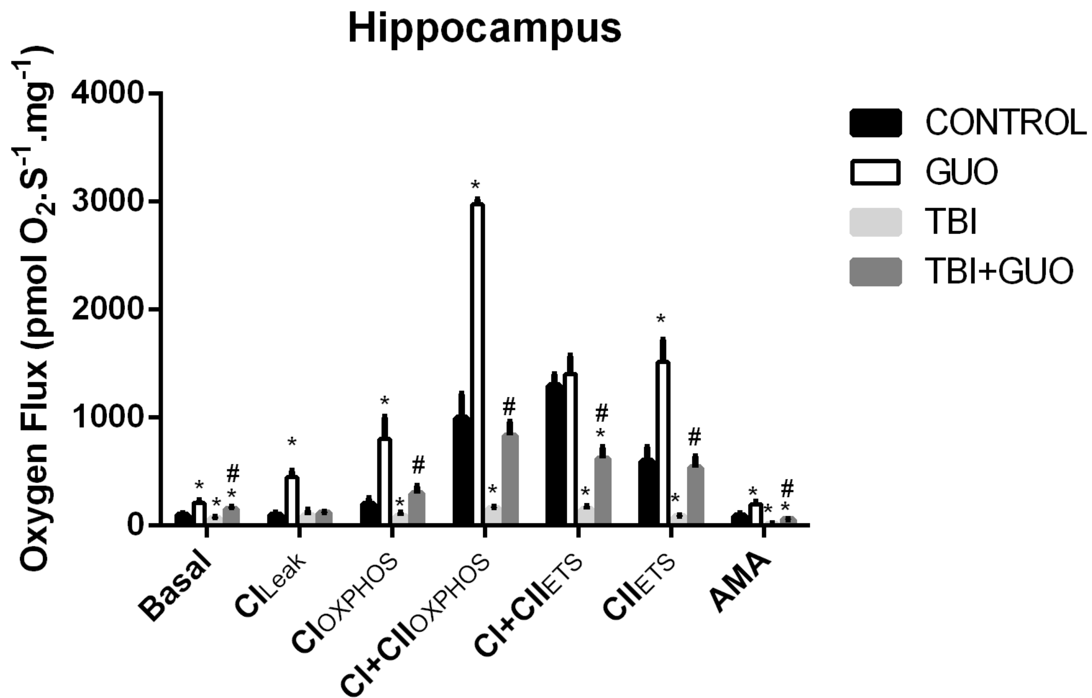

Figure 3: Effect of Guanosine (GUO) treatment on mitochondrial function (HRR) 24 h after mild traumatic brain injury (TBI). O_2 flux was measured in the mitochondria of hippocampus homogenate of the rats. Mitochondrial function is presented with the abbreviation(s) of the complex(es) involved followed by the state of respiration measured in the presence of Pyruvate + Glutamate + Malate (CI_{Leak}), + ADP (CI_{OXPHOS}), + succinate ($CI + CII_{OXPHOS}$), + FCCP ($CI + CII_{ETS}$), + rotenone (CII_{ETS}). Antimycin A (AMA) was used to correct for residual O_2 consumption. Results are expressed as the mean ± SEM for five animals per group. (*) indicates statistic difference from control group by one-way ANOVA, followed by Newman Keuls's post-hoc test ($p<0.05$). (#) indicates statistic difference between TBI and TBI + GUO group by one-way ANOVA, followed by Newman Keuls's post-hoc test ($p<0.05$).

