UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas

Programa de Pós-Graduação em Biologia Celular

Caroline Amaral Machado

PAPEL DO SISTEMA RENINA ANGIOTENSINA NO TRAUMATISMO CRANIOENCEFÁLICO

Belo Horizonte
2023

Caroline Amaral Machado

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Tese apresentada ao Programa de Pós-Graduação em Biologia Celular do Departamento de Morfologia da Universidade Federal de Minas Gerais como requisito parcial para a obtenção do título de Doutora em Biologia Celular.

Orientadora: Dra. Aline Silva de Miranda

Belo Horizonte
2023

043 Machado, Caroline Amaral.
Papel do sistema renina angiotensina no traumatismo cranioencefálico [manuscrito] / Caroline Amaral Machado. – 2023.
151 f. : il. ; 29,5 cm.

Orientador: Aline Silva de Miranda.
Tese (doutorado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas. Programa de Pós-Graduação em Biologia Celular.

1. Biologia Celular. 2. Traumatismos Craniocerebrais. 3. Inflamação. 4. Transtornos Cognitivos. 5. Sistema Renina-Angiotensina. I. Miranda, Aline Silva de. II. Universidade Federal de Minas Gerais. Instituto de Ciências Biológicas. III. Título.

CDU: 576

Ficha Catalográfica elaborada pelo bibliotecário Rafael Gonçalves Dias (CRB: 6 - 3277)

UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR

ATA DA DEFESA DE TESE DE DOUTORADO DE CAROLINE AMARAL MACHADO

Às **oito horas** do dia **30 de outubro de 2023**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora da Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho final intitulado: **"PAPEL DO SISTEMA RENINA ANGIOTENSINA NO TRAUMATISMO CRANIOENCEFÁLICO"**, requisito final para obtenção do grau de Doutora em Biologia Celular. Abrindo a sessão, a Presidente da Comissão, **Dra. Aline Silva de Miranda**, após dar conhecimento aos presentes do teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	**Instituição**	**Indicação**
Dra. Aline Silva de Miranda	UFMG	Aprovada
Dra. Vivian Vasconcelos Costa	UFMG	Aprovada
Dr. Antônio Carlos Pinheiro de Oliveira	UFMG	Aprovada
Dr. Luana da Silva Chagas	UFF	Aprovada
Dra. Eliana Cristina de Brito Toscano	UFJF	Aprovada

Pelas indicações, a candidata foi considerada: APROVADA

O resultado final foi comunicado publicamente à candidata pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 30 de outubro de**

2023.

Dra. Aline Silva de Miranda (Orientadora)_____

Dra. Vivian Vasconcelos Costa_____

Dr. Antônio Carlos Pinheiro de Oliveira_____

Dr. Luana da Silva Chagas_____

Dra. Eliana Cristina de Brito Toscano_____

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Documento assinado eletronicamente por **Luana da Silva Chagas**, **Usuária Externa**, em 16/11/2023, às 15:36, conforme horário oficial de Brasília, com fundamento no art. 5º do Decreto nº 10.543, de 13 de novembro de 2020.

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Referência: Processo nº 23072.267527/2023-79 SEI nº 2775910

Criado por soaresgabriel, versão 2 por soaresgabriel em 06/11/2023 10:47:50.

À minha família por todo amor e por acreditarem em mim quando eu mesma não poderia.

AGRADECIMENTOS

À minha orientadora, Profa. Aline, pelo acolhimento, orientação, carinho e por ser um exemplo a ser seguido em toda a minha carreira. Muito obrigada por abrir as portas no seu grupo para mim, pela dedicação e compreensão nos momentos difíceis e pela alegria de compartilhar o seu conhecimento e vivência profissional.

A todos os colaboradores deste projeto, que sem a fundamental ajuda não seria possível a realização deste trabalho. Em especial, a profa. Eliana Toscano, a profa. Milene Rachid, Walison Nunes, Heliana Barros e Brener Carvalho por toda ajuda.

A todos os meus amigos e colaboradores do Laboratório de Neurobiologia Conceição Machado, que ao longo de quase 7 anos tem me auxiliado e me fizeram sentir em casa. Em especial, ao Prof. Victor Rodrigues Santos por todo o apoio, conselhos e por ser tão humano.

A todos os amigos e colaboradores que fizeram ou fazem parte do Grupo de Neuroimunologia Experimental e Clínica. Em especial aos meus queridos amigos João Luís, Cristian Alexandre, Larissa Sabino, Heliana Barros e Emanuele Pozzolini. A minha doce amiga Barbara Zuccolotto pelo carinho e companheirismo nessa jornada.

A todos meus amigos pelo apoio e amizade mesmo que de longe, em especial, Marianna Bastos, Ludmila Souza, Marcela Ribeiro, Jonas Pereira, Júlio Cesar Rodrigues, Lud Milla Lorena e Clara Regina.

À irmã que a ciência me trouxe, Bruna da Silva Oliveira, muito obrigado pela parceria de trabalho e amizade em todos esses anos de Pós-Graduação, fico muito feliz que nossos caminhos se cruzaram.

A todas as políticas públicas de acesso à educação, em especial ao PROUNI, que possibilitaram com que eu pudesse mudar a minha vida e da minha família.

À minha família por todo o suporte, especialmente a minha irmã por ser minha parceira de vida e a minha amada sobrinha por acreditar em mim.

Aos meus pais por todo o amor e compreensão e por serem o meu porto seguro.

Agradeço a Deus pela sabedoria e resiliência nos dias difíceis.

"Quando a mulher negra se movimenta, toda a estrutura da sociedade se movimenta com ela".

Angela Davis

RESUMO

O traumatismo cranioencefálico (TCE) é qualquer lesão dirigida aos vasos sanguíneos, meninges, encéfalo, crânio, causado por uma força mecânica externa. O TCE atinge principalmente, a faixa etária economicamente ativa da população nos países desenvolvidos e em desenvolvimento, acarretando significativo impacto socioeconômico. A importância do TCE no contexto de saúde pública tem aumentado fortemente nos últimos anos, devido a estudos que têm relacionado essa doença com danos comportamentais e cognitivos que podem surgir após o evento traumático, como depressão, ansiedade e prejuízos de memória e aprendizado. A fisiopatologia do TCE não foi completamente elucidada, mas sabe-se que processos inflamatórios estão relacionados com a evolução de lesões secundárias nessa doença. Portanto, acreditamos que o Sistema Renina-Angiotensina (SRA) possa participar da fisiopatologia do TCE, bem como, que o eixo contra regulatório desse sistema pode desempenhar papel neuroprotetor por meio da regulação desses processos inflamatórios. Nesse sentido, o presente trabalho tem como objetivo principal investigar o papel do SRA em modelo experimental de TCE. Bem como, caracterizar as alterações cognitivas e comportamentais transitórias e ou permanentes, associando-as com alterações morfológicas e níveis de mediadores inflamatórios após o TCE. Além disso, o nosso estudo proposto permitirá também a identificação de potenciais biomarcadores inflamatórios, possibilitando o desenvolvimento de novas estratégias terapêuticas.

Palavras-chave: traumatismo cranioencefálico; sistema renina angiotensina; neuroinflamação; transtornos cognitivos, alterações comportamentais.

ABSTRACT

Traumatic brain injury (TBI) is any injury directed to the vessels, meninges, brain, or skull, caused by an external mechanical force. TBI mainly affects the economically active age group of the population in developed and developing countries, causing significant socioeconomic impact. The importance of TBI in the public health context has increased strongly in recent years, due to studies that have linked this disease to neuropsychiatric and cognitive deficits that may arise after the traumatic event, such as depression, anxiety and memory and learning impairments. The pathophysiology of TBI is still poorly understood, but it is known that inflammatory processes are related to the development of secondary injuries in this disease. Therefore, we believe that the renin-angiotensin system (RAS) may participate in the pathophysiology of TBI, as well as that the counter-regulatory axis of this system may play a neuroprotective role through the regulation of these inflammatory processes. In this sense, the main objective of the present work is to investigate the role of the RAS in an experimental model of TBI. As well as, characterize the transient and/or permanent cognitive and behavioral changes, associating them with morphological changes and levels of inflammatory mediators after TBI. Furthermore, our proposed study will also allow the identification of potential inflammatory biomarkers, enabling the development of new therapeutic strategies.

Keywords: traumatic brain injury; renin-angiotensin system; neuroinflammation; cognitive disorders, behavioral changes.

LISTA DE ABREVIATURAS E SIGLAS

Ang I - Angiotensina I

Ang II - Angiotensina II

ApoE - apolipoproteína E

ApoE−/− - knockout para ApoE

AT1 - receptor de angiotensina do tipo I

AT2 - receptor de angiotensina do tipo II

ECA 1 - enzima de conversão do angiotensina I

ECA 2 - enzima de conversão da angiotensina II

ICC - impacto cortical controlado

IL - interleucina

iNOS - óxido nítrico-sintase induzida

PLF - percussão lateral por fluido

KO - *Knockout*

SRA - Sistema Renina-Angiotensina

SNC- Sistema nervoso central

TCE - Traumatismo cranioencefálico

TNF-α - fator de necrose tumoral

APRESENTAÇÃO

O presente texto tem como objetivo apresentar os resultados obtidos durante o doutorado, no qual investigamos o potencial papel do Sistema Renina-Angiotensina (SRA) em um modelo de Traumatismo cranioencefálico (TCE) leve, através da investigação do estado e níveis desses componentes em regiões cerebrais, bem como a partir do uso de moduladores desse sistema para diminuir alterações comportamentais e cognitivas após a lesão. Para tanto demos continuidade na caracterização do modelo de trauma leve em camundongos que foi padronizado durante o mestrado. Para caracterização do modelo nós continuamos a estudar as alterações comportamentais e cognitivas após a lesão e investigamos a associação dessas alterações com danos nas lesões primárias e secundárias após o TCE. Bem como, buscamos na literatura as principais estratégias imunomoduladoras em TCE para pautar nosso trabalho com o SRA e também apresentamos esses dados nesse texto. Também incluímos nesse texto alguns resultados com o uso do nosso modelo de TCE em animais *knockout* para a apolipoproteína E (ApoE−/−) que foram obtidos durante o doutorado, mas que não compuseram os artigos. É importante mencionar que pude contribuir como coautora do artigo de revisão intitulado *"Potential Role of Adult Hippocampal Neurogenesis in Traumatic Brain Injury",* (Marzano et al., Curr Med Chem. 2022; 29(19):3392-3419) onde analisamos estudos pré-clínicos atuais que avaliaram diferentes abordagens para melhorar a neurogênese após o TCE. Além disso também tive a oportunidade de ser coautora de um trabalho do nosso grupo, intitulado "*The potential role of renin-angiotensin system in mild traumatic brain injury*", (Barros et al., Neurol Sci. 2022 May;43(5):3353-3359) em que mostramos a evidência do envolvimento de moléculas do SRA em pacientes acometidos com TCE leve.

A tese foi estruturada em 10 partes principais: i) Apresentação; ii) Introdução; iii) Hipótese; iv) Justificativa; v) Objetivos; vi) Métodos; vii) Resultados, sob a forma de artigos publicados, submetido e em preparação e dados adicionais ainda não publicados; viii) Considerações finais; ix) Conclusões.

No artigo 1 intitulado "*Immune-Based Therapies for Traumatic Brain Injury: Insights from pre-clinical studies*" publicado na revista *Current Medicinal Chemistry* (Machado et al., 2019) discutimos estratégias terapêuticas para tratar TCE com foco em abordagens imunomoduladoras e/ou anti-inflamatórias no contexto da fase pré-clínica.

No artigo 2 intitulado "*Weight-Drop Model as a Valuable Tool to Study Potential Neurobiological Processes Underlying Behavioral and Cognitive Changes Secondary to Mild Traumatic Brain Injury*" publicado na revista *Journal of Neuroimmunology* (Machado et al., 2023), empregamos um modelo de queda de peso para investigar os mecanismos secundários ao dano cognitivo e comportamental após TCE leve em camundongos. Observamos que os camundongos submetidos ao TCE leve apresentaram comportamento do tipo ansioso, comportamento do tipo depressivo, dano na memória espacial e dano locomotor. Essas alterações foram associadas a alterações em níveis de mediadores inflamatórios, ativação microglial e de astrócitos e aumento do estresse oxidativo. Fizemos uma análise cinética que forneceu evidências originais e robustas de que o TCE leve pode afetar não apenas o lado ipsilateral, mas também o lado contralateral da lesão.

No artigo 3 intitulado "*Involvement of Renin-Angiotensin System (RAS) components in Mild Traumatic Brain Injury*", que está submetido no momento (janeiro/2024) na revista *Neuroscience Bulletin*, demonstramos que o nosso modelo de TCE leve é capaz de induzir alterações nos componentes do eixo clássico e alternativo do sistema renina-angiotensina em regiões cerebrais chave para aprendizagem e memória. Além disso, mostramos que o perindopril, foi capaz de diminuir o dano locomotor agudo após o TCE leve em camundongos.

No artigo 4 intitulado "*Striatal Renin-Angiotensin System and Oxidative Stress activation is associated with motor learning impairment after experimental mild traumatic brain injury*", em preparação para submissão, nós mostramos que o nosso modelo de TCE leve é capaz de induzir danos na aprendizagem motora de camundongos. Esses danos foram associados com alterações nos componentes do sistema renina-angiotensina e de mediadores inflamatórios e com aumento do estresse oxidativo no estriado.

SUMÁRIO

1 – INTRODUÇÃO 15
2 – HIPÓTESE 24
3 – JUSTIFICATIVA 24
4 – OBJETIVOS 25
5 – MATERIAL E MÉTODOS 26
6 – RESULTADOS 39
6.1 ARTIGO CIENTÍFICO I publicado na revista *Current Medicinal Chemistry* 39
Título: *Immune-Based Therapies for Traumatic Brain Injury: Insights from pre-clinical studies*
6.2 - ARTIGO CIENTÍFICO II publicado na revista *Journal of Neuroimmunology* 67
Título: *Weight-Drop Model as a Valuable Tool to Study Potential Neurobiological Processes Underlying Behavioral and Cognitive Changes Secondary to Mild Traumatic Brain Injury* ... 67
6.3- ARTIGO CIENTÍFICO III submetido na revista *Neuroscience Bulletin* 94
Título: *Involvement of Renin-Angiotensin System (RAS) components in Mild Traumatic Brain Injury*.....
7.4 - ARTIGO CIENTÍFICO IV 124
Título: *Striatal Renin-Angiotensin System and Oxidative Stress activation is associated with motor learning impairment after experimental mild traumatic brain injury* 124
6.5 – Resultados produzidos durante o doutorado que não foram incluídos nos artigos 144
7 – CONSIDERAÇÕES FINAIS 147
8 – CONCLUSÃO 148
9 - REFERÊNCIAS 149
PRODUÇÃO BIBLIOGRÁFICA E OUTRAS ATIVIDADES 149

1 – INTRODUÇÃO

1.1 Definição do Traumatismo cranioencefálico

O traumatismo cranioencefálico (TCE) pode ser definido como uma lesão cerebral que causa danos ou alteração na função cerebral e que é causada por uma força mecânica externa. A definição de TCE ainda hoje não é unanimidade e isso impacta diretamente nos dados epidemiológicos (Menon et al. 2010).

1.2 Epidemiologia do TCE

O TCE é uma das principais causas de morbidade e mortalidade em todo o mundo. Ele afeta adultos jovens em idade economicamente ativa, que muitas vezes não conseguem retornar a vida funcional após a lesão, ampliando os danos causados pelo TCE para sociedade. Nesse contexto ele tem sido chamado de “epidemia silenciosa’’, devido as sequelas de longo prazo deixadas pela lesão, tanto no aspecto clínico quanto socioeconômico (Coburn, 1992).

Dados globais mostram que o TCE afeta aproximadamente 69 milhões de pessoas anualmente (Dewan et al., 2018) (Figura 1). De maneira geral, por mais que em algumas regiões a incidência de TCE seja muito reportada, a falta de padronização na coleta de dados torna difícil traçar o perfil epidemiológico de algumas áreas. Um estudo retrospectivo realizado entre 1997 e 2003 mostrou que a incidência de TCE foi de 453 por 100 mil habitantes em uma unidade de urgência britânica (Yates et al., 2006). Enquanto que um estudo prospectivo que ocorreu entre 2000 a 2001 mostrou que na Alemanha a incidência foi de 332 por 100 mil habitantes (Rickels et al., 2010).

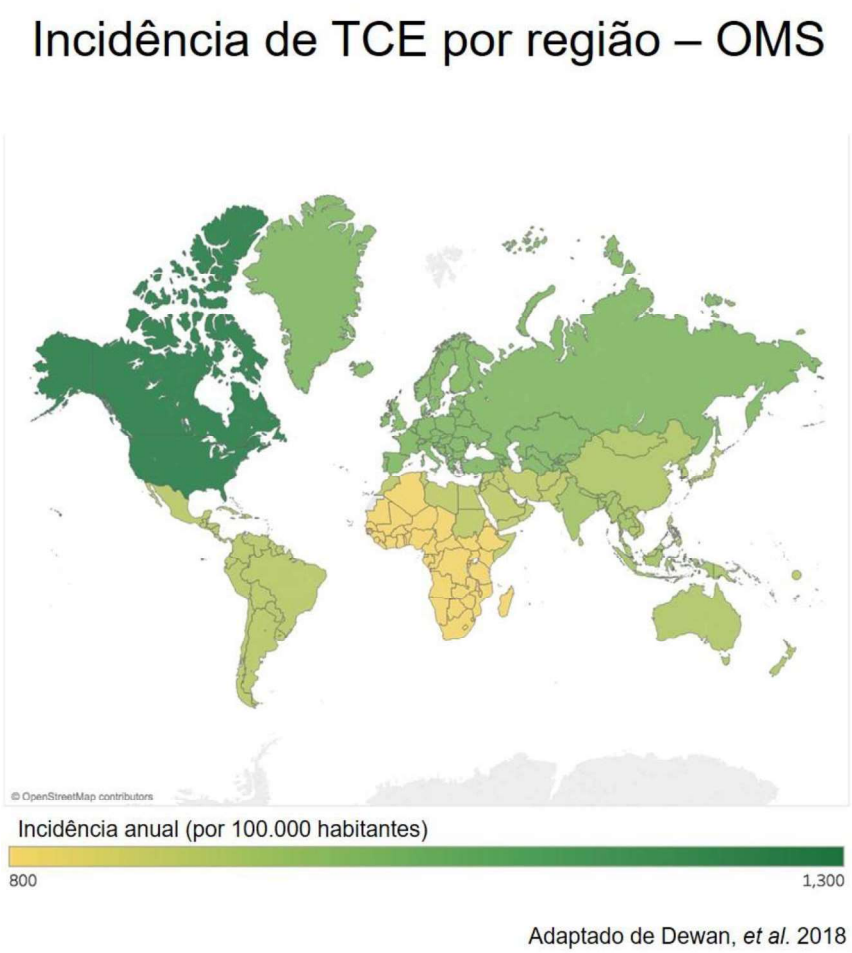


Figura 1: Mapa mostrando a incidência do TCE (casos por 100.000 habitantes) em diferentes regiões subdivididas pela Organização Mundial da Saúde (OMS).

No Brasil, os dados epidemiológicos são muito escassos (Gaudêncio e Leão, 2013). Os estudos realizados mostram custos de despesas hospitalares que chegam a uma média de 70.960.000 de dólares por ano, ocorrência anual de 125.000 internações hospitalares relacionadas ao TCE e taxa de mortalidade de aproximadamente 5.1 por 100 mil habitantes (Almeida et al., 2016). Um trabalho de revisão sistemática conduzido pelo nosso grupo selecionou 7 estudos realizados em diferentes regiões do Brasil e mostrou que os dados epidemiológicos acerca do TCE no Brasil são muito heterogêneos, com mortalidade variando de 2 a 22,9 % e faixa etária entre 15 e 40 anos (Magalhães et al., 2017). Além de poucos levantamentos, a metodologia heterogênea dos estudos faz com que seja difícil chegar a conclusões sobre o perfil epidemiológico do TCE no Brasil.

Os gastos com o TCE têm aumentado todos os anos. Nos Estados Unidos (EUA) onde os dados epidemiológicos são mais robustos e permitem interpretações mais abrangentes, os

custos diretos e indiretos à lesão eram estimados em 4.2 bilhões em 1980 (Kalsbeek et al., 1980), aumentando para 12.5 bilhões em 1990 (Parkerson et al., 1990) e saltando para 60 bilhões em estimativas mais recentes (Thurman, 2001). Ainda nos EUA são estimados cerca de 1.7 milhões de ocorrências, 275 mil hospitalizações e 52 mil óbitos por TCE anualmente (Faul et al., 2010). O principal mecanismo de lesão na população geral são quedas e acidentes automobilísticos (Faul et al., 2010). Um levantamento com dados dos EUA mostrou que enquanto que as quedas estão muito relacionadas com a ocorrência de TCE entre crianças de até 14 anos e adultos com mais de 65 anos, os acidentes automobilísticos estão muito correlacionados com os óbitos relacionados ao TCE (Faul et al., 2010).

Apesar do TCE leve representar mais de 80 % dos casos, ele acaba sendo muito negligenciado, em relação ao TCE moderado e grave, devido ao fato de que muitos pacientes não procuram uma Unidade de Emergência quando ocorre a lesão, mesmo com o potencial para desenvolvimento de sequelas psiquiátricas após a ocorrência do trauma (Feigin et al., 2013, Tabish et al., 2014, Yates et al., 2006). Pacientes que sofreram TCE leve podem apresentar distúrbios do sono (Mani et al., 2015) e alterações cognitivas e de comportamento (Cardoso et al., 2019, L'Ecuyer-Giguère et al., 2020) comprometendo a sua qualidade de vida.

1.3 Classificação e Fisiopatologia do TCE

A Escala de coma de Glasgow (ECG) é utilizada para determinar o nível de consciência e a gravidade do TCE e auxiliar na interpretação do estado clínico do paciente. A partir da avaliação utilizando ECG é que se classifica o TCE em leve (14 e 15 pontos), moderado (9 a 13 pontos) ou grave (3 a 8 pontos) (Oliveira et al., 2014; Settervall et al., 2011).

