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Efeito neuroprotetor do JM-20 em ratos submetidos a traumatismo cranioencefálico leve

Andrezza Bond Vieira Furtado

Orientador: Dr. Félix Alexandre Antunes Soares
Coorientador: Dr. Luiz Fernando Freire Royes

Santa Maria, RS
2021

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EFEITO NEUROPROTETOR DO JM-20 EM RATOS SUBMETIDOS A TRAUMATISMO CRANIOENCEFÁLICO LEVE

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.

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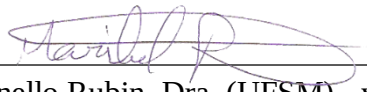

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Santa Maria, RS
2021

DEDICATÓRIA

Dedico este trabalho ao meu irmão, Mauro, cuja breve passagem terrena inspira e ampara todas as minhas vitórias.

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Disse Tom Jobim: é impossível ser feliz sozinho. Vou além: é impossível ser sozinho. Apesar de o processo de doutoramento ser bastante solitário, no qual cada um precisa trilhar, com autonomia, a sua trajetória, seria impossível fazer isso sem amparo. Muitas pessoas e instituições alicerçaram essa caminhada.

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Aos meus amigos, por, de diversas formas, terem contribuído para que eu chegasse até aqui, por terem sido ombro e ouvidos nos momentos que as dificuldades se apresentaram. Por terem me incentivado, terem tido compreensão nos momentos que eu não pude estar tão presente e permanecerem, até agora, fazendo dessa conquista uma vitória compartilhada.

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A todas as instituições, a todos os órgãos e a todas as políticas públicas que financiam a educação e a ciência, áreas que, nos últimos anos, sofrem imensuráveis ataques e tristes retrocessos, mas que se mantêm, através de luta e resistência. Concordo com Luiz Inácio Lula da Silva ao dizer que *"*por mais profundas que sejam as crises, por mais escuro que faça, depende de nós acender a luz nas trevas. E creio que nunca foi tão necessário sonhar e seguir lutando para construir um mundo melhor do que este em que vivemos". Concordo, também, com Leonel Brizola, quando disse que "a educação é o único caminho para emancipar o homem, desenvolvimento sem educação é criação de riquezas apenas para alguns privilegiados." Ao citar esses dois líderes, asseguro que, sendo filha de instituições públicas, jamais recuarei na luta pela educação e pela pesquisa de qualidade em nosso país — e continuarei sonhando com tempos mais justos e felizes.

Muito obrigada!

“Reze e trabalhe, fazendo de conta que esta vida é um dia de capina com sol quente, que às vezes custa muito a passar, mas sempre passa. E você ainda pode ter muito pedaço bom de alegria... Cada um tem a sua hora e a sua vez: você há de ter a sua."

Guimarães Rosa

RESUMO

EFEITO NEUROPROTETOR DO JM-20 EM RATOS SUBMETIDOS A TRAUMATISMO CRANIOENCEFÁLICO LEVE

AUTORA: Andrezza Bond Vieira Furtado
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O traumatismo cranioencefálico (TCE) é uma patologia multissistêmica que envolve interações entre o sistema nervoso central, o sistema nervoso periférico e o sistema imune, sendo uma das principais causas de morte e de condições incapacitantes no mundo. A fisiopatologia do TCE envolve mecanismos complexos relacionados à morte celular e à perda tecidual, que levam à neurodegeneração e a déficits na função neurológica. Estima-se que 75 a 90% dos casos de TCE sejam classificados como leves e gerem danos que podem ser subestimados. Apesar da sua alta incidência, ainda não há tratamento farmacológico padrão para essa patologia. Nesse contexto, a molécula 3- etoxicarbonil-2-metil-4-(2-nitrofenil)-4,11-di-hidro-1H-pirido[2,3-b][1,5] benzodiazepina (JM-20) surge como uma droga neuroprotetora por possuir, como alvo, mediadores envolvidos em eventos de morte celular: os canais de cálcio, devido à porção di-hidropirídinica; e os receptores GABAa, devido à ação benzodiazepínica. Neste estudo, investigamos os efeitos neuroprotetores do tratamento com JM-20 (8 mg/kg) administrado 1h após o modelo de queda de peso em ratos *Wistar*. O tratamento com JM-20 24h após TCE leve foi capaz de atenuar déficits na locomoção e na memória de curto prazo; diminuir o edema cerebral; controlar a ativação exacerbada das células gliais e, consequentemente, a sinalização pró-inflamatória, comprovada pela menor liberação de citocinas pró-inflamatórias e pela maior liberação de neurotrofinas; evitar a disfunção mitocondrial, através de maior consumo do fluxo de oxigênio durante a fosforilação oxidativa e, com base nisso, melhorar um parâmetro relacionado à funcionalidade mitocondrial; e, ainda, preservar a ativação de uma importante via relacionada a cascatas de sobrevivência celular. Com base nos resultados aqui obtidos, pode-se dizer que o JM-20 apresentou efeitos neuroprotetores ao atuar em importantes pontos da lesão secundária induzida por TCE, tornando-se, dessa maneira, um composto com potencial para uso no tratamento do TCE.

Palavras-chave: Neuroproteção; Neurodegeneração**;** Droga multi-alvo; Neuroinflamação; Disfunção mitocondrial; Sobrevivência neuronal.

ABSTRACT

NEUROPROTECTIVE EFFECT OF JM-20 IN RATS SUBMITTED TO MILD TRAUMATIC BRAIN INJURY

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Traumatic brain injury (TBI) is a multisystemic pathology with interactions between central nervous system, peripheral nervous system and immune system. It is one of the main causes of death and desability worldwide. The pathophysiology of TBI involves cell death pathways and tissue loss that lead to neurodegeneration and deficits in neurological function. It is estimated that 75 to 90% of TBI cases are classified as mild, and the damage following TBI may be underestimated. Despite the large numbers of TBI cases, there is no effective pharmacological treatment available. This study aimed to investigate the effects on TBI outcomes of the new hybrid molecule 3-ethoxycarbonyl-2-methyl-4-(2-nitrophenyl)-4,11-dihydro1H-pyrido[2,3-b][1,5]benzodiazepine (JM-20). JM-20 emerge as a neuroprotective drug because it targets mediators involved in cell death events: calcium channels, due to the dihydropyridine portion; and GABAa receptors, due to benzodiazepine action. Male *Wistar* rats were submitted to a weight drop model of mild TBI and treated with a single dose of JM-20 (8 mg/kg). 24 h following TBI, JM-20 treatment was able to attenuate locomotor and short-term memory deficits; decrease brain edema; avoid the exacerbated activation of glial cells and consequently the pro-inflammatory signaling by decreasing release of pro-inflammatory cytokines and higher release of neurotrophins; avoid mitochondrial dysfunction through increased oxygen flux consumption in oxidative phosphorylation, and then, improve mitochondrial functionality; JM-20 treatment was also able to mantain the activation of an important pathway related to cellular cascades. Based on these, is possible to confirm that JM-20 has neuroprotective effects by modulating important secondary injury targets and corroborate our hypothesis that JM-20 may become a promising treatment strategy to TBI.

Keywords: Neuroprotection. Neurodegeneration. Multi-target drug. Neuroinflammation. Mitochondrial dysfunction. Neuronal survival.

LISTA DE ABREVIAÇÕES

AChE – acetilcolinesterase

AKT – proteína quinase B

ATP – adenosina trifosfato

BDNF – fator neurotrófico derivado do cérebro

GABA - ácido gama-aminobutírico

GFAP – proteína fibrilar ácida da glia

IGF-1 – fator de crescimento semelhante à insulina 1

IL – interleucina

NGF - fator de crescimento neural

PI3K - fosfatidilinositol 3-quinase

SNC – sistema nervoso central

SNP – sistema nervoso periférico

TCE – traumatismo cranioencefálico

TGF-β – fator de transformação de crescimento beta

TNF-α – fator de necrose tumoral alfa

SUMÁRIO

1. INTRODUÇÃO

O traumatismo cranioencefálico (TCE) é considerado uma das principais causas de morte e de incapacidade no mundo, estima-se que cerca de 10 milhões de pessoas são afetadas anualmente (HYDER; WUNDERLICH; PUVANACHANDRA; GURURAJ *et al.*, 2007). Devido à alta incidência, ao potencial para gerar incapacidade nos indivíduos acometidos e ao impacto na população economicamente ativa, o TCE, além de definido como grave problema social e econômico, é considerado uma "epidemia silenciosa" (ROOZENBEEK; MAAS; MENON, 2013). Ao considerar que o TCE é uma das principais causas de morbimortalidade, especialmente na população jovem, e que ele leva a incapacidades — como problemas motores, cognitivos, comportamentais e mudanças emocionais, que acompanharão o indivíduo pelo resto de sua vida e alterarão sua rotina —, percebe-se que este é um problema de grande relevância para a saúde pública (PONSFORD; SPITZ; CROMARTY; GIFFORD *et al.*, 2013).

A patologia do TCE é multissistêmica, pois envolve interações entre o sistema nervoso central (SNC), o sistema nervoso periférico (SNP) e o sistema imune (SCHWULST; TRAHANAS; SABER; PERLMAN, 2013). Os mecanismos envolvidos na morte celular e na perda tecidual após TCE são complexos, pois há interações bioquímicas e moleculares agudas e crônicas, além de eventos fisiológicos, que, juntos, levam à neurodegeneração e a déficits na função neurológica (MCINTOSH; SAATMAN; RAGHUPATHI; GRAHAM *et al.*, 1998). Dentre as estruturas e vias afetadas pelo TCE, neste trabalho, destacaremos as células gliais e a sinalização pró e anti-inflamatória, assim como a mitocôndria e a sinalização de sobrevivência celular.

Os astrócitos e a micróglia são células abundantes no SNC, que contribuem para a homeostase, pois dão suporte trófico aos neurônios através da regulação da função e da formação de sinapses. Apesar dos efeitos benéficos, muitos estudos mostram que a ativação microglial e astrocitária pode levar a efeitos anormais, capazes de gerar mudanças patológicas no tecido cerebral após lesão (BARRES, 2008; SOFRONIEW; VINTERS, 2010; VERKHRATSKY; ZOREC; RODRIGUEZ; PARPURA, 2017). Após TCE, astrócitos e micróglia são normalmente as primeiras células a iniciar as cascatas inflamatórias, sendo que ambas ativações celulares são consideradas marcadores da resposta inata do SNC à lesão

(DIAZ-ARRASTIA; WANG; PAPA; SORANI *et al.*, 2014; HERNANDEZ-ONTIVEROS; TAJIRI; ACOSTA; GIUNTA *et al.*, 2013).

As mitocôndrias são organelas intracelulares com dupla membrana que são fundamentais no metabolismo energético celular, tendo como função predominante a geração de ATP através da fosforilação oxidativa (LETTS; SAZANOV, 2017). Além da geração de ATP, elas estão envolvidas em mecanismos regulatórios, apoptóticos e, ainda, na capacidade de tamponamento de cálcio. A bioenergética mitocondrial tem papel importante na manutenção da homeostase e na função neuronal (YONUTAS; VEKARIA; SULLIVAN, 2016), pois essas organelas são extremamente sensíveis a mudanças no estado fisiológico da célula, fazendo com que elas tenham papel importante no desenvolvimento de danos (FINKEL, 2001). A disfunção mitocondrial é um evento característico da fisiopatologia do TCE e está intrinsicamente envolvida na progressão da lesão secundária (HIEBERT; SHEN; THIMMESCH; PIERCE, 2015). A partir da disfunção mitocondrial, eventos necróticos e apoptóticos são desencadeados (YONUTAS; VEKARIA; SULLIVAN, 2016).

Apesar do grande número de casos de TCE, ainda não há tratamento farmacológico efetivo disponível. Ensaios experimentais têm apresentado resultados promissores, porém nenhuma das novas drogas foi capaz de mostrar efeitos clínicos significativos em pacientes com TCE (ZAFONTE; BAGIELLA; ANSEL; NOVACK *et al.*, 2012). Devido à complexa fisiopatologia do TCE e ao não esclarecimento de suas consequências a longo prazo, é importante considerar o uso de terapias com alvos múltiplos, com o objetivo de acelerar a recuperação e, também, impedir que haja complicações em decorrência da fase secundária do TCE. Descrito pela primeira vez por Figueredo (FIGUEREDO; RODRIGUEZ; REYES; DOMINGUEZ *et al.*, 2013), o JM-20 (3- etoxicarbonil-2-metil-4-(2-nitrofenil)-4,11-di-hidro-1H-pirido[2,3-b][1,5]benzodiazepina), faz parte de uma nova família de 1,5-benzodiazepinas, estruturalmente diferente das já conhecidas pela presença de uma 1,4-di-hidropiridina na porção fundida ao anel benzodiazepínico. Essa molécula surgiu como promissora para o tratamento de doenças neurodegenerativas por possuir, como alvo, mediadores envolvidos nos eventos de morte celular: os canais de cálcio, devido à porção di-hidropiridinica, e os receptores GABAa, devido à ação benzodiazepínica.

Já se sabe que o JM-20 tem efeitos multimodais que englobam elementos-chave comuns a doenças neurodegenerativas, como: excitotoxicidade glutamatérgica, disfunção mitocondrial, estresse oxidativo, neuroinflamação e apoptose. A capacidade que o JM-20 possui de proteger não só neurônios, como também células gliais faz com que ele seja promissor na proteção de toda a unidade neurovascular (NUNEZ-FIGUEREDO; RAMIREZ-SANCHEZ; PARDO

ANDREU; OCHOA-RODRIGUEZ *et al.*, 2018). Os resultados de estudos anteriores e a natureza semelhante do desenvolvimento das doenças neurodegenerativas sugerem que os efeitos neuroprotetores da molécula JM-20 se expandem para modelos de TCE leve.

1.1 OBJETIVOS

1.1.1 Objetivo Geral

Investigar os efeitos do tratamento com JM-20 no modelo de TCE leve.

1.1.2 Objetivos Específicos

No modelo proposto, avaliar os efeitos neuroprotetores do JM-20 24h após modelo de TCE leve sobre:

- Alterações comportamentais locomotoras, exploratórias e cognitivas;
- Ativação das células gliais e sua relação com a sinalização pró-inflamatória;
- Fisiologia mitocondrial e sua relação com a sobrevivência celular.

2. REVISÃO DE LITERATURA

2.1 TRAUMATISMO CRANIOENCEFÁLICO

O TCE pode ser definido como uma alteração da função cerebral ou uma evidente patologia cerebral causada por uma força externa (MENON; SCHWAB; WRIGHT; MAAS *et al.*, 2010). Nos Estados Unidos, são de cerca de 2,2 milhões de casos de visitas ao departamento de emergência e 50.000 mortes anuais devido ao TCE, de acordo com o Centro de Controle e Prevenção de Doenças (TAYLOR; BELL; BREIDING; XU, 2017). Estima-se que cerca de 125.500 admissões de pacientes com TCE ocorram anualmente no Brasil, isso corresponde à incidência de 65,5/100.000 habitantes/ano. Quanto à mortalidade, apesar de não ser possível acessar os dados pré-hospitalares dos casos de TCE, o que tornaria o número ainda maior, estimam-se 9.700 mortes hospitalares por ano (DE ALMEIDA; DE SOUSA FILHO; DOURADO; GONTIJO *et al.*, 2016). O TCE é uma das principais causas de morbimortalidade, especialmente na população jovem, e leva a incapacidades, como problemas motores, cognitivos, comportamentais e mudanças emocionais, que podem ser contínuas ao longo da vida do indivíduo e que, com certeza, alterarão sua rotina (PONSFORD; SPITZ; CROMARTY; GIFFORD *et al.*, 2013).

Dois importantes processos fisiopatológicos contribuem para a lesão cerebral após trauma, são eles: 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 continua a se desenvolver após o insulto traumático inicial (MAAS; STOCCHETTI; BULLOCK, 2008). A lesão primária mecânica causada pelo TCE leva à ruptura da membrana celular, de vasos sanguíneos e, ainda, da barreira hematoencefálica; já os eventos secundários de lesão começam segundos a minutos após o insulto primário e podem continuar por dias, semanas e meses, progressivamente contribuindo para o agravamento da função neurológica.

Os principais mecanismos conhecidos da patogênese no dano celular se dão, principalmente, devido à liberação de neurotransmissores, à sobrecarga de cálcio, ao estresse oxidativo, à inflamação e à permeabilização da barreira hematoencefálica, levando a quadros de disfunção mitocondrial, neuroinflamação e morte celular (ANGELONI; PRATA; DALLA SEGA; PIPERNO *et al.*, 2015; LOANE; BYRNES, 2010; SIMON; MCGEACHY; BAYIR;

CLARK *et al.*, 2017). O TCE não é um simples fenômeno fisiopatológico, mas, sim, um complexo processo de doença, que gera dano estrutural e funcional a partir dos seus eventos primários e secundários. Após o TCE, as lesões cerebrais não se restringem à área do trauma primário, ocorre expansão progressiva e centrífuga do dano. A lesão secundária é dependente da resposta do organismo a essa lesão primária (MASEL; DEWITT, 2010; PAVLOVIC; PEKIC; STOJANOVIC; POPOVIC, 2019).

A epidemiologia do TCE está em constante mudança. Em países desenvolvidos com alta renda, a prevalência está na população envelhecida e ocorre em decorrência de quedas. Já em países em desenvolvimento, a maior prevalência se dá por acidentes automobilísticos. Hoje, os conflitos armados têm menor importância na incidência do TCE, e os esportes têm, cada vez mais, aumentado o número desses, casos devido ao forte contato físico (MAAS; MENON; ADELSON; ANDELIC *et al.*, 2017). A incidência e a prevalência do TCE leve é provavelmente subestimada devido ao fato de muitos indivíduos acometidos não buscarem atendimento clínico, impossibilitando, dessa forma, a documentação e o monitoramento (MCCREA; NELSON; GUSKIEWICZ, 2017; PRINCE; BRUHNS, 2017). Em média, 75 a 90% dos casos de TCE são classificados como leves (FEHILY; FITZGERALD, 2017) e resultam de quedas, acidentes automobilísticos, violência e, também, de atividades esportivas e recreativas (CASSIDY; CARROLL; PELOSO; BORG *et al.*, 2004; MCGEE; ALEKSEEVA; CHERNYSHEV; MINAGAR, 2016). Estima-se que, por ano, cerca de 100-300 pessoas em cada 100.000 precisem de atendimento de saúde em decorrência de TCE leve (CANCELLIERE; CASSIDY; LI; DONOVAN *et al.*, 2014).

Dentre os critérios operacionais para definição do TCE leve, o indivíduo deve apresentar um ou mais dos seguintes itens: confusão ou desorientação; perda de consciência por 30 minutos ou menos; amnésia pós-traumática por menos de 24h ou outras anormalidades neurológicas, como sinais focais, convulsões e alterações intracranianas; e, ainda, precisa ser uma lesão que não necessite de intervenção cirúrgica (CARROLL; CASSIDY; HOLM; KRAUS *et al.*, 2004). O Centro de Controle e Prevenção de Doenças dos Estados Unidos da América define o TCE leve como “qualquer período, observado ou relatado, de: confusão transitória, desorientação ou consciência comprometida; disfunção de memória próximo ao evento traumático; perda de consciência por até 30 minutos”; e, ainda, “sinais observados de disfunção neurológica ou neuropsicológica”. Pessoas afetadas por TCE leve não costumam apresentar anormalidades em tomografias, porém podem passar por prolongados déficits cognitivos, emocionais e funcionais, com importantes impactos na qualidade de vida (CONTROL, 2003; EME, 2017).

A grande maioria dos pacientes com TCE leve se recupera em 1 a 2 semanas, porém certos casos chegam a 3 meses para atingir a completa recuperação (MCCREA; IVERSON; MCALLISTER; HAMMEKE *et al.*, 2009). Cerca de 15% dos indivíduos são acometidos por sintomas pós-concussivos persistentes, ainda que o termo "síndrome pós-concussiva" seja dedicado a indivíduos com múltiplos sintomas que persistem por muitos meses e até anos após o TCE. Apesar de o mecanismo de desenvolvimento da síndrome pós-concussiva não estar claro, fatores sociais, biológicos e psicológicos parecem estar envolvidos. Os principais sintomas são dores de cabeça, desordens vestibulares, disfunção visual ou espacial, irritabilidade e labilidade emocional (MCCREA; NELSON; GUSKIEWICZ, 2017; TAPIA; EAPEN, 2017).

Há dois subtipos de lesão craniana, aberta por perfuração ou fechada, que levam ao desenvolvimento de lesão focal ou difusa e aumento da pressão intracranial. A lesão difusa é resultado de forças de aceleração e desaceleração (MCGEE; ALEKSEEVA; CHERNYSHEV; MINAGAR, 2016). O impacto de modelos comuns de TCE, como a lesão por percussão fluida e a lesão cortical controlada, produzem contusão cerebral focal com pouca lesão axonal. Por outro lado, os modelos de queda de peso ou *weight drop* visam a reproduzir a lesão cerebral difusa e ganham destaque dadas as suas semelhanças com o TCE humano, pois são capazes de simular o espectro total do trauma, variando de concussão leve a TCE grave (KALISH; WHALEN, 2016).