Figure 4

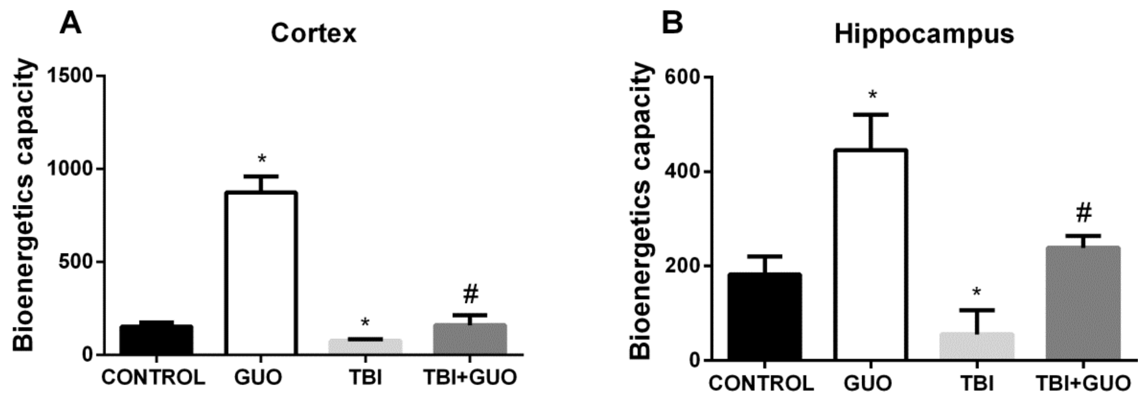

Figure 4: Effect of Guanosine (GUO) treatment on mitochondrial bioenergetics capacity in the cortex (A) and hippocampus (B) 24 h after mild traumatic brain injury (TBI). Results are expressed as the mean ± SEM for five animals per group. (*) indicates statistic difference from control group by one-way ANOVA, followed by Newman Keuls's post-hoc test ($p<0.05$). (#) indicates statistic difference between TBI and TBI + GUO group by one-way ANOVA, followed by Newman Keuls's post-hoc test ($p<0.05$).

Table 1

Effect of Guanosine (GUO) treatment on the values of respiratory control ratio in the cortex and hippocampus 24 h after mild traumatic brain injury (TBI).

	RCR	
	Cortex	Hippocampus
CONTROL	2.47 ± 0.37	2.31 ± 0.49
GUO	2.68 ± 0.34	1.97 ± 0.25
TBI	1.85 ± 0.06*	1.60 ± 0.53*
TBI + GUO	2.41 ± 0.32#	3.02 ± 0.18*#

Respiratory Control Ratio (RCR) for complex I (RCR = CI_{OXPHOS}/CI_{Leak}). Results are expressed as the means ± SD, for five animals per group. (*) indicates statistic difference from control group by one-way ANOVA, followed by Newman Keuls's post-hoc test ($p<0.05$). (#) indicates statistic difference between TBI and TBI + GUO group by one-way ANOVA, followed by Newman Keuls's post-hoc test ($p<0.05$).

Figure 5

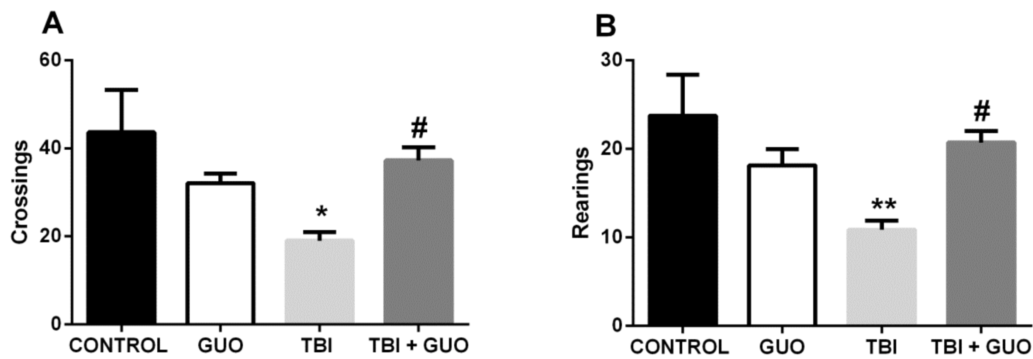

Figure 5: Effect of Guanosine (GUO) treatment (7.5 mg/Kg) on locomotor and exploratory activity 24 h post mild traumatic brain injury (TBI) in rats. Spontaneous locomotor activity of rats from control-saline (CONTROL), control-guanosine (GUO), TBI-saline (TBI), and TBI-guanosine (TBI + GUO) groups on number of crossings (A) and number of rearings (B) on open field test. Values are expressed as the mean ± SEM for eight animals per group. ** $p<0.0005$, * $p<0.0002$ (Kruskal-Wallis followed by the Dunn's post hoc test) were considered significant when compared to control group. # indicates significant difference between TBI and TBI + GUO.