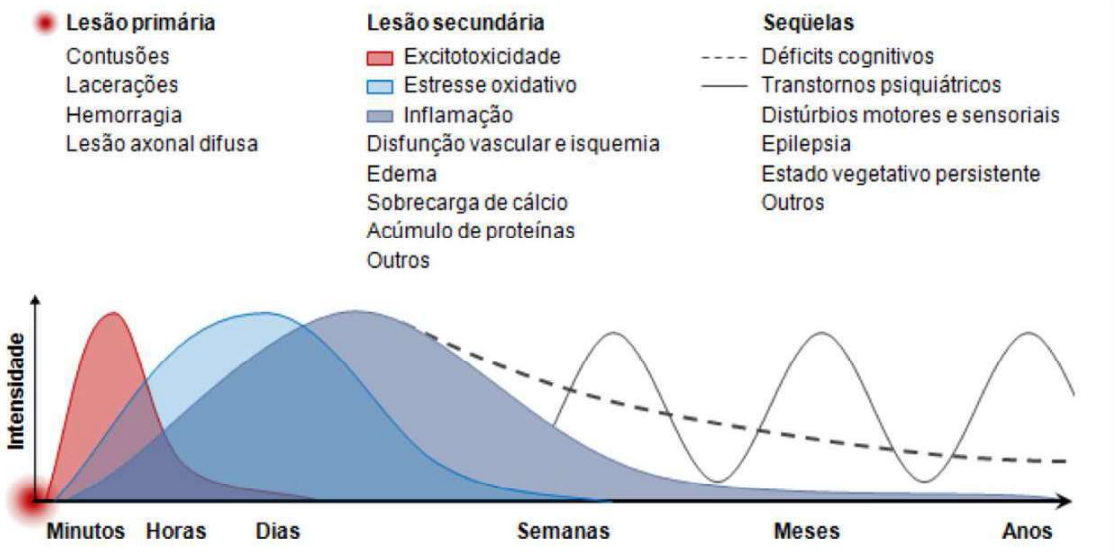
Quadro 1: Parâmetros da Escala de Coma de Glasgow

Variáveis		Escore
ABERTURA OCULAR	Espontânea	4
	À voz	3
	À dor	2
	Nenhuma	1
RESPOSTA VERBAL	Orientada	5
	Confusa	4
	Palavras inapropriadas	3
	Palavras incompreensivas	2
	Nenhuma	1
RESPOSTA MOTORA	Obedece a comandos	6
	Localiza dor	5
	Movimento de retirada	4
	Flexão anormal	3
	Extensão anormal	2
	Nenhuma	1

Baseado em Teasdale e Jennett, 1974

A fisiopatologia do TCE é muito complexa e ainda não foi completamente elucidada. Os processos fisiopatológicos envolvem uma lesão primária que ocorre no momento em que a força mecânica externa é aplicada sobre o cérebro, causando danos em estruturas do sistema nervoso central como axônios, vasos sanguíneos e células da glia (Bramlett et al., 2007) e que pode levar a lesão axonal difusa (Guarnizo et al., 2021, Zhong et al., 2021), alteração na pressão intracraniana (Pérez-Bárcena et al., 2020) e danos na barreira hematoencefálica (Yoen et al., 2021). A lesão secundária por sua vez pode ser compreendida como o conjunto de alterações celulares, patológicas e bioquímicas que se desenvolvem a partir da lesão primária e que podem ser encontradas dias, meses e anos após a lesão primária (Figura 2) (Johnson et al., 2013). Os principais mecanismos de lesão secundária envolvidos no trauma são apoptose (Miñambres et al., 2008), neuroinflamação (Ebert et al., 2019) e estresse oxidativo

(Mojtahedzadeh et al., 2014). De maneira interessante estudos tem mostrado a associação entre esses mecanismos de lesão secundária com o desenvolvimento de alterações comportamentais e cognitivas após o TCE leve em pacientes. Um estudo clínico com pacientes que sofreram TCE leve mostrou que o aumento nos níveis de interleucina (IL-10) no plasma foi associado a aumento de sintomas depressivos e de transtorno de estresse pós-traumático 6 meses após a lesão (Vedantam et al., 2021). Em outro estudo, níveis elevados de IL-1β no soro de pacientes foram relacionados com dano cognitivo um mês após o TCE leve (Sun et al., 2019).



Fonte: Schwarzbold, 2010.

Figura 2: Esquema da Fisiopatologia do TCE, mostrando as lesões primária e secundaria e suas relações.

Apesar das evidências da associação entre os diferentes mecanismos de lesão secundária e o desenvolvimento de alterações comportamentais e cognitivas ainda não há uma intervenção terapêutica que tenha foco na lesão secundária. Todas as intervenções realizadas na clínica são para contenção da lesão primária. Nesse sentido, diferentes modelos animais têm disso desenvolvidos e empregados para o estudo das lesões primária e secundária, bem como de estratégias terapêuticas para melhora nas alterações comportamentais e cognitivas encontradas após o TCE.

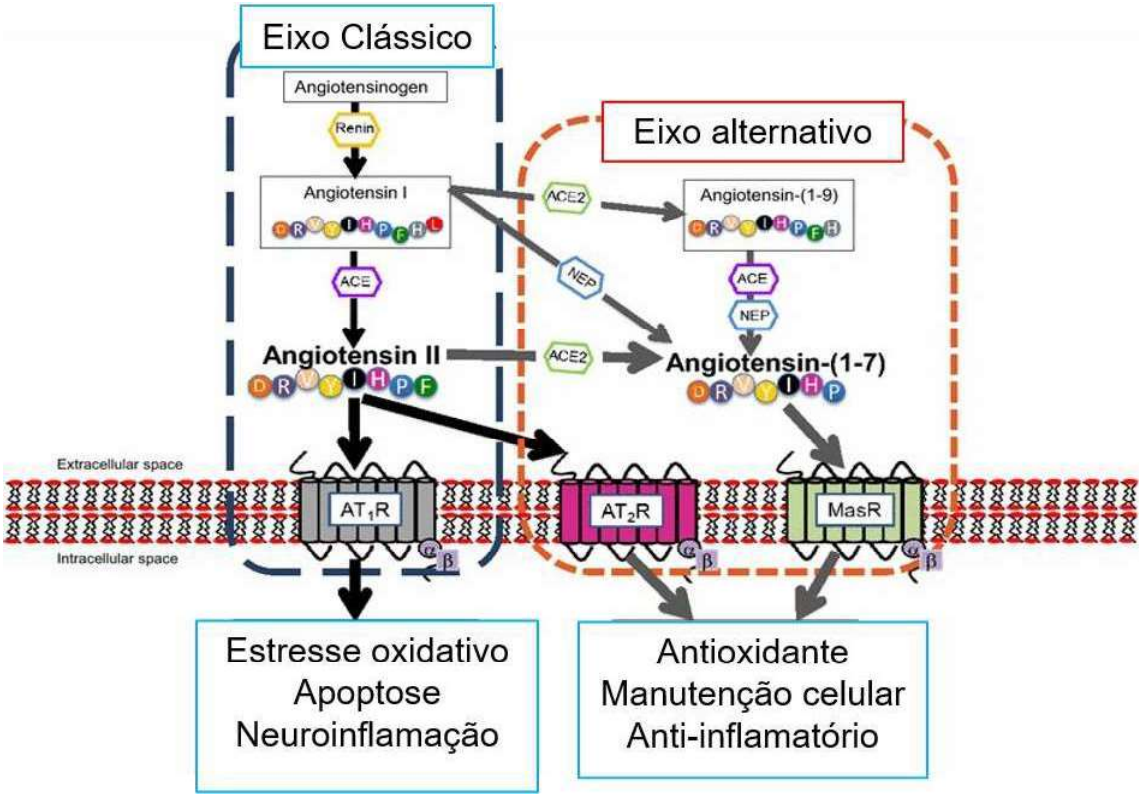
1.4 Modelos animais de TCE

Os principais modelos de TCE, que têm sido utilizados, são o modelo de queda de peso, o modelo de impacto cortical controlado (ICC) e o modelo de percussão lateral por fluido (PLF). No modelo de queda de peso a lesão é causada quando um projétil de determinado peso posicionado a determinada altura incide, em queda livre, sobre o crânio exposto do animal. Esse modelo possui algumas vantagens quando comparado com os modelos de ICC e PLF. Com o modelo de queda de peso é possível trabalhar com o trauma leve, moderado e grave a partir do ajuste do peso e altura da queda do projétil (Albert Weissenberger e Siren, 2010). Além disso, ele pode ser construído no laboratório, não necessita de elaborada preparação pré-lesão e não requer conhecimento técnico avançado para realização da cirurgia, fazendo com que seu uso seja mais simples de ser implementado no laboratório (Flierl et al., 2009). Muitos estudos mostraram que o modelo de queda de peso foi capaz de reproduzir em roedores alterações cognitivas e de comportamento que são vistas na clínica em pacientes que sofreram TCE leve, como depressão, ansiedade e prejuízos na memória e aprendizagem (Bader et al., 2020, McAteer et al., 2016, Milman et al., 2008, Rachmany et al., 2013, Shapira et al., 2007). Por exemplo, o comportamento do tipo depressivo foi mostrado 24 e 72 horas após TCE leve em camundongos C57BL/6 e ICR, respectivamente, usando o modelo de queda de peso (Tweedie et al., 2007; Shapira et al., 2007). O modelo de queda de peso também foi capaz de induzir comportamento do tipo depressivo 7, 60 e 90 dias após a lesão em camundongos ICR (Milman et al., 2008). Em outro estudo usando esse modelo, foi encontrado comportamento do tipo ansioso em camundongos NMRI 11 dias após o TCE leve (Kosari-Nasab et al., 2018[a]).

1.5 O Sistema Renina-Angiotensina

Muitos sistemas biológicos têm sido implicados na modulação dos mecanismos de lesão secundária no TCE (Machado[a] et al., 2019), dentre eles, o Sistema Renina-Angiotensina. O Sistema Renina-Angiotensina (SRA) é conhecido, principalmente pelo seu papel no controle da pressão arterial, hormonal e sua atuação em doenças cardiovasculares (Santos et al. 2003). Entretanto, desde que receptores desse sistema foram identificados no sistema nervoso central (SNC), estudos começaram a investigar o papel que o SRA teria no SNC, especialmente em condições patológicas (Janatpour et al., 2019). Nesse sistema o

peptídeo angiotensinogênio é clivado pela enzima renina, gerando a angiotensina I (Ang I) (Figura 3). A enzima de conversão da angiotensina I (ECA 1) realiza a conversão da angiotensina I em Ang II (Guo et al., 2001). A angiotensina II (Ang II) é o componente central desse sistema e atua regulando a expressão de fatores de crescimento e mediadores inflamatórios que induzem apoptose, estresse oxidativo e inflamação (Benigni et al., 2010). A Ang II atua principalmente, através da ligação ao receptor de Angiotensina do tipo I (AT1), que é encontrado no cérebro e em outros órgãos do corpo, mas também pode se ligar ao receptor de angiotensina do tipo II (AT2). O AT2 é um receptor que se liga a uma proteína G diferente da qual se liga o AT1. Enquanto o AT2 tem papel na vasodilatação, o AT1 se relaciona com a vasoconstrição (Hallberg et al., 2017). A ação da ECA, Ang II e do receptor AT1 é comumente referido como eixo clássico do SRA, entretanto, outros peptídeos relacionados a esse sistema têm sido identificados (Chiu et al., 1976; Jackman et al., 2002; Le et al., 2002; Santos et al., 2013). Um desses peptídeos é a Angiotensina-(1-7) (Ang-(1-7)) que é produzida a partir da clivagem da Ang II por meio da enzima de conversão da angiotensina II (ECA 2). A Ang-(1-7) tem sido estudada porque através de seu receptor Mas ela atua como antagonista da Ang II, sendo capaz de modular os receptores AT1, interferindo na sua atividade e exercendo efeitos opostos ao eixo clássico do SRA. Devido a sua função, a ação da ECA 2 e Ang-(1-7) via receptor Mas tem sido denominado de eixo alternativo ou contra regulatório do SRA (Santos et al., 2003).



Adaptado de Arroja. *et al.*, Exp & Trans Stroke Med (2016) 8:8

Figura 3: Esquema dos componentes principais do Sistema Renina-Angiotensina.

1.6 O Sistema Renina-Angiotensina no TCE

Poucos estudos até o momento investigaram a expressão dos componentes do SRA após o TCE. Foi visto aumento nos níveis de renina, angiotensinogênio e do receptor AT2 no córtex de camundongos C57BL/6N após as primeiras horas depois da lesão pelo modelo de ICC (Timaru et al., 2012; Timaru et al., 2019). A maior parte dos estudos sobre o papel do SRA no TCE têm sido realizada com o uso de moduladores do sistema, principalmente candesartan e telmisartan, que são antagonistas do receptor AT1 (Timaru-Kast et al., 2012; Villapol et al., 2015). A administração intraperitoneal de candesartan (0,1 mg/kg) às 6 horas após a lesão reduziu o estresse oxidativo, a ativação de micróglias e astrócitos, além de diminuir a apoptose e infiltração de neutrófilos no córtex de camundongos C57BL/6NCr 3 dias após TCE moderado pelo modelo de ICC (Villapol et al., 2015). Resultados semelhantes foram obtidos com a administração de telmisartan (1 mg/kg) via gavagem oral, entretanto, somente o candesartan foi capaz de diminuir a ativação da micróglia (Villapol et al., 2015). Candesartan (0,1 mg/kg) também reduziu a expressão de óxido nítrico-sintase induzida

(iNOS), interleucina 6 (IL-6), IL-1β e fator de necrose tumoral (TNF-α) 12 horas após a lesão por ICC, quando administrado 30 minutos depois do TCE (Timaru-Kast et al., 2012). Nesse mesmo estudo, quando o tratamento se iniciou aos 30 minutos após a lesão, mas foi continuado diariamente durante 4 dias, foi capaz de reduzir a lesão cerebral e a ativação da micróglia 5 dias após o TCE. Em contraste com as evidências dos trabalhos experimentais com antagonistas de AT1, os poucos trabalhos que usaram inibidores da ECA para modular o SRA, apresentaram resultados que sugerem que esses inibidores podem agravar as sequelas do TCE em roedores e na clínica (Catapano et al., 2019; Harford-Wright et al., 2010). Por exemplo, um estudo mostrou uma piora nos déficits motores em ratos Sprague-Dawley tratados com captopril (5 mg/kg) e enalapril (5 mg/kg) durante 7 dias antes do TCE moderado pelo modelo de queda de peso (Harford-Wright et al., 2010). De acordo com esses resultados, um estudo retrospectivo mostrou associação entre aumento da mortalidade e o uso de inibidores de ECA antes da lesão em pacientes que sofreram TCE grave (Catapano et al., 2019). Os mecanismos moleculares que explicam a diferença entre a resposta de inibição de AT1 e ECA ainda não estão elucidados.

Receptores do SRA também já foram encontrados no estriado, que é um importante componente do núcleo basal. O estriado faz conexões com o córtex sensório-motor e está associado ao controle motor, aprendizagem e função cognitiva (Cataldi et al., 2021; Mello e Villares, 1997). Evidências na literatura tem sugerido que o estresse oxidativo no estriado pode estar relacionado com alterações na função motora após TCE leve. Estudos com roedores mostraram que o TCE pode induzir prejuízo na função motora associado com alterações em níveis de estresse oxidativo no estriado 24 horas (Huang et al., 2018) e 6 dias (Harmon et al., 2017) após a lesão. Além disso, um estudo mostrou que pacientes que sofreram TCE leve entre 50 e 70 anos de idade, apresentaram comprometimento na capacidade de aprendizagem motora entre 3 a 24 meses após a lesão (Bourassa et al., 2021).

2 – HIPÓTESE

O Sistema Renina-Angiotensina participa na fisiopatologia do TCE e o eixo alternativo tem um efeito neuroprotetor sobre as alterações comportamentais e cognitivas agudas e subcrônicas associadas ao TCE leve.

3 – JUSTIFICATIVA

Os mecanismos envolvidos na fisiopatologia das alterações comportamentais e cognitivas, agudas e subcrônicas, associadas ao TCE permanecem pouco elucidados. A limitada compreensão da patogênese do TCE dificulta o reconhecimento e desenvolvimento de novos alvos terapêuticos. Nas últimas décadas o SRA, em especial o eixo clássico, tem adquirido crescente reconhecimento por seu papel em inúmeras doenças acometendo diferentes órgãos, incluindo o cérebro. Poucos estudos investigaram o papel da modulação do SRA em transtornos neuropsiquiátricos no TCE. Entretanto, devido a capacidade desse sistema de regular a resposta imune e vascular, é razoável a hipótese de que o SRA possa estar pelo menos em parte, associado a fisiopatologia do TCE e possuir papel neuroprotetor em relação às sequelas cognitivas e comportamentais. Nesse contexto, o presente estudo permitiu a investigação de potenciais alterações nos componentes do SRA em resposta ao TCE bem como a identificação de biomarcadores, possibilitando o desenvolvimento de novas estratégias terapêuticas focadas especialmente nas lesões secundárias ao trauma.

4 – OBJETIVOS

4.1 Objetivo geral

Investigar o papel do SRA em modelo experimental de traumatismo cranioencefálico (TCE) leve.

4.2 Objetivos específicos

4.2.1 Caracterizar as alterações cognitivas e comportamentais agudas e ou subcrônicas após o TCE em camundongos.

4.2.2 Caracterizar alterações morfológicas resultantes do TCE no córtex pré-frontal e hipocampo de camundongos machos da linhagem C57BL6.

4.2.3 Avaliar o processo inflamatório via análise dos níveis de citocinas, quimiocinas e de fatores neurotróficos e estresse oxidativo no córtex pré-frontal e hipocampo de camundongos machos da linhagem C57BL6 submetidos ao TCE.

4.2.4 Avaliar a expressão dos componentes do SRA (Angiotensina I, Angiotensina II e Angiotensina-(1-7)) no córtex pré-frontal e hipocampo de camundongos machos após indução do TCE.

4.2.5 Analisar a expressão dos receptores do SRA (AT_1R, AT_2R e Mas) no córtex pré-frontal e hipocampo de camundongos após TCE.

4.2.6 Investigar o efeito neuroprotetor de moduladores do SRA (Perindopril, Telmisartan e AVE0991) em resposta ao TCE no córtex pré-frontal e hipocampo de camundongos.

5 – MATERIAL E MÉTODOS

5.1 Animais e Aspectos éticos

Neste estudo, todos os experimentos que envolveram animais foram conduzidos de acordo com o Comite de Ética em Experimentação Animal da Universidade Federal de Minas Gerais (UFMG), sob o protocolo 250/2017. Camundongos machos da linhagem C57BL/6 selvagem, com o peso médio entre 20 a 30g e com idade entre 10 e 12 semanas, foram obtidos do Centro de Bioterismo (CEBIO) da UFMG, Belo Horizonte, Brasil. Os animais foram mantidos em ciclo claro-escuro de 12 horas, com alimentação e água *ad libitum*. Antes dos testes comportamentais e cognitivos, os animais foram aclimatados na sala de testes por pelo menos 30 minutos.

5.2 TCE leve

O modelo de queda de peso construído (Figura 4) foi baseado nos modelos descritos por Flierl et al. (2009) e Marmarou et al. (1994) e foi padronizado como consta no trabalho Machado et al., 2019[b]. Os animais foram anestesiados com cetamina/xilazina e colocados em posição pronada. O crânio dos camundongos foi exposto através de uma incisão de linha média e um projétil de resina de 15 gramas foi deixado cair livremente por um tubo guia de 60 centímetros no lado parietal direito, entre as suturas sagital e coronal (Figura 5). Cada animal recebeu um único TCE leve. Após a lesão, os animais foram suturados e tiveram os sinais clínicos acompanhados. A seguir, os animais foram levados para a recuperação em uma manta térmica e tiveram os olhos hidratados. O grupo *sham* ou controle foi submetido a todos os procedimentos, exceto ao trauma por queda de peso. Os animais foram divididos em pelo menos 5 grupos diferentes (5-10 camundongos por grupo): 1) *sham* ou controle; 2) TCE 6 horas; 3) TCE 24 horas: 4) TCE 72 horas; 5) TCE 30 dias.

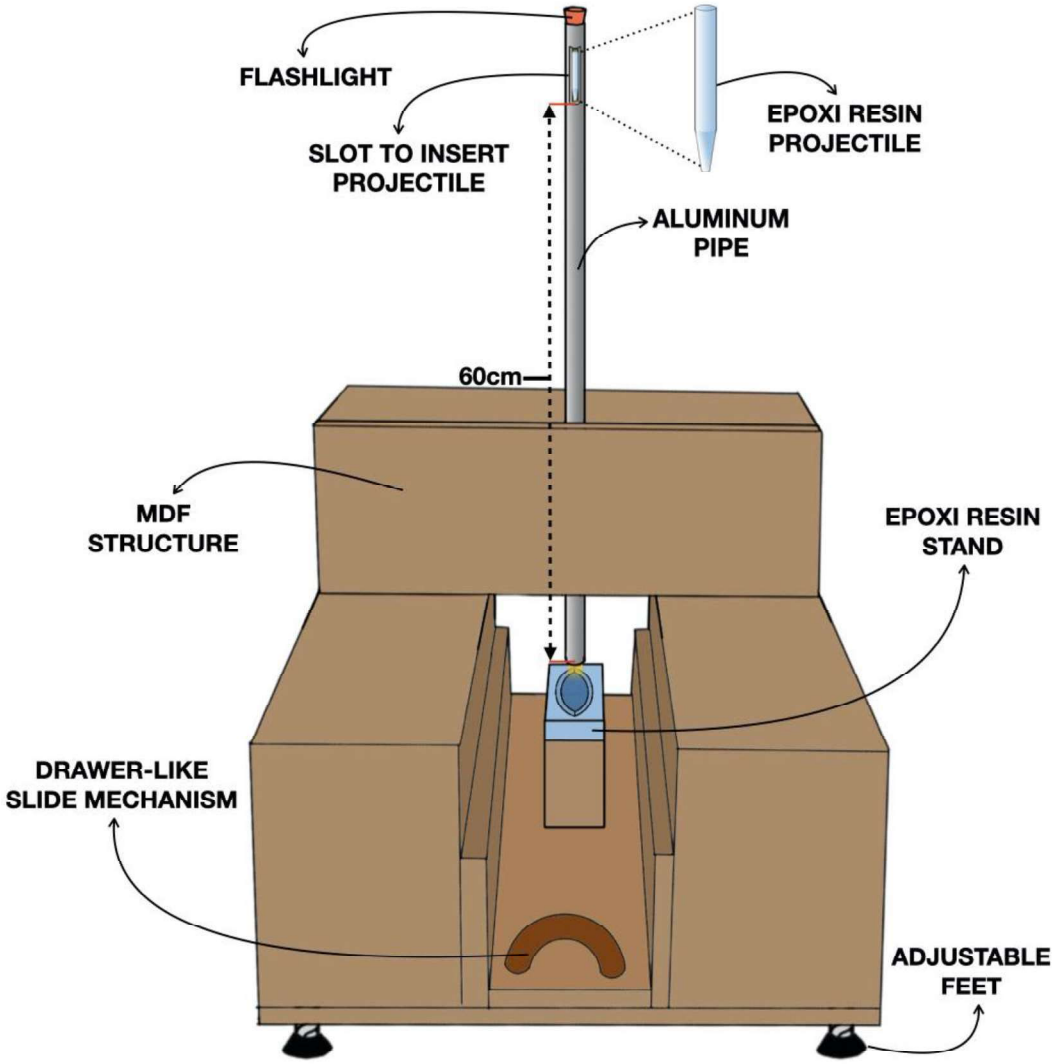


Figura 4: Esquema do modelo de queda de peso.