O insulto inicial após lesão difusa resulta na deformação do tecido devido a forças inerciais de aceleração e desaceleração da cabeça (GENNARELLI; THIBAULT; ADAMS; GRAHAM *et al.*, 1982). A lesão difusa é mais difícil de ser detectada pelos métodos de neuroimagem, principalmente em estados agudos, porém a deformação tecidual causa danos subletais aos neurônios, glia e células vasculares, assim como produz dano crônico progressivo. Como consequência disso, há dano oxidativo, desbalanço osmótico, isquemia e inflamação (BURDA; BERNSTEIN; SOFRONIEW, 2016). Os modelos que incluem a aceleração da cabeça e a aceleração rotacional são capazes de produzir hemorragia, perda celular, lesão axonal difusa e astrogliose de leve a grave escala (ALBERT-WEISSENBERGER; VARRALLYAY; RASLAN; KLEINSCHNITZ *et al.*, 2012).

Para o sucesso na translação entre estudos experimentais com animais e estudos com humanos, é necessário o desenvolvimento e o uso de modelos que consigam exemplificar a lesão primária e a secundária, e que, assim, permitam o entendimento de mecanismos e de como limitar a progressão neurodegenerativa e melhorar a função neurológica pós TCE (PEARN; NIESMAN; EGAWA; SAWADA *et al.*, 2017). Considerando a predominância dos casos

classificados como leves e de lesão craniana fechada, o modelo *weight-drop* é capaz de simular situações cotidianas, como quedas, acidentes automobilísticos ou esportivos (CASSIDY; CARROLL; PELOSO; BORG *et al.*, 2004; MCGEE; ALEKSEEVA; CHERNYSHEV; MINAGAR, 2016).

2.2 AS CÉLULAS GLIAIS E A SINALIZAÇÃO PRÓ E ANTI-INFLAMATÓRIA

A neuroinflamação é o maior processo patológico durante a lesão secundária do TCE (CEDERBERG; SIESJO, 2010). A resposta inflamatória pode ser chamada de "inflamação estéril" ou "inflamação na ausência de estimulo patogênico" e envolve muitos tipos celulares pertencentes ao SNC (ROCK; LATZ; ONTIVEROS; KONO, 2010). Essa resposta ocorre minutos após o TCE e inclui sinalização local de neurônios, glia e células imunes periféricas recrutadas, que induzem a cascata inflamatória (CORPS; ROTH; MCGAVERN, 2015). A resposta das células gliais inclui secreção de citocinas pró e anti-inflamatórias, quimiocinas e fatores de crescimento; formação de barreira ao redor das áreas lesionadas; fagocitose de células mortas e restos celulares; e modulação de respostas celulares (KARVE; TAYLOR; CRACK, 2016).

Os astrócitos fazem parte da macróglia juntamente com os oligondendrócitos, a glia radial e as células ependimárias. Eles são muito importantes na manutenção da homeostase fisiológica do SNC, por ter papel crucial na função neuronal, na transmissão glial e na sinalização via liberação e captação de cálcio (CHEN; SWANSON, 2003; KIMELBERG; NEDERGAARD, 2010). Astrócitos são as células mais populosas no SNC de mamíferos, são altamente polarizados e atuam em diversos fatores, como metabólico e energético, função iônica, função antioxidante, liberação de neurotrofinas e aminoácidos, estando associadas com a modulação da transmissão sináptica excitatória (ARAQUE; PARPURA; SANZGIRI; HAYDON, 1999). Os astrócitos formam uma barreira funcional, via interação dos seus processos, semelhantes a "pés", com a membrana basal do parênquima, chamada de glia limitante. Essa barreira separa o SNC das veias, os espaços perivasculares e as meninges, servindo, ainda, como controle da entrada de células imunes derivadas do sangue. Os astrócitos são, também, uns dos responsáveis pela reparação da barreira hematoencefálica e pela manutenção da homeostase, por fornecer suporte metabólico para neurônios e suas sinapses (SOFRONIEW, 2015).

Após dano cerebral, os astrócitos passam por uma mudança drástica, chamada de astrogliose ou reatividade astrocitária (ZAMANIAN; XU; FOO; NOURI *et al.*, 2012). A reatividade astrocitária é uma resposta dos astrócitos a lesões cerebrais ou doenças como trauma, infecção, neurodegeneração e isquemia. A capacidade dos astrócitos de dar suporte aos neurônios, controlar a barreira hematoencefálica, remodelar o espaço extracelular e regular tanto as células imunes quanto as sinapses são de grande importância para definir como o cérebro reagirá durante e após o insulto degenerativo (PEKNY; NILSSON, 2005; SOFRONIEW, 2009). A reatividade astrocitária é importante na formação da cicatriz pós-trauma, que é responsável por conter o dano e a resposta inflamatória na área lesionada, limitando, desta forma, o alastramento do dano para áreas não afetadas (BURDA; BERNSTEIN; SOFRONIEW, 2016). Em situações de neuroinflamação e condições isquêmicas, os astrócitos se apresentam de duas possíveis formas durante a reatividade: fenótipo A1, que contribui para a morte neuronal, e fenótipo A2, que é neuroprotetor (LIDDELOW; GUTTENPLAN; CLARKE; BENNETT *et al.*, 2017).

Níveis elevados de filamentos intermediários de astrócitos estão relacionados à gravidade da lesão e ao nível de perturbação celular que ela desencadeia, visto que a expressão da proteína fibrilar ácida da glia (GFAP) é maior em astrócitos próximos ao local de lesão e menor em regiões perilesionais (WANNER; ANDERSON; SONG; LEVINE *et al.*, 2013). Uma das primeiras ações dos astrócitos após um insulto é a liberação de ATP — em estudos *in vitro*, a sinalização de cálcio pelos astrócitos é dependente de ATP e, logo após, há liberação do mesmo (GUTHRIE; KNAPPENBERGER; SEGAL; BENNETT *et al.*, 1999; VERDERIO; MATTEOLI, 2001). O ATP extracelular induz a um aumento rápido do cálcio citoplasmático em astrócitos reativos ao redor das lesões cerebrais. Essa sinalização precede fatores como: a polarização astrocitária em direção ao local da lesão e o recrutamento da micróglia e dos neutrófilos, que ocorrem minutos após o trauma e são eventos dependentes de ATP (DAVALOS; GRUTZENDLER; YANG; KIM *et al.*, 2005; KIM; DUSTIN, 2006; ROTH; NAYAK; ATANASIJEVIC; KORETSKY *et al.*, 2014). Os astrócitos podem liberar ou responder a diversas moléculas imunomodulatórias, como citocinas, quimiocinas e mediadores inflamatórios (BURDA; SOFRONIEW, 2014). A resposta pró ou anti-inflamatória é dependente de diversos contextos, como a combinação de moléculas inflamatórias e o momento de sua liberação, a partir disso, poderão ser produzidas moléculas imunomoduladoras, citocinas, quimiocinas, fatores de crescimento e proteases extracelulares (SOFRONIEW, 2014). Com base nessas informações, pode-se concluir que o primeiro dano tecidual induzido pelo TCE desencadeia rapidamente a liberação de ATP pelos astrócitos e outras células, o que leva a uma

onda sinalizadora de cálcio entre astrócitos e mais liberação de ATP pelos mesmos, ativando, dessa forma, a resposta microglial nos locais de lesão e a subsequente reatividade dessas células (BURDA; BERNSTEIN; SOFRONIEW, 2016).

As células microgliais possuem semelhança com macrófagos e contribuem para a vigilância imunológica no SNC (KETTENMANN; HANISCH; NODA; VERKHRATSKY, 2011). A micróglia é composta por pequenas células gliais encontradas no cérebro e no cordão espinal de origem mesodérmica, ao contrário da neuroglia clássica, como astrócitos, células ependimárias e oligodendrócitos (GRAEBER; STREIT, 2010). Ela foi descrita, pela primeira vez, no século XX, é derivada de células mieloides da periferia (RIO-HORTEGA; SOCIEDAD ESPAÑOLA DE HISTORIA NATURAL MADRID. [FROM OLD CATALOG], 1932) e corresponde a cerca de 10 a 20% das células do sistema nervoso central adulto (SPRANGER M, 1996). Durante o desenvolvimento da rede neuronal, a micróglia tem papel importante através da promoção da proliferação e da sobrevivência de células precursoras neurais (MICHELL-ROBINSON; TOUIL; HEALY; OWEN *et al.*, 2015), já durante a vida adulta, a micróglia atua na estruturação da plasticidade neuronal (HONG; DISSING-OLESEN; STEVENS, 2016). Em um cérebro adulto, a micróglia geralmente se encontra em estado de repouso e apresenta morfologia ramificada, que tem como objetivo monitorar o microambiente, ou seja, atua em constante inspeção, a fim de encontrar agentes nocivos e processos prejudiciais (NIMMERJAHN; KIRCHHOFF; HELMCHEN, 2005).

Quando em contato com estes agentes ou processos, como lesão cerebral ou estímulo imunológico, a micróglia se torna ativa (FETLER; AMIGORENA, 2005), assumindo, portanto, morfologia ameboide (CHO; SONG; SUGAMA; SHIN *et al.*, 2006). Embora os outros tipos celulares cerebrais — como neurônios, astrócitos, oligodendrócitos e células endoteliais — possam produzir citocinas pró-inflamatórias, as micróglias têm papel central na neuroinflamação, pois são as principais células imunes do SNC (GINHOUX; LIM; HOEFFEL; LOW *et al.*, 2013). A curto prazo, a ativação microglial pode ser benéfica devido a sua função fagocítica, capaz de manter a homeostase celular e preservar tecidos saudáveis (FRASER; PISALYAPUT; TENNER, 2010).

Após o TCE, a micróglia produz mediadores anti-inflamatórios e atua como *scavenger* de células danificadas, a fim de promover a recuperação. Por outro lado, em caso de ativação exacerbada, a micróglia produz mediadores pró-inflamatórios que acentuam os danos cerebrais e impedem tanto o reparo cerebral quanto a recuperação funcional neurológica (LOANE; KUMAR, 2016). Em um estudo realizado com humanos *post-mortem*, foi mostrado que a ativação da micróglia é persistente por anos após um único episódio de TCE (JOHNSON;

STEWART; BEGBIE; TROJANOWSKI *et al.*, 2013). A ativação microglial prolongada é associada à piora da patologia e dos desfechos secundários (RAMLACKHANSINGH; BROOKS; GREENWOOD; BOSE *et al.*, 2011).

Há dois tipos de estados de ativação microgliais possíveis: a ativação clássica, o estado M1, relacionado à sinalização pró-inflamatória; e a ativação alternativa, o estado de M2, relacionada ao reparo (HANISCH; KETTENMANN, 2007). Quando em seu estado de polarização M1, pode produzir citocinas pró-inflamatórias, espécies reativas de oxigênio e óxido nítrico, o que pode contribuir para a disfunção da rede neural no SNC. Por outro lado, quando em seu estado de polarização M2, a micróglia pode expressar citocinas e receptores relacionados à inibição da inflamação e na restauração da homeostase (SAIJO; GLASS, 2011).

Na ativação clássica, o processo de neurotoxicidade e dano é caracterizado pela liberação de citocinas pró-inflamatórias como fator de necrose tumoral alfa (TNF-α), as interleucinas (IL) IL-1β, IL-6, IL-12 e as quimiocinas, o aumento da atividade da NADPH oxidase e o consequente aumento de espécies reativas de oxigênio, liberação de ácido araquidônico, de glutamato e de ATP. Já na ativação alternativa, o processo é de neuroproteção e reparo, logo, ocorre liberação de citocinas anti-inflamatórias, como IL-4, IL-10 e IL-13, e os fatores de crescimento, como o fator de transformação de crescimento beta (TGF-β), o fator neurotrófico derivado do cérebro (BDNF) e o fator de crescimento semelhante à insulina 1 (IGF-1). Além disso, ocorre a fagocitose de restos celulares e substâncias tóxicas (LOANE; BYRNES, 2010).

Quando superativa, em seu modo clássico ou M1, a micróglia atua amplificando o dano neuronal, e isso, por sua vez, induz a danos mais generalizados aos neurônios vizinhos, esse processo é conhecido como reatividade microglial (BLOCK; HONG, 2005). A micróglia pode levar à neurotoxicidade progressiva através de dois mecanismos: inicialmente, a micróglia estabelece o dano neuronal após reconhecer o estímulo pró-inflamatório, como, por exemplo, lipopolissacarídeo, e, a partir disso, torna-se ativa e produz fatores pró-inflamatórios; após, a micróglia pode ficar superativada em resposta ao dano neuronal, o que é tóxico aos neurônios vizinhos e resulta em um ciclo de morte neuronal perene (BLOCK; ZECCA; HONG, 2007).

A comunicação intercelular entre astrócitos e micróglia ocorre através da liberação de diversas moléculas, incluindo citocinas, ATP e fatores de crescimento. Essa comunicação tem um papel importante no desenvolvimento cerebral, na manutenção de funções e na homeostase, porém, por outro lado, uma comunicação deficitária pode resultar em neuropatologias. A micróglia, que geralmente reage mais rápido ao estímulo patológico, induz a ativação astrocitária e define o destino dos astrócitos. Similarmente, os astrócitos têm potencial não só

para desencadear a ativação microglial, como também para controlar as suas funções celulares. As células microgliais normalmente atenuam a neuroinflamação, porém, em situações de ativação exacerbada da micróglia, há liberação de citocinas pró-inflamatórias. Através dessa liberação, a micróglia é capaz de induzir o fenótipo A1 dos astrócitos e contribuir para o ambiente citotóxico. Em condições patológicas, ocorre a transdução de sinal intracelular para os astrócitos, através da liberação de sinalizadores pró-inflamatórios – como NF-κB e MAPK – pela micróglia. Astrócitos reativos, por sua vez, secretam substâncias que promovem mudanças na permeabilidade da barreira hematoencefálica, o que resulta em recrutamento de células imunes para o parênquima cerebral (JHA; JO; KIM; SUK, 2019; KARVE; TAYLOR; CRACK, 2016; LIDDELOW; GUTTENPLAN; CLARKE; BENNETT *et al.*, 2017; ZIEBELL; MORGANTI-KOSSMANN, 2010).

2.3 A MITOCÔNDRIA E A SINALIZAÇÃO DE SOBREVIVÊNCIA CELULAR

A estrutura mitocondrial surgiu de uma alfa-proteobactéria absorvida por um progenitor eucariótico (LANE; MARTIN, 2010). Como seu ancestral bacteriano, as mitocôndrias são compostas por duas membranas: externa e interna, separadas e funcionalmente distintas, que encapsulam o espaço intermembranas e os compartimentos da matriz (LECRENIER; VAN DER BRUGGEN; FOURY, 1997). A mitocôndria é classificada como essencial na vida eucariótica, pois são potências dentro das células e produzem a maior parte do ATP necessário para a sua manutenção. A mitocôndria possui um genoma próprio (DNA mitocondrial), que é replicado independentemente do genoma do organismo hospedeiro. Em humanos, o DNA mitocondrial codifica 13 proteínas, em geral relacionadas à fosforilação oxidativa (ANNESLEY; FISHER, 2019).

As mitocôndrias são organelas importantíssimas, pois controlam inúmeros processos fisiológicos vitais no organismo. Cerca de 98% do oxigênio inalado é consumido pelas mitocôndrias, e sua geração de energia precisa ser eficiente para manter diversos tipos de atividades celulares (CHAN, 2006). Esta organela é conhecida pelo seu papel primordial na produção de ATP via fosforilação oxidativa. Na matriz mitocondrial, as enzimas do ciclo do ácido cítrico geram carreadores de elétrons (NADH e $FADH_2$), que doam elétrons ao sistema de transferência de elétrons, localizado no espaço intermembranas.

O sistema de transferência de elétrons é composto por proteínas que sofrem mudanças conformacionais através de reações redox sequenciais, com o objetivo de bombear prótons da matriz para o espaço intermembranas (DIAZ; KOTARSKY; FELLMAN; MORAES, 2011; EFREMOV; SAZANOV, 2011). Em mamíferos, este sistema é formado por cinco enzimas multiproteicas – ou complexos - e dois carreadores de elétrons na membrana mitocondrial interna. As quatro primeiras enzimas (CI – CIV) compõem a cadeia respiratória mitocondrial, que facilita a transferência de elétrons, através da redução de equivalentes a oxigênio acoplada à geração de um gradiente de prótons, por uma membrana interna mitocondrial, que será utilizada pela quinta enzima, ATP sintase, para síntese de ATP (LOBO-JARNE; UGALDE, 2018).

Os complexos se encontram incorporados na membrana mitocondrial interna, e suas reações são organizadas espacialmente de uma maneira vetorial extremamente precisa. Como consequência disso, a transferência de elétrons resulta em cargas e prótons sendo movidos através da membrana mitocondrial interna, o que gera a força próton-motriz, composta por gradiente de pH (mais ácido no meio externo) e uma diferença de carga eletrostática (positiva externamente). Sendo assim, o potencial de energia liberado pela transferência de elétrons é retido nessa carga e gradiente de prótons e pode ser utilizado, subsequentemente, na síntese de ATP via ATP sintase (RICH; MARECHAL, 2010). A disposição dos complexos mitocondriais se encontra de forma resumida na figura a seguir:

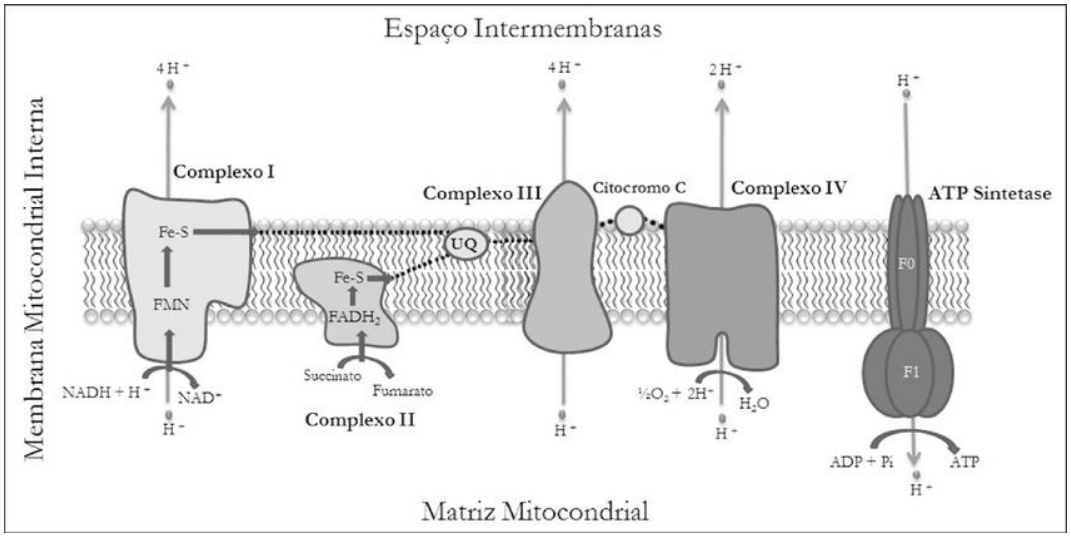


Figura 1: o sistema de transferência de elétrons (PEREIRA, 2012). O complexo I, NADH:ubiquinona oxirredutase, oxida NADH, transfere dois elétrons para ubiquinona e transloca quatro prótons através da membrana. O complexo II, succinato desidrogenase, faz parte do ciclo do ácido cítrico e do sistema de transferência de elétrons, contribui com elétrons adicionais para a ubiquinona, originados do succinato. O complexo III, ubiquinol:citocromo c oxidorredutase, transfere elétrons da ubiquinona para o citocromo c periférico

e transloca quatro prótons. O complexo IV, citocromo c oxidase, que transfere elétrons do citocromo c ao oxigênio molecular e transloca dois prótons. No total, 10 prótons por molécula de NADH são translocados através da membrana mitocondrial interna (SOUSA; D'IMPRIMA; VONCK, 2018).

De uma forma mais detalhada, o complexo I tem papel central no metabolismo celular, ele oxida NADH em NAD+ na matriz mitocondrial e, por isso, supre os redutores equivalentes necessários para manter o ciclo do ácido cítrico e a β-oxidação. NADH vindo da glicólise é transferido para dentro da matriz, por um mecanismo de transporte de substrato. Isso, juntamente com o NADH produzido pelo ciclo do ácido cítrico, é reoxidado a NAD+, acoplado com a redução da ubiquinona localizada na membrana pela NADH:Ubiquinona oxirredutase. Esse complexo representa a porta de entrada dos elétrons na cadeia respiratória e o gradiente de prótons gerado por ele representa cerca de 40% da força próton-motriz utilizada para a síntese de ATP. Além disso, o complexo I é uma das principais fontes de espécies reativas de oxigênio, por isso tem grande impacto no funcionamento da mitocôndria e no estresse oxidativo. O complexo II difere dos outros complexos em diversas características. Esse é o único complexo do sistema que não bombeia prótons através da membrana mitocondrial interna. Ele faz parte do ciclo do ácido cítrico, o que o faz ser uma conexão entre o ciclo e o sistema de transferência de elétrons independente de NADH. A oxidação do succinato a fumarato, ainda no ciclo do ácido cítrico, resulta na redução do seu grupo prostético flavina, FAD (RICH; MARECHAL, 2010; SOUSA; D'IMPRIMA; VONCK, 2018).