Figure 6

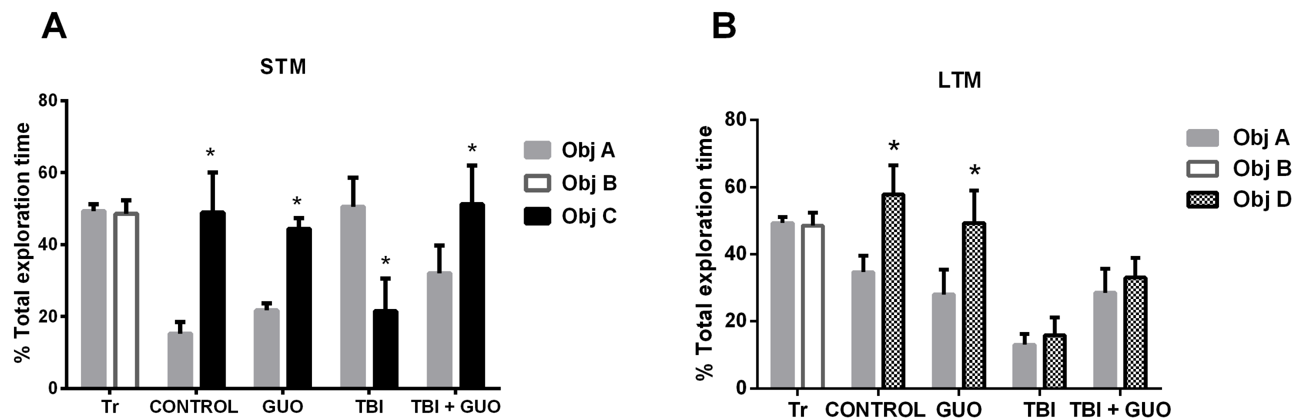

Figure 6: Effect of Guanosine (GUO) treatment (7.5 mg/Kg) on object recognition memory test 24 h post mild traumatic brain injury (TBI) in rats. Short-term (STM) (A) and long-term memory (LTM) (B) of rats from control-saline (CONTROL), control-guanosine (GUO), TBI-saline (TBI), and TBI-guanosine (TBI + GUO) were evaluated. Values are expressed as the mean ± SEM for eight animals per group. * $p<0.005$ (followed by the unpaired Student's t-test) were considered significant when compared with object A of its respective group.

Figure 7

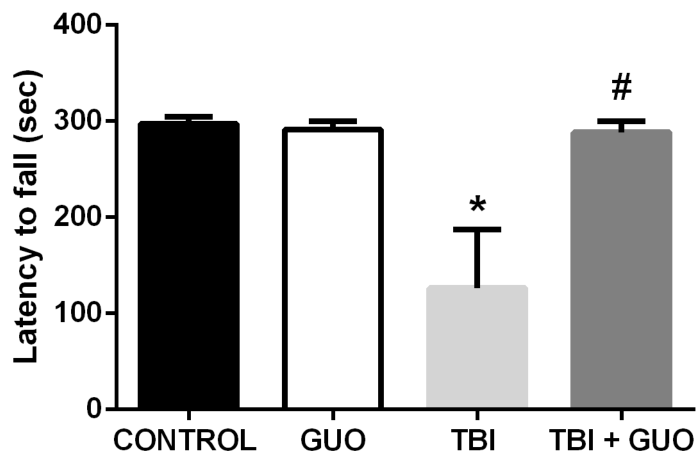

Figure 7: Effect of Guanosine (GUO) treatment (7.5 mg/Kg) on motor coordination 24 h post mild traumatic brain injury (TBI) in rats. Latency to fall of rats from control-saline (CONTROL), control-guanosine (GUO), TBI-saline (TBI), and TBI-guanosine (TBI + GUO) during the rotarod test were analyzed using the Kruskal-Wallis test followed by Dunn's post hoc test. Values are expressed as the mean ± SEM for eight animals per group. * $p<0.0001$ was considered significant when compared to control group. # $p<0.05$ indicates significant difference between TBI and TBI + GUO.