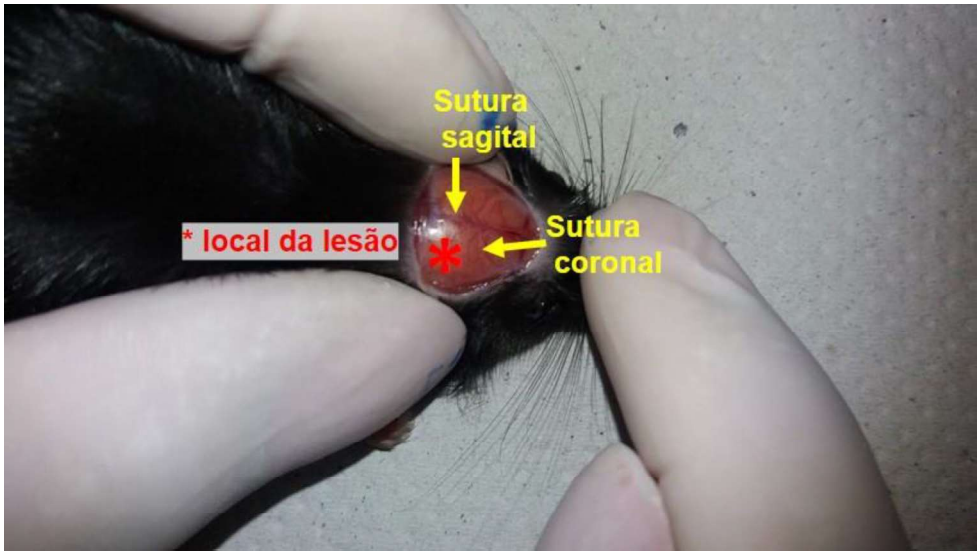


Imagem da autora

Figura 5: Imagem mostrando o local da lesão (*) entre as suturas sagital e coronal na região parietal direita do crânio do camundongo.

5.3 Análise de parâmetros cognitivos e comportamentais

5.3.1 Avaliação Neurológica pelo escore de gravidade neurológica – NSS

O escore neurológico de gravidade (*Neurological severity escale* - NSS) foi utilizado para avaliação neurológica por meio de 10 testes diferentes, que avaliaram a resposta à função locomotora, estado de alerta e estímulo após TCE conforme Flierl., 2009 (Figura 6). Os 10 parâmetros clínicos podem ser observados no quadro 2. A avaliação do escore NSS 1 hora após o TCE tem sido utilizada para estabelecer a gravidade da lesão em modelos experimentais (Sharma et al., 2012, Khalin et al., 2016, Shen et al., 2017, Dai et al., 2018, Lan et al., 2019). Uma pontuação NSS menor ou igual a 5 pontos é indicativa de TCE leve. Uma pontuação máxima de 10 pontos após a primeira hora pós-lesão indica comprometimento neurológico grave e trauma grave Flierl et al., 2009. Realizamos uma avaliação neurológica usando NSS antes do TCE e nos momentos 1, 3, 6, 12, 24, 48 e 72 horas e 4, 5, 6, 7, 10, 12 e 14 dias após TCE leve, conforme descrito anteriormente por Flierl et al. (2009). Os camundongos que não conseguiram realizar o teste receberam 1 ponto, os camundongos que completaram metade da tarefa receberam meio ponto e os camundongos que completaram a tarefa com sucesso receberam 0 ponto.

Quadro 2: Parâmetros do Escore Neurológico de Gravidade (NSS)

Parâmetros do Escore Neurológico de Gravidade (NSS)
1. Presença de paresia
2. Inabilidade em andar em linha reta
3. Perda do comportamento exploratório
4. Falha em sair do círculo (até 3 min)
5. Inabilidade em andar na trave de 3 cm (até 2 min)
6. Inabilidade em andar na trave de 2 cm (até 2 min)
7. Inabilidade em andar na trave de 1 cm (até 2 min)
8. Inabilidade em se equilibrar na trave de 0,7 cm (até 10 s)
9. Inabilidade em se equilibrar na trave redonda (até 10 s, 3 tentativas)
10. Perda do reflexo de sobressalto

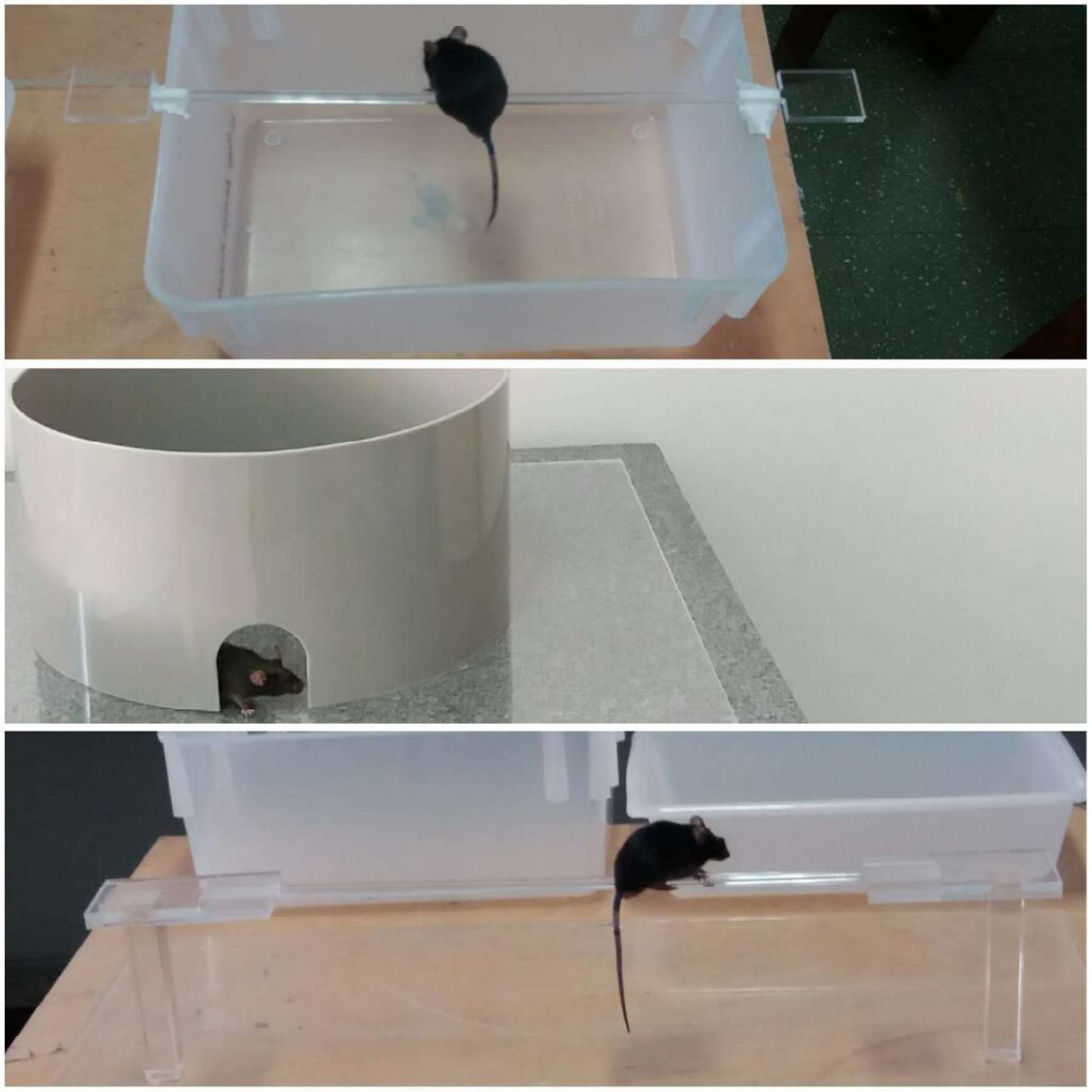


Imagem da autora

Figura 6: escore neurológico de gravidade (*Neurological severity escale* - NSS

5.3.2 Teste de Campo Aberto

O teste de campo aberto foi realizado para avaliar a atividade locomotora após TCE leve usando o sistema *PhenoTyper (Noldus Information Technology, Leesburg,* VA, EUA) Miranda et al., 2017. Os camundongos foram colocados para explorar uma arena aberta (30 cm x 30 cm x 35 cm) durante 30 minutos. A distância total percorrida na arena foi analisada utilizando o *software Ethovision XT (Noldus Information Technology, Leesburg*, VA, EUA).

5.3.3 Teste do Nado Forçado

O teste do nado forçado foi realizado para avaliar o comportamento do tipo depressivo conforme descrito em Miranda et al. (2017). Os camundongos foram colocados num béquer de vidro com capacidade total para 5 litros (18 cm de diâmetro e 28 cm de altura) com água a uma altura de 18 cm e o nado foi registado durante 6 minutos. Para a análise foram considerados apenas os últimos 4 minutos do teste. Nesse período, o tempo de imobilidade, o tempo de escalada ou *climbing* e a latência até a primeira imobilidade foram registrados e utilizados para avaliar o comportamento do tipo depressivo. O tempo de escalada ou *climbing* foi considerado como movimentos ascendentes contra a parede do copo de vidro (Costa et al., 2013).

5.3.4 Teste do Labirinto em cruz elevado

O teste do Labirinto em cruz elevado foi realizado para avaliar o comportamento do tipo ansioso. Nesse teste os animais foram colocados para explorar um labirinto que consiste em uma plataforma elevada 30 cm do chão com 2 braços abertos (30 cm x 7 cm x 0,25 cm) e 2 braços fechados (30 cm x 7 cm x 15 cm). O teste foi realizado durante 5 minutos e analisado no software Ethovision XT (Noldus Information Technology, Leesburg, VA, EUA). A porcentagem de tempo gasto no braço aberto do labirinto foi usada para medir o comportamento do tipo ansioso (Fernandes et al., 2020).

5.3.5 Teste de *Marble burying* ou enterrar o mármore

O teste de *Marble burying* foi realizado para avaliar o comportamento compulsivo após TCE leve, conforme descrito em Tucker et al., 2017. Os animais foram colocados em uma caixa *Phenotyper* (30 cm x 30 cm) coberta com maravalhas a uma altura de 12 cm e que continha na sua superfície 25 bolinhas de gude de 1 cm com espaço de aproximadamente 3 cm ente elas. Os camundongos foram deixados para explorar individualmente a caixa durante 30 minutos. As bolinhas que estavam 2,5 cm dentro da maravalha foram consideradas enterradas. O aumento no número de bolinhas de gude enterradas em comparação ao grupo *sham* foi usado para indicar o comportamento do tipo compulsivo (Tucker et al., 2017).

5.3.6 Teste do Labirinto de Barnes

O teste do Labirinto de Barnes foi realizado para avaliação da memória espacial dependente do hipocampo 72 horas e 30 dias após TCE leve. O protocolo utilizado foi adaptado de Pitts et al. (2018). O labirinto de Barnes foi realizado em uma plataforma circular (90 cm de diâmetro) com 20 buracos e elevado a uma altura de 15 cm em relação ao chão. Foi utilizado 3 pistas visuais (triângulo, círculo e quadrado), uma caixa e escape e uma caixa preta. Para a fase de habituação, os camundongos foram colocados na caixa de escape por 1 minuto e depois no centro do labirinto por 5 minutos ou até retornar a caixa de escape. A primeira sessão de treinamento iniciou 1 hora após a fase de habituação. O treinamento foi realizado 2 vezes diariamente, durante 9 dias, com intervalos de 1 hora entre os treinamentos diários. Em cada tentativa, os camundongos foram contidos em uma caixa preta por 15 segundos, que era colocada em um dos quadrantes do labirinto. Os camundongos eram então liberados para explorar o labirinto e encontrar a caixa de escape em 3 minutos. Os camundongos que não localizaram a caixa de escape em 3 minutos foram conduzidos de volta e mantidos na caixa de escape por 15 segundos para aprenderem a localização. O teste final foi realizado 24 horas após o último treinamento. Os camundongos foram colocados para explorar o labirinto por 1 minuto. O tempo para os camundongos encontrarem a caixa de escape (latência) no teste final foi avaliado e usado como medida da memória espacial dependente do hipocampo Pitts et al., 2018.

6.3.7 Teste do Labirinto em Y

O teste do Labirinto em Y foi realizado para avaliação da memória de trabalho. O labirinto em Y foi realizado em uma plataforma com 3 braços fechados (40 cm x 7 cm x 15 cm): A, B e C. O animal foi posicionado num braço inicial livre para mover-se pelos outros braços durante 5 minutos. O teste foi gravado e a análise foi feita visualmente. A porcentagem de alternâncias espontâneas entre os braços durante 5 minutos foi obtida através do índice: [total alternado/ (total de entradas nos braços -2) X 100] (Salari et al., 2018).

5.3.8 Teste de reconhecimento de novos objetos

O teste de reconhecimento de novos objetos foi realizado para avaliar a memória de trabalho de curta duração (1,5 horas) e de longo prazo (24 horas) após TCE leve, conforme descrito em Miranda et al., 2017. Nesse teste foi realizada uma sessão de habituação durante 30 minutos sem objetos na caixa. Após 24 horas da sessão de habituação, os camundongos foram colocados na caixa com dois objetos idênticos (A1 e A2) por 10 minutos (fase de adaptação). A avaliação da memória de curta duração foi realizada 1,5 horas após o teste de adaptação e um objeto antigo (A2) foi substituído por um objeto novo (A3). A avaliação da memória de longo prazo foi realizada 24 horas após a sessão de adaptação e um objeto (A3) foi substituído por um objeto novo (A4). O teste de memória de curto e longo prazo foi realizado durante 10 minutos. A porcentagem de tempo de exploração do novo objeto foi analisada utilizando o *software Ethovision XT (Noldus Information Technology, Leeburg*, VA, EUA).

5.3.9 Teste do Rotarod

O teste do Rotarod (Insight, Ribeirão Preto, Brasil) foi realizado para avaliar a aprendizagem e coordenação motora após TCE leve, conforme descrito em Kao et al., 2015. O teste foi realizado durante 5 dias, sendo o primeiro e o segundo dias realizados antes da lesão. O terceiro dia de teste foi realizado 24 horas após a lesão, o quarto dia de teste foi realizado 48 horas após a lesão e o quinto dia de teste foi realizado 72 horas após a lesão.

No primeiro dia do teste, os animais passaram por uma fase de habituação, em que foram colocados no aparelho a uma velocidade constante de 4 rpm por 30 segundos antes do primeiro treinamento. Essa etapa ocorre apenas no primeiro dia do protocolo e se realiza um intervalo de 30 minutos antes do treinamento.

Após o tempo de habituação, se iniciou o treinamento, onde os animais foram colocados no aparelho por 5 minutos em velocidade acelerada (4-40 rpm) em três tentativas diárias com intervalo de 30 minutos entre as tentativas. Esse treinamento se repetiu durante 5 dias consecutivos.

A média da latência da primeira queda foi usada como medida para avaliação da coordenação motora. O desempenho ou index da aprendizagem motora foi obtido pelo percentual de habilidades aprendidas em dois dias consecutivos de treinamentos através da

fórmula do índice de aprendizagem motora: (T n - T n - 1)/T n-1 x 100 %, onde T n é a latência decrescente no n dia de teste.

5.4 Avaliação da permeabilidade da barreira hematoencefálica

A permeabilidade da barreira hematoencefálica (BHE) foi analisada pelo marcador azul de Evans às 6, 24 e 72 horas e 30 dias após TCE leve. Camundongos sob anestesia com cetamina/xilazina receberam injeção intravenosa de solução de azul de Evans a 0,5% (Sigma-Aldrich, St. Louis, MO). Após 1 hora, os camundongos foram decapitados e o cérebro foi incubado em formamida durante 24 horas (1 mL/100 mg de tecido). A quantidade de Azul de Evans nos extratos de tecido cerebral foi medida pela absorbância a 610 nm e os resultados apresentados como o nível de Azul de Evans (pg) em 100 mg de tecido cerebral, conforme descrito em Toscano et al., 2016.

5.5 Análise do fluxo sanguíneo cerebral

A imagem de perfusão Laser Doppler (Moor Instruments, Ltd., Inglaterra) foi utilizada para estimar o fluxo sanguíneo cerebral (FSC) às 72 horas e 30 dias após TCE leve. Os animais anestesiados (cetamina/xilazina) foram colocados em decúbito ventral e o crânio foi exposto através de uma incisão na linha média para análise conforme descrito por Valle et al. (2020). A perfusão tecidual foi indicada por cores. Pouco ou nenhum fluxo sanguíneo foi indicado em azul escuro, enquanto que a perfusão máxima foi indicada em vermelho. Após a digitalização, as imagens foram analisadas no *software* Moor LDI Laser Doppler 5.3 para quantificação do fluxo sanguíneo.

5.6 Análise histológica

Após avaliação comportamental e cognitiva, os camundongos foram anestesiados com injeção intraperitoneal de cetamina/xilazina e submetidos à cirurgia de perfusão transcardíaca às 6, 24 e 72 horas e 30 dias após TCE leve. A cirurgia foi realizada com soro fisiológico (0,9%) e formalina (10%) para coloração com hematoxilina-eosina (H&E) e paraformaldeído (4%) para coloração por imunofluorescência.

5.6.1 Coloração com hematoxilina-eosina (H&E)

Os cérebros foram cuidadosamente removidos e pós-fixados em formalina tamponada a 10%. Seções de 5 μm em intervalos de 10 μm foram cortadas e montadas para coloração H&E de rotina. As seções foram examinadas em nível óptico (Olympus, Japão, JP). Imagens digitais foram adquiridas para documentação utilizando uma objetiva planacromática × 400.

5.6.2 Coloração por imunofluorescência

A coloração por imunofluorescência foi realizada em cérebros fixados de camundongos. Após a fixação, cada hemisfério cerebral foi cortado em fatias coronais de 2 milímetros e em seguida os cérebros foram parafinizados. Cada bloco de parafina foi cortado em fatias de 5 mícrons e montado em lâminas de microscópio IHC de imuno-histoquímica (Agilent Technologies Singapore). Em seguida, as lâminas foram desparafinizadas e realizada a coloração por imunofluorescência. Resumidamente, as lâminas foram lavadas com PBS e incubadas no anticorpo primário *overnight* na temperatura de 8 graus *Celsius*. Os seguintes anticorpos primários foram utilizados para quantificação de dano axonal, ativação microglial e ativação astrocítica: Proteína Precursora Anti-Amilóide de coelho-APP (1:200; ab32136), anti-Iba-1 de coelho (1:200; PA5-21274) e anti-GFAP de camundongo (1:150; MAB3402), respectivamente. No dia seguinte, as lâminas foram lavadas com PBS e incubadas com anticorpos secundários durante 4 horas, protegidas da luz a 22-26 graus Celsius. Foram utilizados os seguintes anticorpos secundários: anti-coelho (1:1000; A21245) e anti-camundongo (1:750; A11001). As lâminas foram então incubadas com DAPI para marcação nuclear por 30 minutos, montadas e observadas em microscópio confocal LSM 880 ZEISS (CAPI – ICB/UFMG). O número total de células positivas para Iba-1/DAPI e GFAP/DAPI foi contado em cinco campos diferentes do córtex e hipocampo (área padronizada). Para a quantificação do APP, foi contado o valor médio da intensidade de fluorescência em cinco diferentes campos do córtex e hipocampo (área padronizada). Além do número total de células positivas, o valor médio da intensidade de fluorescência também foi realizado para os marcadores Iba-1 e GFAP.

5.7 Análise dos mediadores inflamatórios, peroxidação lipídica e níveis de expressão de CX3CR1 e óxido nítrico sintases

5.7.1 Análises CBA e ELISA de citocinas e fatores neurotróficos

Após análise comportamental e cognitiva, os camundongos foram anestesiados com injeção intraperitoneal de cetamina / xilazina e sacrificados às 6, 24 e 72 horas e 30 dias após TCE leve. Os animais *sham* também foram anestesiados e eutanasiados imediatamente após análises comportamentais e cognitivas. Os cérebros foram cuidadosamente removidos para dissecção do hemisfério ipsilateral (direito) e contralateral (esquerdo) do córtex pré-frontal e do hipocampo. Após a dissecção, o tecido foi homogeneizado em solução extratora (100 mg de tecido por mililitro), contendo NaCl, Tris-Hcl 20 mM, glicerol a 10%, PMSF 1 mM, Pepstatina A 1 μM, EDTA 10 mM, E-64 10 μM e vanadato de sódio a 0,5 mM. Os lisados foram centrifugados a 14.000 g por 10 min a 4 ° C, os sobrenadantes foram coletados e estocados a -70 ° C até o uso. Os níveis de interleucina (IL)-6, IL-10, IL12p70, interferon-gama (IFN-γ), fator de necrose tumoral alfa (TNF-α) e proteína quimiotática de monócitos (MCP-1) foram medidos por *Cytometric Bead Array* (CBA) (BD Biosciences, San Diego, CA) e a concentração de mediadores inflamatórios foi determinada por FACSCanto (Becton Dickinson, San José, CA, EUA). Os níveis de IL-1β, quimiocina derivada de queratinócitos (KC), fractalquina (CX3CL1), fator neurotrófico derivado do cérebro (BDNF), fator neurotrófico derivado de linhagem celular glial (GDNF) e fator de crescimento nervoso (NGF) foram avaliados por Ensaio de imunoabsorção enzimática (ELISA), de acordo com o protocolo do fabricante (R&D Systems, Minneapolis, MN, EUA). Os resultados foram expressos como picograma (pg) por 100 mg de tecido. O limite de detecção dos ensaios de CBA e ELISA foram em torno de 0,2 e 5 pg/mL, respectivamente.

5.7.2 Expressão de mRNA do hipocampo e do córtex pré-frontal de CX3CR1 e óxido nítrico sintases

A expressão de mRNA de CX3CR1 e as isoformas da enzima óxido nítrico sintase (NOS), conhecidas como isoenzimas neuronal 'n'NOS, indutível 'i'NOS e endotelial 'e'NOS, no hipocampo e no córtex pré-frontal camundongos submetidos ao TCE leve foram estimados por reação em cadeia da polimerase (PCR) quantitativo em tempo real de acordo com o protocolo do fabricante (Biorad, EUA). O RNA total foi obtido utilizando o reagente Trizol (Life Technologies, ThermoFisher Scientific, MA, EUA) seguindo as instruções do fabricante. O RNA obtido foi ressuspendido em água tratada com pirocarbonato de dietila e estocado a -70°C até o uso. A transcrição reversa foi realizada utilizando 2 microgramas de

RNA total, 200U de transcriptase reversa, tampão RT 5X (4 ul), dNTPs 10 mM (1 ul), RNAsina 10.000 U (0,2 ul) e oligo dT 15 50 uM (1,0 ul) em um volume de reação final de 20 ul. O cDNA resultante foi usado para qPCR. A síntese de cDNA foi realizada a partir de 2 microgramas de RNA, utilizando transcriptase reversa M-MLV (Invitrogen, ThermoFisher Scientific, MA, EUA). A PCR quantitativa foi realizada utilizando o kit RT2 SYBR® Green qPCR Mastermix (Qiagen) no sistema de detecção CFX96 Touch™ Real Time (BioRad). As sequências iniciadoras utilizadas (sense e antisense) foram Cx3cr1 (TGCCTTCTTCCTCTTCTGGA; TAAAGGGGTTGAGGCAACAG), nNos (CTGGTGAAGGAACGGGTCAG; CCGATCATTGACGGCGAGAAT), eNos (CTCTACCGGGACGAGGTACT); (GAGGTCTTGCACGTAGGTCT), iNos (GCTTGCCCCAACAGGAGAAG; GCTGCCCGGAAGGTTTGTAC) e Rpl32 (GCTGCCATCTGTTTTACGG; TGACTGGTGCCTGATGAACT). Os dados de expressão foram calculados como 2 elevado à potência de −ΔCt (onde ΔCt é a diferença entre Cts do gene alvo e o gene de referência Rpl32). Os resultados são apresentados como valores normalizados relativos ao grupo *sham*.

5.7.3 Avaliação da peroxidação lipídica no hipocampo e no córtex pré-frontal

A análise da peroxidação lipídica nos homogeneizados do hipocampo e do córtex pré-frontal foi realizada pela avaliação do conteúdo de malondialdeído (MDA) através dos níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS), conforme descrito em Mihara e Uchiyama (1978). As amostras foram incubadas a 100 °C durante 60 min em meio ácido contendo 0,45% de dodecilsulfato de sódio e 0,67% de ácido tiobarbitúrico. Após centrifugação, o produto da reação foi determinado a 532 nm utilizando MDA como padrão. Os resultados foram dados como nmol/100mg de tecido.