A redução de FAD é, na sequência, reoxidada pela ubiquinona na membrana mitocondrial interna. Diferentemente dos outros complexos, essa reação não resulta na translocação de prótons através da membrana. Além da oxidação do succinato em fumarato, ocorre a redução de ubiquinona a ubiquinol, que é o substrato para o complexo III. O ubiquinol produzido pelos complexos I e II é capaz de se difundir ao longo e através do espaço intermembrana mitocondrial, até encontrar o sítio de oxidação do ubiquinol no complexo III, onde é oxidado novamente a ubiquinona. O complexo IV é a última oxidase no sistema de transferência de elétrons e reduz O_2 a H_2O enquanto bombeia prótons pela membrana interna mitocondrial. Ele catalisa a transferência de elétrons a partir da redução do citocromo c a oxigênio pelo complexo III. Essa redução a duas moléculas de água requer quatro transferências de elétron do citocromo c, juntamente com quatro prótons retirados da matriz. Essa reação gera uma mudança no gradiente de prótons através da membrana mitocondrial interna (RICH; MARECHAL, 2010; SOUSA; D'IMPRIMA; VONCK, 2018).

A respirometria de alta resolução é a medição da variação do consumo de oxigênio dependente do fluxo elétrons do sistema de transferência de elétrons. Este método é possibilita a descrição da funcionalidade mitocondrial em tempo real (PUURAND; TEPP; KLEPININ; KLEPININA *et al.*, 2018). O protocolo SUIT, realizado no equipamento OROBOROS O2k®, consiste em uma série de titulação de substratos, desacopladores e inibidores, o que permite avaliar o fluxo de elétrons em pontos específicos do sistema de transferência de elétrons mitocondrial de maneira sensível, a fim de realizar diagnóstico integrado da função mitocondrial (DOERRIER; GARCIA-SOUZA; KRUMSCHNABEL; WOHLFARTER *et al.*, 2018).

A disfunção mitocondrial pode se desenvolver após o TCE leve (GOVINDARAJU; GAUGER; MANLEY; EBEL *et al.*, 2004), ela se inicia com o impacto na cabeça, as forças de aceleração-desaceleração e rotacionais levam à deformação do tecido neural e desencadeiam fluxo de saída de potássio do espaço intracelular para o extracelular, com maior liberação dependente da severidade do impacto (KATAYAMA; BECKER; TAMURA; HOVDA, 1990). O TCE não leva só ao aumento de cálcio mitocondrial e de níveis de glutamato, mas também à regulação negativa dos transportadores gliais de glutamato GLT-1 e GLAST (RAO; BASKAYA; DOGAN; ROTHSTEIN *et al.*, 1998).

A estimulação excessiva dos receptores NMDA e o aumento dos níveis de cálcio geram a despolarização da membrana mitocondrial, a abertura do poro de transição de permeabilidade mitocondrial e o aumento do fluxo de cálcio e, por consequência, a dano mitocondrial, devido ao desequilíbrio no gradiente eletroquímico necessário para a produção de ATP. Numa tentativa de reestabelecer a homeostase, as bombas de cálcio dependentes de ATP são ativadas (SCHINDER; OLSON; SPITZER; MONTAL, 1996; SULLIVAN; THOMPSON; SCHEFF, 1999). Fisiopatologicamente, o TCE aumenta as concentrações intracelulares de cálcio, que, por sua vez, é sequestrado pela mitocôndria. A entrada de cálcio na matriz mitocondrial é mediada pelo *uniporter* mitocondrial de cálcio, que importa cálcio através da membrana interna e, dessa forma, dissipa o potencial de membrana. Esse tamponamento de cálcio causa diminuição da capacidade de síntese de ATP e consequente diminuição no metabolismo, que, por sua vez, é capaz de alterar a funcionalidade celular e, concomitantemente, possibilitar a formação de espécies reativas de oxigênio (JAFRI; KUMAR, 2014; PANDYA; NUKALA; SULLIVAN, 2013).

A mitocôndria regula diversos processos biológicos a fim de promover o funcionamento adequado e a sobrevivência celular, como, por exemplo, transformação de energia, sistema de transferência de elétrons, sinalização celular e apoptose. Por esse motivo, uma mitocôndria

disfuncional tem consequências sérias para a saúde (PICARD; TAIVASSALO; GOUSPILLOU; HEPPLE, 2011). A sobrevivência neuronal está intimamente ligada à homeostase mitocondrial devido ao papel de suprir energeticamente o SNC via ATP e a regulação de cálcio dentro da célula (KHODOROV; PINELIS; STOROZHEVYKH; VERGUN *et al.*, 1996). Nos neurônios, a habilidade da mitocôndria de modular o fluxo de cálcio é essencial para o controle da liberação de neurotransmissores, neurogênese e plasticidade neuronal. Além disso, a mitocôndria fornece grandes quantidades de ATP, bem como intermediários do ciclo do ácido cítrico para a síntese dos neurotransmissores GABA e glutamato (SIBSON; DHANKHAR; MASON; ROTHMAN *et al.*, 1998; WAAGEPETERSEN; SONNEWALD; GEGELASHVILI; LARSSON *et al.*, 2001). Alterações na homeostase mitocondrial parecem alterar os níveis de neurotransmissores e deixar o cérebro vulnerável aos déficits energéticos (PERRY; HAWORTH; ROBINSON, 1985).

Em caso de disfunção, há falha no sistema de transferência de elétrons e na transdução energética em células cerebrais (XIONG; GU; PETERSON; MUIZELAAR *et al.*, 1997). A inibição dos complexos respiratórios mitocondriais e a redução do fluxo de elétrons no sistema de transferência de elétrons já foram observados no TCE (SINGH; SULLIVAN; DENG; MBYE *et al.*, 2006). Pequenos desequilíbrios no fluxo metabólico podem resultar em grandes alterações cumulativas no estado do sistema metabólico (PESTA; GNAIGER, 2012). A disfunção mitocondrial está associada ao aumento nos níveis de espécies reativas de oxigênio e caspases, o que leva a processos apoptóticos (SONG; GAO; WANG; LI *et al.*, 2013). A mitocôndria contém várias proteínas pró-apoptose, como o citocromo c, que está contido na membrana mitocondrial interna como parte do sistema de transferência de elétrons e pode mediar sua própria liberação, assim como de outros fatores apoptóticos. A disfunção mitocondrial induzida por TCE leva ao aumentos desses fatores e, consequentemente, a ativação das caspases (BUKI; OKONKWO; WANG; POVLISHOCK, 2000; KROEMER; GALLUZZI; BRENNER, 2007). Apoptose e lesão axonal são consequências neurológicas bem estabelecidas do TCE. Além da diminuição de ATP necessário para a manutenção das funções axonais, a mitocôndria ativa as caspases, que desencadeiam a morte de neurônios e a fragmentação de proteínas estruturais que mantêm a membrana axonal. A ruptura do potencial transmembrana mitocondrial e a abertura do poro de transição de permeabilidade são fatores que antecipam a apoptose (HILL; COLEMAN; MENON, 2016; HIRSCH; SUSIN; MARZO; MARCHETTI *et al.*, 1998).

Além da morte celular por apoptose, pode ocorrer sinalização pró-inflamatória, que está intimamente relacionada ao estresse oxidativo mitocondrial, ao ciclo inflamatório e à formação de inflamassomas (LOPEZ-ARMADA; RIVEIRO-NAVEIRA; VAAMONDE-GARCIA; VALCARCEL-ARES, 2013). A bioenergética mitocondrial desempenha um papel importante na manutenção da homeostase e na função neuronal, porém, em caso de disfunção mitocondrial, há disfunção celular que pode levar a morte celular (YONUTAS; VEKARIA; SULLIVAN, 2016). Os eventos celulares de necrose e apoptose há muito tempo são reportados em TCE humano (NG; YEO; TANG; SOONG *et al.*, 2000) e em modelos animais (BITTIGAU; SIFRINGER; POHL; STADTHAUS *et al.*, 1999).

Dentre as vias pró-sobrevivência, destacamos a proteína quinase b (Akt). Ela participa de múltiplos processos celulares, especialmente na modulação de morte e sobrevivência celular (DASH; JOHNSON; CLARK; ORSI *et al.*, 2011; GUO; ZHAO; YANG; WANG *et al.*, 2013), além disso, promove sobrevivência celular mediada por fatores de crescimento direta e indiretamente (DATTA; DUDEK; TAO; MASTERS *et al.*, 1997). A Akt foi descrita como neuroprotetora contra diversas doenças neurodegenerativas, incluindo o TCE (NOSHITA; LEWEN; SUGAWARA; CHAN, 2002). Um estudo mostrou que a Akt fosforilada (p-Akt), sua forma ativa, diminui drasticamente após o TCE (FAROOK; SHIELDS; TAWFIK; MARKAND *et al.*, 2013).

Sabe-se, ainda, que a Akt, em sua forma ativa, é capaz de proteger os neurônios dos danos induzidos pelo TCE (WANG; JIANG; PU; ZHANG *et al.*, 2013). Um dos mecanismos de ação já conhecidos da Akt na mitocôndria é a capacidade de regular a enzima hexoquinase II, através de sua fosforilação, e, com isso, há maior associação da enzima com a membrana mitocondrial via ligação com o canal aniônico dependente de voltagem (MAJEWSKI; NOGUEIRA; BHASKAR; COY *et al.*, 2004; PASTORINO; HOEK, 2008). Estudos prévios mostraram que a dissociação da hexoquinase II da mitocôndria está relacionada a eventos de morte celular, como a liberação de citocromo c (PASTORINO; SHULGA; HOEK, 2002; RATHMELL; FOX; PLAS; HAMMERMAN *et al.*, 2003). As isoformas I e II da hexoquinase são capazes de interagir diretamente com a mitocôndria, o que permite a vantagem de acesso direto a altas concentrações de ATP mitocondrial. O uso do ATP mitocondrial permite conectar a glicólise com a fosforilação oxidativa quando os níveis de ATP citosólico são baixos. Esse mecanismo comprovou ser eficiente para a sobrevivência celular durante situações de hipóxia (ROBEY; HAY, 2006; STILES, 2009).

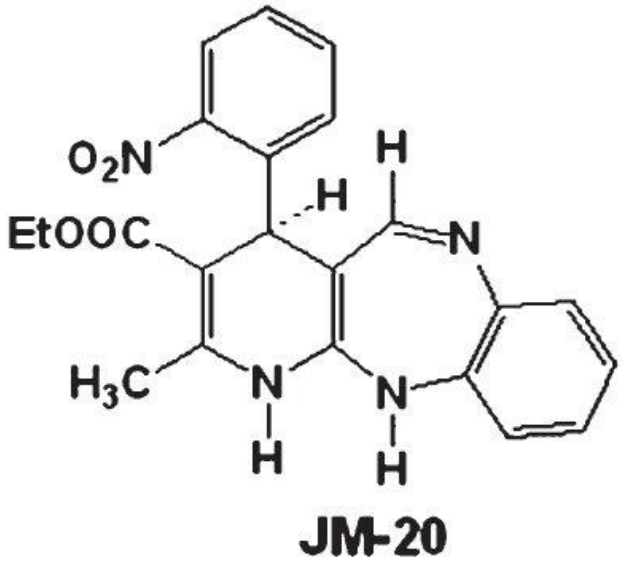
2.4 O JM-20 COMO TRATAMENTO PARA TCE LEVE

Para que ocorra a reabilitação após TCE, é necessário que o metabolismo cerebral seja normalizado o mais rápido possível pós-evento traumático, para evitar posteriores déficits funcionais (KRISHNA; AGRAWAL; ZHUANG; YING *et al.*, 2017). Por isso, é de suma importância que haja uma abordagem multidisciplinar para elucidar os mecanismos, identificar os fatores de risco de desenvolver tratamentos que sejam focados em evitar os danos decorrentes da lesão (MANLEY; MAAS, 2013; SHARMA; VAVILALA, 2012). Com o objetivo de minimizar o dano cerebral após o TCE, as intervenções terapêuticas são direcionadas para a prevenção do dano do primeiro impacto e para restringir as cascatas moleculares e celulares que levam ao dano celular contínuo. Por enquanto, não há tratamento efetivo para o dano causado pelo primeiro impacto (GALGANO; TOSHKEZI; QIU; RUSSELL *et al.*, 2017).

Diversos estudos têm objetivado a busca por um tratamento que previna o dano neuronal causado pelo TCE e que também seja capaz de promover reorganização das redes neuronais e recuperação funcional. Infelizmente, esses estudos experimentais não têm tido sucesso em se tornar terapias de uso clínico (GALGANO; TOSHKEZI; QIU; RUSSELL *et al.*, 2017). Por sua característica tardia, a lesão secundária se torna o potencial alvo para tratamento do TCE, com objetivo de prevenir a progressão da morte celular, reiniciar a neuroplasticidade e atenuar os danos motores e cognitivos causados pela lesão. As intervenções farmacológicas para o tratamento de TCE, atualmente, incluem agentes anti-inflamatórios, inibidores de ciclos celulares e agentes capazes de aumentar AMP cíclico. Além disso, estratégias não invasivas (como exercício físico e estimulação magnética transcranial) e estratégias biológicas (como células-tronco, terapia genética ou peptídica) (PEARN; NIESMAN; EGAWA; SAWADA *et al.*, 2017).

Moléculas sintéticas que são capazes de interagir com alvos múltiplos já demonstraram ser efetivas para tratamentos com o objetivo de neuroproteção em modelos experimentais que mimetizam eventos de isquemia cerebral (KLECZKOWSKA; KAWALEC; BUJALSKA-ZADROZNY; FILIP *et al.*, 2015; LORRIO; GOMEZ-RANGEL; NEGREDO; EGEA *et al.*, 2013; ZHANG; ZHANG; SUN; SZETO *et al.*, 2016). Baseado no paradigma de desenvolvimento de drogas multimodais, uma nova família de 1,5-benzodiazepinas foi desenvolvida por Figueredo et al. (figura 2) (FIGUEREDO; RODRIGUEZ; REYES; DOMINGUEZ *et al.*, 2013). Essa molécula difere das outras benzodiazepinas já disponíveis pela presença de uma 1,4-dihidropiridina fundida ao anel benzodiazepínico. O JM-20 (3-

etoxicarbonil-2-metil-4-(2-nitrofenil)-4,11-di-hidro-1H-pirido[2,3-b][1,5] benzodiazepina) foi desenvolvido com o objetivo de ser uma droga que alcançasse o sucesso no tratamento de doenças neurodegenerativas, por combinar, em sua estrutura, dois compostos capazes de atingir diferentes mediadores de morte celular: cálcio, devido à porção dihidropiridínica; e receptores GABAa, devido à porção benzodiazepínica (DIRNAGL; SIMON; HALLENBECK, 2003). A combinação de mais de um ligante neuroprotetor em uma só estrutura estimulou a hipótese de que o JM-20 poderia ser um composto adequado para neuroproteção nesses modelos experimentais.



JM-20

Figura 2: fórmula estrutural do JM-20. Disponível em Figueredo; Rodriguez; Reyes; Dominguez et al., 2013.

Os derivados benzodiazepínicos apresentam efeitos como relaxamento muscular, anti-convulsivante e sedativos (ROBL; CIMARUSTI; SIMPKINS; BROWN *et al.*, 1996; VINKERS; OLIVIER, 2012). O uso dessa classe para fins terapêuticos tem sido interessante não só em condições de ansiedade e estresse, mas também para fins neuroprotetores (IWATA; INOUE; KAWAGUCHI; FURUYA, 2012; WYATT; ALLEN; CHILCOTT; HICKIN *et al.*, 2001), devido ao efeito de inibição da atividade glutamatérgica, que conhecida por sua ação em eventos que resultam em morte celular, via ativação do sistema gabaérgico (GREEN; HAINSWORTH; JACKSON, 2000). A ação do JM-20 como bloqueador dos canais de cálcio, devido a sua porção di-hidropiridínica, pode estar relacionada a eventos anti-inflamatórios, como foi observado em estudos que utilizaram outros compostos da família 1,4-dihidropiridina (KOMODA; INOUE; NODE, 2010).

JM-20 já mostrou possuir efeitos ansiolíticos e sedativos em roedores, semelhantes aos encontrados na administração de Diazepam (FIGUEREDO; RODRIGUEZ; REYES; DOMINGUEZ *et al.*, 2013). Foi capaz de reduzir tanto o dano isquêmico causado por oclusão da artéria cerebral média (NUNEZ-FIGUEREDO; RAMIREZ-SANCHEZ; HANSEL; SIMOES PIRES *et al.*, 2014), quanto as concentrações de glutamato no fluido cerebrospinal (NUNEZ-FIGUEREDO; PARDO ANDREU; OLIVEIRA LOUREIRO; GANZELLA *et al.*, 2015). Em estudos *in vitro*, exerceu atividade citoprotetiva em modelos de isquemia cerebral (NUNEZ-FIGUEREDO; RAMIREZ-SANCHEZ; DELGADO-HERNANDEZ; PORTO-VERDECIA *et al.*, 2014), menor ativação da micróglia, acompanhada pela redução das citocinas pró-inflamatórias (TNF-α, IL-1β e IL-6), e o aumento da liberação da citocina anti-inflamatória IL-10. O tratamento com JM-20 foi capaz também de diminuir a reatividade astrocitária, comprovada pela redução de GFAP (RAMIREZ-SANCHEZ; PIRES; MENEGHETTI; HANSEL *et al.*, 2018).

A presença de carga positiva na fração benzodiazepínica, juntamente com baixo peso molecular (404,15 g/mol) e alta lipofilicidade, conferem ao JM-20 propriedades físico-químicas suficientes para passar a barreira hematoencefálica e alcançar a mitocôndria, processo facilitado pela carga negativa da matriz dessa organela (NUNEZ-FIGUEREDO; RAMIREZ-SANCHEZ; PARDO ANDREU; OCHOA-RODRIGUEZ *et al.*, 2018). Ao considerar essas propriedades, hipotetizou-se que a mitocôndria poderia ser um alvo para as ações neuroprotetoras do JM-20. Em um estudo realizado com mitocôndrias e sinaptossomas isolados de cérebro de ratos, o JM-20 foi capaz de inibir a geração de H_2O_2 induzida por succinato, preveniu o influxo de cálcio e também a transição de permeabilidade do poro de abertura mitocondrial induzida por cálcio, a dissipação do potencial de membrana e a liberação de citocromo c (NUNEZ-FIGUEREDO; PARDO-ANDREU; RAMIREZ-SANCHEZ; DELGADO-HERNANDEZ *et al.*, 2014). O JM-20 foi capaz de inibir a atividade hidrolítica da ATP sintase tanto em partículas submitocondriais quanto na mitocôndria intacta em concentrações micromolares baixas, o que sugere que essa molécula é capaz de cruzar a membrana mitocondrial e atingir a enzima — e, em consequência disso, preservar o ATP durante a isquemia, promovendo, dessa forma, a sobrevivência celular neuronal (NUNEZ-FIGUEREDO; RAMIREZ-SANCHEZ; DELGADO-HERNANDEZ; PORTO-VERDECIA *et al.*, 2014).

Estudos utilizando o JM-20 como tratamento para outras doenças neurodegenerativas já foram iniciados. Em um estudo *in vitro* e *in vivo* de modelo de doença de Parkinson induzido por rotenona, o tratamento com JM-20 preservou a viabilidade das células SH5Y-SY, aumentou a atividade de enzimas antioxidantes, evitou a disfunção mitocondrial cerebral, a perda de peso

corporal e a mortalidade induzida por esta neurotoxina (FONSECA-FONSECA; WONG-GUERRA; RAMIREZ-SANCHEZ; MONTANO-PEGUERO *et al.*, 2019). Quando administrada como pré-tratamento, a molécula mostrou-se capaz de atenuar déficits em um modelo de Alzheimer induzido por escopolamina, reduzindo os déficits de memória, a disfunção mitocondrial, o estresse oxidativo e a hiperatividade da enzima acetilcolinesterase (AChE), provavelmente em decorrência da inibição específica dessa enzima (WONG-GUERRA; JIMENEZ-MARTIN; FONSECA-FONSECA; RAMIREZ-SANCHEZ *et al.*, 2019).

A cascata de eventos patológicos tardios ou secundários subsequentes à lesão causada pelo TCE é de extrema complexidade. Por isso, há necessidade de que a droga neuroprotetora aja em amplo espectro, ou seja, com diferentes alvos, para que haja mais de um mecanismo de proteção ao SNC (SAMINI; SAMARGHANDIAN; BORJI; MOHAMMADI *et al.*, 2013). Visto que a fisiopatologia do TCE engloba eventos isquêmicos, como, por exemplo, a redução da pressão de reperfusão cerebral e do fluxo sanguíneo na região devido à lesão primária e as suas consequências, como hemorragia e edema, criou-se a hipótese de que o JM-20 seja capaz de atuar como droga neuroprotetora também no TCE (BRAMLETT; DIETRICH, 2004; HACKENBERG; UNTERBERG, 2016).