4 DISCUSSÃO

A disfunção mitocondrial tem sido associada nos últimos tempos a uma grande variedade de doenças degenerativas e metabólicas, câncer e envelhecimento, uma vez que todas essas manifestações clínicas resultam do papel central da bioenergética na biologia celular (Teresa, 2014; Bhatti, Jasvinder Singh *et al.*, 2017; Janikiewicz *et al.*, 2018). As moléculas e compostos atuais cujo alvo são as mitocôndrias cobrem uma vasta gama de agentes farmacológicos, os quais atuam de forma direta ou indireta sobre a organela (Thomaz, D. T. *et al.*, 2016; Marques *et al.*, 2019). O estudo de agentes terapêuticos, com propriedades neuroprotetoras já descritas, pode auxiliar na compreensão das desordens mitocondriais e permitir novas perspectivas para sua aplicação (Zhang *et al.*, 2018).

Na presente tese avaliaram-se os efeitos protetores da guanosina sobre alterações na funcionalidade e bioenergética mitocondrial em ratos. Através de um estudo *in vitro* frente a um dano induzido pelo cálcio, e *in vivo*, frente a um dano mitocondrial e comportamental causados pelo traumatismo crânio encefálico leve. Os resultados obtidos corroboram com achados anteriores que demonstraram o efeito protetor da guanosina em diferentes modelos experimentais (Ramos, D. B. *et al.*, 2016; Lanznaster *et al.*, 2017; Dobrachinski *et al.*, 2018). Dados do nosso estudo *in vitro*, mostraram que a guanosina apresentou um efeito protetor contra o dano mitocondrial hepático induzido pelo cálcio, através da redução do inchaço mitocondrial, diminuição dos níveis de EROs e H_2O_2, aumento da atividade da enzima superóxido dismutase e fosforilação oxidativa. O tratamento com a guanosina sozinha parece modular o inchaço mitocondrial. Apresentando um efeito protetor em diferentes concentrações quando exposta ao inchaço induzido pelo Ca^{2+}, protegendo a mitocôndria contra a geração excessiva de EROs, como o H_2O_2 e aumento da defesa antioxidante. Outros estudos demonstraram que esse efeito protetor da guanosina está relacionado com a estabilização do potencial da membrana mitocondrial através da modulação dos níveis de expressão de proteínas apoptóticas intrínsecas envolvidas na via apoptótica mitocondrial (Li *et al.*, 2014; Thomaz, D. T. *et al.*, 2016). No entanto, em nosso estudo a guanosina não protegeu contra a perda do $\Delta\Psi m$ causada pelo Ca^{2+}, acredita-se que a perda inicial do potencial não foi desencadeada pela abertura do mPTP, mas talvez por uma falha energética causada pelo aumento da demanda de energia. A guanosina aumentou a fosforilação oxidativa e a produção de ATP relacionada ao consumo de oxigênio, sugerindo uma possível ação regulatória a nível mitocondrial, impulsionada principalmente pelos efeitos da guanosina através dos complexos I e II.

Assim, acreditamos que a presença de MCU, uma proteína da membrana mitocondrial interna responsável por regular a captação de Ca^{2+} (Horn *et al.*, 2017), possa ser o local pelo qual a guanosina induza um efeito protetor contra a disfunção mitocondrial desencadeada pelo Ca^{2+}, regulando e/ou reduzindo o volume da matriz mitocondrial como forma de aliviar o estresse, consequentemente evitando o aumento dos níveis de EROs (Javadov *et al.*, 2018). Estudos demonstraram que a captação de Ca^{2+} induzida pelo MCU é dependente da atividade da PI3K, evidenciada pelo uso de um agente terapêutico no processo de reperfusão, o qual causou inibição da ativação do NF-κB via MAPK e fosforilação da Akt (Sun *et al.*, 2010; Panahi *et al.*, 2018). Os mecanismos elucidados nesses estudos são semelhantes às vias que conferem neuroproteção pela guanosina, sustentando nossa hipótese da interação guanosina e MCU.