5.8 Análise estatística

As análises estatísticas utilizadas nos testes de comportamento, cognição e parâmetros clínicos foram realizadas no software GraphPad Prism 8 (GraphPad, La Jolla, CA, EUA). Os resultados foram analisados quanto à distribuição normal pelo teste de Kolmogorov-Smirnov. Variáveis com distribuição normal foram comparadas por meio de análise de variância (One away ou Two away ANOVA). No caso de variáveis não normais, a análise foi realizada pelo teste de Kruskal-Wallis. O nível de significância foi estabelecido em $p < 0,05$.

Os níveis de expressão de citocinas e mediadores inflamatórios foram analisados usando código R personalizado no software R. Inicialmente, a análise de componentes principais (PCA) utilizando as medidas de todas as áreas, hemisfério do cérebro em relação ao trauma e tempos de análise em relação à lesão foi realizada usando a função *prcomp* no pacote *stats*. PCA é um método de redução de dimensionalidade que extrai metavariáveis das semelhanças na variação das variáveis originais (Jolliffe et al., 2016). Aqui, cada componente principal (PC) foi interpretado como a expressão de um mediador metainflamatório, compreendendo a expressão de vários mediadores inflamatórios que variam em direção semelhante. Citocinas, quimiocinas e fatores neurotróficos que aumentaram ou diminuíram a expressão juntos foram agrupados no mesmo componente. Em seguida, foram realizados modelos aditivos generalizados (GAM) para modelar a variação temporal de PC1 e PC2 em diferentes regiões cerebrais. A identidade animal entrou como variável aleatória nos modelos para controlar a pseudo-replicação. As análises GAM foram realizadas utilizando o pacote *mgcv*.

6 – RESULTADOS

6.1 ARTIGO CIENTÍFICO I publicado na revista *Current Medicinal Chemistry*

Título: ***Immune-Based Therapies for Traumatic Brain Injury: Insights from pre-clinical studies***

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Current Medicinal Chemistry, 2019, *26*, 1-28

REVIEW ARTICLE

Immune-Based Therapies for Traumatic Brain Injury: Insights from Pre-Clinical Studies

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ARTICLE HISTORY

Received: December 12, 2018
Revised: April 24, 2019
Accepted: May 22, 2019

DOI:
10.2174/0929867326666190710173234

Abstract: Traumatic brain injury (TBI) is a major public health problem. It is the leading cause of death and disability, especially among children and young adults. The neurobiology basis underlying TBI pathophysiology remains to be fully revealed. Over the past years, emerging evidence has supported the hypothesis that TBI is an inflammatory based condition, paving the way for the development of potential therapeutic targets. There is no treatment capable to prevent or minimize TBI-associated outcomes. Therefore, the search for effective therapies is a priority goal. In this context, animal models have become valuable tools to study molecular and cellular mechanisms involved in TBI pathogenesis as well as novel treatments. Herein, we discuss therapeutic strategies to treat TBI focused on immunomodulatory and/or anti-inflammatory approaches in the pre-clinical setting.

Keywords: Traumatic brain injury, inflammation, treatment, immunomodulatory agents.

1. INTRODUCTION

Traumatic brain injury (TBI) is a critical public health and socio-economic problem worldwide. It is the leading cause of death and disability, especially among children and young adults. The incidence in the elderly population has also been rising [1]. Data from the Centers for Disease Control and Prevention (CDC) indicate that each year in the USA, 1.7 million people exhibit a TBI, being 1.4 million of these injured individuals treated in emergency departments, with around 275,000 hospitalizations and 52,000 fatalities. The annual cost of TBI was estimated to be around US$ 77 billion, comprising both direct and indirect medical expenses [2, 3].

TBI is defined as an acute brain injury resulting from external mechanical forces to the head. The damage caused at the moment of the trauma is regarded as the primary injury, leading to anatomical and functional disturbance in structures of the central nervous system (CNS). Biochemical, cellular, oxidative stress and inflammatory events characterize the secondary injury and might occur hours, days, months, or even years after the primary lesion [1]. The therapeutic management of TBI is often focused on the primary injury and relies on the neurological evaluation with the Glasgow Coma Scale (GCS), neuroimaging techniques to determine the extension of the lesion, and intracranial pressure (ICP) monitoring and control. Currently, there is no treatment that prevents or minimizes TBI-associated secondary injury and neuropsychiatric sequelae, with the search for effective therapies a priority goal [4].

The molecular and cellular mechanisms involved in TBI pathophysiology are not fully understood, which

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0929-8673/19 $58.00+.00 © 2019 Bentham Science Publishers

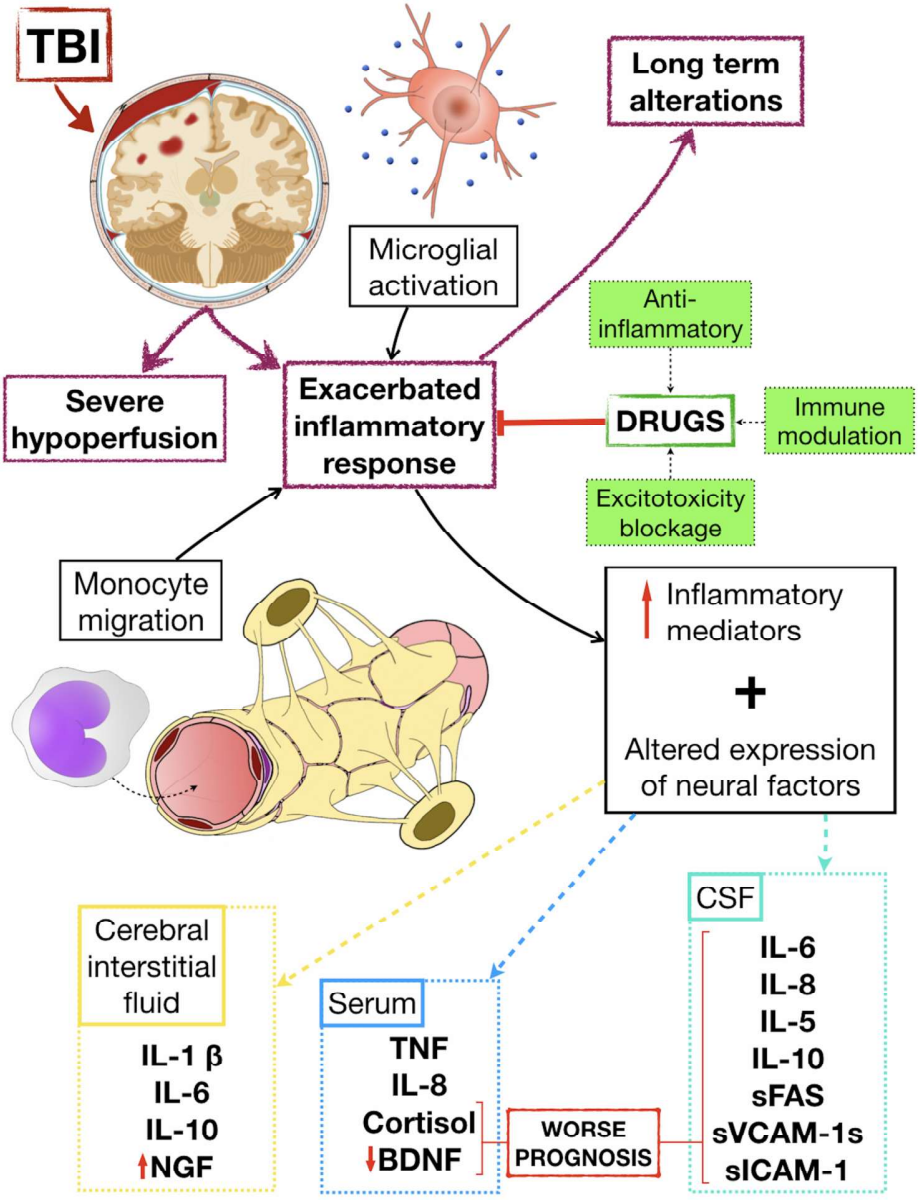

Fig. (1). Inflammatory response and potential immune-based therapies in traumatic brain injury (TBI). After TBI, severe hypoperfusion and inflamatory response occur in the brain. Microglial activation and monocyte recruitment increase the inflammatory response through the release of several inflammatory mediators and changes in neurotrophic factors expression. It has been reported that inflammation may play a role in TBI-associated long-term cognitive and behavior outcomes. Emerging therapies have been focusing on the modulation of inflammatory response secondary to the brain injury in order to improve TBI prognosis.

hampers the search and development of new therapy targets. Over the past years, a growing body of evidence has pointed out inflammation as a complex component of TBI-associated secondary injury cascade that may play an important role in TBI poor outcomes, such as cognitive, motor, and behavioral disorders [4-15]. TBI normally induces a complex inflammatory response, mainly characterized by fast proliferation and migration of brain-resident immune cells (microglia) and circulating leukocytes to the lesion site, with consequent production of inflammatory mediators like cytokines and chemokines [5, 14]. Microglia remain activated for weeks, months, or even years following a TBI event, which has been associated with increased expression of inflammatory cytokines, such as interleukin-1 beta (IL-1β) and tumor necrosis factor (TNF), and long-term cognitive dysfunction [6, 8, 11, 12].

By employing an intracerebral microdialysis approach, one study revealed an elevation of the levels of inflammatory cytokines as IL-1β and Interleukin-6 (IL-6) and nerve growth factor (NGF) in the interstitial fluid of the brain of patients 36 hours after severe head injuries [15]. Using microdialysis analysis of the cerebral extracellular space, an independent study found high levels of IL-6 at 48 hours and of IL-1β and IL-10 at 72 hours after severe TBI in humans [9]. The association between increased serum levels of TNF and of IL-8 with the severe status of cerebral hypoperfusion after TBI in humans was also reported [13]. Importantly, increased levels of IL-6 and IL-8 in the cerebrospinal fluid (CSF) of patients 5 days after a severe TBI were significant predictors of worse 6-month neurologic outcome [10]. A poor outcome 6 months after severe TBI was also associated with increased CSF concentrations of adhesion molecules (sICAM-1, sVCAM-1s) and other inflammatory mediators as IL-10, IL-6, IL-5, and IL-8 alongside higher serum cortisol and lower serum brain-derived neurotrophic factor (BDNF) levels [7]. It is worth noticing that despite the fact that IL-10 exerts mainly anti-inflammatory and neuroprotective effects, its CSF concentration has consistently shown to be increased after TBI, being associated with an unfavorable outcome [16-18]. For example, high CSF levels of IL-10 were associated with increased mortality following severe TBI [16]. Taken together, these studies support the concept that TBI is an inflammatory condition, and that inflammatory mechanisms might be potential therapeutic targets.

In this scenario, we aim to discuss the emerging data on the therapeutic strategies to treat TBI focused on immunomodulatory and/or anti-inflammatory approaches in the pre-clinical setting.

2. ANIMAL MODELS OF TBI

Animal models are valuable tools in biological and medical research. Among other advantages, they allow the study of underlying neurobiological mechanisms of diseases, the possibility of genetic manipulation as well as the assessment of novel therapeutic strategies in preclinical settings. However, animal models have intrinsic limitations and often do not reflect the complex pathophysiology of human diseases. Accordingly, caution regarding extrapolation to human conditions is highly recommended [19]. Different animal models, especially the weight-drop model, the controlled cortical impact model (CCI), and the lateral fluid percussion (LFP) model, have been employed for the study of TBI [20].

In the weight-drop model, a projectile of a predetermined weight is dropped in free fall on the skull of the animal [21]. Studies using this model have already shown cognitive, behavioral, and locomotor changes in mice [22, 23]. The severity of the brain injury in this model can be controlled by changes in the weight of the projectile used and the height that the projectile is dropped [24]. There are noticeable variations in this model regarding the lesion site and the pathological features [20].

The CCI model generally causes a focal lesion that, in a histopathological point of view, closely resembles the brain lesions of human patients. Of note, this model has often been employed for the study of molecular and genetic mechanisms related to TBI [25]. In this model, a device with pneumatic track contacts the exposed dura mater. This model allows the pre-definition of the depth of brain injury depth and speed of the device [26, 27].

In the LFP model, the skull and brain parenchyma are exposed, and the injury is caused by the impact of a high-velocity fluid jet [28]. The magnitude of the injury can be controlled by variations in the height of the pendulum used to generate pressure in the fluid jet, which may produce mild, moderate and severe brain injuries [28]. The application of a rapid pressure pulse on the exposed and intact dura mater causes a brief displacement and focal deformation of the brain tissue. The LFP model reproduces focal contusion and hemorrhage accompanied by axonal injury in the fimbria and internal capsule commonly found in human patients [29]. Importantly, the LFP model has been very efficient in reproducing the neurological impairment observed in the clinical practice [30-32].

3. MECHANISM OF THE HARMFUL ROLE OF INFLAMMATION IN TRAUMATIC BRAIN INJURY

TBI is a complex and multifactorial condition. The limited knowledge regarding the cellular and molecular mechanisms underlying TBI pathogenesis has hampered the development of potential effective therapies [33]. Herein, we will briefly discuss the main immune-based mechanisms triggered by TBI (for a complete review, please see Jassam, Izzy *et al.*, 2017).

The inflammatory response is an important TBI-associated event and has been a major target in studies focusing on strategies to control post-injury cell death and tissue degeneration [4, 11]. A sterile inflammatory response occurs shortly after TBI and relies on local signaling in neuronal and glial cells as well as periph-

eral immune cells activation and recruitment [34]. In response to TBI-related cerebral edema and increased permeability of the blood-brain barrier (BBB), dying or damaged cells within the lesion and peri-lesion areas in the brain release the so-called damage-associated molecules patterns (DAMPs), including the alarmins as high mobility group box 1 protein (HMGB1), heat shock proteins (HSPs), low-density lipoprotein and adenosine triphosphate (ATP), priming microglia and astrocytes [35-37]. Neuronal and resident immune cells (microglia) up-regulate innate immunity receptors, also known as "danger" sensors, such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD) like receptors (NLRs), purinergic and scavenger receptors. These immune receptors initiate the inflammatory cascade by recognizing the DAMPs [38-40]. After recognition of DAMPs, a variety of cellular responses, orchestrated by neurons, microglia and astrocytes, increases the release of cytokines (IL-1β, IL-6, IL-12, TNF-α), chemokines (CXCL1 and CCL2), growth factors, nitric oxide (NO) and oxygen reactive species (ROS) and the phagocytosis of dying cells and cellular debris [37, 41, 42]. For instance, following TBI, the transcript factor NFκB translocates to the nuclei of glial cells and induces cellular proliferation and production of inflammatory mediators like the cytokines IL-6 and TNF [41, 43]. Moreover, the activation of the cytosolic NLRs that integrate a macromolecular complex named inflammasome expressed in microglia, astrocytes, neurons, and peripheral immune cells promotes the cleavage of pro-caspase 1 into its active form, the caspase 1. Active caspase 1 then leads to the formation of mature inflammatory cytokines as IL-1β and IL-18, which, in turn, seem to induce sterile immune responses following TBI [44-46]. Interestingly, increased levels of inflammasome proteins, like caspase-1, was found in the CSF of patients following moderate or severe TBI and were associated with poor neurological outcomes [44].

The binding of ATP or uridine diphosphate (UDP) released from dead cells in the purinergic receptors (P2Y6, P2Y12, P2X4, P2X7, P2RY1) also modulates microglia and astrocytes functions. Particularly, the signaling of P2Y6 and P2X7 receptors in microglia mediates phagocytosis and cell activation and proliferation, respectively, in response to the acute brain injury [47, 48]. The activation of P2X7 receptors in microglia and potentially in astrocytes, following the release of ATP by damaged neurons, activates the inflammasome complex leading to TBI-associated cerebral edema and poor neurological outcome [48, 49]. Paradoxically, the expression of P2RY1 specifically in astrocytes in response to TBI reduced edema, neuronal swelling, and reactive gliosis through an IP3-signaling pathway [50]. It is worth mentioning that the role of glial cells in TBI is complex and should be considered in a time- and context-dependent manner. On the one hand, neurons and glial cells seem to interact *via* inflammatory mediators production to amplify the brain inflammation and damage, whereas, on the other hand, these cells may also promote tissue healing after TBI by releasing various growth factors, like IGF1 and nerve growth factors [33, 51, 52].

In parallel with the CNS local inflammatory response, peripheral immune cells are also recruited to the brain parenchyma. The neutrophils are usually the first peripheral immune cells to arrive in the lessoned brain a few hours following the injury [48, 53]. The infiltration of neutrophils to sites of sterile injury depends on purinergic receptors (P2X7), formyl-peptide receptor-dependent signaling, and release of cytokines and chemokines [54, 55]. High concentration of inflammatory cytokines, like TNF and IL-1β from damage brain in response to TBI, lead to the production of neutrophils chemoattractant factors such as the chemokine CXCL1 by the choroid plexus epithelium along with an increase in the expression of adhesion molecules, like ICAM-1 by endothelial cells [55]. Taken together, these events culminate in neutrophils migration to the brain parenchyma by means of several routes including across the BBB or inflamed vessels, *via* leptomeninges, through the choroid plexus located in the ventricular system or even by entering the subarachnoid space *via* pial microvessels located near to the site of brain lesion [55, 56]. Although several studies have shown a deleterious effect of neutrophils activation and recruitment [57-60], the role of these immune cells in TBI remains controversial. Emerging evidence supports a beneficial role for neutrophils in wound-healing responses and in neurological outcome improvements [61, 62].

During TBI, neutrophils are not the only peripheral immune cells to be recruited to the brain injury site. Approximately, one or two days after the traumatic event, circulating monocytes, expressing the receptor CCR2, in response to the production of the chemokine CCL2 by the choroid plexus epithelium, infiltrate the perivascular spaces and brain parenchyma differentiating in macrophages [63-65]. Similar to neutrophils, monocytes also reach the CNS *via* several routes, including the leptomeninges, choroid plexus, subarachnoid spaces, and even by crossing the damaged BBB [55, 65]. The role of monocytes-derived-macrophages

in TBI seems also to be controversial and depends on the time and context of the injury. Monocytes may take part in the clearance of debris and damaged cells, thus initiating the inflammation resolution and tissue repair. In contrast, monocytes may also promote inflammatory response amplification and further neuronal damage [63, 66]. Interestingly, the deletion of the CCR2 in mice subjected to acute mild TBI, induced by the LFP, decreased monocyte infiltration, lesion cavity volume, and axonal damage. However, the CCR2 absence was also associated with increased levels of phosphorylated tau, a marker of neurodegeneration, in the mice hippocampus. These findings support a complex and potential dual role of infiltrated macrophages in TBI [63].

Over the past years, some studies provided evidence that the adaptive immune system may also participate in TBI pathogenesis, as revealed by the infiltration of T-lymphocytes in the brain parenchyma following experimental TBI and by the presence of circulating T-lymphocytes markers in the serum of TBI patients [67-69]. However, the type of T-lymphocytes related to TBI pathophysiology and whether these cells promote beneficial or deleterious effects or may represent a potential therapeutic target remain to be fully addressed.

It is worth mentioning that the sterile immune response to TBI, which included local and peripheral cells, is an evolutionarily conserved process that ultimately aims to restore tissue homeostasis. For instance, the signaling provided by programmed death cells, a process known as apoptosis, generally induces an anti-inflammatory response in order to promote apoptotic cells clearance by engulfers cells like microglia or peripheral macrophages. In turn, the uptake of apoptotic cells also leads to the release of anti-inflammatory molecules like transforming growth factor (TGF)-β and interleukin (IL)-10 by engulfing macrophages that will amplify and preserve the anti-inflammatory state, ultimately restoring tissue homeostasis [70, 71]. Despite the fact that many factors and signaling pathways activated during an inflammatory response are involved in the regulation of cell apoptosis, the programmed cell death is often considered a protective process. In fact, the removal of apoptotic cells by phagocytosis is normally efficient enough to prevent secondary necrosis and the consequent release of cells' contents that would induce inflammation [70-72]. Accordingly, a failure to resolve the inflammatory reaction or to promote an immune-mediated wound-healing response may exacerbate TBI injury, leading to a chronic inflammatory scenario. Thus, the success of potential therapies for TBI may rely on the stimulation of immune wound-healing response in order to restore the CNS function rather than in completely abrogating the immune reaction to TBI [33].

4. IMMUNE-BASED THERAPIES: INSIGHTS FROM PRE-CLINICAL STUDIES

The current treatment for TBI focuses on the acute consequences of the injury and includes the control of vital signs and intracranial pressure. As mentioned, TBI-related immune response has been associated with the development of neuropsychiatric sequelae [73-75]. Preclinical studies have investigated the role of immune-based therapies in the physiopathology of TBI as well as in potential therapeutic targets (Table **1**). In these studies, an immunomodulator is administered before (pre-treatment) and/or after (treatment) brain injury [76]. Another approach is to genetically interfere with a target molecule or pathway by means of overexpression [77, 78], down-regulation, or deletion (knock-out) [79] in order to investigate possible neuroprotective roles. In the following sections, we will discuss pre-clinical evidence regarding immune-based therapies in experimental models of TBI. The chemical structure of the compounds used in these pre-clinical studies is shown in Fig. (**2**). The proposed molecular and cellular immune-based mechanisms of the therapy approaches reported by the pre-clinical studies are summarized in Figs. **3**, **4** and **5**.

4.1. Administration of Drugs with Anti-Inflammatory Properties

4.1.1. Ibuprofen

Ibuprofen is a potent nonsteroidal anti-inflammatory drug (NSAID) that has been evaluated as a neuroprotective agent in CNS conditions, such as Parkinson's disease [80]. In TBI, a study using the CCI model showed that oral administration of ibuprofen from 5 days to 4 weeks after brain injury in C57BL6 mice decreased microglial activation and monocyte infiltration in the lesion area. The anti-inflammatory environment induced by ibuprofen administration also allowed the migration and survival of transplanted neural stem and progenitor cells near the contusion and in the ipsilateral hippocampus [81]. An effect of ibuprofen was also observed in Wistar rats submitted to the weight-drop model. Rats received ibuprofen by oral route one hour before the injury, resulting in a significant decrease of brain and serum levels of IL-1β [82]. On the other hand, the intraperitoneal administration of ibuprofen immediately after brain injury induced by midline LFP model of concussion did not change cortical levels of

Table 1. Pre-clinical studies of immune-based therapies in traumatic brain injury.