3. DESENVOLVIMENTO

O desenvolvimento que faz parte dessa tese está apresentado sob a forma de dois artigos científicos. 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 Molecular Neurobiology e encontra-se no formato da mesma. O artigo 2 encontra-se submetido na revista Neurochemical Research e está apresentado em forma de manuscrito, na formatação para publicação na revista.

3.1 ARTIGO CIENTÍFICO 1

JM-20 Treatment After Mild Traumatic Brain Injury Reduces Glial Cell Pro-inflammatory Signaling and Behavioral and Cognitive Deficits by Increasing Neurotrophin Expression

Andrezza Bond Vieira Furtado, Débora Farina Gonçalves, Diane Duarte Hartmann, Aline Alves Courtes, Gustavo Cassol, Yanier Nunez-Figueredo, Deivison Silva Argolo, Ravena Pereira do Nascimento, Silvia Lima Costa, Victor Diogenes Amaral da Silva, Luiz Fernando Freire Royes, Félix Alexandre Antunes Soares.

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JM-20 Treatment After Mild Traumatic Brain Injury Reduces Glial Cell Pro-inflammatory Signaling and Behavioral and Cognitive Deficits by Increasing Neurotrophin Expression

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Abstract

Traumatic brain injury (TBI) is considered a public health problem and is often related to motor and cognitive disabilities, besides behavioral and emotional changes that may remain for the rest of the subject's life. Resident astrocytes and microglia are the first cell types to start the inflammatory cascades following TBI. It is widely known that continuous or excessive neuroinflammation may trigger many neuropathologies. Despite the large numbers of TBI cases, there is no effective pharmacological treatment available. This study aimed to investigate the effects of the new hybrid molecule 3-ethoxycarbonyl-2-methyl-4-(2-nitrophenyl)-4,11-dihydro1H-pyrido[2,3-b][1,5]benzodiazepine (JM-20) on TBI outcomes. Male Wistar rats were submitted to a weight drop model of mild TBI and treated with a single dose of JM-20 (8 mg/kg). Twenty-four hours after TBI, JM-20–treated animals showed improvements on locomotor and exploratory activities, and short-term memory deficits induced by TBI improved as well. Brain edema was present in TBI animals and the JM-20 treatment was able to prevent this change. JM-20 was also able to attenuate neuroinflammation cascades by preventing glial cells—microglia and astrocytes—from exacerbated activation, consequently reducing pro-inflammatory cytokine levels (TNF-α and IL-1β). BDNF mRNA level was decreased 24 h after TBI because of neuroinflammation cascades; however, JM-20 restored the levels. JM-20 also increased GDNF and NGF levels. These results support the JM-20 neuroprotective role to treat mild TBI by reducing the initial damage and limiting long-term secondary degeneration after TBI.

Keywords JM-20 · Neuroprotection · Neuroinflammation · Multi-target · Astrocytes · Microglia

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Introduction

Traumatic brain injury (TBI) is defined as a brain function alteration or when other brain pathology evidences caused by an external force are found [1]. Due to its high incidence and the potential to generate disability in affected individuals besides its impact on the economically active population, TBI is defined as a serious social and economic problem and is considered a "silent epidemic" [2]. TBI is a major cause of morbidity and mortality, especially regarding the young population. TBI triggers motor and cognitive disabilities, besides behavioral and emotional changes that may remain for the rest of the subject's life, being considered a public health problem [3]. It is estimated that 100 to 300 out of 100,000 of the population needs health assistance for mild TBI every year [4].

Over 80% of TBI cases are classified as mild and close head injury; in other words, it is the result of falls, car accidents, violence and assault, sport injuries, and recreational activities falls or impacts on the head [4, 5]. The primary injury nature and severity occurring at the moment of the impact, which is characterized by mechanical rupture events—such as disruption in blood–brain barrier, cell membrane, and vascular components—will determine the clinical outcomes following TBI [6]. In secondary injury events, the main known pathogenesis mechanisms in cell damage happen due to neurotransmitter release, calcium homeostasis, oxidative stress, inflammation, and blood–brain barrier permeabilization [7]. The secondary damage delayed nature suggests that there is a window for therapeutic intervention to avoid progressive damage [8]. It is necessary that the brain metabolism is normalized soon after a traumatic event to avoid subsequent functional deficits and it is also important that experimental TBI researches focus on therapies to trigger mechanisms and restore function and behavior, since one goal of clinical trial is to improve neurologic, motor, and cognitive functions [9, 10].

Neuroinflammation is recognized as a common factor among many different pathological conditions and it is defined by glial cell activation, leukocyte recruitment, upregulation, and cytokine and chemokine release [11, 12]. Resident astrocytes and microglia are normally the first cell types to start inflammatory cascades following TBI and both activations are considered biomarkers of the brain's innate response to injury [13, 14]. Microglia is the most involved structure in secondary injury following TBI [15] and plays a crucial role in neuroinflammation and it is the main component of innate immune system in CNS, besides being the first line of defense after injury or disease events [16]. These cells are often the first to react to any inflammatory event; they are able to respond, in a dynamic way, to exogenous and endogenous signals. This response can be harming or neuroprotective, depending on the situation, but it is widely known that when the neuroinflammation is continuous or excessive it may be the main cause of numerous neuropathologies [17].

Despite the large numbers of TBI cases, there is no effective pharmacological treatment available. Most of the trials target single secondary injury mechanism ignoring the secondary injury process multifactorial nature that may not result in significant recovery because just one point is being aimed [8]. Firstly described by Figueredo [18], JM-20 (3-ethoxycarbonyl-2-methyl-4-(2-nitrophenyl)-4,11-dihydro-1H-pyrido [2,3- b] 1,5] benzodiazepine) is part of a novel family of 1,5-benzodiazepines, structurally different from the 1,5-benzodiazepines, which are already known by the presence of a 1,4-dihydropyridine in the fused portion of the benzodiazepine ring. This molecule has emerged as a promising one for the treatment of neurodegenerative diseases because it targets the mediators involved in cell death events: calcium, due to the dihydropyridine portion, and GABAa receptors due to the benzodiazepine action [19]. JM-20 already presented positive results in ischemia, Parkinson's, and Alzheimer's disease models [20–23].

These findings support our hypothesis that JM-20 may be an auspicious option for TBI therapy. Knowing that TBI is capable of inducing motor and cognitive deficits and brain edema and triggers acute classical neuroinflammation and cytokine release [5, 24–26], our investigation aimed to evaluate JM-20 treatment after a mild close head TBI model, more specifically TBI-induced behavioral changes, brain water content, glial cell activation, and subsequent pro- and anti-inflammatory signaling and cascades.

Materials and Methods

Animals and Reagents

Adult male Wistar rats weighing 200–220 g were used in this study. During the experimental protocol, animals were kept in cages with 3 animals each, with food and water ad libitum. Rats were maintained in a room with controlled temperature with a 12-h light/dark photoperiod. The room where the experimental procedures were performed had a controlled temperature of 22 °C ± 2. Assay reagents were purchased from Sigma (St. Louis, MO, USA) and biochemical kits were obtained from the standard commercial supplier Labtest (Lagoa Santa, Brazil). All procedures with animals followed the Committee on Care and Use of Experimental Animal Resources Guidelines of the Federal University of Santa Maria, Brazil (9,426,190,418).

Drug Treatment and Experimental Groups

Animals were randomly assigned to four groups: (1) control, animals were not submitted to any protocol; (2) JM-20, treated with 8 mg/kg JM-20; (3) TBI, submitted to weight drop protocol and treated with vehicle; (4) TBI + JM-20, submitted to weight drop protocol and treated with 8 mg/kg JM-20. A single drug dose or vehicle was orally administered (by gastric gavage) 1 h after TBI. JM-20 was suspended in a 0.05% carboxymethylcellulose (CMC) solution (vehicle) immediately before use. The dose of JM-20 was chosen based on previous studies [22].

Traumatic Brain Injury

Animals were submitted to a single TBI using a weight drop model, following Meehan et al. [27] with modifications. Before the TBI procedure, the animals received topical lidocaine on the head [28]. The rats were anesthetized (isoflurane, 1% inhaled, Baxter) and maintained on an aluminum paper sheet, which had small cuts along its extension. Under the aluminum paper, there were sponges to cushion the drop of the animal. An acrylic weight of 54 g (2.5-cm diameter) was used to perform a free fall to reach the animal head, observing 100 cm of height (28 in.). A fishing line (0.30 mm, Mazzaferro) supported the weight. On the moment of the impact, the animal's weight tore the aluminum paper and allowed sudden acceleration movement, consequently tearing the paper and allowing the animal to roll over and fall on a sponge. All the experiments were performed 24 h after TBI procedure.

Behavior Tests

Open-Field Test

Open-field apparatus was made of plywood and 30-cm high walls surround it. The open-field floor was 45 cm in length and 45 cm in width. It was divided into 9 squares (3 rows of 3) by masking tape markers. Each animal was individually placed in the center of the apparatus and observed for 5 min to register the spontaneous locomotor (number of segments crossed with the four paws) and exploratory activities (expressed by the number of times rearing on hind limbs) [29].

Object Recognition Memory Test: Short-term Memory

Training and testing in the object recognition task were carried out in an open-field arena built with black-painted wood [30]. Firstly, the rats were individually habituated in the apparatus and left to freely explore it some days before the training session. In the training session, 22 h and 30 m after TBI, two different objects (A and B) were placed in the apparatus, and rats were allowed to freely explore it 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 was not considered an exploratory behavior. After 1 h 30 min of training session, one of the objects was randomly exchanged for a novel object (C), and the rats were reintroduced to the apparatus for 5 min. The time spent exploring the familiar and the novel objects was recorded. To avoid confusing effects of lingering olfactory stimuli and preferences, the object and the arena were cleaned with 70% ethanol after testing each animal.

Brain Edema

Brain edema was determined by measuring brain water content with the wet–dry method 24 h after TBI [31]. After the rats were anesthetized and sacrificed by decapitation, the brains were quickly removed and separated through the interhemispheric fistula into left and right hemispheres. Tissue samples from injured hemispheres were immediately weighed to get the wet weight. After drying in an oven for 48 h at 100 °C, the tissues were weighed again to yield the dry weight. Brain water content was then calculated using the following formula: % H_2O = (1—dry weight/wet weight) × 100%.

Protein Expression Analysis

Western blot analysis was performed according to Gerbatin et al. [32] with some modifications. Cerebral hippocampus samples were lysed on ice in RIPA (radio-immunoprecipitation assay) and centrifuged for 20 min at 12,700 × *g* and 4 °C. The protein concentration of each sample was determined by the bicinchoninic acid protein assay (Thermo Fisher Scientific). Samples (30 µg protein) were then subjected to a 4–12% SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane using Trans-Blot® Turbo™ Transfer System and equal protein loading was confirmed by Ponceau S solution (Sigma Aldrich—P7170). After specific blocking, the blots were incubated overnight at 4 °C with rabbit anti-Iba-1 ionized calcium binding adapter molecule 1 (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA) glial fibrillary rabbit anti-GFAP acid protein (1:1000; Dako).

Mouse anti-β-actin (1:10,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was stained as protein loading additional control. After primary antibody incubation, membranes were washed with TBS-T (TBS plus 0.1% Tween 20) two times at room temperature for 10 min and incubated with anti-rabbit (Sigma Aldrich – A6154) or anti-mouse (Santa Cruz Biotechnology – sc-2005) secondary antibodies conjugated with horseradish peroxidase (1:5000) for 2 h at room temperature. Bands were visualized by enhanced chemiluminescence using ECL Western Blotting Substrate (Pierce ECL, BioRad) and signals were captured with a photodocumenter ChemiDoc

XRS + (BioRad). In sequence, the bands were quantified by using Image Lab software (Bio-Rad).

mRNA Level Quantification

The relative abundance of tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and nerve growth factor (NGF) mRNA was measured in the hippocampus by quantitative real-time PCR (qPCR) following the described method with some modifications. The samples were macerated with liquid nitrogen in Eppendorf tubes and re-suspended in Trizol reagent (Invitrogen), then they were settled on ice. The sample pellet was followed by chloroform extraction and isopropanol precipitation. Total RNA isolation was performed according to the manufacturer's suggested protocol.

Total RNA samples were treated with DNase I (Promega) to eliminate DNA contamination. High-capacity cDNA reverse transcription kits of approximately 2 µg total RNA was performed with random primer, dNTPs, and M-MLV reverse transcriptase enzyme (Invitrogen) according to the manufacturer's suggested protocol. The following gene-specific primers were used:

Actin (forward, 5′-GTGTGACGACGAGGTTGCCGC TCTTGTTGTAGAC-3′; and reverse, 5′-GGTAAGGAT CTTCATGAGGTAATCAGTAAGATCAC-3′),
BDNF (forward, 5′CTTTGGGGCAGACGAGAAAGC-3′; and reverse, 5′CACCTGGTGGAACTCAGGGTC-3′),
TNF-α (forward, 5′ ATGGGCTCCCTCTCATCAGT-3′; and reverse, 5′- GCTTGGTGGTTTGCTACGAC 3′), and
IL-1β (forward, 5′CCAGTCAGGCTTCCTTGTGC–3′; and reverse, 5′-ACAGGTCATTCTCCTCACTGTCG-3′)

We used 10 µl PCR mixture containing 5 µl cDNAs (1:100), 1X PCR buffer, 0,1 mM dNTPs, 0.2 µM of each primer, 3 mM $MgCl_2$, 0.1 X SYBR Green I (Molecular Probes), and 0.5 U Platinum *Taq* DNA Polymerase (Invitrogen).

The qPCR conditions were the following: 95 °C for 5 min followed by 40 cycles of 15 s at 95 °C, 15 s at 60 °C, and 20 s at 72 °C for extension in a Thermocycler StepOne Plus (Applied Biosystems). After amplification, samples were heated from 60 to 95 °C at a 0.3 °C/s temperature gradient to construct the denaturing curve of the amplified products. All samples were analyzed in triplicate with a non-template control included as well. SYBR Green fluorescence (molecular probes) was analyzed by StepOne Plus Software version 2.0 (Applied Biosystems) and Cq value (ΔCq) was calculated for each sample and reported using the ΔΔCq method [33]. Briefly, for each well, a ΔCq value was obtained by the difference in Cq values (ΔCq) between the target gene and reference gene. The ΔCq mean value obtained from the control group of each gene was used to calculate the ΔΔCq of the respective gene ($2^{-\Delta\Delta Cq}$).

To perform the quantification of GNDF and NGF mRNA levels, a different protocol was used. Total RNA was isolated using Trizol reagent (AmbiomTM 15,596,018) and subjected to DNase treatment using TURBO DNA-freeTM Kit (Invitrogen, AM1906), following the manufacturer's instructions. The RNA sample concentration was spectrophotometrically determined. Complementary DNA was generated from 2.5 mg total RNA using the SuperScript1 VILOTM Master Mix according to the manufacturer's recommendations. mRNA expression of target genes and the endogenous control genes Actin B and HPRT1 were assessed by real-time PCR (with TaqMan Gene Expression Assay products, Applied Biosystems), according to the manufacturer's recommendations. Expression levels for each gene of interest were calculated by normalizing the quantified mRNA amount to Actin b and HPRT1. Relative gene expression was determined and used to test significance among different groups. Real-time PCR was performed in QuantStudioTM 7 Flex Real-Time PCR System (Applied Biosystems, CA, USA) using TaqMan Universal PCR Master Mix II (Applied Biosystems™ 4,440,044), TaqMan probes, and primers provided by Applied Biosystems. The assay ID provided by the manufacturer is the following: NGF (Rn01533872_m1), GDNF (Rn00569510_m1, Actin B (ACTB; Rn00667869_m1).

Statistical Analysis

GraphPad Prism 7.0 software (GraphPad Software Inc., USA) was used to determine significant differences among experimental groups. Data normality was analyzed using D'Agostino and Pearson's normality test. Data were expressed as mean ± standard error of the mean (SEM) and were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test when they pass the normality test and by Kruskal–Wallis test followed by Dunn's post hoc test when data did not pass the normality test. $p < 0.05$ was considered statistically significant.

Results

JM20 Treatment Improved Behavioral Assays

Effects of JM-20 Treatment on Locomotor and Exploratory Activity Alterations Induced by TBI

Animals in the TBI + vehicle group showed a decrease in the number of crossings ($p < 0.05$) (Fig. 1a) and rearings

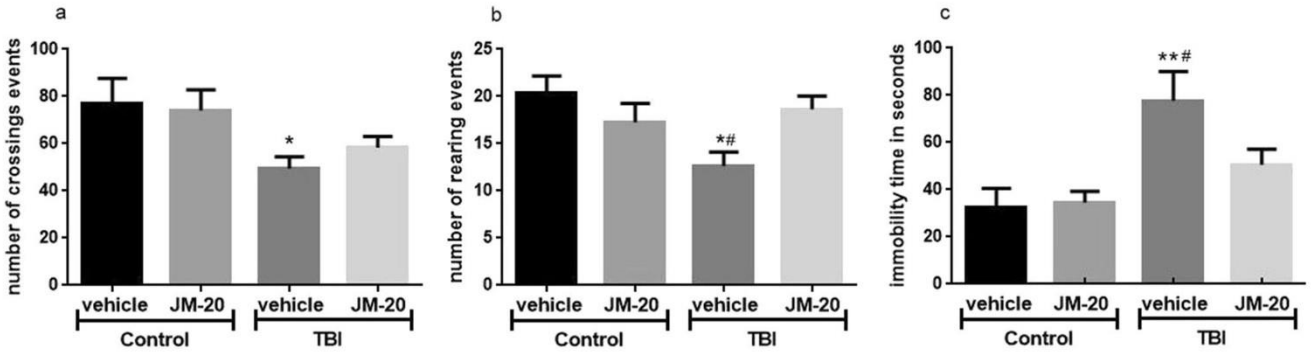

Fig. 1 Effects of TBI and JM-20 treatment on open-field test performance. (**A**) Crossing events. (**B**) Rearing events. (**C**) Immobility time. Data are mean ± SEM (n = 8–11). *Different from control, $p < 0.05$. **Different from control + vehicle, $p < 0.01$. #Different from TBI + JM-20. One-way ANOVA followed by Tukey's post hoc test

($p < 0.05$) (Fig. 1B) when compared to the control + vehicle group, such as TBI + vehicle and TBI + JM-20 groups in rearing events ($p < 0.05$) (Fig. 1a). Immobility time was increased in the TBI + vehicle group compared with those of the control + vehicle group ($p < 0.01$) (Fig. 1c). In addition, JM-20 treatment protected against locomotor and exploratory deficits induced by TBI.

Effects of JM-20 Treatment on Short-term Memory After TBI

In Fig. 2, as was expected, the rats explored each object (A and B) for a similar percentage of total time in the training session. The control groups—vehicle and JM-20—and TBI + JM-20 groups did not show deficits in short-term memory (STM) in object recognition test ($p < 0.005$). They spent more time exploring a new object (object C). Animals of the TBI + vehicle group presented deficits in STM; they spent more time exploring the familiar object than the new one.

JM-20 Search for a Mechanism of Action

Effects of JM-20 Treatment on Brain Edema

To investigate JM-20 potential effect, we detected brain water content 24 h after TBI injury (Fig. 3). The TBI + vehicle group presented an increase in water brain content in comparison to the control + vehicle and TBI + JM-20 groups ($p < 0.05$). This increase was not observed when the animals were treated with JM-20 after TBI.

Effects of JM-20 Treatment on Glial Cell Activation After TBI

Figure 4a shows that GFAP expression was increased in the TBI + vehicle group in comparison to the control + vehicle group ($p < 0.05$). In this way, Iba1 expression was also increased in the TBI + vehicle group when compared to both control groups—vehicle and JM-20 ($p < 0.01$) (Fig. 4b). In the TBI + JM-20 groups, these increases did not occur,

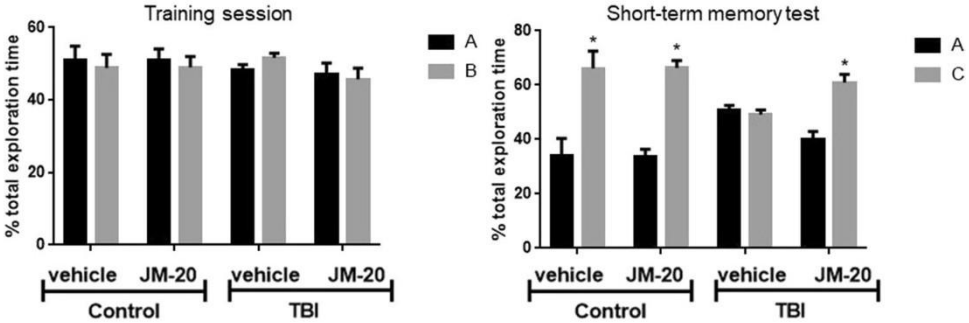

Fig. 2 Effect of TBI and JM-20 treatment on object recognition memory test 24 h post mild traumatic brain injury (TBI) in rats. Data are reported as mean ± SEM (n = 7). Training session was performed 22 h and 30 m after TBI. There is no difference among the groups. *Different from object A of its respective group ($p < 0.0005$)

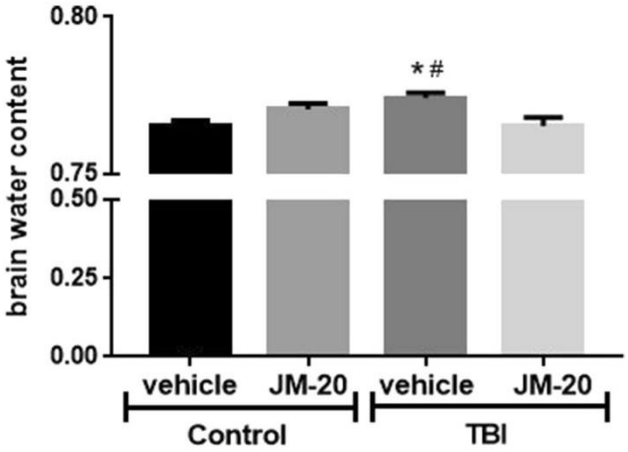

Fig. 3 Effects of TBI and JM-20 treatment on brain water content. Data are mean ± SEM (n = 6). *Different from control, # different from TBI $p < 0.05$ (one-way ANOVA followed by Tukey's post hoc test)

which suggest that JM-20 treatment avoids astrocyte reactivity and microglial activation cascades following TBI.