Existem poucos estudos focados no efeito protetor da guanosina em tecidos ou mitocôndrias hepáticas, visto que a maioria é relacionada ao cérebro (Dalla Corte *et al.*, 2012; Schmidt *et al.*, 2015; Bettio, Neis*, et al.*, 2016; Gerbatin *et al.*, 2016). Considerando estudos prévios que relatam os efeitos neuroprotetores da guanosina em modelos experimentais de doenças cerebrais (Lanznaster *et al.*, 2016), através deste estudo *in vitro,* podemos postular que os efeitos benéficos causados pela guanosina em mitocôndrias hepáticas podem auxiliar em novos estudos envolvendo neuroproteção, é possível correlacionar os mecanismos patológicos mitocondriais envolvidos no fígado e cérebro. Uma vez que o tratamento com a guanosina, segundo dados mostrados neste e em outros estudos (Paniz *et al.*, 2014), reage de forma similar nos tecidos.

Através do nosso estudo *in vivo,* foi possível observar que o tratamento com a guanosina após o TCE, aumentou a fosforilação oxidativa, o sistema de transporte de elétrons na presença de um desacoplador, o consumo do oxigênio residual, a razão do controle respiratório e a capacidade bioenergética em córtex e hipocampo de ratos, observados através de respirometria de alta resolução. Embora alguns dos processos fisiopatológicos associados ao TCE tenham sido amplamente descritos (Patel *et al.*, 2016; Collins-Praino *et al.*, 2018), há limitações nas estratégias terapêuticas disponíveis para neutralizar as sequelas neuropatológicas. Aqui mostramos que a guanosina pode amenizar o dano secundário associado com o TCE em um modelo experimental *in vivo*.

Nossos resultados revelaram que o tratamento com a guanosina após o TCE causou um aumento no fluxo de oxigênio referente à respiração basal e a fosforilação oxidativa tanto em córtex quanto no hipocampo. Esse efeito pode estar relacionado à atividade *scavenger* exercida pela guanosina sobre espécies reativas (Quincozes-Santos *et al.*, 2014) provenientes

da disfunção mitocondrial causada pelo TCE.

Estudos relatam que o complexo I é um alvo seletivo para o dano oxidativo mediado por peroxinitrito, especialmente após um trauma, podendo contribuir para a inibição do sistema redox mitocondrial (Singh *et al.*, 2006). O tratamento com a guanosina após TCE mostrou um aumento no fluxo de oxigênio em córtex e hipocampo, através do sistema de transporte de elétrons. Observamos também um aumento parcial no consumo do oxigênio residual (ROX) em córtex e hipocampo; é provável que o tratamento com a guanosina tenha induzido uma tentativa mitocondrial de adaptabilidade ao estresse para reduzir os níveis de EROs pós-TCE, tendo em vista que o estado de ROX pode ser associado com a geração de EROs pela mitocôndria (Gonçalves *et al.*, 2019). O aumento da capacidade bioenergética e da razão do controle respiratório induzidos pelo tratamento com a guanosina pós-TCE, sugere um aumento na fosforilação do ATP, aliada a melhora na funcionalidade através do acoplamento mitocondrial.