Immunomodulator	Reference	Study approach	Population	TBI model/Severity	Dose	Experimental conditions	Main results
Ibuprofen	Browne et al., 2006	*In vivo*	Sprague–Dawley rats	LFP/No mention	40 mg/kg	Ibuprofen administration daily for 4 months after injury	Decreased astrocyte activation
	Wallenquist et al., 2012	*In vivo*	C57BL6 mice with grafted stem cells	CCI /No mention	30 mg/kg	Oral administration of ibuprofen 5 days after injury, daily for 4 weeks	Decreased microglia activation and macrophages infiltration
	Keshavarzi et al., 2012	*In vivo*	Wistar rats	Weight drop/moderate	10 mg/kg	Oral administration of ibuprofen 1 h before the injury	Reduction of IL-1 ß levels
	Cao et al., 2012	*In vivo*	Sprague–Dawley rats	LFP/moderate	20 mg/kg	Intraperitoneal injection 30 minutes after TBI and then daily for 7 days	Attenuated the expression of microglial activation genes
	Harrison et al., 2014	*In vivo*	C57BL/6 mice	LFP/mild	60 mg/kg	Ibuprofen administration immediately after injury	Decreased of IL-6 and TNF levels
Indomethacin	Chao et al., 2012	*In vivo*	Wistar rats	Weight drop/No mention	2.5 mg/kg	Intraperitoneal injection of indomethacin immediately after injury	Prevented IL-1β from overloading
	Girgis et al., 2013	*In vivo*	Swiss mice	Weight drop/No mention	5 mg/kg	Intraperitoneal injection de indomethacin 10 min after TBI	Improved neurological symptoms
Substantia P	Donkin et al., 2009	*In vivo*	Sprague–Dawley rats	Weight drop/severe	0.25 at 25 mg/kg	NAT administration 30 minutes after injury	Decreased of vascular permeability and edema formation
	Donkin et al., 2011	*In vivo*	Sprague–Dawley rats	Weight drop/moderate to severe	2.5 mg/kg	NAT administration at different time points	Decreased of axonal injury and improvement of cognitive outcome
	Corrigan et al., 2012	*In vivo*	Female Sprague–Dawley rats	Weight drop/No mention	2.5 mg/kg	NAT administration 30 minutes after injury	Decreased of axonal injury and improvement of motor skills
Minocycline	Homsi et al., 2009	*In vivo*	Swiss mice	Weight drop/No mention	45 or 90 mg/kg	Minocycline single administration of 5 minutes after injury	Reduction of IL-1 ß levels
						Minocycline single administration of 5 minutes after injury + Second injection of minocycline 3 hours after TBI	Reduction of IL-1 ß levels and brain edema formation

(Table 1) Contd….

Immunomodulator	Reference	Study approach	Population	TBI model/Severity	Dose	Experimental conditions	Main results
	Homsi et al., 2010	*In vivo*	Swiss mice	Weight drop/No mention	45 or 90 mg/kg	Minocycline were administered three times post-insult, at 5 min (90 mg/kg.), 3 h, and 9 h post-TBI (45 mg/kg)	Early inhibition of microglia activation provided long-lasting motor recovery
	Baki et al., 2010	*In vivo*	Sprague–Dawley rats	CCI/moderate	45 mg/kg	Minocycline administration + NAC administration 1 hour after injury	Decreased of IL-1β release
	Siopi et al., 2011	*In vivo*	Swiss mice	Weight drop/No mention	45 or 90 mg/kg	Intraperitoneal injections of mynocicline at 5 min (90 mg/kg) and at 3 and 9 hours (45 mg/kg) post-TBI	Restauration of brain levels of the endogenous neuroprotector sAPP α
	Ng et al., 2012	*In vivo*	C57BL/6 mice	Weight drop/No mention	45 or 22,5 mg/kg	Minocycline administration + NAC administration 3 hours before injury	Decrease in the accumulation and activation of microglia
	Lam et al., 2013	*In vivo*	Sprague–Dawley rats	CCI/severe	25 mg/kg	Intraperitoneal injection of minocycline 24 hours (and daily at 7 days, 12 days, and 16 days) after injury	Inhibition of microglial activation
	Haber et al., 2013	*In vivo*	Sprague–Dawley rats	CCI/mild	45 mg/kg	Intraperitoneal injections of minocycline + NAC 1 hour and 1 and 2 days after injury	Inhibition of microglial activation
	Kumar et al., 2013	*In vivo*	Wistar rats	Weight drop/moderate	50 mg/kg	Oral administration of minocycline + Ginseng	Decreased of IL-6 and TNF levels
	Taylor et al., 2017	*In vivo*	Male and Female Sprague–Dawley rats	CCI/moderate	50 mg/kg	Intraperitoneal injection of minocycline 1 hour (and daily for 3 days) after injury	No changes in TNF and IL-6 levels in the brain of male rats Increased the levels of IL-1β in the brain of female rats

(Table 1) Contd….

Immunomodulator	Reference	Study approach	Population	TBI model/Severity	Dose	Experimental conditions	Main results
	Haber et al., 2018	*In vivo*	Sprague–Dawley rats	CCI/mild	45 mg/kg	Intraperitoneal injections of minocycline + NAC 1 hour and 1 and 2 days after injury	Promoted protection of oligodendrocytes
EPO	Yatsiv et al., 2005	*In vivo*	Sabra mice	Weight-drop/No mention	5000 U/kg	rhEpo administration 1 and 24 h after	Prevented neurodegeneration and activation of glial cells
	Lu et al., 2005	*In vivo*	Wistar rats	CCI/No mention	5000 IU/kg	rhEpo administration 24 hours and daily for 14 days after injury	Improved spatial memory and enhanced neurogenesis
	Chen et al., 2007	*In vivo*	Wistar rats	Weight-drop/No mention	5000 U/kg	Intraperitoneal injections of rhEpo 1 hour (and daily for 3 days) after injury	Decreased brain expression of NF-kB, IL-1 ß, TNF and ICAM-1. Prevented brain edema, increase in BBB permeability and apoptosis.
	Verdonck et al., 2007	*In vivo*	Wistar rats	Weight-drop/No mention	5000 IU/kg	rhEpo administration 30 min after injury	Reduced brain edema
	Valable et al., 2010	*In vivo*	Wistar rats	Weight-drop/No mention	5000 IU/kg	rhEpo administration 30 min after injury	Prevented brain edema trough inhibition of extracellular-regulated kinase (ERK)-1/-2 phosphorylation
	Jin et al., 2011	*In vivo*	ICR mice	Weight-drop/No mention	5000 IU/kg	rhEpo administration 30 min after injury	Decreased severity of neurological deficit, brain edema, and cortical apoptosis by activating Nrf2
	Kertman et al., 2015	*In vivo*	Wistar rats	Weight-drop/moderate	30 µg/kg	A single intraperitoneal injection of darbepoetin-alfa immediately after injury	Prevented cell death and oxidative stress
	Zhou et al., 2017	*In vivo*	C57BL/6 mice	LFP/ No mention	5000 U/kg	EPO injected intraperitoneally at 1 hour and at 1, 2, and 3 days after TBI	Increased brain levels of IL-10 and decreased IL-1 ß, and TNF concentration Enhanced Treg cells number in the brain and inhibitted neutorphils and lymphocytes infiltration and microglial activation

(Table 1) Contd….

Immunomodulator	Reference	Study approach	Population	TBI model/Severity	Dose	Experimental conditions	Main results
Lithium	Zhu et al., 2010	*In vivo*	CD-1 mice	CCI/moderate	1 mmol/kg	Intraperitoneal injection with lithium daily for 2 weeks after injury	Reduction of IL-1 ß levels
	Dash et al., 2011	*In vivo*	Sprague-Dawley rats	CCI/No mention	1 mEq/kg	Lithium administration at 30 minutes and daily for 5 days after injury	Prevented neuronal loss
	Yu et al., 2012	*In vivo*	C57BL/6 mice	CCI/No mention	1.5 mEq/kg	Lithium administration at 15 min and daily for 3 days after injury	Inhibition of microglial activation and neuronal apoptosis
	Yu et al., 2013	*In vivo*	C57BL/6 mice	CCI/No mention	1 mEq/kg	Administration of lithium + valproate at 15 min and daily for 3 days after injury	Decreased lesion size and neurodegeneration
Heat shock protein	Zhao et al., 2013	*In vivo*	C57BL/6 mice	CCI/moderate	800 mg/kg	Treatment with a HSP70 inducer – Geranylgeranylacetone (GGA) at 48 hours before injury	Decreased lesion size and neuronal loss
	Kim et al., 2013	*In vivo*	Hsp70 KO mice	CCI/No mention	-	-	Reduction of lesion size and of brain hemorrhage
	Eroglu et al., 2014	*In vivo*	Hsp110-/- deficient mice and Hsp70i-/- deficient mice	CCI/ No mention	-	-	Lack of HSP70 leads to increased levels of the chemokines CCL2, CCL3 and LIF
			C57BL/6 mice		1mg/kg (celastrol) Or 15mg/kg (BGP-15)	Treatment with a Celastrol at 30 minutes and 6 hours, and then once daily for 5 days after injury or Treatment with a BGP-15 immediately before, and then at 6 hours, and once daily for 5 days	Decreased brain edema
	Gu et al., 2016	*In vivo*	Sprague-Dawley rats	CCI/moderate to severe	80 mg/kg	A single intraperitoneal injection of 17AAG immediately after injury	Reduction of IL-1 ß, IL-6 and TNF concentration

(Table 1) Contd….

Immunomodulator	Reference	Study approach	Population	TBI model/Severity	Dose	Experimental conditions	Main results
Statins	Qu et al., 2005	*In vivo*	Female Wistar rats	CCI/No mention	1 mg/kg/day	Atorvastatin administration at 1 day and daily for 7 days after injury	Improvement of spatial memory
	Lu et al., 2007	*In vivo*	Wistar rats	CCI/No mention	1 mg/kg	Atorvastatin and simvastatin administration at different times points	Prevented neurodegeneration and post traumatic hypoperfusion
	Wang et al., 2007	*In vivo*	C57BL/6 mice	Weight - drop/No mention	20 mg/kg	Atorvastatin administration at 30 min and daily for 3 days after injury	Decreased the number of F4/80-positive microglia and neurodegeneration
	Xu et al., 2017	*In vivo*	C57BL/6 mice	CCI/moderate	1 mg/kg	Atorvastatin administration at 1 hour and daily for 3 days after injury	Reduction of neuronal apoptosis and brain inflammatory cytokines levels
Memantine	Lamprecht et al., 2015	*In vitro*	-	In vitro /moderate	-	Memantine administration immediately after the injury or Memantine + 17β-estradiol administration immediately after the injury	Decreased neuronal death
	Kelestemur et al., 2016	*In vivo*	Balb/C mice	No mention	20 mg/kg	Memantine administration immediately after the injury	Reduction of iNOS expression
	Wang et al., 2018	*In vivo*	Sprague-Dawley rats	LFP/moderate	20 mg/kg and 1 mg/kg	Memantine treatment immediately after injury and drug administration twice for 3 days	Prevented microglial and astrocytes activation and neuronal damage Decreased iNOS levels
Endocannabinoid system	Cohen Yeshurun et al., 2011	*In vivo*	Sabra mice	Weight-drop/No mention	3mg/kg	Intraperitoneal injection with a CB2R agonist – AraS – at 1 hour after injury	Prevented brain edema
	Tchantchou and Zhang et al., 2013	*In vivo*	C57BL/6 mice	CCI/No mention	5 mg/kg or 10 mg/kg	Treatment with an ABHD6 inhibitor – WWL70 at 30 min after TBI, and then once a day for 3, 7, or 21 days	Reduction of lesion volume, neurodegeneration, BBB dysfunction and of iNOS and COX-2 expression
	Amenta et al., 2014	*In vivo*	C57BL/6 mice	CCI/No mention	-	Treatment with a CB2R agonist – JWH 133 at different times points	Reduction of iNOS, TNF and ICAM-1 mRNA expression.

(Table 1) Contd….

Immunomodulator	Reference	Study approach	Population	TBI model/Severity	Dose	Experimental conditions	Main results
	Tchantchou et al., 2014	*In vivo*	C57BL/6 mice	CCI/No mention	2 mg/kg, 5 mg/kg or 10 mg/kg	Treatment with a FAAH inhibitor – PF3845 – at 30 min after TBI, and then once daily for 3 or 14 days	Prevented neurodegeneration and decreased the expression. of iNOS and COX-2
	Katz et al., 2015	*In vivo*	Sprague-Dawley rats	LFP/mild	JZL184, 16 mg/kg URB597, 0.3 mg/kg	Treatment with inhibitor of 2-AG or AEA degradation - JZL184 or URB597 at 30 min after injury	Blocked astrocyte and microglia activation
	Braun et al., 2017	*In vivo*	CD-1 mice	CCI/No mention	1–5 mg/kg	Treatment with a CB2R agonist – GP1a – at 10 min after injury	Prevented anxiety like-behavior and brain edema.
Hormones	He et al., 2004	*In vivo*	Sprague-Dawley rats	No mention//severe	8 mg/kg	Administration of progesterone or allopregnanolone 1 hora after injury (followed by subcutaneous injections at 6 and daily for 5 days)	Reduction of IL-1 ß and TNF levels
	Chen et al., 2008	*In vivo*	Wistar rats	Weight drop/No mention	16 mg/kg	Injections of progesterone at 1 and 6 hours and daily for 5 days	Reduction of IL-1 ß, IL-6 and TNF levels
	Cekic et al., 2012	*In vivo*	Sprague-Dawley rats	CCI/No mention	16 and 8 mg/kg	Intraperitoneal injection of progesterone 1 after lesion (followed by subcutaneous injections at 6 and 24 hours)	Decreased the expression of proBDNF, TrkA, TrkB and p75NTR receptors Increased the levels of neurotrophic factors BDNF and NGF
	Sarkaki et al., 2013	*In vivo*	Female Albino N-Mari rats	Weight drop/ No mention	8 mg/kg 1 mg/kg 1.7 mg/kg	A single dose of 17β-estradiol or progesterone 30 min after injury	Decreased IL-6 levels
						17β-estradiol administration at 6 hours after injury	Enhanced TGF ß concentration
	Zhang et al., 2016	*In vivo*	C57BL/6 Nrf2-/- mouse	Weight drop/No mention	32 mg/kg	Progesterone administration immediately after injury	Reduction of IL-1 ß, IL-6 and TNF levels
Laquinimod	Katsumoto et al., 2018	*In vivo*	Ccr2rfp/+ and Cx3cr1 gfp/+	LFP/moderate	25 mg/kg	Laquinimod administration at 7 days before and 3 days after injury	Reduction of lesion volume and axonal damage

TBI: traumatic brain injury; CCI: controlled cortical impact; LFP: lateral fluid percussion; IL: interleukin; KO: knockout; NAT: N-acetyl-L-tryptophan; NAC: N-acetylcysteine; EPO: Erythropoietin; GGA: Geranylgeranylacetone; HSP: Heart Shock Proteins; ProBDNF: pro-apoptotic BDNF precursor; COX2: Cyclooxygenase 2; BBB: blood brain barrier; 17AAG: 17-allyl-amino-demethoxy geldanamycin; CCL2: chemokine ligand 2; ICAM-1: Intercellular Adhesion Molecule 1; 2-AG: 2-Arachidonoylglycerol; sAPP α: soluble form α of the amyloid precursor protein; Nrf2: nuclear related factor 2.

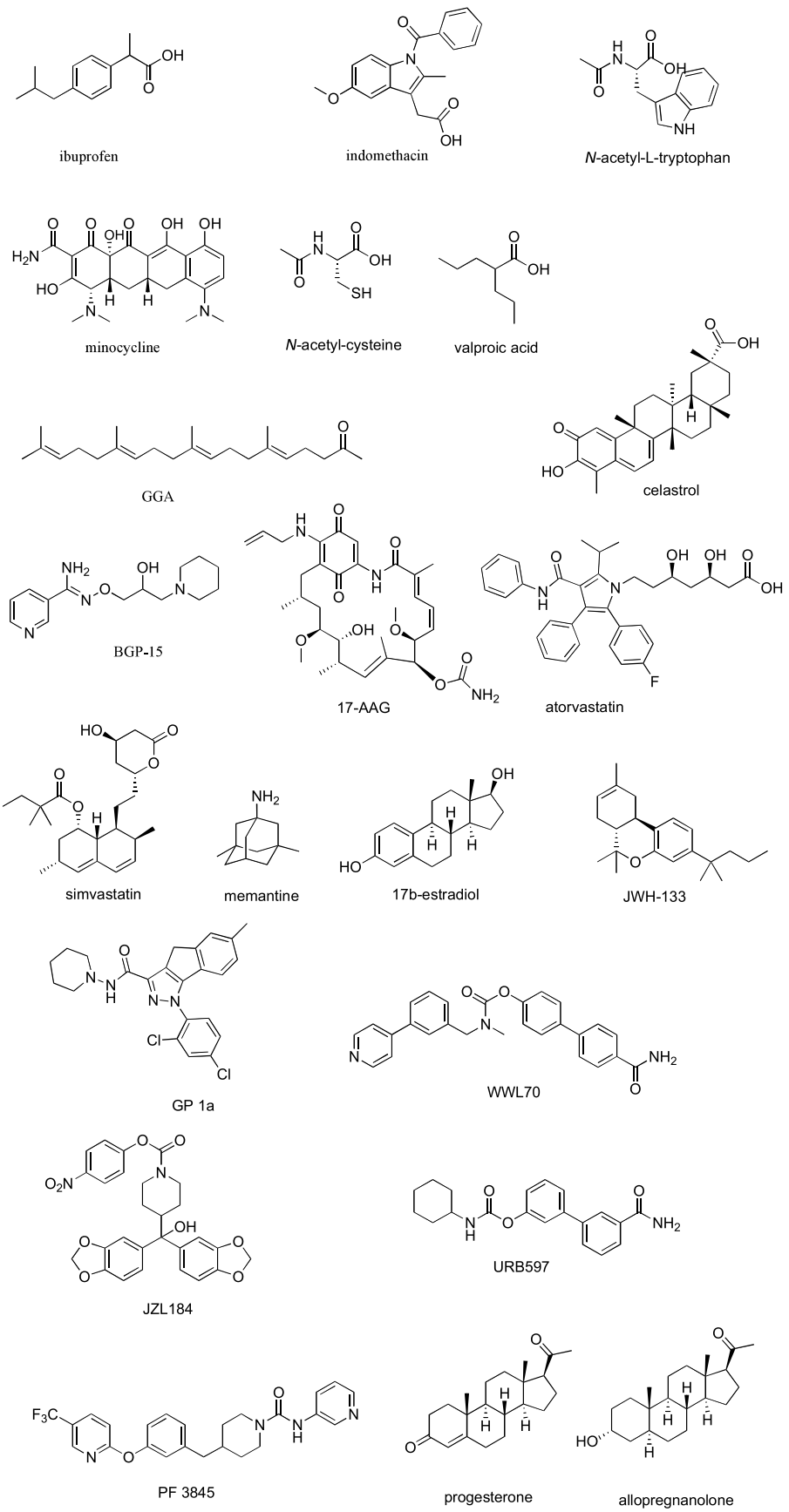

Fig. (2). Chemical structure of compounds employed in pre-clinical studies of traumatic brain injury (TBI).

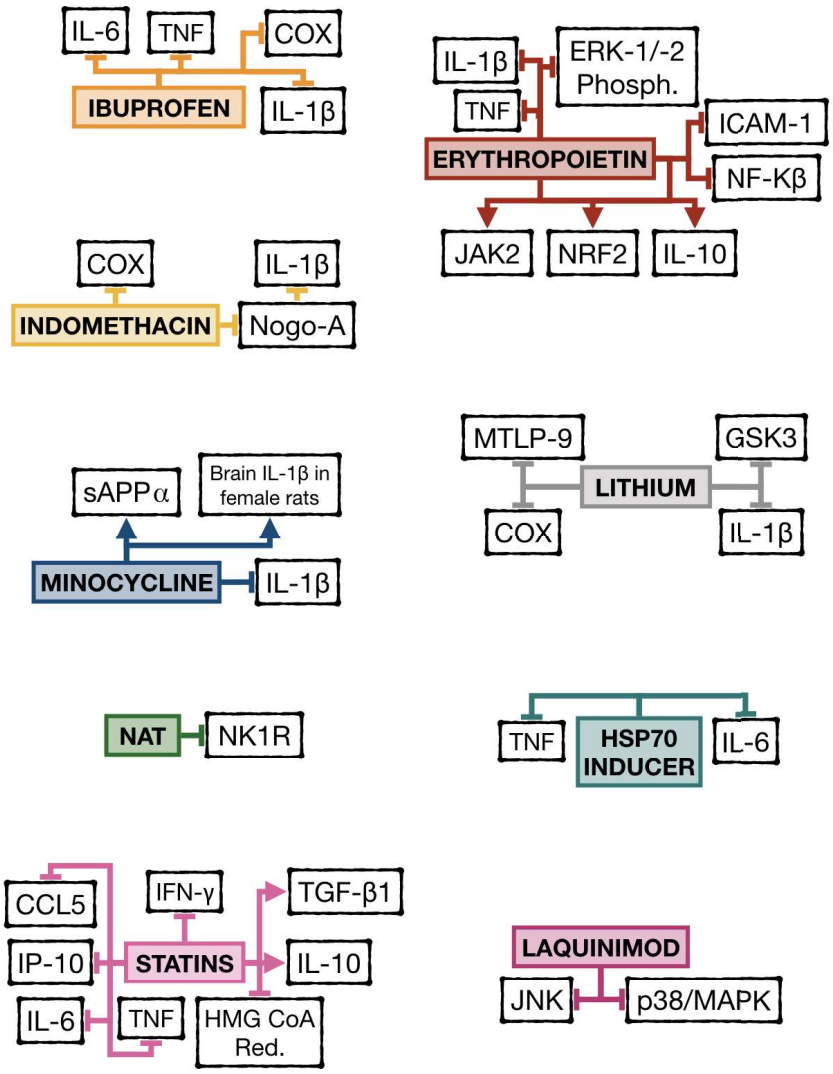

Fig. (3). Proposed molecular immune-based mechanisms of medications with anti-inflammatory properties. The therapies include the administration of Ibuprofen, Erythropoietin, Indomethacin, Minocycline, Lithium, N-acetyl-L-tryptophan (NAT), Inducers of 70Kdaltons heat shock protein (HSP70), Statins, Laquinimod. Molecular mechanisms including inhibition of inflammatory mediators (CCL5, COX, IL-1β, IL6, TNF, IFN-γ), of signaling pathways (ERK-1/-2 PHOSPH, JAK2, JNK, P38/MAPK), as well as induction of anti-inflammatory factors like IL-10, soluble form α of the amyloid precursor protein (sAPP α) and nuclear-related factor 2 (Nrf2) are reported.

IL-6 and TNF in C57BL6 mice. A decrease in the cortical concentration of these cytokines was observed 24 hours after the traumatic event. Moreover, this pharmacological intervention did not attenuate or exacerbate TBI-induced deficits, as measured by rotarod and a neurological score [83]. By employing the same TBI model in rats, a previous study showed that ibuprofen (20 mg/kg, i.p.) administered 30 minutes after TBI and then daily for 7 days decreased the expression of genes associated with microglial activation (CD45, major histocompatibility complex I, major histocompatibility complex II, translocator protein 18 kDa [TSPO]) in ventral posterior medial nucleus of the thalamus [84]. Chronic administration of ibuprofen for months at doses of 20 mg/kg body weight/day or 40 mg/kg body weight/day, after TBI induced by LFP in Sprague–Dawley rats led to long-term cognitive dysfunction despite the reduction of astrocyte activation in the CA3 region of the hippocampus [85]. These latter authors speculated on the potential negative effects of long-term prescription of NSAIDs to TBI patients, often used to treat chronic pain. Taken together these studies suggest potentially a protective effect of Ibuprofen in pre-treatment but none or even harmful effect in the post- and chronic administrations.