Effects of JM-20 Treatment on Cytokine and Neurotrophin Release After TBI

Figure 5 shows that IL-1β mRNA levels were increased in the TBI + vehicle group in comparison to those of the control + vehicle group ($p < 0.05$) (Fig. 5a). TNF-α mRNA levels were increased in the TBI + vehicle group in comparison to those in the control + vehicle ($p < 0.05$) and TBI + JM-20 ($p < 0.01$) groups (Fig. 5b). These results suggest that JM-20 treatment is able to avoid pro-inflammatory cytokine release.

In Fig. 6, BDNF mRNA was significantly decreased in the TBI + vehicle group in comparison to that in the control + vehicle group and TBI + JM-20 ($p < 0.05$) (Fig. 6a). GDNF was increased in the JM-20 groups in comparison to that in both vehicle groups ($p < 0.05$) (Fig. 6b). NGF was increased in the TBI + JM-20 group in comparison to that in both vehicle groups (Fig. 6c).

Discussion

In the present study, we evaluated JM-20 neuroprotective effects on TBI-induced behavioral changes, as motor and memory alterations, brain edema, glial cell activation, and neurotrophin and cytokine release. JM-20–treated animals presented improvements in the behavioral assays performed; besides, a brain edema was avoided by JM-20 treatment. The TBI-induced neuroinflammation reduction triggered by the JM-20 treatment provides evidence that it exerts a neuroprotective role on mild TBI.

TBI is a multifaceted condition; the trauma episode is the first moment of a continuous, maybe permanent, process that affects multiple systems and may lead to a neurodegenerative process. TBI physiopathology is characterized by the combination of cellular and physiologic disturbances such as increase in lesion volume, neurological and cognitive impairment, and motor dysfunction. It requires a wide and multidisciplinary approach to enlighten its mechanisms, identify risk factors, and develop treatments [34–36]. Neurodegenerative diseases claim for the development of novel multifunctional ligands, which are designed to simultaneously attach mechanisms, being able to delay disease progress and promote neuro-restorative effects instead of only giving a symptomatic control [37, 38]. JM-20 is a multipotent molecule that has multi-target effects by combining the GABAergic activity of benzodiazepines with anti-calcic effects of dihydropyridines [23].

The results of the primary injury are brain tissue damage, cerebral blood flow impairment, brain metabolism alterations, inflammatory marker upregulation, oxidative events,

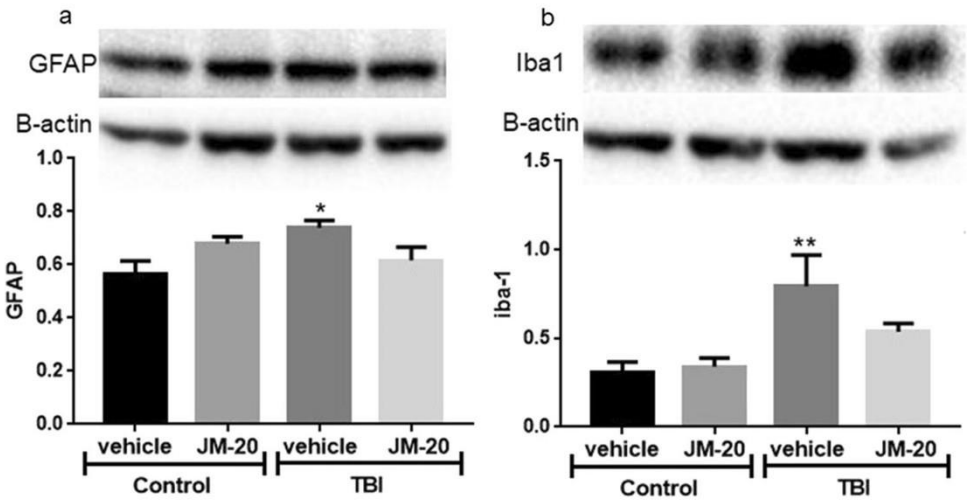

Fig. 4 Effects of TBI and JM-20 treatment on GFAP and iba-1 expression in hippocampus by Western blotting analysis. Data are mean ± SEM (n = 6). *Different from control + vehicle, $p < 0.05$ **Different from control + vehicle and control + JM-20 groups, $p < 0.01$. One-way ANOVA followed by Tukey's post hoc test

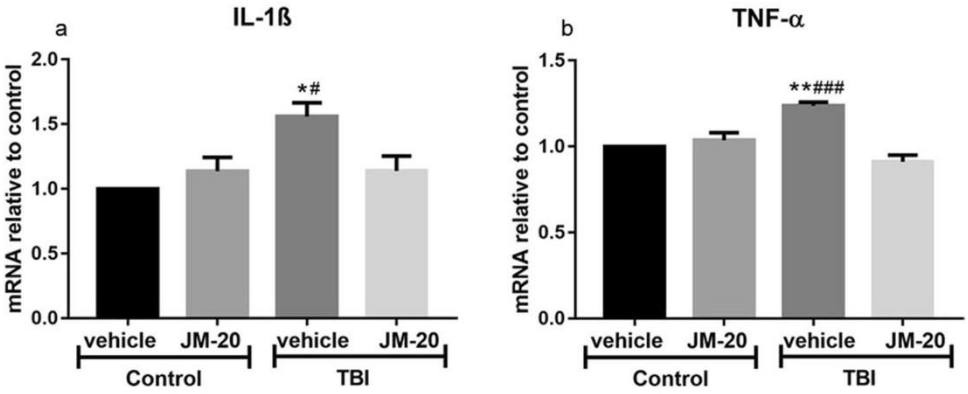

Fig. 5 Effects of TBI and JM-20 treatment on IL-1β (**A**) and TNF-α (**b**) mRNA levels in hippocampus. Data are mean ± SEM (n = 5). *Different from control + vehicle ($p < 0.05$). #Different from TBI + JM-20 ($p < 0.05$). ## Different from TBI + JM-20 ($p < 0.01$). One-way ANOVA followed by Tukey's post hoc test

and vasospasm. All these processes are strictly correlated with cell death and generalized brain edema [26]. Brain edema, inflammatory responses, and increase of intracranial pressure are closely associated with mortality and neurological disturbance caused by TBI [39, 40]. TBI-related increase in brain edema is caused by direct trauma to the head or from accelerated injury and decelerated injury [41]. Our results showed an increase in brain water content in TBI animals and a decrease in TBI rats after the JM-20 treatment. Brain edema development following TBI contributes to injury process evolution. It derives from the blood–brain barrier disruption—vasogenic edema—or cellular ionic pump dysfunction—cytotoxic edema. Blood–brain barrier alterations play a role in edema formation by exacerbating the cascade of secondary injury events including the neuroinflammatory process. These mechanisms are closely correlated with morbidity and mortality [42].

A few minutes after TBI, a potent inflammatory response begins in the brain. The resident glial cell activation, astrocytes, and microglia and the infiltration of blood leukocytes are of concern in the complexity of post-traumatic response [24]. Astrocytes are the most abundant glial cell type of CNS and are directly correlated with brain homeostasis by providing metabolites and growth factors to neurons, supporting synapse formation and plasticity and controlling the extracellular balance of ions, fluids, and neurotransmitters. GFAP expression is commonly used to identify in vivo and in vitro astroglia. The upregulation of this marker is associated with several CNS pathologies such as infection, trauma, ischemia, and neurodegenerative diseases [43]. When activated in controlled ways, it is considered benign to neurons; however, when astrocytes are activated in a reactive way, there is probably a loss in the neuroprotective function and a gain in neurotoxic properties. Strategies to prevent astrocyte reactivity have emerged as a promising neuroprotection alternative [23]. In our study, there was an increase in GFAP expression in TBI-submitted group in comparison to the control group. JM-20 treatment following TBI is able to mitigate the astrocyte reactivity. Similar results were found in an ischemia model [23]. Remarkably, impaired astrocyte behavior intensifies neuronal dysfunction following brain injury. Therefore, preventing astrocyte reactivity is substantial to avoid its upregulation that triggers cytokine and chemokine production and may contribute to concomitant resident microglia and peripheral immune cell activation, which increases neuronal cell death and promotes a worse outcome after TBI [16].

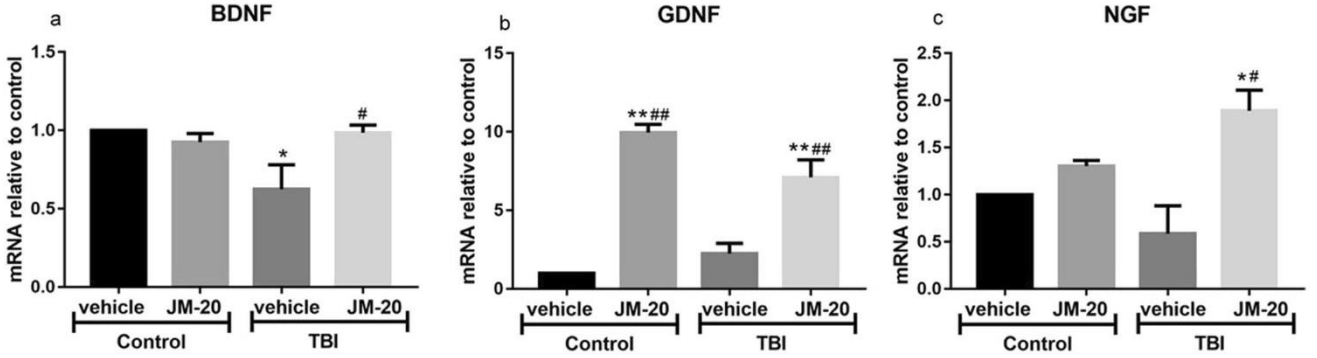

Fig. 6 Effects of TBI and JM-20 treatment on BDNF (**A**), GDNF (**B**), and NGF (**C**) mRNA levels in the hippocampus. Data are mean ± SEM (n = 5). *Different from control + vehicle ($p < 0.05$). #Different from TBI + vehicle ($p < 0.05$). Two-way ANOVA followed by Tukey's post hoc test

Microglia release different factors, including pro- and anti-inflammatory cytokines, chemokines, nitric oxide, prostaglandins, growth factors, and superoxide species, which are able to modulate secondary injury and recovery after TBI [44]. A highly reactive microglial activation state outcome is the pro-inflammatory and cytotoxic mediator release that leads to neuronal dysfunction and cell death [8]. Iba1 upexpression is a known marker of microglial activation [45]. In this study, we showed that TBI increases Iba1 expression in comparison to the control group. JM-20 treatment following TBI is able to prevent microglia activation. Microglia activation composes a central point of regulation in neuroinflammation that may lead to neurotoxic or neurosupportive environments, being critical for neuron death or survival [46]. Taken together, GFAP and Iba1 results indicate one of the possible neuroprotective mechanisms of the JM-20 treatment. Such treatment could modulate the astrocyte reactivity and microglia activation, which is the starting point of the neuroinflammation process.

An extensive number of different cytokines with many different functions build a complex network of inflammatory signaling. Each cytokine expression after brain injury can provide information about tissue damage development. IL-1 can be considered an early biomarker of neuroinflammation that plays an important role in the onset and development of hormonal and cellular inflammatory cascades and is known to be involved in BBB disruption, edema formation, and apoptosis. TNF-α is assigned as a potent pro-inflammatory cytokine that is produced by microglia and astrocytes, and is able to induce cerebral inflammation, BBB disruption, and leukocyte recruitment [24, 47, 48]. In our study, IL-1β and TNF-α mRNA expression were increased 24 h following a non-treated TBI. When treated with JM-20, these increases did not occur, providing the information that JM-20 is able to modulate inflammatory cascades. Distinct TBI models presented an increase in IL-1β and TNF-α in different time points following the TBI episode [49, 50]. IL-1 neurotoxic effects have shown that it synergistically acts with TNF-α [51], suggesting that these are two crucial cytokines in post-traumatic inflammation and in the development of secondary damage. Both IL-1β and TNF- α are markers of classically activated microglia [52]. Notably, there is a bidirectional relation between microglia and astrocytes. Microglia-astrocytes crosstalk demonstrates that the communication occurs via molecules secreted by them, including cytokines, chemokines, ATP, and growth factors. The first to react to pathological stimuli is commonly the microglia, and then it induces astrocyte activation by the exposure to inflammatory stimuli and release of proinflammatory mediators such as IL-1β, TNF-α, and neurotoxic factors [53, 54]. We believe

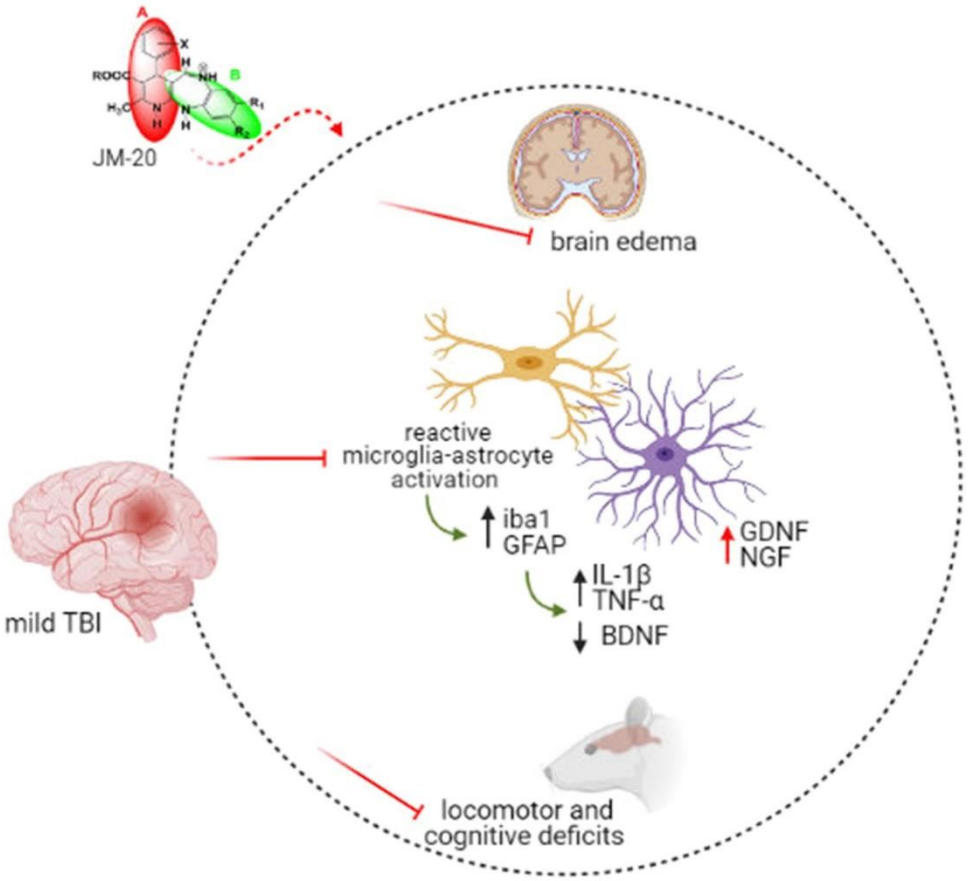

Fig. 7 Overview of the neuroprotective mechanisms of JM-20 treatment after mild TBI. Inside the circle: TBI outcomes. Green arrows: TBI outcomes mechanisms. Red bar-headed lines: JM-20- mediated inhibition of TBI outcomes. Red arrows: JM-20 treatment effects on neurotrophins

that by not allowing the astrocyte reactivity and microglia activation, JM-20 treatment is able to reduce pro-inflammatory cytokine release.

Neurotrophic factors have fundamental importance in the maintenance of normal brain function not only in a healthy CNS environment, but also in the neuroprotection against neurodegenerative diseases [55]. Previously, Oyesiku [56] described the beneficial effects of neurotrophin expression increase due to its several known functions, such as apoptosis process inhibition, control of calcium homeostasis, and ischemia mechanism attenuation.

BDNF is one of the most studied neurotrophic factors and has numerous effects in neurogenesis and inflammation modulation [57]. Upon "controlled" activation, astrocytes upregulate neurotrophic factors, such as BDNF, aiming to support and protect against injury-induced cell death. BDNF elevated expression was found in healthy in vitro astrocytes, which suggests that it is involved in the beneficial/pro-survival response [58, 59]. BDNF is also secreted by microglia in "steady" basal state, promoting continuous CNS surveillance [60]. It has been proposed that pro-inflammatory overproduction inhibits BDNF release [61]. GDNF is a member of the transforming growth factor (TGF) superfamily, which has demonstrated neuroprotective effects, specifically over dopaminergic neurons [62]. It has been shown that GDNF has neuroprotective effects in different neurodegenerative diseases, including TBI [63]. In addition, GDNF is a potent inhibitor of microglial activation [64] and able to downregulate pro-inflammatory cytokine expression [65], which may explain the increase in expression of Iba1, TNF-α, and IL-1β in TBI animals, but not in animals treated with JM-20 following TBI. NGF is known for its association with cholinergic neuron survival and growth and to exert a role in maintenance of neuronal proliferation by its interaction with another growth factor [66, 67]. Previous researches proved that NGF has protective effects in neurons, such as protecting against cytotoxic agents and excitotoxicity [68], that information lead us to believe that NGF expression increase induced by JM-20 treatment was able to protect the brain against secondary injury events. In studies with inflammatory induction, CNS cells presented reduction in BDNF, GDNF, and NGF and that response is related with neuroinflammatory and neurodegenerative cascades [69, 70]. In line with this, we may indicate that JM-20 is able to prevent BDNF reduction induced by TBI and increase GDNF and NGF levels to modulate neuroinflammatory responses.

Taking into account the behavioral changes, TBI outcomes are cognitive decline, neurologic and physical deficits, and impairment of psychosocial activities and function [5]. In the present study, TBI induced deficits in locomotor and exploratory activity and short-term memory. Motor function deficits observed soon after TBI are reliable indicators of the neurological function preservation degree [10]. TBI has decreased the spontaneous locomotor activity [32]. In our study, spontaneous locomotor activity was analyzed in open-field task. These results demonstrate that our TBI model induced locomotor and exploratory deficits 24 h after injury. There is an established relation between decrease in exploratory activity and increase of TNF-α levels 24 h after TBI [71]. Studies using anti-inflammatory drugs presented TBI-induced motor deficit rehabilitation [72, 73]. Our treatment with JM-20 was effective in attenuating it and, in some cases, reverting TBI-induced behavioral deficits, this could be directly correlated with the neuroinflammation suppression that JM-20 promoted.

Another common dysfunction induced by TBI is cognitive impairment, such as memory deficits [74]. Behavioral tests that use previously presented stimulus ability of recognition are used to simulate human amnesia tests [75]. In our study, we used the Object Recognition Test to evaluate short-term memory following TBI. Short-term memory, defined as capacity of learning and remembering recent information, is usually affected after TBI [76]. In our research, animals submitted to TBI explore the familiar and the new object at the same time, which means that TBI induced short-term memory deficits. This behavioral pattern was not present in other groups, which suggests that JM-20 treatment after TBI preserves memory capacity. In a previous study using an Alzheimer's disease model, JM-20 preserved the acquisition and consolidation memory process [21]. This result could be explained by the decrease of BDNF mRNA in the hippocampus of non-treated TBI animals and the increase of GDNF and NGF in treated animals. It has become conspicuous that neurotrophic factor impairment in the central nervous system intensifies functional decline following TBI [77]. The hippocampus and eyesight are tightly intertwined, which is majorly responsible for providing access to brief-interval memory signals; this signaling is required for efficient visual exploration [78]. Object recognition memory reactivation is hippocampus-dependent and the reconsolidation of the familiar object memory only occurs with the simultaneous novel object information. This process depends on gene expression and de novo protein synthesis in the hippocampus for restoration following recovery [79]. A previous study proved that BDNF is enough for this process and able to control the integration of new information in the recognition trace [80]. In another study, BDNF ± mice presented an impairment in object-place memory and object-place exploration [81]. Concurrently, GDNF and NGF increase in JM-20 treated groups may be associated with this result; previous studies associated GDNF with improvements in spatial memory process in adult-born hippocampal neurons and also in Alzheimer's disease model [82, 83]. Growth factors like NGF have an important role in neuronal survival and plasticity of forebrain cholinergic neurons, which are present in related-memory areas, such as the hippocampus

[84]. In studies using NGF as treatment following injury, the spatial memory performance test was improved 1 week after TBI [85] and cognitive function presented enhancements even after 1 month of TBI event [86].