Alterações comportamentais relacionadas à função motora observadas após o TCE em roedores são importantes indicadores do grau da preservação das funções neurológicas (Dobrachinski *et al.*, 2018). Assim, demonstramos que o TCE prejudicou a coordenação motora, parâmetros exploratórios e memória de curto prazo. Uma única dose de guanosina administrada intraperitonealmente em ratos, após um TCE leve promoveu efeitos neuroprotetores contra déficits locomotores e exploratórios, bem como prejuízos de memória de curto prazo, 24 horas após o TCE. Estes achados estão de acordo com um estudo anterior o qual demonstrou os efeitos da guanosina na recuperação funcional após uma lesão na medula espinhal em ratos (Jiang *et al.*, 2007). Embora diferentes mecanismos estejam envolvidos na patogênese do TCE, o desequilíbrio do sistema glutamatérgico representa um importante mecanismo de lesão que pode causar danos irreversíveis ao SNC (Guerriero *et al.*, 2015; Dorsett *et al.*, 2017). As evidências sugerem que um aumento extracelular nos níveis de glutamato constitui um fator-chave na progressão de vários distúrbios neurológicos (Lara *et al.*, 2001; Molz, Decker*, et al.*, 2008). Alguns autores relataram a captação de glutamato como o alvo da guanosina para proteger o desenvolvimento neuronal em condições excitotóxicas (Gerbatin *et al.*, 2016; Dobrachinski *et al.*, 2017).

Vários fatores podem ser responsáveis pela associação entre disfunção mitocondrial e excitotoxicidade glutamatérgica na cascata secundária do trauma (Walker e Tesco, 2013). Após o TCE, altos níveis de glutamato são observados, resultando na ativação excessiva do NMDA. Consequentemente, a exposição mitocondrial a níveis excessivos de glutamato, acaba despolarizando rapidamente e aumenta a produção de EROs (Andriessen *et al.*, 2010). Esses

eventos podem levar ao comprometimento do sistema redox mitocondrial e à fosforilação oxidativa (Kilbaugh *et al.*, 2015).

As vias de sinalização, bem como alvos moleculares envolvidos nos efeitos neuroprotetores da guanosina permanecem sem serem elucidados. Acredita-se que ela promove a neuroproteção aumentando as defesas antioxidantes (atividade da SOD, GSH e níveis de HO-1) (Bellaver *et al.*, 2015; Hansel *et al.*, 2015; Bettio, Gil-Mohapel*, et al.*, 2016); e principalmente, modulando a captação de glutamato, aumentando a atividade da glutamina sintetase, reduzindo assim os níveis extracelulares de glutamato e protegendo contra a excitotoxicidade (Soares *et al.*, 2004). Além dos efeitos neuroprotetores mencionados anteriormente, o efeito neurotrófico da guanosina também já foi demonstrado. A guanosina promove efeito trófico em cultura de astrócitos (Ciccarelli *et al.*, 2000). O qual parece envolver a HO-1, pois o tratamento de feocromocitomas (PC12) com guanosina e o fator de crescimento neuronal promoveu o crescimento de neuritos através da indução da HO-1(Bau *et al.*, 2005).

Outros estudos já demonstraram a participação da via de sinalização da PI3K nos efeitos biológicos da guanosina, uma via que atua na neuroproteção (Bettio *et al.*, 2012; Dal-Cim *et al.*, 2012). Como citado anteriormente, a guanosina é capaz de induzir um aumento na captação de glutamato, sendo este efeito dependente da via de sinalização intracelular modulada pela PI3K/Akt (Dal-Cim *et al.*, 2011). Além disso, o efeito anti-apoptótico da guanosina em cultura de células SH-SY5Y submetida à incubação com o peptídeo β-amiloide também envolve a ativação das vias PI3K/Akt, MAPK p38 (proteína cinase ativada por mitógeno p38) e ERK (cinase regulada por sinal extracelular) (Pettifer *et al.*, 2004).

Notavelmente, a guanosina é acumulada sob condições fisiológicas, bem como em resposta a uma lesão (Ciccarelli *et al.*, 1999). Aqui, mostramos que esse nucleosídeo pode ser uma molécula protetora em situações patológicas envolvendo a mitocôndria. Demonstramos através do estudo *in vitro* que os efeitos da guanosina nas mitocôndrias hepáticas não foram associados com suas propriedades antioxidantes diretas *per se* ou estabilização do potencial de membrana mitocondrial. Em ambos os estudos, *in vitro* e *in vivo*, a guanosina aumentou a fosforilação oxidativa como um mecanismo compensatório para manter a homeostase energética mitocondrial; a fim de aumentar a produção de ATP, limitar o aumento de EROs e proteger contra danos neurológicos e comportamentais após o TCE.