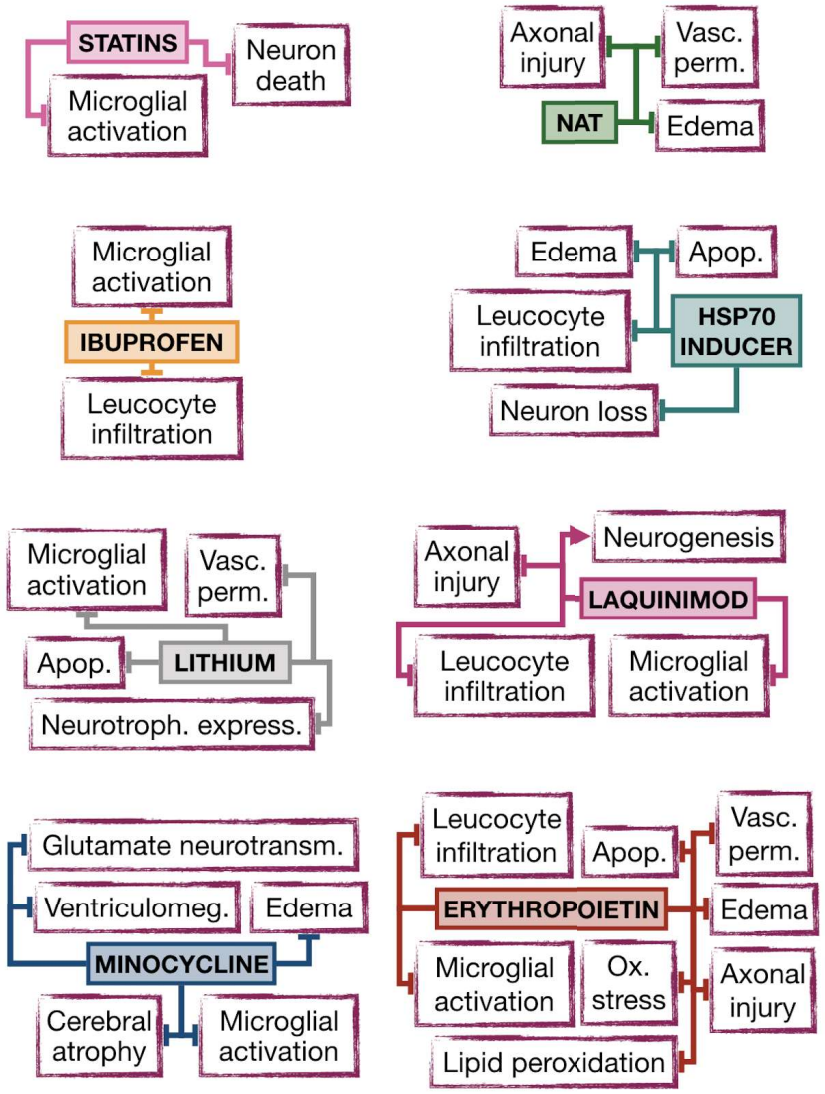

Fig. (4). Proposed cellular and clinical effects of medications with anti-inflammatory properties. The therapies include the administration of Ibuprofen, Erythropoietin (EPO), Indomethacin, Minocycline, Lithium, N-acetyl-L-tryptophan (NAT), Inducers of 70Kdaltons heat shock protein (HSP70), Statins, Laquinimod. Cellular and clinical repercussions such as inhibition of microglial activation, leucocytes infiltration, neuronal death, and axonal injury as well as reduction of brain edema, cerebral atrophy, vascular permeability (vasc. perm.), glutamate neurotransmission, oxidative stress (ox. stress), and lipid peroxidation are described.

4.1.2. Indomethacin

Indomethacin is also an NSAID, a non-selective cyclooxygenase (COX) inhibitor, that has been evaluated in different CNS conditions such as stroke and hepatic encephalopathy [86, 87]. Intraperitoneal injection of indomethacin immediately after TBI induced by weight-drop decreased the levels of IL-1β in the hippocampus of Wistar rats eight hours after the event, resulting in less axonal demyelination and neuronal damage. This effect might be associated with inhibition of Nogo-A, a member of the reticulon family of membrane-associated proteins that plays an important role in axonal remodeling [84]. By employing the same TBI model, Girgis *et al.* [88] showed that intraperitoneal injection of indomethacin 10 min after TBI significantly promoted neurological recovery at 6 and 24 hours after injury in Swiss mice, without improving brain edema. The neuroprotective mechanisms of indomethacin in TBI remain to be elucidated.

4.1.3. NK1 Antagonist

Substance P (SP) is a neuropeptide member of the tachykinin family, a group of structurally related pep-

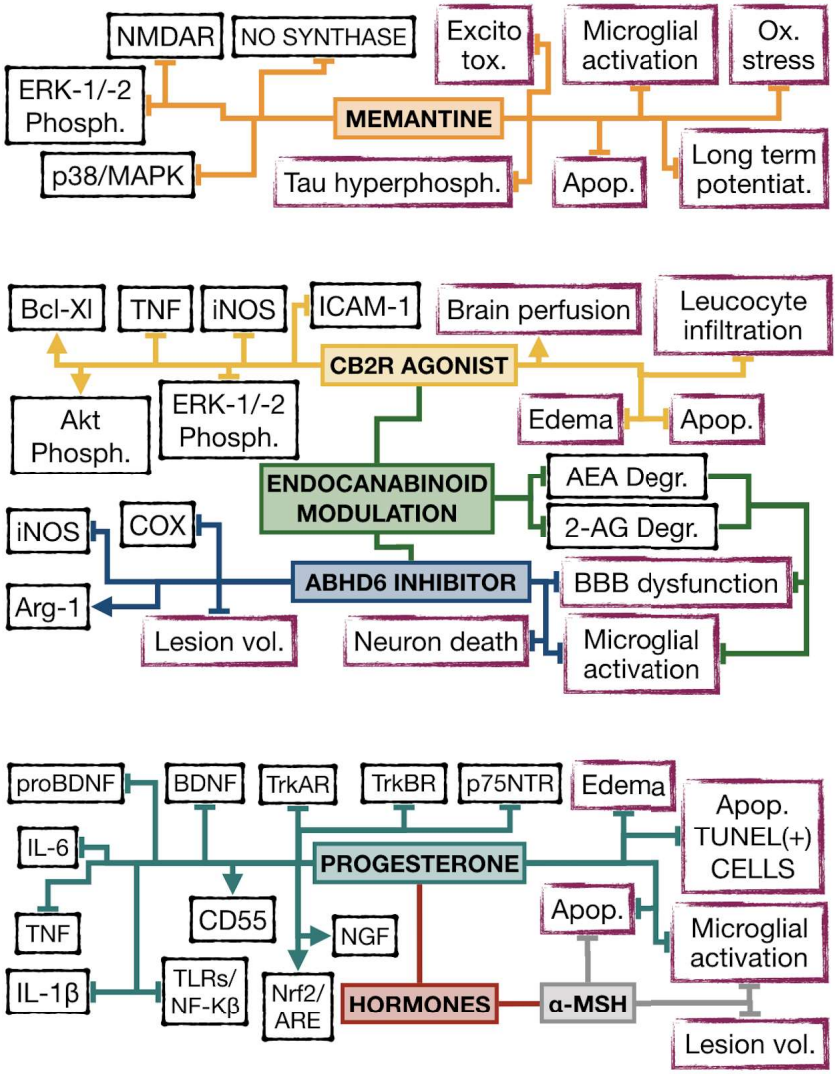

Fig. (5). Molecular, cellular and clinical effects of medications with anti-excitotoxicity properties (memantine and endocannabinoid system) and hormones with anti-inflammatory properties (progesterone and alpha-melanocyte-stimulating hormone, α-MSH). The proposed molecular mechanisms include inhibition of inflammatory and oxidative stress mediators (COX, IL-1β, iNOS, NO synthase, IL6, pro-BDNF, TNF, IFN-γ), and signaling pathways (ERK-1/-2 PHOSPH, Akt PHOSPH, P38/MAPK), as well as induction of anti-inflammatory and neurotrophic factors like NGF and nuclear-related factor 2 (Nrf2). Cellular and clinical repercussions including inhibition of microglial activation, leukocytes infiltration, neuronal death, and blood-brain barrier (BBB) dysfunction as well as reduction of brain edema, lesion volume, Tau hyperphosphorylation, glutamate neurotransmission, and oxidative stress (ox. stress) are reported.

tides sharing the same carboxyl-terminal sequence, which also includes neurokinin A and neurokinin B [89]. SP is a potent initiator of neurogenic inflammation, especially by increasing blood-brain barrier (BBB) permeability with subsequent plasma protein extravasation and edema formation [90]. SP seems also to potentiate the inflammatory response by stimulating the release of inflammatory mediators such as histamine, NO, and cytokines and the migration of leukocytes to the lesion site [91]. The actions of SP are mediated by the high-affinity binding with the tachykinin NK1 receptor. The NK1 receptor is a typical G-protein coupled receptor (GPCR) with seven transmembrane domains that is expressed by neurons and glial (astrocytes and microglia) cells. Binding of SP to NK1 receptor leads to activation of phospholipase C (PLC), which catalyzes the hydrolysis of phosphoinositides into inositol 1,4,5-trisphosphate and diacylglycerol, which then allow mobilization of calcium from internal stores affecting neuronal survival [92].

A growing body of evidence has shown that pharmacological blockage of the NK1 receptor with its an-

tagonist N-acetyl-L-tryptophan (NAT) protects the brain against edema formation and increased vascular permeability in response to TBI, also improving neurological symptoms [93, 94]. Male Sprague-Dawley rats submitted to TBI weight-drop model, in which NAT was intravenously administered 30 minutes after the trauma, reduced vascular permeability and edema formation 5 hours after TBI. Treatment with NAT attenuated motor and cognitive impairment as measured by rotarod task and Barnes maze test, respectively [94]. Similar findings were reported in female Sprague-Dawley rats, indicating that sex differences did not influence the neuroprotection associated with the blockage of SP signaling in response to TBI [93]. Moreover, administration of NAT attenuated vascular permeability and edema formation at 5 hours after TBI [94] and also improved cognitive outcome in the Morris water maze and novel object recognition tests and reduced axonal injury 5 and 24 hours after the trauma in male Sprague-Dawley rats subject to acceleration-induced diffuse TBI model [95].

4.1.4. Minocycline

Minocycline, a safe and well-tolerated second-generation tetracycline with antimicrobial and anti-inflammatory properties, is reported to be capable of inhibiting activated microglia [96] and enhancing glutamate neurotransmission [97]. Accordingly, previous studies have shown a protective effect of minocycline in CNS conditions such as schizophrenia-related cognitive impairment, anxiety, and depression [98-100].

A neuroprotective effect of minocycline was observed in a study using three different regimens of intraperitoneal minocycline administration in mice submitted to weight-drop TBI model. Minocycline was administered once (5 min), twice (5 min and 3 h), or three times (5 min, 3 h, and 9 h) following TBI. An anti-inflammatory effect of minocycline, as indicated by a reduction in IL-1β levels in the brain, was found at 6 hours following TBI in the one and two doses administration. The three-dose regimen did not influence IL-1β levels but resulted in brain edema reduction and neurological recovery, indicating that beneficial effects of minocycline may depend on the treatment regimen following the brain injury [101]. The same research group showed that minocycline administered 24 hours after TBI induced by weight-drop model provided long-lasting motor recovery that lasted up to 8 weeks after TBI [102]. In line with these findings, a regime of intraperitoneal injection of minocycline at 5 min (90 mg/kg) and then at 3 and 9 hours (45 mg/kg) post-TBI induced by weight-drop model was able to attenuate corpus callosum and striatal atrophy, ventriculomegaly, astrogliosis, and microglial activation up to 3 months after the traumatic event in Swiss mice. These long-term protective effects of minocycline were, at least in part, associated with the restoration of brain levels of the endogenous neuroprotector soluble form α of the amyloid precursor protein (sAPP α) [103]. A decrease in the accumulation and activation of microglia in the hippocampus and peri-contusion cortex along with an early and sustained improvement in neurological outcome were also observed in mice treated with minocycline (i.p 22.5 mg/kg) 1 week after TBI induced by the weight drop model [104].

The effect of minocycline in TBI has also been investigated in the CCI model. Intraperitoneal injection of minocycline daily for 2 weeks, beginning 1 day after the trauma, attenuated microglial activation in the peri-contusion cortex and hippocampus of rats, and improved spatial learning and memory in the water maze test [105]. Conversely, female and male Sprague Dawley rats intraperitoneally treated with minocycline 1 hour after TBI induced by CCI model and with a single daily dose for the next 3 days did not have changes in brain levels of IL-6 and TNF at 35 days after injury. However, minocycline induced an increase in IL-1β expression in the hippocampus of female rats, suggesting that sex might influence minocycline effects in response to TBI [3].

The combination of minocycline with other compounds has also been investigated following TBI. When minocycline was administrated with N-acetylcysteine, an antioxidant compound, 1 hour after moderate TBI induced by CCI model in rats, the combined therapy improved acquisition and long-term retention on an active place avoidance task [106, 107]. Examination of brain histology two weeks after TBI revealed that the combined therapy preserved the white matter. However, the association of minocycline with NAC did not influence IL-1β concentration in the hippocampus measured 1 hour after brain injury [107]. In a mild CCI model of TBI, the administration of minocycline in combination with NAC 1 hour, 1 day and 2 days after the trauma improved long-term memory in the active place avoidance task and attenuated CD68-expressing phagocytic microglia without altering neutrophil infiltration or astrocyte activation in the lesion site. Moreover, at distal white matter tracts including the corpus callosum, the combined therapy decreased CD68 expression, but increased overall microglial activation as measured by Iba-1, suggesting a modulation rather than a suppression of the immune response

[108]. The same research group recently reported that the combined administration of minocycline and NAC might improve cognitive impairment following mild TBI induced by CCI model by modulating inflammation and inducing remyelination by means of the protection of resident oligodendrocytes [109].

The oral administration of minocycline combined with ginseng, a plant extract with antioxidant and anti-inflammatory properties, for a period of 2 weeks improved memory impairment in Morris water maze and decreased the levels of TNF and IL-6 in the cerebral cortex and hippocampus of Wistar rats submitted to the weight-drop model of TBI. The protective effect was less significant when each compound was administered separately [110].

4.1.5. Erythropoietin

Erythropoietin (EPO) belongs to the cytokine superfamily and has traditionally been viewed as an erythropoiesis-regulating hormone with a role in inflammatory processes, especially by inhibiting apoptosis and maintaining cell survival *via* Janus tyrosine kinase 2 (JAK2) pathway [111]. EPO exerts a protective role in a wide range of CNS conditions such as stroke [112], Parkinson's disease [113, 114], and epilepsy [115].

In TBI, Wistar rats submitted to the weight-drop model and treated with 5,000 units/kg injection of recombinant human erythropoietin (rhEPO) 1 hour and at days 1, 2, and 3 post-trauma reduced brain expression of inflammatory mediators including IL-1β, TNF, and ICAM-1. The rhEPO treatment also decreased brain edema, BBB permeability, and apoptotic cells in the injured brain [116]. The reduction of brain edema in the neocortex and caudate-putamen was also found six hours after diffuse TBI induced by the impact-acceleration model in Wistar rats treated with rhEpo (5000 IU per kg of body weight) 30 minutes after the injury [117]. Sabra mice submitted to the TBI weight-drop model that received rhEpo (5000 U per kg of body weight, intraperitoneally) 1 hour and 24 hours after injury exhibited a significant recovery of motor and cognitive functions along with a decrease in neuronal apoptosis, axonal damage, microglial and astrocyte activation at the lesion site. All the behavioral and histological parameters were evaluated up to 14 days after TBI [118]. A mechanism by which rhEPO exerts its protective effect in experimental TBI seems to involve the activation of nuclear-related factor 2 (Nrf2), an antioxidant transcription factor and the inhibition of extracellular-regulated kinase [119]-1/-2 phosphorylation in the injured brain [120, 121].

More recently, the administration of single intraperitoneal dose darbepoetin-alfa (30 μg/kg), a hypersialylated analogue of EPO that activates EPO receptors, immediately after moderate TBI induced by the weight-drop model decreased oxidative stress, lipid peroxidation and neuronal apoptosis in the brain of Wistar albino rats [122]. Apart from its anti-oxidant activity, EPO (5000 U/kg body weight) injected intraperitoneally at 1 hour and at 1, 2, and 3 days after TBI induced by LFP in mice was able to decrease TBI-associated inflammatory response. The administration of EPO increased brain concentration of the anti-inflammatory cytokine IL-10, while decreased the levels of IL-1β and TNF. EPO also enhanced the number of Treg cells in the brain and spleen, inhibited the infiltration and activation of peripheral immune cells like neutrophils and lymphocytes, and decreased microglial activation. The anti-inflammatory effect of EPO was also associated with reduced brain edema and improvement of cognitive function, suggesting EPO as a potential therapy in TBI [123].

4.1.6. Lithium

Lithium is the lightest metal in nature. Clinically, lithium has been traditionally used as a mood-stabilizer [124]. The mechanisms underlying lithium neuroprotective effects remain to be fully understood. There is evidence that by inhibiting the glycogen synthase kinase-3 (GSK3), a serine/threonine kinase pathway, lithium may decrease cellular apoptosis, inflammation, whereas enhances the expression of neurotrophins and cell-survival molecules, leading to neuroprotection [125, 126].

The potential neuroprotective role of lithium was investigated in the CCI model of TBI [127-129]. Daily intraperitoneal administration of lithium (1 mmol/kg) for 2 weeks before TBI until mice sacrifice enhanced spatial learning and memory performance in the Morris water maze. The restoration of cognitive function was associated with a significant decrease in cortex levels of IL-1β, cerebral edema, and neuronal degeneration in hippocampal CA3 and dentate gyrus sub-regions [129]. Daily intraperitoneal administration of lithium 15 minutes after TBI until day 3 attenuated neuronal apoptosis in the dentate gyrus of the hippocampus and decreased microglial activation, COX2 induction, metalloproteinase-9 expression, and BBB disruption in cortical areas close to the lesion site. Regarding behavioral outcomes, lithium treatment reduced anxiety-like behavior in open-field test, and improved short- and long-term motor coordination in rotarod and beam-walk tests [128]. The same authors also reported beneficial effects of the

combination of low doses of lithium and valproate, another mood-stabilizer, for TBI. The combined therapy significantly reduced lesion volume, attenuated BBB disruption, and prevented hippocampal neurodegeneration 3 days after TBI and also improved motor coordination up to 21 days after injury [127].

4.1.7. Heat Shock Proteins

Heat shock proteins (HSPs) are conserved molecules in prokaryotes and eukaryotes with low expression under physiological conditions. However, the synthesis of these proteins increases in response to environmental challenges, including hyperthermia, excitotoxic exposure, and other stressful conditions [130]. HSPs act as intracellular chaperones and play a crucial role in the maintenance of cellular integrity and viability. One of the major HSPs is the 70 kD heat shock protein (HSP70) which regulates pathways of apoptosis and inflammation [131]. Accordingly, the genetic and/or pharmacological overexpression of HSP70 protected neurons from lethal insults, including cerebral focal ischemia and intracerebral hemorrhage [132, 133].

In TBI, the genetic deletion of HSP70 in mice submitted to the CCI model increased brain lesion size and hemorrhage [79, 134]. In addition, the lack of HSP70 enhanced the expression and activation of matrix metalloproteinases, brain edema, macrophage infiltration, apoptosis, and levels of inflammatory mediators such as CCL2, CCL3, and leukemia inhibitory factor (Lif), thereby worsening brain damage and motor function in the CCI model of TBI [134]. The absence of HSP70 was also associated with an increase in oxidative stress and ROS production as indicated by the elevated levels of p53-induced genes (Pigs) in the injured brain [134]. On the other hand, genetic overexpression of HSP70 protected mice from brain damage and related behavioral changes, supporting the use of HSP70 for the treatment of TBI [79].

In line with these findings, the administration of HSP70 inducers, Celastrol and BGP-15, protected against TBI-induced brain apoptosis, inflammation, cellular infiltration, and gliosis, thus improving behavioral and cognitive dysfunctions [134]. The administration of other HSP70 inducers such as the 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) and the Geranylgeranylacetone (GGA) also produced neuroprotective effects. Intraperitoneal (IP, 2 mg/kg) or intracerebroventricular (ICV, 1 μg/kg) administration of 17-AAG to mice subjected to CCI model of TBI increased microglial and neuronal, but not an astrocytic expression of HSP70, decreased brain lesion size and hemorrhage, and improved motor function [78]. In the same TBI model, intraperitoneal injection of 17-AAG (80 mg/kg) immediately after trauma reduced brain edema, motor deficits, and down-regulated the levels of IL-1β, IL-6, and TNF [77]. Moreover, oral administration of GGA (800 mg/kg), an HSP70 inducer already used in a clinical setting as an antiulcer agent, 48 hours before and 3 hours after TBI induced by CCI prevented motor and cognitive impairment and depressive-like behavior. The neuroprotective effects included a decrease in neuronal loss in the hippocampus, cortex, and thalamus, and less microglial activation in the cortex [135].

4.1.8. Statins

Statins are 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors with anti-inflammatory, anti-oxidant, and anti-excitotoxicity actions. Beneficial effects of statins have been reported in TBI models [76, 136-138]. It is worth mentioning that statin administration has practical advantages for TBI treatment, especially due to wide availability, safety, few side effects, and easy monitoring [139].

The administration of statins by different routes and time points in experimental rodent models of TBI, especially CCI, significantly improved cognitive and behavioural outcomes by decreasing brain inflammation and neuronal death [76, 136-138]. Atorvastatin (1 mg/kg/day) orally administered for 7 consecutive days in female Wistar rats, starting at 24 hours after TBI induced by CCI, restored spatial memory deficits and increased neuronal survival in CA3 region of the hippocampus. However, atorvastatin was not able to improve sensorimotor functional impairment [137]. Similar findings were also reported in rats treated with atorvastatin and simvastatin one day after TBI and daily for 14 days [136]. Mice subjected to TBI *via* CCI that received subcutaneous injections of simvastatin (20 mg/kg) or atorvastatin (20 mg/kg) daily for 3 days following head injury decreased mRNA expression of TNF and IL-6. Less microglial cells activation and neuronal death in the hippocampus alongside improvement in motor and cognitive deficits were also found [138]. A more recent study showed that acute treatment with atorvastatin at doses of 1 mg/kg/day, administered orally starting at one-hour post-TBI for three consecutive days, decreased the levels of inflammatory cytokines (IFN-γ and IL-6) and chemokines (CCL5 and IP-10) in the brain of mice subjected to the CCI model. Atorvastatin treatment also increased the proportion of regulatory T cells (Tregs) in both spleen and brain, in

parallel with enhanced expression of regulatory cytokines (IL-10 and TGF-β1). These changes were associated with a reduction in neuronal apoptosis and behavioral deficits, supporting the use of statins for TBI [76].

4.1.9. Laquinimod

Laquinimod is an immunomodulatory oral drug originally developed for the treatment of multiple sclerosis [119]. Although the mechanisms of action of laquinimod remain to be revealed, there is evidence that it modulates inflammatory pathways like p38/MAPK and JNK and might regulate microglia profile and monocyte infiltration [119, 140, 141]. A recent study investigated the effects of laquinimod in a murine model of moderate TBI induced by fluid percussion. Daily oral administration of laquinimod (25 mg/kg) for 7 days before and 3 days after TBI decreased hippocampal axonal damage and restored neurogenesis. Laquinimod also inhibited macrophage activation and infiltration to the brain and led microglia phenotype toward a neuroprotective profile, supporting the role of laquinimod for TBI [142].