One of the most common TBI outcomes is the loss of hippocampal GABAergic neurons [87]. As a consequence, an impairment in inhibitory and excitatory neuronal transmission and subsequent damage in hippocampal function may occur [88]. JM-20 acts on GABAa receptors because of its benzodiazepine portion [19]. In a recent study, JM-20 treatment after ischemia attenuated the exacerbation of neurotransmitter release [23]; we strongly believe that the same mechanism occurred in this study. Another drug, galantamine, was able to stabilize the hippocampal GABAergic neuron loss and improve memory function; however, it was not efficient to control neuroinflammation [87]. We already emphasized the TBI multifaceted pathology and the demand for developing multifunctional ligands as a potential treatment. Besides having action in GABAa receptors, JM-20 further acts in calcium mechanisms due to the dihydropyridine portion [19]. 1,4-Dihydropyridine family represents one of the most important groups of calcium channel blockers [89]. Anti-inflammatory properties of dihydropyridines have already been presented [90]. We found here that the combination of these two mechanisms, GABAergic activity of benzodiazepines with anti-calcic effects of dihydropyridines, was potentially able to handle TBI outcomes and explain both functional and biochemical improvements found.

Nonetheless, there are still important questions that should be answered in the future to improve the knowledge about JM-20 treatment mechanisms following TBI. Multiple points are involved in the neuroinflammation pathway and some were not approached in this study, for example, p38 MAKP and NF-κB, that are strongly correlated with inflammatory cascades developed after TBI [91], mitochondrial dysfunction, which are well-established mechanisms that mediate inflammation, and subsequent neurodegenerative disorders due to reactive oxygen and nitrogen species production [92]. Further research is needed regarding moderate and severe TBI models. In conclusion, we provided evidence to support the neuroprotective role of JM-20 for treatment of mild TBI. We clearly demonstrated in a TBI model that JM-20 treatment is efficient in preventing locomotor deficits, preserving short-term memory, and avoiding microglia activation and astrocyte reactivity. Additionally, the treatment may reduce pro-inflammatory cytokine release and increase the expression of important neurotrophins involved in neuroprotection (BDNF, GDNF, and NGF). An overview on neuroprotective mechanisms of JM-20 treatment after mild TBI is in Fig. 7. We credit the benefits of JM-20 treatment to its multi-targeting features. All these results support the multi-ligand molecule neuroprotective effects and corroborate our hypothesis that JM-20 may become a promising treatment strategy to TBI.

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Data Availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethics Approval All procedures with animals followed the Committee on Care and Use of Experimental Animal Resources Guidelines of the Federal University of Santa Maria, Brazil (9426190418).

Consent to Participate Not applicable.

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3.2 ARTIGO DE CIENTÍFICO 2

JM-20 improves pro-survival signaling and avoids mitochondrial dysfunction after mild traumatic brain injury

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JM-20 IMPROVES PRO-SURVIVAL SIGNALING AND AVOIDS MITOCHONDRIAL DYSFUNCTION AFTER MILD TRAUMATIC BRAIN INJURY

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ABSTRACT

Traumatic brain injury (TBI) temporarily or permanently impairs brain function, besides, injury events may be detectable and sometimes they may not. TBI pathology includes primary and secondary injuries. Because of primary injury untreatable nature, the secondary injury gradually develops, progressing by hours or even days. Due to this, secondary injury becomes the focus of TBI treatment interventions. Mitochondrial damage following TBI is closely related to the development and progression of secondary injury, impaired cellular bioenergetics, initiating a wide array cascade of events that culminate on cellular damage that may lead to cellular death. Despite the large numbers of TBI cases, there is no effective pharmacological treatment available. This study aimed to investigate the effects on mitochondrial TBI outcomes of the new hybrid molecule 3-ethoxycarbonyl-2-methyl-4-(2-nitrophenyl)-4,11-dihydro1H-pyrido[2,3-b][1,5]benzodiazepine (JM-20). Male Wistar rats were submitted to a weight drop model of mild TBI and treated with a single dose of JM-20 (8 mg/kg). 24 h after TBI, the following secondary injury mechanisms were assessed: mitochondrial dysfunction, cell pro-survival signaling and behavioral tasks. JM-20 treatment avoided mitochondrial dysfunction, such as the decrease of oxygen flux consumption during different states of mitochondrial respiration and there was an improvement in mitochondrial functionality after TBI, cell survival pathway (p-Akt) was enhanced and behavioral changes related to balance impairment were prevented. Regarding the importance of mitochondria on TBI development and the consequences of mitochondrial dysfunction on cell death and functional parameters, JM-20 is a potential strategy to treat TBI and its secondary injury outcomes.

KEYWORDS: JM-20; Neuroprotection; Mitochondrial dysfunction; Multi-target; Apoptosis.

INTRODUCTION

The most ordinary traumatic brain injury (TBI) definition is: alteration in brain function - or other brain pathology evidence - that is caused by an external force [1]. Other definition established by The National Institute of Neurological Disorders and Stroke (NINDS) said that TBI is a form of acquired brain injury and occurs when sudden trauma causes damage to the brain [2]. The TBI symptoms can be mild, moderate or severe; this fact depends on the damage's extent. It is estimated that 100-300/100.000 of population needs health assistance for mild TBI by year. In mild cases the subject may remain conscious or unconscious, present headache, confusion and other symptoms like dizziness and difficulty in balance [2, 3]. Despite the definition, TBI temporarily or permanently impairs brain function, besides, injury events may be detectable; however, sometimes they may not. [4]. TBI pathology includes primary and secondary injuries [5]. Mechanical shift of brain tissue at the impact moment causes the primary injury, which is relatively impossible to treat [6]. Because of primary injury untreatable nature, the secondary injury gradually develops, progressing by hours or even days. Due to this, secondary injury becomes the focus of TBI treatment interventions. [7, 8].

Mitochondria are strongly correlated to cellular homeostasis and regulatory pathways such as apoptosis and oxidative stress, both associated with secondary injury pathophysiologic process following TBI [9]. From this point forward, mitochondria become dysfunctional and lead to necrosis and apoptosis events [10]. Cellular events as necrosis and apoptosis have been reported in both human and animals models of TBI [11, 12]. Neuronal survival is strongly related to mitochondrial homeostasis because the mitochondria supply the central nervous system with energy (ATP), also regulating calcium influx in cells [13]. Considering cellular survival, one of the most popular pro-survival pathways is Akt, a protein which is known by its involvement in many cellular processes, especially in death and survival cell modulation [14, 15]. Akt has been described as a neuroprotective protein in many neurodegenerative diseases including TBI [16]. In its active state – phosphorylated - Akt is able to protect neurons against TBI-induced damage by targeting proteins involved in cell survival, cell cycling and angiogenesis. On the other hand, after a TBI event p-Akt is drastically decreased [17-19]. Exactly how it happens with Akt, mitochondria is a target of TBI injury development, experimental models of TBI proved that mitochondrial damage develops fast, respiratory changes appears in 15-30 minutes following TBI and the peak of mitochondrial dysfunction occurs between 12h to 24 hours [20, 21]. Once mitochondria become dysfunctional, the cell also becomes, and these events can lead to cell death. Although there is a wide range of

therapeutic possibilities being researched, mitochondrial dysfunction and cell death have been shown to be a promising target for new treatments approach [5, 10, 22].

Multitargeting ligands have been claimed as an alternative to treat neurodegenerative diseases, these molecules are designed to simultaneously attack injury mechanisms and are capable to delay disease progress and promote neuro-restorative effects instead of only promoting symptomatic control [23, 24]. First described by Figueredo [25], JM-20 (3-ethoxycarbonyl-2-methyl-4- (2-nitrophenyl) -4,11-dihydro-1H-pyrido [2,3- b] 1,5] benzodiazepine) is part of a novel family of 1,5-benzodiazepines, structurally different from the 1,5-benzodiazepines already known because of the presence of a 1,4-dihydropyridine in the fused portion of the benzodiazepine ring. This molecule has emerged as promising for the treatment of neurodegenerative diseases by targeting mediators involved in cell death events: calcium, due to the dihydropyridine portion, and GABAa receptors due to benzodiazepine action [26]. JM-20 already presented positive results in ischemia, Parkinson's, Alzheimer's and TBI models [27-30]. In previous studies with different neurodegenerative models, JM-20 was able to modulate both mechanisms proposed in the present research: mitochondria and the Akt pro-survival pathway. In an ischemia model, JM-20 treatment protected brain mitochondria from ischemic neuronal damage by preventing intramitochondrial calcium overload, opening the permeability transition pores, membrane potential dissipation and cytochrome c release [29]. JM-20 also presented a strong antioxidant action [31]. In an oxygen-glucose deprivation model JM-20 prevents the Akt phosphorylation decrease and consequent inactivation of glycogen synthase kinase-3β (GSK-3β), an important protein related to cell death events [32].

Our group previously studied JM-20 treatment following TBI used here. We demonstrated that JM-20 was able to reduce glial cells pro-inflammatory signaling, behavioral and cognitive deficits by increasing the neurotrophins expression [33]. In this study, we aimed to investigate other important points of secondary injury cascade as mitochondrial dysfunction and death/survival mechanisms. In the present study, high-resolution respirometry (HRR) and activation/deactivation of survival mechanism was performed and the influence on behavior related to animal balance 24 h after TBI was analyzed.

MATERIALS AND METHODS

Animals and reagents

Adult male Wistar rats weighing 200-220g were used in this study. During the experimental protocol, animals were kept in cages with 3 animals each, with food and water ad libitum. Rats were maintained in a room with controlled temperature with a 12 h light/dark photoperiod. The room where the experimental procedures were performed had controlled temperature of 22ºC ±2. Assay reagents were purchased from Sigma (St. Louis, MO, USA) and biochemical kits were obtained from the standard commercial supplier Labtest (Lagoa Santa, Brazil). All procedures with animals followed the Committee on Care and Use of Experimental Animal Resources Guidelines of the Federal University of Santa Maria, Brazil (9426190418).

Experimental groups

Animals were randomly assigned to the following groups: (1) control+vehicle animals were not submitted to any protocol (2) control+JM-20, treated with 8 mg/kg JM-20 (3) TBI+vehicle, submitted to weight drop protocol and treated with vehicle (4) TBI + JM-20, submitted to weight drop protocol and treated with 8 mg/kg JM-20. A single dose of the drug or vehicle was orally administered by gastric gavage 1 h after TBI. JM-20 was suspended in a 0.05% carboxymethylcellulose (CMC) solution (vehicle) immediately before use. The JM-20 dose and the time following TBI was selected based on previous studies [29].

Traumatic Brain Injury

Animals were submitted to a single TBI using weight drop model, following Meehan et al [34] with modifications. Before the TBI procedure, the animals received topical lidocaine on the head [35]. The rats were anesthetized (isoflurane, 1 % inhaled, Baxter) and maintained on an aluminum paper sheet, which had small cuts along its extension. Under the aluminum paper, there were sponges to cushion the drop of the animal. An acrylic weight of 54g (2.5cm diameter) was used to perform a free fall to reach the animal head, observing 100 cm of height (28 inches). A fishing line (0.30mm, Mazzaferro) supported the weight. On the moment of the impact, the animal weight tore the aluminum paper and allowed the sudden acceleration movement, consequently tearing the paper and allowing the animal to roll over and fall on a sponge. All the experiments were performed 24 h after TBI procedure.

High-resolution respirometry (HRR)

The oxygen flow in the mitochondrial respiratory chain was measured by high-resolution respirometry (Oroboros Oxygraph-O2K) using the DataLab software program version 4.2.0.73 (Oroboros Instruments, Innsbruck, Austria). Hippocampus were homogenized in mitochondrial respiration buffer - MIR05 (0.5 mM EGTA, 3 mM MgCl2, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose, 1 g / l serum albumin bovine - BSA). 20 µl of sample were added to the device chamber, containing the breathing buffer MIR05 (final volume of 2 mL). Following a previous research, we chose to analyze the homogenates instead of isolated mitochondria [36]. Isolation techniques can remove about 60% of mitochondrial population in comparison to homogenates and may disrupt mitochondrial structure and function more than permeabilization/homogenization of the whole tissue [37-39].

When indicated the substrates, uncouplers or inhibitors were added respectively to the vat containing the sample, in accordance to SUIT protocol [40]: 5 mM Pyruvate, 5 mM Glutamate and 5 mM Malate, to determine the electron flow independent of ATP synthase without the use of a specific inhibitor (proton LEAK); 1 mM ADP, to measure oxidative phosphorylation capacity mediated by complex I (CI OXPHOS); 10 mM succinate, to measure the maximum oxidative phosphorylation capacity mediated by complexes I and II (CI + CII OXPHOS); 2.5 µM oligomycin, to demonstrate mitochondrial respiration independent of ATP production (LEAK); 0.5 µM FCCP, to determine maximum respiration by the electron transfer system of complexes I and II (CI + CII ETS); 0.5 µM rotenone, to measure complex I respiration through ETS (CI ETS); 5 mM malonate, to determine the ETS ability by complex II (CII ETS); and 2.5 µM antimycin A, to measure residual oxygen consumption (ROX). All data related to the SUIT protocol were normalized by the activity of the citrate synthase enzyme of each sample [41]. Respiratory acceptor control ratio (RCR = CI OXPHOS/LEAK) was used as mitochondrial quality control [40].

Protein expression analysis

Western blot analysis was performed according to Gerbatin et al [42] with some modifications. Cerebral hippocampus samples were lysed on ice in RIPA (radio-immunoprecipitation assay) and centrifuged for 20 min at 12.700×g and 4 °C. The protein concentration of each sample was determined by the bicinchoninic acid protein assay (Termo Fisher Scientific). Samples (30µg protein) were then subjected to a 4-12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane using Trans-Blot® Turbo™ Transfer System and equal protein loading was confirmed by Ponceau S solution (Sigma

Aldrich - P7170). After specific blocking, the blots were incubated overnight at 4°C with rabbit anti phospho-Akt (1:1000; Cell Signaling).

Mouse anti-β-actin (1:10.000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was stained as protein loading additional control. After primary antibody incubation, membranes were washed with TBS-T (TBS plus 0.1% Tween 20) two times at room temperature for 10 min and incubated with anti-rabbit (Sigma Aldrich – A6154) or anti-mouse (Santa Cruz Biotechnology – sc-2005) secondary antibodies conjugated with horseradish peroxidase (1:5000) for 2h at room temperature. Bands were visualized by enhanced chemiluminescence using ECL Western Blotting Substrate (Pierce ECL, BioRad) and signals were captured with fotodocumenter ChemiDoc XRS+ (BioRad). In sequence, the bands were quantified by using Image Lab software (Bio-Rad).

Behavioral assays

Rotarod Test

Before the TBI event, the animals were habituated and trained to remain in the rotarod (Insight, Brazil) under a constant speed of 8 rpm for 300 s, the same criteria was used for the following tests. Before TBI, after habituation on the rotarod for 1 min, the animals who failed to stay on the rotarod were disqualified to avoid a false positive. Twenty-four hours after TBI, the rotarod 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 [36].

Beam-walking test

The beam-walk test described by Hausser et al [43] consists of the animal walking on a suspended wooden beam with a width of 2.5 cm and a length of 100 cm to reach a black wooden box at the end of the apparatus. First, the rats were placed in the wooden box for 1 minute for setting. Soon after, they were placed at the other end and encouraged to walk along the beam to reach the opposite end. Three attempts were made. The animals were pre-trained to TBI to adapt to the apparatus. On the test day, the time of each of the three trials was recorded and an average of these values was recorded.

Statistical Analysis

GraphPad Prism 7.0 software (GraphPad Software Inc., USA) was used to determine significant differences among experimental groups. Data normality was analyzed using D'Agostino and Pearson's normality test. Data were expressed as mean ± standard error of the mean (SEM) and were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's post hoc test when passed in normality test and by Kruskal-Wallis test followed by Dunn's post hoc test when data did not pass the normality test. $p < 0.05$ was considered statistically significant.

RESULTS

JM-20 treatment increases mitochondrial O2 flux consumption

TBI-vehicle group presented a decrease in O2 flux consumption in OXPHOS CI-linked state (Fig. 1B) and OXPHOS CII-linked state (Fig. 1C) in comparison to control JM-20 group ($p <0.05$ and 0.01, respectively) and in comparison to TBI+JM-20 group in OXPHOS CI& CII-linked state (Fig. 1D). There is no difference between groups in Proton LEAK state (Fig. 1A). TBI-vehicle group presented a decrease in O2 flux consumption in LEAK-state with oligomycin (Fig. 2A) in comparison to JM-20 control group. The same parameter occurred when respiration was uncoupled by FCCP addition: ETS CI&CII-linked (Fig. 2B), ETS CI-linked (Fig. 2C) and ETS CII-linked (Fig. 2D) TBI-vehicle O2 flux consumption were diminished in comparison to JM-20 control group ($p <0.05$).

JM-20 treatment following TBI improves mitochondrial functionality

Respiratory control ratio was used as a measure of mitochondrial quality and functionality [36]. RCR was decreased in TBI-vehicle group in comparison to TBI-JM-20 group (Fig. 3) which suggest that JM-20 treatment following TBI is able to restore mitochondrial function.

Effects of JM-20 treatment on cell pro-survival signaling

Figure 4 shows that p-Akt expression was decreased in TBI+vehicle group in comparison to control+vehicle group and TBI-JM+20 group ($p <0.05$). In TBI+JM-20 group,

this decrease did not occur in comparison to control groups, which suggest that JM-20 treatment reverse the impairment in pro-survival signaling following TBI.

Effects of JM-20 treatment in behavioral assays

Balance deficit (Fig. 5) was revealed in TBI+vehicle animals by decrease of latency to fall in rotarod test. Statistical analysis revealed TBI+vehicle group presented difference when compared to the control groups – vehicle and JM-20 ($p < 0.01$) and TBI+JM-20 groups ($p < 0.05$).

Figure 6 shows that TBI+vehicle induced balance deficits observed by the increase in time to complete beam-walk test in comparison to the control groups – vehicle and JM-20 ($p < 0.05$).

DISCUSSION

Our study evaluated JM-20 treatment following TBI regarding important points of secondary injury as mitochondrial dysfunction and survival mechanisms and its possible influence on behavioral parameters. Our data clearly indicates that JM-20 treatment has the ability to avoid mitochondrial dysfunction, as the decrease of oxygen flux consumption and improvement in mitochondrial functionality after TBI. In line with this, JM-20 demonstrate the capacity to enhance cell survival pathway and prevent behavioral changes related to balance impairment.

Although brain contribution in human's body mass is about 2%, it uses more than 20% of the whole-body oxygen consumption. As a result, a high aerobic capacity is required for brain mitochondria to maintain all functions [44, 45]. Cellular homeostasis requires complex orchestration of innumerous processes that depend on proper energetic supply. Mitochondria is known as the cells "Power Plant" and provide the greater part of energetic substrates to make these processes happen [22]. Mitochondrial damage following TBI is closely related to development and progression of secondary injury, ROS generation and impaired cellular bioenergetics initiate a wide array cascade of events that culminate on cellular damage [5]. Our results indicate that JM-20 treatment increases mitochondrial oxygen flux consumption in different respiration states. During TBI development, damaged neurons demand higher energy supply [46]. Although there are no differences between control+vehicle group and TBI+vehicle group, we suggest that JM-20 boost on cellular respiration is extremely important to prevent the bioenergetic decline caused by TBI and attenuate secondary injury outcomes. TBI-induced

bioenergetics capacity reduction is a process that is not exclusively related to failure of mitochondrial membrane potential (ΔΨm) but also to OXPHOS and ETS capacity impairment [47].

In our HRR assessment, TBI+vehicle group showed a decrease in OXPHOS CI, CII and CI&CII in comparison to JM-20 groups. The OXPHOS is defined as the respiratory state with ADP saturated concentrations, respiratory fuel substrates and O2, in the absence of exogenous uncouplers. This state estimates the maximal mitochondrial respiratory and coupled capacity [48, 49]. Observing this result is possible to conclude that oxygen flux dependent on OXPHOS system is impaired and as consequence, it can compromise ADP phosphorylation [36]. Our treatment is able to increase the oxygen flux during OXPHOS, which results in mitochondrial dysfunction prevention. When compared to other cell types, neurons are more OXPHOS-dependent to completely supply their energy demand. This limited capacity to upregulate glycolysis leads to energy failure, which is one of the major responsible for neuronal cell loss in brain disorders [50, 51]. Previous studies using Cortical Contusion Injury model, complex I and II respiration were significantly affected by TBI and considering that neurons need ATP for survival, as a result of this decrease, a failure may be developed [52]. With JM-20 treatment this failure may be prevented, which reflects on the maintenance of mitochondrial functionality.