Sendo assim, o presente trabalho indica que essa molécula apresentou efeitos neuroprotetores em estabilizar o dano mitocondrial, tornando-se um alvo potencial para o tratamento após um TCE. Ainda, este trabalho pode futuramente auxiliar na produção de

novas moléculas capazes de atuar similarmente à guanosina ou mesmo a produção de antagonistas específicos para as ações desempenhadas por ela na bioenergética mitocondrial.

5 CONCLUSÕES

No presente estudo, avaliamos os efeitos da guanosina sobre alterações na funcionalidade e bioenergética mitocondrial em ratos, através de um estudo *in vitro* frente a um dano induzido pelo cálcio, e *in vivo*, frente a um dano causado pelo TCE leve. Os resultados obtidos nos permitem concluir que a guanosina apresentou um efeito protetor em diferentes concentrações quando exposta ao inchaço mitocondrial induzido pelo Ca^{2+}, protegendo contra a geração excessiva de EROs, como o H_2O_2, aumentando a defesa antioxidante e esses efeitos não foram associados com suas propriedades antioxidantes diretas *per se* ou estabilização do potencial de membrana mitocondrial. Ainda, a guanosina restaurou a atividade mitocondrial, através do aumento da fosforilação oxidativa como um mecanismo compensatório para manter a homeostase energética mitocondrial; a fim de aumentar a produção de ATP, aumentou a atividade do sistema de transporte de elétrons e o consumo do oxigênio residual, a capacidade bioenergética e a razão do controle respiratório, esses efeitos podem estar relacionados à estabilização do complexo I por esta molécula. Observamos efeitos neuroprotetores da guanosina contra déficits locomotores e exploratórios, bem como prejuízos na memória de curto prazo induzidos pelo TCE. O presente trabalho indica que a guanosina pode fornecer uma abordagem terapêutica para controlar danos à função mitocondrial associada a diferentes patologias. Assim, compostos endógenos que protegem contra o estresse oxidativo em situações patológicas envolvendo a bioenergética mitocondrial podem ser considerados como estratégias para proteger contra danos neurológicos em doenças associadas ao sistema nervoso central, tornando essa molécula um atrativo terapêutico para o tratamento do TCE.

5.1 CONCLUSÕES PARCIAIS

- A guanosina preveniu a disfunção mitocondrial induzida pelo Ca^{2+} *in vitro*, evidenciada pela redução do inchaço mitocondrial, dos níveis de EROs e através da manutenção da bioenergética mitocondrial.
- A guanosina foi capaz de manter o fluxo de oxigênio e de elétrons através dos complexos mitocondriais, promovendo um aumento da fosforilação oxidativa, consequentemente estabilizando a função mitocondrial frente a uma condição patológica.

6 PERSPECTIVAS

Tendo em vista os resultados obtidos nesta tese, as perspectivas para trabalhos futuros são:

- Investigar a possível interação do efeito protetor da guanosina com o canal uniporter mitocondrial de Ca^{2+} (MCU);
- Avaliar se a melhora na função mitocondrial induzida pela guanosina após o TCE pode estar relacionada com a ativação da via PI3K / Akt;
- Elucidar a interação do efeito protetor da guanosina entre cérebro e fígado na disfunção mitocondrial induzida pelo TCE.

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ANEXO

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Possiveis efeitos da administração de guanosina em ratos com traumatismo cranioencefálico sobre a suscetibilidade a crises epilépticas."

Número do Parecer: 153/2014

Pesquisador Responsável: Prof. Dr. Felix Alexandre Antunes Soares

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

OBS: Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

*** Foram Aprovados 140 animais (Rato heterogênico).**

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DE APROVAÇÃO: 25/03/2015.

Santa Maria, 25 de março de 2015.

Prof.ª Dr.ª Vania Lucia Loro
Coordenadora da Comissão de Ética no Uso de Animais- UFSM

Comissão de Ética no Uso de Animais - UFSM - Av. Roraima, 1000 – Prédio da Reitoria - 2º andar - Campus Universitário 97105-900 – Santa Maria – RS - - Tel: 0 xx 55 3220 9362