4.2. Administration of Drugs with Anti-Excitotoxicity Properties

4.2.1. Memantine

Memantine is a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist, protecting neurons from excessive glutamate activity. Memantine is also able to enhance long-term potentiation and decrease tau hyperphosphorylation, thereby exerting neuroprotection. This drug is currently approved for the treatment of Alzheimer's disease [143, 144]. Additionally, studies have been supporting the efficacy of memantine for cognitive improvement in other neurodegenerative and neuropsychiatric disorders, like Parkinson's disease and bipolar disorder [106, 145]. Due to its well-established safety and efficacy in clinical practice, especially regarding the cognitive outcome, it is quite reasonable to hypothesize that memantine might be a promising therapy for TBI.

A study using the LFP model to induce TBI in Sprague Dawley rats showed that intraperitoneal administration of memantine (initial dose of 20 mg/kg and then 1 mg/kg every 12 hours until 6 doses) attenuated neuronal apoptosis, astrogliosis, and microgliosis in the brain cortex at 72 hours after TBI. Treatment also decreased oxidative stress as indicated by a reduction in the inducible NO synthase expression in microglia and astrocytes in the ischemic cortex. These molecular and cellular changes were associated with improvement in motor and proprioception deficits after TBI [146]. Memantine was also able to protect mice brain against cold injury-induced trauma model by decreasing local inflammatory pathways signaling such as phosphorylation of p38/MAPK, ERK-1, and ERK-2 [147].

4.2.2. Endocannabinoid System

Endocannabinoids represent a class of molecules released from post-synaptic neurons to control the release of classical neurotransmitters from pre-synaptic terminals. These 'neuromessengers' are endogenous ligands (*i.e.* the fatty acid derivatives: anandamide and 2-arachydonoyl glycerol, 2-AG) that participate in the endocannabinoid system found in the mammalian brain. This system also comprises at least two G-protein coupled receptors, CB1R and CB2R, and the enzymes responsible for endocannabinoid synthesis and catabolism. Endocannabinoids modulate a wide range of brain functions, including emotion regulation [148]. More recently, endocannabinoids have emerged as powerful anti-inflammatory compounds, although the molecular and cellular mechanisms underlying these effects are still unclear [149].

It has been proposed that the endocannabinoid system may play a protective role in TBI. The administration of synthetic selective CB2R agonists (0-1966 and JWH-133) decreased the levels of TNF and mRNA expression of the intracellular adhesion molecule-1 (ICAM-1) and iNOS in the brain of mice subjected to CCI model of TBI. Double-label immunohistochemistry confirmed that iNOS was expressed by macrophage/microglia in the injured cortex. Importantly, the pharmacological blockade or genetic deletion of the CB2R worsened post-traumatic inflammation and neurovascular responses [150]. Corroborating these findings, the activation of CB2R with GP1a, the most specific CB2R ligand currently available, skewed infiltrating macrophages toward an anti-inflammatory profile in CCI mice model 3 days after TBI. Of note, in response to TBI, CB2R expression increased in infiltrating macrophages within peri-contusion cortex. Treatment with GP1a also decreased brain edema, improved cerebral perfusion, and enhanced motor and neurological outcomes after TBI [149]. Accordingly, the administration of AM630, a CB2R antagonist, blunted all neuroprotective effects of GP1a administration [149].

Anti-apoptotic and anti-inflammatory effects were reported following the administration of N-arachidonoyl-L-serine (AraS), a brain component struc-

turally related to the endocannabinoid system, in Sabra mice submitted to weight-drop TBI model. AraS treatment-induced elevation of the prosurvival factor Bcl-xL levels 24 hours after injury and 30% reduction in caspase-3 activity 3 days after injury. AraS also attenuated the phosphorylation of ERK 1/2 and increased the phosphorylation of Akt in both ipsilateral and contralateral cortices. The neuroprotective effects of AraS seem to be associated with indirect signaling *via* the CB2R and the transient receptor potential vanilloid 1 (TRPV1) since specific antagonists to these receptors reversed them [151].

Apart from modulation of CB2R activity, the administration of compounds that interfere with components of the endocannabinoid system also seems to confer protection against TBI. For instance, mice submitted to CCI model of TBI treated with a selective inhibitor (WWL70) of Alpha/beta hydrolase domain 6 (ABHD6), a 2-AG hydrolytic enzyme that accounts for 2-AG hydrolysis, improved motor coordination and working memory performance and reduced BBB dysfunction, the volume of lesion in the cortex and neurodegeneration in the dentate gyrus. WWL70 also abrogated the expression of iNOS and cyclooxygenase-2 while enhanced the expression of arginase-1 in the ipsilateral cortex, resulting in microglia/macrophage anti-inflammatory profile. The WWL70-associated neuroprotection may also involve up-regulation and activation of both CB1 and CB2 receptors and the subsequent phosphorylation of the ERK and the serine/threonine protein kinase AKT [152]. Since fatty acid amide hydrolase (FAAH) is an enzyme responsible for the hydrolysis of the N-arachidonoyl-ethanolamine (anandamide, AEA), a major endocannabinoid in the brain, the administration of the FAAH inhibitor PF3845 prolonged the protective effects of AEA in TBI [153]. Pharmacological inhibition of the degradation of the endocannabinoids 2-AG and AEA by the administration of JZL184 and URB597, respectively, also induced neuroprotective effects in rats after TBI induced by LFP. Selective inhibition of 2AG and AEA degradation was associated with improved behavioral and neurological function, BBB integrity and blockage of microglia and astrocyte activation without changing the mRNA expression for inflammatory and oxidative mediators in the ipsilateral cortex [154].

4.3. Administration of Hormones with Anti-Inflammatory Properties

The neuroprotective role of female sexual hormones, like progesterone, has also been investigated in experimental models of TBI. The neuroprotection associated with these hormones relies mainly on anti-inflammatory effects [155-159]. Intraperitoneal administration of progesterone (8 mg/kg) or its metabolite allopregnanolone (4 mg/kg) decreased the levels of TNF and IL-1β in the brain of rats submitted to a bilateral prefrontal cortical contusion, a type of CCI model. The anti-inflammatory effects of both hormones were observed 3 hours after TBI, suggesting an early control of TBI-associated inflammation. This mechanism may reduce cerebral edema and promote functional recovery [157]. Progesterone and allopregnanolone may also exert neuroprotection by enhancing the production of CD55, a single-chain type 1 cell surface protein that acts as a potent inhibitor of complement convertases, thereby blocking the inflammatory response in the cerebral cortex of rats 48 hours after TBI [160]. In addition, intraperitoneal administration of progesterone (16 mg/kg) 1 hour after TBI followed by subcutaneous injections at 6 and 24 hours after the trauma was able to influence neurotrophic factor signaling. Treatment with progesterone resulted in significant improvement of motor activity, increase in the levels of NGF and reduction in the expression of BDNF, its pro-apoptotic precursor (proBDNF) and receptors TrkA, TrkB, and p75NTR in the cerebral cortex of rats at 72 hours after TBI. These changes in neurotrophin-related pro-survival and pro-apoptotic signaling induced by progesterone remained at least 7 days following the brain injury and may represent another mechanism by which this hormone protects the brain after trauma [155].

A single intraperitoneal injection of the alpha-melanocyte-stimulating hormone (α-MSH), a neuropeptide with immunomodulatory properties, 30 min after TBI induced by CCI, exerted an anti-inflammatory effect as revealed by a decrease in neuronal apoptosis and microglial activation along with a reduction in the volume of brain lesion [159].

The protective role of hormones has been also reported in the weight-drop model of TBI. Rats receiving progesterone (16 mg/kg) at 1 and 6 hours postinjury and on days 1, 2, 3, 4, and 5 after TBI, decreased brain concentration of inflammatory cytokines (IL-1β, IL-6, and TNF) and the number of apoptotic TUNEL-positive cells. Based on the fact that rats submitted to TBI had up-regulation of toll-like receptor (TLR)2, TLR4, NF-kappaB, pro-inflammatory cytokines, and ICAM-1 expression in the peri-contusion area, the protective effects of progesterone might be associated with inhibition of TLRs/NF-kappaB signaling pathway [156]. More recently, neuroprotective effects of proges-

terone in TBI were related to the activation of Nrf2/ARE signaling pathway [161]. Intraperitoneal administration of progesterone (32 mg/kg) immediately after TBI reduced brain edema, apoptosis, cytokine release, and microglial activation. Conversely, these beneficial effects were blunted in mice with genetic deletion of Nrf2 [161].

There is evidence that the anti-inflammatory effects of sexual hormones seem to be dependent on the type, dose, and time of administration of the hormone after TBI. The intraperitoneal administration of different doses of 17β-estradiol or progesterone (30 min after TBI) influenced the expression of IL-1β, IL-6, TNF, and TGFβ in the brain of female albino rats submitted to the weight-drop model of TBI. Both hormones exerted anti-inflammatory effects and were able to protect the brain, although the efficacy was dependent on the dose and administration time after TBI [158].

CONCLUDING REMARKS

Despite important advances in critical medical care, TBI is still a major public health problem with a significant socio-economic burden. The cellular and molecular mechanisms underlying TBI pathophysiology remain to be fully elucidated. Over the past years, emerging evidence has pointed to a crucial role of the immune system in TBI. The inflammatory response following TBI seems to be orchestrated by complex interactions between neurons, glial cells and peripheral immune cells, mainly neutrophils and monocytes-derived macrophages. The sterile inflammatory response may result in beneficial or deleterious effects to TBI according to the time and context of the injury. Local and peripheral cells may amplify inflammation or, in a different way, may promote an immune-mediated wound healing. The better understanding of TBI-associated immune-based mechanisms may allow the identification of potential therapeutic targets and the development of novel therapies.

Preclinical studies play a critical role in this context. Accordingly, the reduction of exacerbated inflammation, cellular toxicity, oxidative stress, and apoptosis emerges as promising strategies for neuroprotection. Despite several compounds have been investigated in preclinical studies, there is still a long road to translate these findings into clinical practice.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The authors would like to thank 2016 NARSAD Young Investigator Grant from Brain & Behavior Research Foundation, FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Brazil), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for financial support. ACSS and ALT is a CNPq productivity fellowship recipient. ASM is a 2016 NARSAD Young Investigator Grant Awardee from the Brain & Behavior Research Foundation.

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6.2 - ARTIGO CIENTÍFICO II publicado na revista *Journal of Neuroimmunology*

Título: ***Weight-Drop Model as a Valuable Tool to Study Potential Neurobiological Processes Underlying Behavioral and Cognitive Changes Secondary to Mild Traumatic Brain Injury***

Journal of Neuroimmunology 385 (2023) 578242

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Weight-drop model as a valuable tool to study potential neurobiological processes underlying behavioral and cognitive changes secondary to mild traumatic brain injury

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ARTICLE INFO

Keywords:
Mild traumatic brain injury
Neuroinflammation
Lipid peroxidation
Behavioral changes
Cognitive damage
Weight-drop model

ABSTRACT

The pathophysiology of post-traumatic brain injury (TBI) behavioral and cognitive changes is not fully understood, especially in its mild presentation. We designed a weight drop TBI model in mice to investigate the role of neuroinflammation in behavioral and cognitive sequelae following mild TBI. C57BL/6 mice displayed depressive-like behavior at 72 h after mild TBI compared with controls, as indicated by a decrease in the latency to first immobility and climbing time in the forced swim test. Additionally, anxiety-like behavior and hippocampal-associated spatial learning and memory impairment were found in the elevated plus maze and in the Barnes maze, respectively. Levels of a set of inflammatory mediators and neurotrophic factors were analyzed at 6 h, 24 h, 72 h, and 30 days after injury in ipsilateral and contralateral hemispheres of the prefrontal cortex and hippocampus. Principal components analysis revealed two principal components (PC), which represented 59.1% of data variability. PC1 (cytokines and chemokines) expression varied between both hemispheres, while PC2 (neurotrophic factors) expression varied only across the investigated brain areas. Our model reproduces mild TBI-associated clinical signs and pathological features and might be a valuable tool to broaden the knowledge regarding mild TBI pathophysiology as well as to test potential therapeutic targets.

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https://doi.org/10.1016/j.jneuroim.2023.578242
Received 1 September 2023; Received in revised form 27 October 2023; Accepted 5 November 2023
Available online 7 November 2023
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1. Introduction

Traumatic brain injury (TBI) is one of the leading causes of death worldwide. In the United States alone, over 280,000 TBI-related hospitalizations and 56,800 deaths were reported in 2014 (*Surveillance Report of Traumatic Brain Injury-related Emergency Department Visits, Hospitalizations, and Deaths*, 2014). TBI is a cerebral lesion caused by a mechanical force applied to the skull. Falls and car accidents are the main external causes of TBI (Faul et al., 2010). Mild TBI accounts for approximately 80% of TBI cases and, among all TBI severities, is the most neglected as most patients are not followed-up on the potential development of TBI-related cognitive and neuropsychiatric sequelae (Feigin et al., 2013; Tabish and Syed, 2014; Yates et al., 2006). Patients with mild TBI may exhibit sleep disturbances (Mani et al., 2015), behavioral and cognitive changes (de Freitas Cardoso et al., 2019; L'Ecuyer-Giguère et al., 2020), which might affect their quality of life. Despite the high prevalence and clinical importance, the pathophysiological mechanisms of mild TBI are not fully understood.

The primary injury occurs immediately after the traumatic event and disrupts structures in the central nervous system (CNS) (Bramlett and Dietrich, 2007). This process may lead to diffuse axonal injury (Guarnizo et al., 2021; Zhong et al., 2021), altered intracranial pressure (Pérez-Bárcena et al., 2020) and blood-brain barrier (BBB) disruption (Yoen et al., 2021). The secondary injury involves a sequence of cellular and pathological events triggered by the primary insult that can be observed within a few hours or days of the traumatic event and can persist for years (Johnson et al., 2013). The main mechanisms of the secondary TBI-related injury involve apoptosis (Miñambres et al., 2008), neuroinflammation (Ebert et al., 2019) and oxidative stress (Mojtahedzadeh et al., 2014).

Patients with mild TBI can display cognitive impairment at the acute phase (de Freitas Cardoso et al., 2019) that can last for years after the injury (Theadom et al., 2018). Anxiety and depression are also sequelae that have been reported both acutely and years following mild TBI (Ma et al., 2019). Recent evidence supports the role of secondary injury mechanisms in the development of persistent behavioral and cognitive alterations in these patients (Ebert et al., 2019; Sun et al., 2019; Vedantam et al., 2021). For instance, Vedantam et al. (2021) showed that increased plasma levels of interleukin (IL)-2 at 24 h after a mild TBI were associated with more severe 1-week post-concussive symptoms, as assessed by the Rivermead Post-Concussion Symptoms Questionnaire (RPCSQ) (Vedantam et al., 2021). In addition, elevated plasma concentrations of IL-10 were associated with more depressive and post-traumatic stress disorder (PTSD) symptoms at 6 months in these patients with mild TBI (Vedantam et al., 2021). Elevated serum levels of IL-1β were also related with cognitive impairment one month after mild TBI (Sun et al., 2019).

Different experimental models have been employed to study mechanisms underlying the secondary injury in mild TBI (Taib et al., 2017; Kosari-Nasabb et al., 2018). The most used TBI models are the weight-drop, the controlled cortical impact (CCI) and the lateral fluid-percussion injury (LFP). In the weight-drop model, the injury is caused by the controlled fall of a weight on the animal's skull. This model has some advantages in comparison with the CCI and LFP models. It does not require any pre-trauma manipulation, making this model faster and easier technique to implement (Flierl et al., 2009). Several studies have also shown that the weight-drop model can reliably induce in rodents the typical behavioral and cognitive changes seen in mild TBI patients, such as depression, anxiety and deficits in memory and learning tasks (Bader et al., 2020; McAteer et al., 2016; Milman et al., 2008; Rachmany et al., 2013; Shapira et al., 2007). Previous studies have also demonstrated the involvement of inflammatory pathways in mild TBI pathogenesis in rodents subjected to weight drop. However, they often investigated TBI-related inflammation in the ipsilateral side of the lesion in a single time point [25,30,31). Accordingly, there is still limited evidence regarding the effects on the contralateral site as well as of the kinetic of the injury.

We hypothesize that mild TBI sequelae results from secondary lesions, mainly inflammatory and oxidative stress pathways alongside disruptions in neuroprotective processes, not restrict to the brain region directed affected by the physical trauma and/or to the ipsilateral side of the lesion. Herein, we took advantage of a weight-drop model to characterize clinical, behavioral, and cognitive changes related to primary and secondary TBI-associated injuries. We focused on the role of inflammatory mediators and neurotrophic factors in key brain regions associated with mild TBI-related behavioral and cognitive impairment. To the best of our knowledge, this is the first study that investigated the kinetic of secondary lesions, by measuring inflammation at several time points after the injury, as well as both ipsilateral and contralateral hemispheres response to the experimental mild TBI. We also provided additional evidence of the potential involvement of isoforms of the enzyme nitric oxide synthase (NOS) in mild TBI outcomes.

2. Material and methods

2.1. Animals

Male wild type C57BL/6 mice weighing 20 to 30 g and aged 10–12 weeks were obtained from the Bioterism Center at Federal University of Minas Gerais (UFMG), Belo Horizonte, Brazil. The study was conducted according to the Ethics Committee on Animal Experimentation of UFMG (under the protocol 250/2017). The animals were kept in a 12-h light-dark cycle, with food and water ad libitum. Given the higher incidence of TBI in men compared to women [4], herein, sex was not studied as a biological variable and we used male mice for all the experiments.

Before behavioral and cognitive tests, the animals were acclimated in the test room for at least 30 min. Fig. 1 summarizes the experimental design.

2.2. Mild TBI injury

The weight-drop model built (Fig. S1) here was based on the models described by Flierl et al. (2009) (Flierl et al., 2009) and Marmarou et al. (1994) (Marmarou et al., 1994). Animals were put under ketamine/xylazine anesthesia and placed in a prone position. Mice skulls were exposed through a midline incision and a 15 g weight was dropped by a guide tube of 60 cm on the right parietal side, between the sagittal and coronal sutures. Importantly, in our model we place the mouse in the device with the head fixed to guarantee that the weight will fall at the exactly same region of the skull, decreasing the variability of the model seen in other weight-drop models. Each animal received a unique mild TBI or sham injury. After the mild injury, the animals were sutured, and clinical signs were observed. Next, the animals were placed in a thermal blanket and were submitted to eye hydration during follow-up recovery. The severity of TBI as mild was defined mainly by the projectile weight and falling height alongside the absence of mortality, skull fracture and loss of body mass. All procedures were carried out with the sham or control group, except the weight-drop trauma. The animals were divided in at least 5 different groups: 1) sham; 2) TBI 6 h; 3) TBI 24 h: 4) TBI 72 h; 5) TBI 30 days. As no significant differences were found between sham mice in the open field task in the evaluated time points, we conducted all the analysis with sham animals at 72 h. For the behavior and cognitive tasks 10 animals per group were used and for pathological and neurochemical/inflammatory analysis 5 mice per group were used. A total of 10 mice were followed up for mortality, neurological severity score (NSS) and body weight recordings. Four different cohorts of mice were employed to perform behavioral analyses totaling 180 mice: 1) Open field and novel object recognition task: 50 mice, 10 per each of the five groups; 2) Elevated plus maze and forced swim test: 50 mice, 10 per each group: sham, TBI 6 h, TBI 24 h, TBI 72 h, TBI 30 days; 3) Y maze and marble burying: 50 mice being 10 per each group; 4) Barnes maze: 30 mice, 10 per each time point (sham, TBI 72 h and TBI 30 days). Each

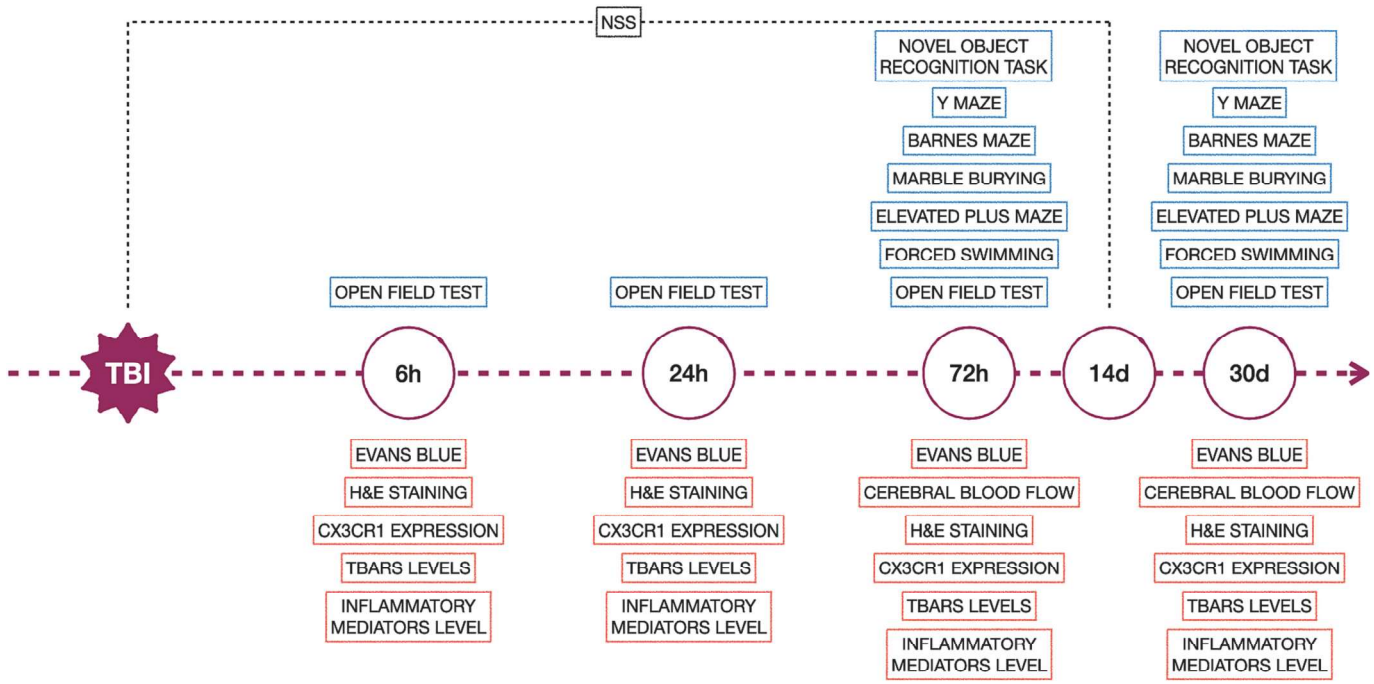

Fig. 1. Experimental design.

cohort was subjected to two behavioral tests with an hour of interval between the tasks. The more aversive task was always the last one to be performed. From the 180 mice, 50 animals from the first cohort were used for BBB permeability (total of 25 mice, being 5 per group) and histological analysis (total of 25 mice, being 5 per group); 50 animals from the second cohort were used for the analysis of inflammatory/oxidative stress markers and neurotrophic factors by ELISA, Citometric Bead Array (CBA) or real-time PCR; 45 animals from the third cohort were used in the lipid peroxidation analysis and immunofluorescence staining; 15 animals from the fourth cohort was used for the cerebral flow experiments.