LEAK is the respiratory state in absence of ATP synthesis with O2 flux compensated by the ions leaks with saturating concentrations of respiratory fuels and O2 [40, 48]. LEAK stage can measure an estimate of intrinsic uncoupling without uncoupler addition [53]. We evaluated the LEAK state in the presence of all fuel substrates with addiction of oligomycin as ATP synthase inhibitor because this inhibition is linked to the oxygen flux coupled to ATP synthesis [54]. Our results demonstrated a significant LEAK state decrease on TBI group supporting our idea of a lower coupling efficiency related to ATP synthesis. On the other hand, the treatment with JM-20 was able to restore LEAK state at control levels, which could be related to a better coupling efficiency for ATP generation. Our hypothesis is supported because the JM-20 treatment was able to increase oxygen flux dependent on OXPHOS after a TBI. Following an injury, state V, uncoupled by FCCP, is important to be assessed due to neurons and supporting cells dependence on mitochondria to produce ATP and sequester calcium influx [52]. The uncoupled state, or ETS state, yields an estimate of electron transfer capacity through mitochondrial complexes [48], TBI-induced mitochondrial dysfunction have been associated to ETS impairment and energy transduction consequently to mitochondrion-associated calcium overload [55]. In our results, in the presence of FCCP, TBI+vehicle group presented a decrease in electron flow when compared to control+JM-20 group in multiple states: ETS CI, CII and

CI&CII-linked. These analyses represented the maximum O2 consumption capacity and indicated the ETS disruption [36]. TBI-induced changes in electrons flow can be related to ROS levels increase and impairment in the mitochondrial membrane fluidity, as a consequence, radical leakage can occur [5]. JM-20 showed efficiency in maintaining electron flow through all ETS states. The maintenance or restoration of a functional mitochondrial ETS is an essential therapeutic target [56]. The traffic flow of electrons determines the healthy, alarming or disease cell state. When ETS is disrupted, there is an alteration in membrane potential and an increase in ROS production, these factors can lead to loss of mitochondrial structural integrity and function [57, 58].

As a consequence of homeostasis loss followed by TBI and the highest energy demand, mitochondria are doubtless working near its maximal capacity [52]. A loss of mitochondrial function reflects in a wide range of vital cellular events after TBI [59]. Here, we used RCR to measure mitochondrial functionality [36]. This control ratio is an indicator of mitochondrial coupling state, it represents ADP-activated flux to measure coupled OXPHOS capacity (state III) divided by leak flux (state IV) [60]. Mitochondrial dysfunction is an extremely engaging target in TBI models, it has a crucial importance in both cellular homeostasis and cell death [10]. TBI non-treated group presented lower RCR when compared to TBI+JM-20 group. Low RCRs demonstrate a compromised ability to produce ATP, which is deleterious for injured neurons that are working to achieve homeostasis [55]. JM-20 already presented protective effects on mitochondria in an ischemia model, it was able to prevent multiple mitochondrial events related to ischemic neuronal damage, especially related to calcium overload, transition pores, membrane potential, antioxidants and anti-apoptotic pathways [29, 31].

Although mitochondria are generally associated to ATP production, they also have an important role in the cell death process. One well-known cell death mechanism is necrosis, which occurs when water crosses the mitochondrial membranes leading to mitochondrial swelling, dysfunction and ATP depletion. Another important process is apoptosis, which is triggered by the release of mitochondrial pro-death proteins in the cytosol and leads to the activation of various caspases, as a consequence, cell death occurs. Both of these cell death pathways occur following a brain injury and, because of this, it is important to consider them when studying mitochondrial therapeutics [61]. Mitochondria homeostasis is strongly related to cell survival; then, mitochondrial dysfunction can culminate in cell death through necrotic or apoptotic events by triggering apoptosis cascades including Bcl-2 family and cytochrome C, which induces protein and lipid peroxidation and DNA damage [62-64]. Changes in mitochondrial functions have decisive outcomes for cellular function and disease progression,

there are a great number of neurological disorders influenced by mitochondrial dysfunction [65-68]. As we presented here, JM-20 treatment showed efficiency to maintain mitochondrial function, which we believe to be related to the prevention of the pro-survival signaling decrease promoted by the molecule.

The Akt pathway is a well-known pro-survival factor. Its action is crucial considering a variety of circumstances such as angiogenesis, protein synthesis, metabolism and proliferation [69, 70]. Akt exerts neuroprotective effects following TBI by phosphorylating downstream targets and inhibiting calcium dependent apoptosis cascades [71, 72]. Some of these phosphorylation targets are the pro-apoptotic factors BAD, caspase-9 and GSK - all of them are inactivated by phosphorylation -[73, 74] and Mitochondria-Associated endoplasmic reticulum Membranes (MAM) proteins such as IP3Rs, Hexokinase 2, and PACS-2 - activated by phosphorylation and involved in MAM integrity and mitochondrial function [75]. In the present research, p-Akt (its active isoform) is decreased in the TBI+vehicle group, the treatment with JM-20 following TBI prevented this decrease and consequently, it maintained cell survival signaling, avoiding apoptosis. JM-20 treatment have already shown this ability to maintain the Akt phosphorylated state and prevent cell death in an ischemia model [30, 32]. In a subarachnoid hemorrhage model, the activation of Akt signal pathway was able to attenuate brain injury [76]. Another involvement of Akt pathway is the neuroprotective and neuritogenic activities of Nerve Growth Factor (NGF) upon binding to its high affinity receptor TrKA. NGF regulate neuronal survival, growth and differentiation [77-79]. In a previous study of our group using the same TBI model, JM-20 was able to increase NGF mRNA levels [33]. Akt has an important mechanism in mitochondria: the ability to regulate hexokinase II through its phosphorylation. The phosphorylation leads to a greater enzyme association with the mitochondrial membrane via binding with the voltage-dependent anionic channel. Hexokinase isoforms I and II are able to directly interact with the mitochondria, which allows direct access to high mitochondrial ATP. Using mitochondrial ATP permits a connection between glycolysis and OXPHOS in low ATP cytosolic levels situations. This mechanism proved to be efficient during hypoxia [80, 81].

It is important to notice that atypical energy metabolism caused by TBI primary and secondary injuries are associated with functional deficits. Mitochondrial dysfunction, ROS overproduction and neuronal apoptosis are responsible for neurological deficits induced by TBI [82, 83]. Taking into account behavioral changes, TBI outcomes are cognitive decline, neurologic and physical deficits and impairment of psychosocial activities and function [84]. In our previous study, TBI was able to induce cognitive deficit (loss in short-term memory) and

decrease spontaneous locomotor and exploratory activity. Now, we evaluated the TBI effects and JM-20 treatment on balance. Dizziness and balance deficits have a prevalence of 39-62% in TBI patients due to postural instability caused by decline in interactions between sensory, motor and muscular systems [85, 86]. In this experiment, TBI non-treated animals spent more time to cross the beam-balance apparatus and had a decrease in latency to fall on rotarod. These results demonstrate that our TBI model induced balance deficits 24h after of injury. Our treatment with JM-20 was effective in attenuating TBI-induced behavioral deficits. We associate these results with the suppression of mitochondrial dysfunction and the preservation of cell survival signaling.

Several studies associated neuroinflammation and disruptions in brain energy metabolism during TBI second injury development. Mitochondrial dysfunction occurs and promotes excessive reactive oxygen species production and consequent inflammatory pathway activation [87, 88]. Our previous study proved that JM-20 treatment following TBI was able to reduce glial cells pro-inflammatory signaling [33]. Previous research established a direct initiation of apoptotic mechanisms by tumor necrosis factor (TNF) and a bidirectional link between brain mitochondrial dynamics and IL-1β signaling. Strategies that are able to neutralize the excessive release of those cytokines can protect the mitochondrial function [63]. Our TBI model induced an increase in TNF-α mRNA levels and JM-20 treatment prevented pro-inflammatory and apoptotic signaling [33]. Neuroinflammation suppression and the reach of energy homeostasis are related to an enhancement of cognitive function in the brain [88, 89]. We believe that JM-20, by combining multipotent agents have complementary effects on TBI treatment. Compounds that pass through biological membranes are more efficient to prevent mitochondrial dysfunction [90]. It has been already proved that JM-20 20 is able to cross the blood-brain barrier and the mitochondrial membrane as well [26, 29].

The main limitation in developing a successful treatment to TBI is the lack of understanding about the complex cellular and molecular events that characterize secondary injury, such as: cell dysfunction and consequent death, glutamate excitotoxicity, mitochondrial dysfunction, oxidative damage, inflammation and necrotic and apoptotic cell death signaling pathway activation [59]. The Secondary injury develops quickly, 24-48h after injury, fragmented mitochondria were increased in hippocampal neurons [91]. Considering all our results, we can notice that, even a mild TBI model is able to induce mitochondrial dysfunction, interrupt pro-survival signaling and induce balance impairment. Some authors suggested that there is a heterogeneous mitochondrial response following TBI, that is, cortical mitochondrial suffers a massive depletion and irreparable structural changes, while hippocampal

mitochondrial are more susceptible to swelling and lose the calcium buffer capacity [92, 93]. We explain JM-20 beneficial effects considering its multi-targeting features, but here, especially its calcium mechanism, which has already proved to inhibit calcium uptake at mitochondrial level. Due to its advantaged structure, JM-20 has a net positive charge and a high level of lipophilicity, that guarantees the crossing of blood-brain barrier and also cell membranes, selectively targeting mitochondria [31]. Due to the importance of mitochondria on TBI development and the consequences of mitochondrial dysfunction on cell death and functional parameters, JM-20 becomes a potential strategy to treat TBI and its secondary injury outcomes.

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Availability of data and material

All data generated or analyzed during this study are included in this published article.

Consent to participate

Not applicable.

Consent for Publication

Not applicable.

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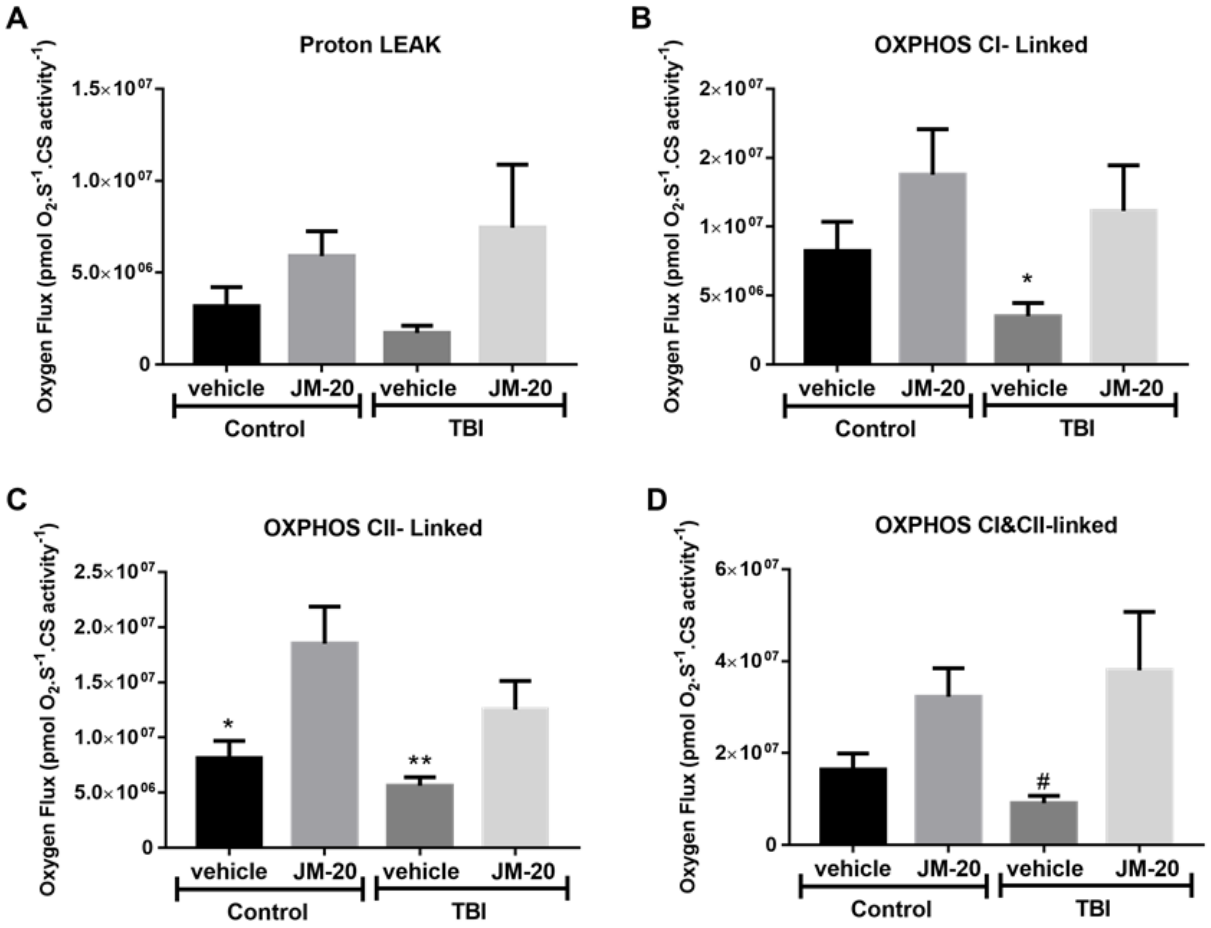

Fig. 1 Effect of JM-20 treatment on mitochondrial function 24 h after TBI. O2 flux was measured in hippocampus homogenate. (A) Proton LEAK. (B) OXPHOS CI-linked. (C) OXPHOS CII-linked. (D) OXPHOS CI&CII-linked. Results are expressed as the mean ± SEM (n= 7). * difference from JM-20 control group ($p < 0.05$). ** difference from JM-20 control group ($p < 0.01$). # difference from TBI+JM-20 group ($p < 0.05$). One-way ANOVA followed by Tukey's post hoc test.

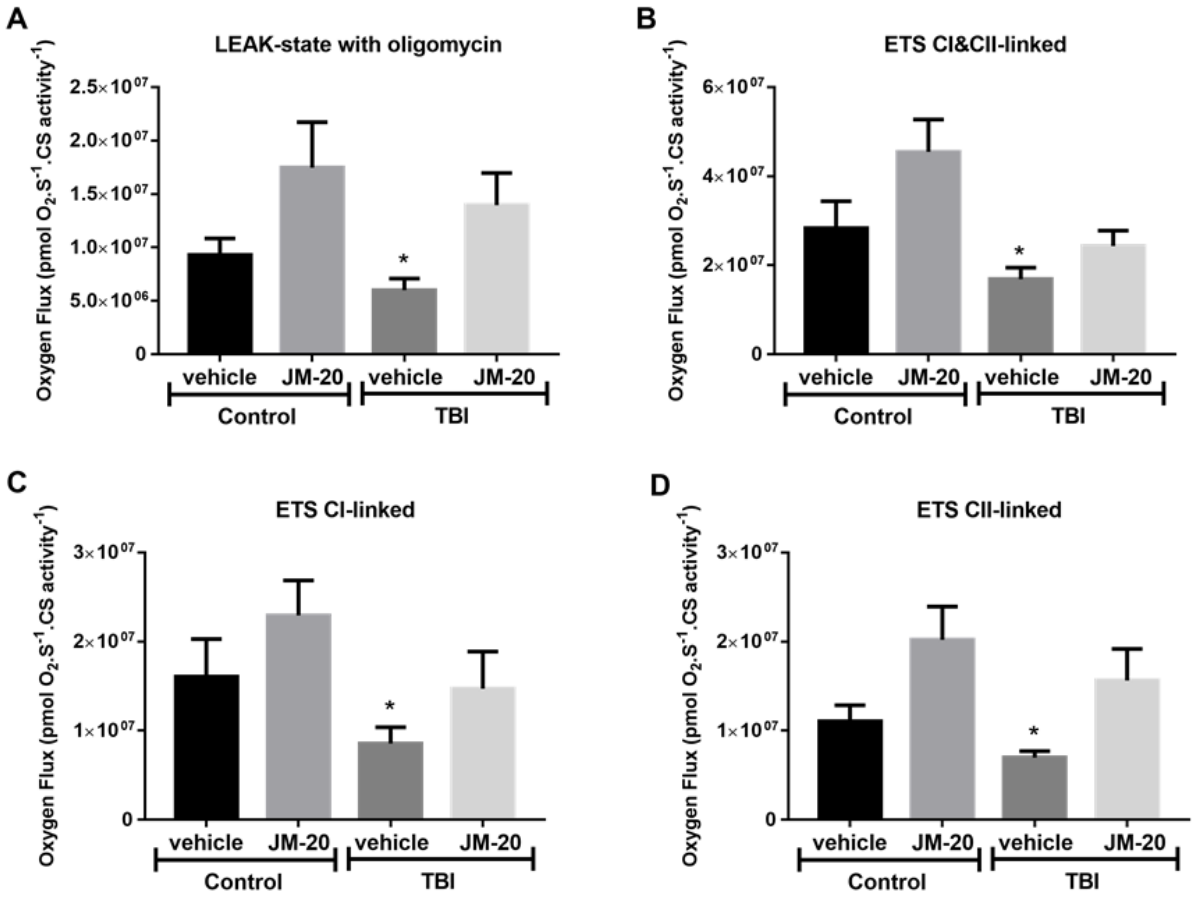

Fig. 2 Effect of JM-20 treatment on mitochondrial function 24 h after TBI. O2 flux was measured in hippocampus homogenate. (A) LEAK state with oligomycin. (B) ETS CI&CII-linked. (C) ETS CI-linked. (D) ETS CII-linked. Results are expressed as the mean ± SEM (n= 7). * difference from JM-20 control group ($p < 0.05$). One-way ANOVA followed by Tukey's post hoc test.

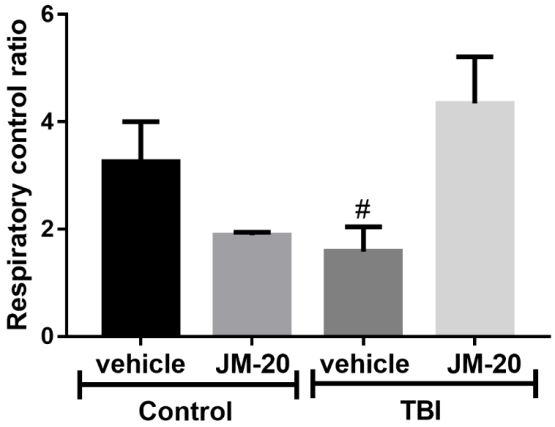

Fig. 3 Effect of JM-20 treatment on respiratory control ratio 24 h after TBI. # difference from TBI+JM-20 group ($p < 0.05$). Results are expressed as the mean ± SEM (n= 7). One-way ANOVA followed by Tukey's post hoc test.

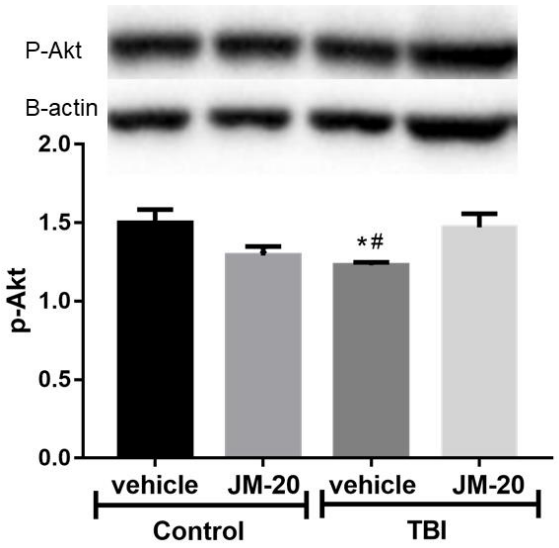

Fig. 4 Effects of TBI and JM-20 treatment on p-Akt expression in hippocampus by Western blotting analysis. Results are expressed as mean ± SEM (n=6). * Different from control+vehicle ($p<0.05$) # Different from TBI+JM-20 group ($p<0.05$). One-way ANOVA followed by Tukey's post hoc test.

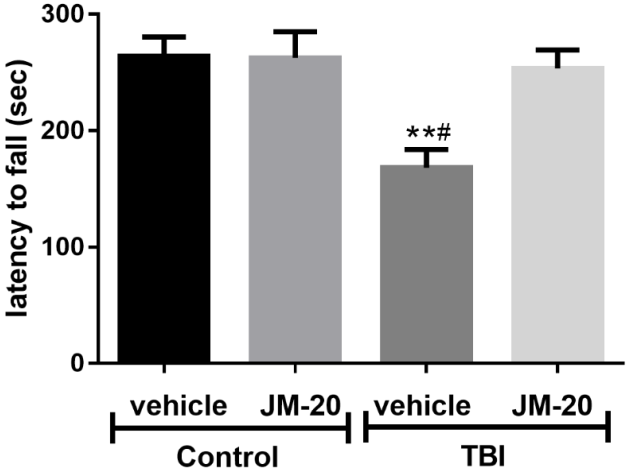

Fig. 5 Effects of TBI and JM-20 treatment on latency to fall during rotarod test. Results are expressed as mean ± SEM (n= 8-11). **different from control+vehicle and control+JM-20 ($p <0.01$) # different from TBI+JM-20. Kruskal-Wallis test followed by Dunn's post hoc test.

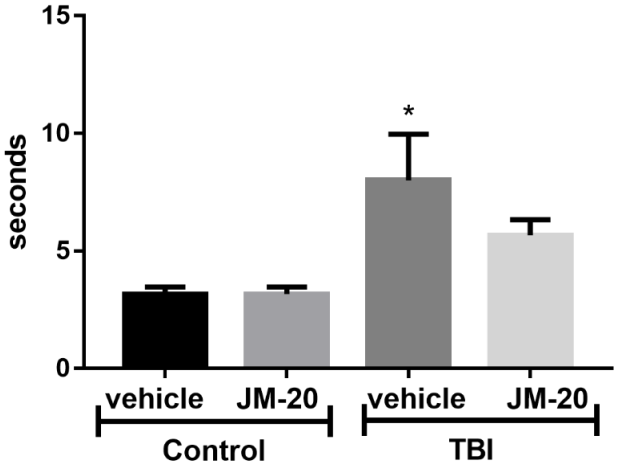

Fig. 6 Effects of TBI and JM-20 treatment on time to complete beam-walk test. Results are expressed as mean ± SEM (n=6). *different from control+vehicle and control+JM-20 ($p < 0.05$). One-way ANOVA followed by Tukey's post hoc test.

4. DISCUSSÃO

Dados apontam o TCE como uma das maiores causas de morbimortalidade mundiais, com grandes gastos de recursos financeiros dos sistemas de saúde, sendo assim considerado um problema de saúde pública (CASSIDY; CARROLL; PELOSO; BORG *et al.*, 2004; NINDS, 2021; PONSFORD; SPITZ; CROMARTY; GIFFORD *et al.*, 2013; ROOZENBEEK; MAAS; MENON, 2013). A incidência do TCE é estimada em 200–300/100.000 pessoas hospitalizadas por ano, e se supõe que esse número seria maior que o dobro se incluísse os indivíduos que não foram hospitalizados (BRAZINOVA; REHORCIKOVA; TAYLOR; BUCKOVA *et al.*, 2021; MAAS; MENON; ADELSON; ANDELIC *et al.*, 2017). Uma pesquisa mostrou que 36,4% dos entrevistados reportaram, pelo menos, um episódio de TCE leve durante a vida, e 6% um episódio de moderada a severa gravidade. Entre todos os entrevistados que relataram terem sofrido TCE, 27,5% não havia procurado atendimento médico (WHITENECK; CUTHBERT; CORRIGAN; BOGNER, 2016).

Em um estudo com indivíduos acometidos por TCE classificados como o nível I de trauma, foi mostrado que cerca de 50% deles não retornaram aos níveis pré-TCE, mesmo após um ano do evento traumático (NELSON; TEMKIN; DIKMEN; BARBER *et al.*, 2019). Danos cognitivos, psicológicos e emocionais após TCE são comumente subestimados, em especial em casos leves. Ainda que sutis, esses problemas podem interferir consideravelmente na rotina do indivíduo (LINSL, 2021). As tendências epidemiológicas recentes apontam que, num futuro próximo, haverá um aumento nas taxas de TCE em pessoas idosas. Essa informação serve de alerta para as autoridades de saúde pública, que precisam encarar esse assunto com mais empenho (LEFEVRE-DOGNIN; COGNE; PERDRIEAU; GRANGER *et al.*, 2021).

A maior dificuldade para desenvolver uma opção de tratamento ideal para o TCE é a característica complexa de sua fisiopatologia, que inclui diversos eventos moleculares e celulares que geram diferentes consequências, como disfunção mitocondrial e celular, dano oxidativo, excitotoxicidade glutamatérgica, inflamação e morte celular por apoptose ou necrose (WANG; SULLIVAN; SPRINGER, 2017). Como dito previamente, a lesão primária causa dano imediato e irreversível na área que absorve a energia mecânica do insulto traumático. Já a lesão secundária apresenta múltiplos mecanismos fisiopatológicos posteriores e com possibilidade de reversão. Por tal motivo, os agentes terapêuticos multialvo são considerados interessantes para o tratamento de TCE, pois são capazes de atingir mais de um dos mecanismos da lesão secundária (LOANE; FADEN, 2010).

Os mecanismos pós-TCE mais bem descritos são: crise metabólica, disfunção glial ou neuronal e dano vascular (HUBBARD; DONG; CRUZ; RUMBAUT, 2021). Esses três mecanismos foram encontrados com nosso modelo de TCE leve. Em nosso trabalho, o JM-20 foi utilizado pela primeira vez como um possível tratamento para modelo de TCE leve. Aqui, os nossos dados mostraram que o tratamento, 24h após o evento traumático, foi capaz de atuar em diversos mecanismos e sinais comuns pós TCE, como: I) reduzir o edema cerebral; II) reduzir a ativação reativa das células gliais, evitando, dessa forma, a liberação de citocinas inflamatórias; III) preservar a ativação de uma das vias responsáveis pela sobrevivência celular; IV) preservar os níveis de BDNF; V) aumentar os níveis de neurotrofinas relacionadas com o fenótipo neuroprotetor das células gliais; VI) evitar a disfunção mitocondrial através de maior consumo do fluxo de oxigênio durante a fosforilação oxidativa; VII) promover a funcionalidade mitocondrial; VIII) prevenir a perda de memória de curto prazo; IX) evitar danos na atividade locomotora e exploratória.

Os dois principais pontos desses estudos são a neuroinflamação e a disfunção mitocondrial, importantes mecanismos desenvolvidos durante a lesão secundária. A neuroinflamação contínua e as disfunções no metabolismo energético cerebral ocorrem com frequência em casos de TCE, e a regulação desses dois processos para a normalidade tem relação com a recuperação da função cognitiva dos indivíduos (ZHANG; GAO; LI; SUN *et al.*, 2020). Cada vez mais, é reconhecido que disfunção mitocondrial e inflamação são danos interdependentes. A disfunção mitocondrial é capaz de induzir à inflamação. Se a função mitocondrial estiver inibida ou comprometida, a inflamação ocorre; o contrário também é verdadeiro, pois a inflamação altera a função mitocondrial. A combinação de disfunção mitocondrial, falha bioenergética e neuroinflamação pode contribuir para o desenvolvimento ou, ainda, para a progressão da neurodegeneração (BROWN, 1997; DI FILIPPO; CHIASSERINI; TOZZI; PICCONI *et al.*, 2010; WILKINS; CARL; GREENLIEF; FESTOFF *et al.*, 2014; ZHOU; YAZDI; MENU; TSCHOPP, 2011).

A importância da mitocôndria vai além da transformação de energia, ela é capaz de atuar na imunidade através da modulação dos estados metabólicos e fisiológicos em diferentes tipos de células imunes (BREDA; DAVANZO; BASSO; SARAIVA CAMARA *et al.*, 2019). Há fortes evidências de que a mitocôndria atua no direcionamento do fenótipo microglial (PARK; CHOI; MIN; PARK *et al.*, 2013). A fosforilação oxidativa e o ciclo do ácido cítrico são necessários para manter as funções de macrófagos anti-inflamatórios, pois estes requerem transfomação adequada de energia e utilizam a fosforilação oxidativa para transcrição de genes de reparação tecidual dependentes de ATP. Além disso, necessitam de produção mínima de

espécies reativas de oxigênio, um mecanismo de *scavenger* funcionando adequadamente e, ainda, oxidação de ácidos graxos e aminoácidos eficiente para produção de fatores de crescimento e para dar suporte à respiração mitocondrial, através do provimento de metabólitos para o ciclo do ácido cítrico. Em condições nas quais a fosforilação oxidativa está comprometida, os macrófagos não são capazes de se repolarizarem em seu fenótipo anti-inflamatório, e a plasticidade fica limitada à ativação pró-inflamatória (DEVANNEY; STEWART; GENSEL, 2020; VAN DEN BOSSCHE; BAARDMAN; OTTO; VAN DER VELDEN *et al.*, 2016).

Evidências apontam que o metabolismo também tem papel importante na determinação da resposta inflamatória microglial. No quesito metabolismo, a micróglia parece se comportar de maneira semelhante aos macrófagos pois possui a mesma infraestrutura genética envolvida tanto na glicólise quanto na fosforilação oxidativa. Após estímulo pró-inflamatório, a micróglia em seu estado primário utiliza a glicólise como fonte de energia principal, enquanto a ativação do fenótipo anti-inflamatório depende da função mitocondrial eficiente e da fosforilação oxidativa (GHOSH; CASTILLO; FRIAS; SWANSON, 2018; NAGY; FEKETE; HORVATH; KONCSOS *et al.*, 2018). Alterações na mitocôndria estão intrinsicamente ligadas ao estímulo pró-inflamatório, pois este suprime a função mitocondrial para facilitar a produção de citocinas pró-inflamatórias. Por outro lado, a respiração mitocondrial e a fosforilação oxidativa eficientes são essenciais para que a micróglia adote fenótipos reparadores/anti-inflamatórios (DEVANNEY; STEWART; GENSEL, 2020; VAN DEN BOSSCHE; BAARDMAN; OTTO; VAN DER VELDEN *et al.*, 2016).

Neste modelo, o TCE foi capaz de induzir o aumento de citocinas inflamatórias, como, por exemplo, o TNF-α. Essa citocina tem um papel importante no desenvolvimento neuronal, na sobrevivência celular, na plasticidade sináptica e na homeostase iônica. Alterações nessa sinalização estão envolvidas em diversos processos fisiopatológicos de doenças que afetam o SNC, especialmente relacionadas à infiltração de células imunes e ao aumento da permeabilidade da barreira hematoencefálica (KIM; WASS; CROSS; OPAL, 1992; RAMILO; SAEZ-LLORENS; MERTSOLA; JAFARI *et al.*, 1990). Alterações na barreira hematoencefálica estão relacionadas ao aumento do edema cerebral, componente crucial para o desenvolvimento exacerbado da lesão secundária, principalmente da neuroinflamação, e que está intimamente ligado à mortalidade e a danos cerebrais (JULLIENNE; OBENAUS; ICHKOVA; SAVONA-BARON *et al.*, 2016; SHAMSI MEYMANDI; SOLTANI; SEPEHRI; AMIRESMAILI *et al.*, 2018). A ruptura mecânica, mesmo sendo a causa mais imediata, não é o único fator importante para o dano da barreira hematoencefálica. Cascatas que incluem

citocinas pró-inflamatórias e neuroinflamação, fatores angiogênicos, moléculas de adesão ou fatores que promovam o extravasamento de proteínas e rearranjos no citoesqueleto são outros fatores adicionais que contribuem para o desenvolvimento do edema vasogênico, que tem seu pico entre 6 e 24h após o trauma. Estes mecanismos podem aumentar a pressão oncótica, ocluir pequenos vasos, causar isquemia local e, com isso, exacerbar o edema citotóxico (WINKLER; MINTER; YUE; MANLEY, 2016). O tratamento com JM-20 foi capaz de reduzir o edema cerebral 24h após o TCE, prevenindo, dessa maneira, o aumento da sinalização inflamatória.

A disfunção mitocondrial também é prejudicial aos astrócitos, pois leva à produção insuficiente de ATP, à desregulação do metabolismo de cálcio, à indução de resposta inflamatória e à desregulação tanto na liberação quanto na captação de glutamato (GOLLIHUE; NORRIS, 2020). Os astrócitos são responsáveis por 20% do consumo de oxigênio total do cérebro, a maior parte disso ocorre durante a fosforilação oxidativa, dentro das mitocôndrias astrocitárias, a caminho da produção de ATP (BLUML; MORENO-TORRES; SHIC; NGUY *et al.*, 2002). Foi mostrado que a mitocôndria em estado não-saudável nos processos astrocíticos está relacionada à perda neuronal após isquemia, mesmo que o número de astrócitos permaneça estável, o que indica que a perda de mitocôndrias nas junções astrócito-sinapse pode desempenhar importante papel na perda neuronal (ITO; HAKAMATA; KAWAKAMI; OYANAGI, 2009).

Assim como em outros tipos celulares, a mitocôndria astrocitária é vulnerável ao envelhecimento, à lesão e a doenças. Grande parte dos estímulos que são gatilho para uma robusta ativação astrocitária — como isquemia, lesão no tecido cerebral, inflamação e liberação de citocinas — também levam à disfunção mitocondrial nos astrócitos. Os sinais incluem desregulação do cálcio, geração excessiva de espécies reativas de oxigênio e cascatas de morte celular. Esses fatores podem ter efeitos de longo alcance através da rede de astrócitos e causar maior ativação dos astrocitária, entrando, dessa forma, em *looping* de ativação astrócitária e desenvolvimento de disfunção mitocondrial (ABRAMOV; CANEVARI; DUCHEN, 2004).

Semelhante aos neurônios, astrócitos ativados apresentam grande desregulação de cálcio durante o processo de envelhecimento, lesão e doença, caracterizado por altos níveis de cálcio citosólico ou, ainda, por oscilações de cálcio (AGULHON; PETRAVICZ; MCMULLEN; SWEGER *et al.*, 2008; KUCHIBHOTLA; LATTARULO; HYMAN; BACSKAI, 2009). O acúmulo excessivo de cálcio dentro da mitocôndria, que ocorre frequentemente quando há alterações nos mecanismos regulatórios, depleta o potencial de membrana mitocondrial, e, como consequência disso, há prejuízo na produção de ATP — ainda,

em casos extremos, há abertura do poro de transição mitocondrial (HUNTER; HAWORTH; SOUTHARD, 1976).

A mitocôndria não tem papel primordial apenas como transformadora de energia, diversos estudos mostram que ela é uma importante reguladora para a sinalização de diversas vias e também está envolvida nos processos que determinam a função e o destino da célula (YARBRO; EMMONS; PENCE, 2020). Há evidência de que a mitocôndria fragmentada, quando liberada pela micróglia aciona a resposta astrocitária de fenótipo A1, ou seja, a resposta pró-inflamatória (JOSHI; MINHAS; LIDDELOW; HAILESELASSIE *et al.*, 2019). A mitocôndria é, também, o centro de diversas funções neuroprotetoras associadas à ativação astrocitária. Para liberação de fatores de crescimento, estabilização das cicatrizes gliais e aumento do suporte sináptico, necessita-se que haja suprimento de energia na mitocôndria astrocitária. Em testes *in vivo*, a morte neuronal aumentada foi relatada após lesão isquêmica quando analisada especificamente a cadeira transportadora de elétrons nas mitocôndrias astrocitárias (FIEBIG; KEINER; EBERT; SCHAFFNER *et al.*, 2019).

Além da relação com a funcionalidade mitocondrial, a fosforilação da proteína Akt parece estar envolvida na redução da sinalização inflamatória. Como uma quinase lipídica, a fosfatidilinositol 3-quinase (PI3K) pode induzir a fosforilação da Akt para regular sobrevivência celular, crescimento e angiogênese em resposta a sinais extracelulares (ZHU; LI; WU; HUANG *et al.*, 2014). A ativação da via PI3K/Akt leva à supressão tanto do apoptose neuronal quanto da neuroinflamação (CHEN; PENG; SHERCHAN; MA *et al.*, 2020).

Em um estudo recente, que induziu hemorragia subaracnóidea em ratos, o tratamento foi capaz de modular positivamente a expressão de PI3K e p-Akt, diminuiu a expressão de p-NF-κB p65, TNF-α, e IL-1β, além de reduzir a infiltração de neutrófilos e a ativação microglial (ZHU; ENKHJARGAL; HUANG; ZHANG *et al.*, 2018). Há, também, relação da Akt com as atividades neuroprotetoras e neurogênicas do fator de crescimento neural (NGF) devido à ligação de alta afinidade ao seu receptor TrKA (JING; TAPLEY; BARBACID, 1992). Os níveis de NGF apresentam diminuição diante da indução de inflamação (ARSENIJEVIC; HERNADFALVI; VON MEYENBURG; ONTENIENTE *et al.*, 2007). Esses resultados mostram que a sobrevivência celular não está relacionada apenas com o aumento da funcionalidade mitocondrial propiciado pelo tratamento com JM-20, mas também com a redução das cascatas de neuroinflamação e comprova que o JM-20 é capaz de proteger toda unidade neurovascular também no modelo proposto.

No nosso estudo, o JM-20 foi capaz de prevenir a reatividade microglial e astrocitária, preservar a Akt em seu estado fosforilado, bem como manter a funcionalidade mitocondrial,

reduzindo, dessa maneira, a retroalimentação entre neuroinflamação e disfunção mitocondrial. Alguns fatores não abordados em nossos estudos podem ser os responsáveis por esses resultados, como, por exemplo, a modulação da enzima hexoquinase pelo JM-20. O aumento da atividade desta enzima já mostrou estar relacionada a melhoras na eficiência mitocondrial (REAL-HOHN; NAVEGANTES; RAMOS; RAMOS-FILHO *et al.*, 2018). A regulação negativa da hexoquinase gera redução da produção de lactato e da glicólise. Uma menor liberação de lactato está sinergicamente relacionada a atividades neurotóxicas dos astrócitos e da micróglia. O lactato liberado pelos astrócitos parece limitar a morte neuronal em situações de estresse, pois ele está envolvido no suporte metabólico dos neurônios (CHAO; GUTIERREZ-VAZQUEZ; ROTHHAMMER; MAYO *et al.*, 2019; CHOI; GORDON; ZHOU; TAI *et al.*, 2012).

Em um estudo utilizando cultura de astrócitos, a ativação das cascatas inflamatórias através do NF-κB parecem estar relacionadas com a supressão da atividade da enzima hexoquinase II e concomitante redução na geração de lactato para o suporte neuronal (CHAO; GUTIERREZ-VAZQUEZ; ROTHHAMMER; MAYO *et al.*, 2019). A atividade da hexoquinase é fortemente ligada à atividade mitocondrial, visto que essa enzima se liga à membrana mitocondrial via canal aniônico dependente de voltagem, e essa ligação pode modular positivamente a atividade de ambas (NAKASHIMA; MANGAN; COLOMBINI; PEDERSEN, 1986; VIITANEN; GEIGER; ERICKSON-VIITANEN; BESSMAN, 1984). A dissociação da hexoquinase do canal aniônico dependente de voltagem sinalizada para a ativação do inflamassoma NLRP3, um complexo citosólico que regula o processamento e a secreção de algumas interleucinas, incluindo a IL-1β (WOLF; REYES; LIANG; BECKER *et al.*, 2016). Com base nisso, podemos supor que o ponto de convergência entre a redução da neuroinflamação e a prevenção da disfunção mitocondrial dadas pelo tratamento com JM-20 seja a regulação da enzima hexoquinase pelo JM-20.

5. CONCLUSÃO E PERSPECTIVAS

Diante dos resultados apresentados, podemos concluir que este trabalho endossa as evidências do papel neuroprotetor do JM-20 em mais uma doença neurodegenerativa. Em linhas gerais, no modelo de TCE proposto, o JM-20 foi capaz de atenuar e, em alguns casos, reverter déficits locomotores e de memória, diminuir o edema cerebral, bem como reduzir a reatividade astrocitária e a ativação microglial, diminuição que se relaciona com a menor liberação de citocinas pró-inflamatórias e com a maior liberação de moléculas neuroprotetoras.

O tratamento também foi capaz de prevenir a disfunção mitocondrial e melhorar a funcionalidade da mitocôndria após o evento traumático, fato que pode estar relacionado com a manutenção da ativação de uma das mais importantes vias de sobrevivência neuronal. Nossos resultados e a literatura prévia mostram que os eventos de inflamação e disfunção mitocondrial são codependentes e se retroalimentam. Ao evitar que esses dois eventos ocorram, o JM-20 exerce grande efeito neuroprotetor no modelo proposto.

Os sintomas e as cascatas de sinalização desencadeadas pelo TCE são comuns a outras doenças neurodegenerativas. Sendo assim, os resultados aqui obtidos indicam que essa molécula apresentou efeitos neuroprotetores ao atuar em dois importantes pontos da lesão secundária induzida por TCE, tornando-se, dessa maneira, um potencial alvo de estudo para o tratamento do TCE. Quanto às perspectivas futuras para o avanço da construção de conhecimento acerca desse tema, os principais pontos são:

1) Investigar mais profundamente as cascatas inflamatórias, como, por exemplo, a p38 MAPK e NFκB;
2) Avaliar vias de morte celular, como citocromo C oxidase e as caspases, a fim de entender a sinalização da mitocôndria em relação a esses eventos;
3) Investigar se os resultados positivos alcançados pelo tratamento com JM-20 estão relacionados à modulação da enzima hexoquinase;
4) Analisar os efeitos do JM-20 no tratamento do TCE leve em diferentes tempos pós-traumatismo cranioencefálico, a fim de investigar a eficácia do tratamento não só a curto prazo, visto que esta condição tem importantes desfechos crônicos.

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