2.3. Neurological severity score (NSS)

NSS was used for neurological assessment using 10 different tests, which evaluated the response to locomotor function, alertness and stimulus after TBI in several animal studies (Sharma et al., 2012; Khalin et al., 2016; Shen et al., 2017; Dai et al., 2018; Lan et al., 2019). The 10 clinical parameters encompass: 1) exit circle, 2) upper and lower monoparesis/hemiparesis, 3) straight walk, 4) startle reflex, 5) seeking behavior, 6) beam balancing, 7) round stick balancing, 8) beam walk: 3 cm, 9) beam walk: 2 cm, 10) Beam walk: 1 cm, as described elsewhere (Flierl et al., 2009). Evaluation of the NSS score 1-h post TBI was used to establish injury severity in experimental models. A NSS score of less than or equal to 5 points is indicative of mild TBI. A maximum score of 10 points after the first hour post-injury indicates severe neurological impairment and severe trauma (Flierl et al., 2009). We performed a neurological evaluation using NSS at baseline and at time-points 1, 3, 6, 12, 24, 48 and 72 h and 4, 5, 6, 7, 10, 12 and 14 days after mild TBI, as previously described by Flierl et al. (2009) (Flierl et al., 2009). Mice that failed to perform the test were assigned 1 point, mice that completed half the task were assigned half a point, and mice that successfully completed the task were assigned 0 point.

2.4. Open field test

Open field test was performed to assess locomotor activity after mild TBI using the PhenoTyper system (Noldus Information Technology, Leesburg, VA, USA) (Miranda et al., 2017). The total distance travelled in the open arena (30 cm × 30 cm × 35 cm) was analyzed for 30 min using the Ethovision XT software (Noldus Information Technology, Leesburg, VA, USA).

2.5. Forced swimming test

The forced swimming test was performed to evaluate depressive-like behavior as described in Miranda et al. (2017) (Miranda et al., 2017). Mice were placed in a glass beaker (18 cm in diameter and 28 cm in height) with water at the height of 18 cm and swimming was recorded for 6 min. For the analysis, only the last 4 min of the test were considered. During this period, immobility time, climbing time and latency to the first immobility were recorded and used to measure the depressive-like behavior. The climbing time was considered as upward movements against the wall of the glass beaker (Costa et al., 2013).

2.6. Elevated plus maze test

The elevated plus maze test was performed to assess anxiety-like behavior. The test was performed for 5 min and analyzed using the Ethovision XT software (Noldus Information Technology, Leesburg, VA, USA). The percentage of time spent in the maze open arm was used to measure the anxiety-like behavior (Fernandes et al., 2020).

2.7. Marble burying test

The marble burying test was performed to assess compulsive-like behavior after mild TBI as described elsewhere (Tucker et al., 2017). The increase in number of marbles buried in comparison to the sham-group was used to indicate the compulsive-like behavior (Tucker et al., 2017).

2.8. Barnes maze test

Barnes Maze test was performed for evaluation of hippocampal-dependent spatial memory at 72 h and 30 days after mild TBI. The protocol used was adapted from Tachiki et al. (2023) (Tachiki et al.,

2023). For the adaptation phase, mice were placed in the escape tunnel for 1 min and then in the center of the maze for 5 min or until it returned to the escape tunnel. The first training session started 1 h after the habituation phase. Training was performed with 2 daily trials, for 9 days, with 1-h intervals between trials. In each trial, mice were contained in a black box for 15 s placed in one of the maze's quadrants. Mice were then released to explore the maze and find the escape tunnel in 3 min. Mice that did not locate the escape tunnel in 3 min were conducted back to and maintained in the tunnel for 15 s to learn the location. The probe trial test was performed 24 h after the last training. Mice were placed in a maze to explore for 1 min. The time for the mice to find the escape tunnel (latency), error number to find the escape tunnel and time in scape zone in the probe test was recorded (Tachiki et al., 2023). The search strategies to resolve the maze during the probe test (i.e. spatial, serial and random) were also analyzed as described elsewhere (Tachiki et al., 2023).

2.9. Y maze

The Y Maze was performed for evaluation of working memory. The percentage of spontaneous alternations between arms during 5 min was obtained through the index: [alternate total/ (total of entries in arms −2) X 100] (Salari et al., 2018).

2.10. Novel object recognition task

The Novel object recognition test was performed to assess short (1.5 h) and long-term working memory (24 h) after mild TBI, as described elsewhere (Miranda et al., 2017). Briefly, a habituation session is performed during 30 min without objects in the box. After 24 h of the habituation session, mice were placed at the box with two identical objects (A1 and A2) for 10 min (adaptation phase). The short memory task was performed 1.5 h after adaptation trial and one old object (A2) was replaced by one novel object (A3). The long-term memory was performed 24 h after adaptation session and one object (A3) was replaced by one novel object (A4). The trial for short- and long-term memory was performed during 10 min. The percentage of time exploring the novel object was analyzed using the Ethovision XT software (Noldus Information Technology, Leeburg, VA, USA).

2.11. Blood-brain barrier integrity

The permeability of the BBB was analyzed by Evans blue dye at 6, 24 and 72 h and 30 days after mild TBI. Mice under ketamine/xylazine anesthesia received intravenous injection of 0.5% Evans blue solution (Sigma-Aldrich, St. Louis, MO). After 1 h mice were decapitated and the brain was incubated in formamide for 24 h (1 mL /100 mg of tissue). The amount of Evans Blue in the brain tissue extracts was measured by absorbance at 610 nm and the results presented as the level of Evans Blue (pg) in 100 mg of brain tissue as described elsewhere (Toscano et al., 2016).

2.12. Cerebral blood flow analysis

Laser Doppler perfusion imaging (Moor Instruments, Ltd., England) was used to estimate cerebral blood flow (CBF) at 72 h and 30 days after mild traumatic brain injury. The animals (anesthetized) were placed in a prone position and the skull was exposed through a midline incision for analysis as described by Valle et al. (2020) (Valle et al., 2020). When the scanning procedure is completed, tissue perfusion is color-coded. Little or no perfusion is shown in dark blue color and maximum perfusion in red. After digitization, the images were analyzed using the Moor LDI Laser Doppler 5.3 software to quantify the blood flow.

2.13. Histological analysis

After behavioral and cognitive analysis, mice were anesthetized with an intraperitoneal injection of ketamine/xylazine and submitted to transcardiac perfusion surgery at 6, 24 and 72 h, and 30 days after mild TBI. The surgery was performed using saline (0.9%) and formalin (10%) for hematoxylin-eosin (H&E) staining and paraformaldehyde (4%) for immunofluorescence staining.

2.13.1. Hematoxylin-eosin (H&E) staining

Brains were carefully removed and post-fixed in 10% buffered formalin. Sections of 5 μm at intervals of 10 μm were cut and mounted for routine H&E staining. The sections were examined at the optical level (Olympus, Japan, JP). Digital images were acquired for documentation using a × 400 planachromatic objective.

2.13.2. Immunofluorescence staining

Immunofluorescence staining was performed on fixed mouse brains. After fixation, each hemisphere was cut into coronal slices of 2 mm and then proceeded to paraffinization processing. Each paraffin block was cut into 5-μm slices and mounted on immunohistochemistry IHC microscope slides (Agilent Technologies Singapore). Then the slides were deparaffinized and the immunofluorescence staining was performed. Briefly, the slides were washed with PBS and incubated in the primary antibody overnight under temperature of 8 degrees Celsius. The following primary antibodies were used for quantification of axonal damage, microglial activation, and astrocytic activation: rabbit anti-Amyloid Precursor Protein-APP (1:200; ab32136), rabbit anti-Iba1 (1:200; PA5–21274) and mouse anti-GFAP (1:150; MAB3402), respectively. The next day, slides were washed with PBS and incubated with secondary antibodies for 4 h, protected from light at 22–26 degrees Celsius. The following secondary antibodies were used: anti-rabbit (1:1000; A21245), and anti-mouse (1:750; A11001). The slides were then incubated with DAPI for nuclear staining for 30 min, mounted, and observed under a confocal microscope LSM 880 ZEISS (CAPI – ICB/UFMG). The total number of Iba-1/DAPI and GFAP/DAPI positive cells was counted in five different fields of cortex and hippocampus (standardized area). For the quantification of APP, the mean value of the fluorescence intensity was counted in five different fields of cortex and hippocampus (standardized area). Apart from the total number of positive cells, the mean value of the fluorescence intensity was also performed for Iba-1 and GFAP markers.

2.14. Measurement of inflammatory mediators, lipid peroxidation and CX3CR1 levels

2.14.1. CBA and ELISA analyses of cytokines and neurotrophic factors

After behavioral and cognitive analysis, TBI mice were anesthetized with an intraperitoneal injection of ketamine/xylazine and euthanized at 6, 24 and 72 h, and 30 days after mild TBI. Sham animals were also anesthetized and euthanized immediately after behavioral and cognitive analyses. The brains were carefully removed for dissection of the ipsilateral (right) and contralateral (left) hemisphere of prefrontal cortex and hippocampus. After the dissection, the tissue was homogenized in an extraction solution (100 mg of tissue per milliliter), containing NaCl, 20 mM Tris-Hcl, 10% glycerol, 1 mM PMSF, 1 μM Pepstatin A, 10 mM EDTA, 10 μM E-64 and 0,5 mM sodium vanadate, sing Ultra-Turrax. Lysates were centrifuged at 13,000*g* for 10 min at 4 °C, supernatants were collected and stocked at −70 °C until use. Levels of IL-6, IL-10, IL12p70, IFN-γ, TNF-α and MCP1 were measured by Cytometric Bead Array (CBA) (BD Biosciences, San Diego, CA) and the concentration of inflammatory mediators was determined by FACSCanto (Becton Dickinson, San Jose, CA, USA). Levels of IL-1β, KC, CX3CL1, BDNF, GDNF, and NGF were assessed by Enzyme-linked immunosorbent (ELISA), according to the manufacturer protocol (R&D Systems, Minneapolis, MN, USA). Results are expressed as picogram (pg) per 100 mg of tissue. The

detection limit of the CBA and ELISA assays was around 0.2 and 5 pg/mL, respectively.

2.14.2. Hippocampal and prefrontal cortex mRNA expression of CX3CR1 and nitric oxide synthases

The mRNA expression of CX3CR1 and the isoforms of the enzyme NOS, known as the isozymes neuronal 'n'NOS, inducible 'i'NOS, and endothelial 'e'NOS, in the hippocampus and prefrontal cortex of TBI-mice and sham animals were estimated by quantitative real-time PCR (polymerase chain reaction) according to the manufacturer protocol (Biorad, USA). Total RNA was obtained using the Trizol reagent (Life Technologies, ThermoFisher Scientific, MA, USA) following manufacturer's instructions. The RNA obtained was resuspended in diethyl pyrocarbonate-treated water and stocked at −70 °C until use. Reverse transcription was performed using 2 microgram of total RNA, 200 U of reverse transcriptase, RT buffer 5× (4 ul), 10 mM dNTPs (1 ul), RNAsin 10,000 U (0.2 ul) and oligo dT 15 50 uM (1.0 ul) in a final reaction volume of 20 ul. Resultant cDNA was used for qPCR. cDNA synthesis was performed from 2 microgram of RNA, using M-MLV reverse transcriptase (Invitrogen, ThermoFisher Scientific, MA, USA). Quantitative PCR was performed using RT2 SYBR® Green qPCR Mastermix kit (Qiagen) in the CFX96 Touch™ Real Time detection system (BioRad). The primer sequences used (sense and antisense) were Cx3cr1 (TGCCTTCTTCCTCTTCTGGA; TAAAGGGGTTGAGGCAACAG), nNos (CTGGTGAAGGAACGGGTCAG; CCGATCATTGACGGCGAGAAT), eNos (CTCTACCGGGACGAGGTACT); (GAGGTCTTGCACGTAGGTCT), iNos (GCTTGCCCCAACAGGAGAAG; GCTGCCCGGAAGGTTTGTAC) and Rpl32 (GCTGCCATCTGTTTTACGG; TGACTGGTGCCTGATGAACT). The expression data were calculated as 2 to the power of −ΔCt (where ΔCt is the difference between Cts of the target gene and Rpl32 reference gene). Results are presented as normalized values relative to sham group.

2.14.3. Lipid peroxidation evaluation in the hippocampus and prefrontal cortex

The analysis of lipid peroxidation in the hippocampus and prefrontal cortex homogenates was performed by malondialdehyde (MDA) content by thiobarbituric acid reactive substances (TBARS) levels as described in Mihara and Uchiyama (1978) (Mihara and Uchiyama, 1978). Briefly, the samples were incubated at 100 °C for 60 min in acid medium containing 0.45% sodium dodecyl sulfate and 0.67% thiobarbituric acid. After centrifugation, the reaction product was determined at 532 nm using MDA as standard. The results are given as nmol/100 mg of tissue.

2.15. Statistical analysis

Statistical analyzes of behavior, cognition and clinical parameters were performed in the GraphPad Prism 6 software (GraphPad, La Jolla, CA, USA). The results were analyzed for normal distribution using the Kolmogorov-Smirnov test. Variables with normal distribution were compared using analysis of variance (One away or Two away ANOVA). In case of non-normally variables, the analysis was performed using Kruskal-Wallis test. The level of significance was set at $p < 0.05$.

Cytokine and inflammatory mediator expression levels were analyzed using custom R code in the R software. First, principal components analysis (PCA) using the measurements of all areas, brain side relative to trauma and time points was performed using the *prcomp* function in the *stats* package. PCA is a dimensionality reduction approach that extracts meta-variables from the similarities in the variation of the original variables (Jolliffe and Cadima, 2016). Here, each PC should be interpreted as the expression of a meta inflammatory mediator, comprising the expression of several inflammatory mediators that vary in a similar direction. Cytokines, chemokines and neurotrophic factors that increase or decrease expression together are grouped in the same component. Then, generalized additive models (GAM) were performed to model the temporal variation of PC1 and PC2 in different brain areas. Animal identity entered as random variable in the models to control for pseudo-replication. GAM analyzes were performed using the *mgcv* package.

3. Results

3.1. Clinical signs and locomotor impairment after mild TBI

In our model, as expected for mild TBI, no mortality (data not shown), no skull fractures and no significant changes in body weight were found over time (Fig. 2A). Following trauma, the animals had the highest NSS within the first 3 h after brain injury with subsequent 'normalization' within 6 h (Fig. 2B).

Open field test revealed locomotor impairment at 6 and 24 h after brain injury, but no changes at 72 h and 30 days post-TBI (Fig. 2C). Given that behavior and cognitive tests rely on preserved locomotor activity (Ramos, 2008; Seibenhener and Wooten, 2015), we focused on TBI-related neuropsychiatric changes at time points when no motor changes were present, i.e. 72 h and 30 days post-TBI.

3.2. Depressive-like and anxiety-like behavior following mild TBI

TBI-mice showed no difference in the immobility time in comparison to sham animals in the forced-swim test (Fig. 3A). However, we found a decrease in the latency to first immobility time at 72 h after TBI (Fig. 3B), indicating a depressive-like behavior. Moreover, TBI-mice also displayed a significant decrease in climbing time at 72 post-injury, supporting the depressive-like behavior at this time-point (Fig. 3C). No significant changes were observed at 30 days post-injury (Fig. 3 A-C).

In the elevated plus maze, a significant decrease in the percentage of time spent in the open arms of the maze was found at 72 h and 30 days after mild TBI, indicating an anxiety-like behavior (Fig. 3D). No significant differences were observed in the marble burying test, which indicates a lack of compulsive-like behavior following mild TBI (Fig. 3E).

3.3. Hippocampal-dependent spatial memory impairment after mild TBI

In the Barnes maze test, TBI-mice showed significant increase in the latency to find a scape tunnel at 72 h and 30 days following the brain injury when compared with sham mice (Fig. 4A), suggesting a hippocampal-dependent cognitive impairment. No significant changes were observed in the other parameters assessed in the Barnes maze such as error number to find the escape tunnel (Fig. 4B) and time in scape zone (Fig. 4C). Most animals, sham and TBI-mice displayed the spatial search strategy to resolve the Barnes maze during the probe test (Fig. 4D). No significant differences were found in the Y Maze test at any of the time-points evaluated (Fig. 4E). TBI-mice displayed no significant impairment in the novel object recognition short and long-term memory compared to sham (Fig. 4F-G).

3.4. Disruption of blood-brain barrier after mild TBI

A significant damage in the BBB was found only at 6 h after TBI, but not at later time points (Fig. 5A). We also investigated potential changes in the CBF, but no significant changes were found at 72 h and 30 days following mild TBI (Fig. 5 B—H).

3.5. Acute microhemorrhage signs in the brain cortex and assessment of diffuse axonal injury after mild TBI

Brains from sham animals showed normal histological appearance (Fig. 6A). Focal and mild lesions characterized by formation of microhemorrhages (asterisks) were observed in the ipsilateral frontal cortex of TBI mice 6 h after the trauma (Fig. 6B). No histopathological alterations were observed at 24 and 72 h and 30 days post-TBI (Figs. 6C-E). A significant increase in APP-immunoreactivity was found in the ipsilateral hippocampus at 30 days post-TBI when compared with sham (Fig. S2A-D

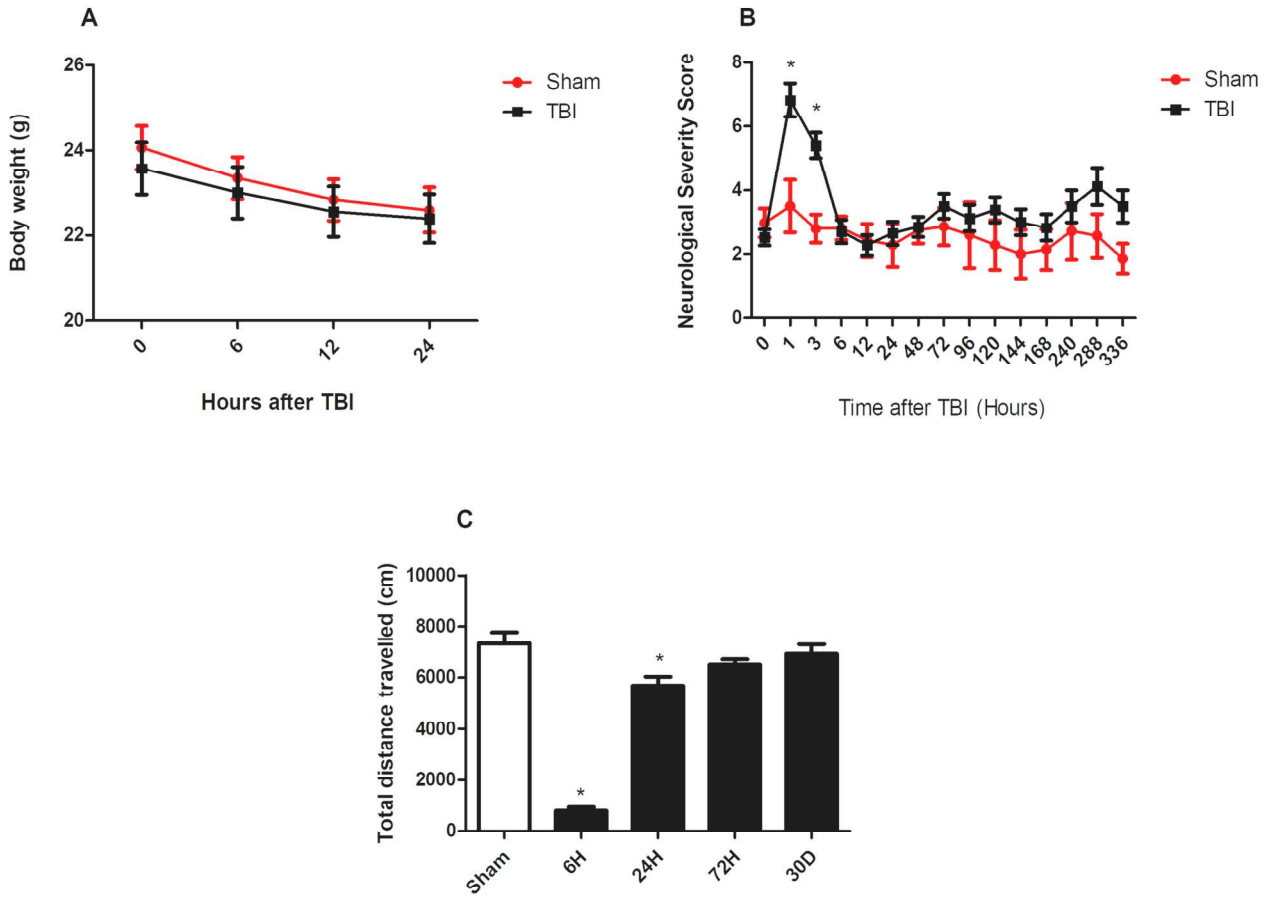

Fig. 2. Evaluation of clinical signs and locomotor changes after experimental mild TBI. A - No alteration in body weight following TBI was detected. B- Neurological Severity Score was performed for 14 days after brain injury. TBI-mice presented impaired neurological function up to 3 h post-injury. C – Open field test revealed impairment in locomotor activity at 6 and 24 h after brain injury. The data is representative of two independent experiments with $n = 10$ mice per experimental group. *means significant difference between TBI and sham group ($p < 0.05$).

and I). No significant changes were found in APP-immunoreactivity in the hippocampus and cortex at any other time-point (Fig. S2-3).

3.6. *Mild TBI induces changes in the glia and in the cytokines, chemokines and neurotrophic factors profile in prefrontal cortex and hippocampus*

TBI-mice displayed a significant increase in the number of Iba-1$^+$ cells, which indicates microglial reaction, at 72 h and 30 days post-TBI in the ipsilateral cortex compared with sham (Fig. 7A-N). No significant differences were found in the contralateral cortex or in both hippocampal hemispheres (Fig. S4). Considering the fluorescence intensity, a significant increase in Iba-1- immunoreactivity was found in the ipsilateral cortex and hippocampus and in the contralateral hippocampus at 30 days post-TBI compared with sham (Fig. 7N and Figure S4PR). No significant changes were observed in the contralateral cortex (Fig. S4N).

Astrogliosis, as revealed by a significant increase in GFAP$^+$ cells, was observed in the ipsilateral hippocampus at 72 h and 30 days post-TBI and in both contralateral cortex and hippocampus at 30 days after injury (Fig. 7E-R and Fig. S5). No significant changes in GFAP$^+$ cells were found in the ipsilateral cortex (Fig. S5). Based on the analysis of fluorescence intensity, mild TBI mice displayed a significant increase in GFAP-immunoreactivity in both ipsilateral and contralateral hippocampus at 72 h and 30 days after injury (Figure 7PR). However, no significant differences were found in the cerebral cortex (Figure S5KL). Representative images in low magnification (x10) of the quantified brain areas for GFAP staining are shown in Figure S6AP.

We measured the expression of 12 cytokines, chemokines and neurotrophic factors in the ipsilateral- and contralateral cortex and hippocampus of TBI mice at 6 h, 24 h, 72 h and 30d after mild trauma and in sham TBI animals. Principal Components Analysis (PCA) of the expression measures in all regions, brain sides and time points revealed two principal components (PCs) which, together, accounted for 59.1% of the data variability (Fig. 8A). PC1 expression, which encompasses 35.5% of the variance of all mediators, correlated with the expression of IL-10, IFN-γ, IL12P70, TNF-α, IL-6 and MCP1 (Fig. 8B). PC2 expression (23.6% of the variance) correlated with the expression of GDNF, BDNF and NGF (Fig. 8B). Individual analyses of inflammatory mediator levels are shown in the supplementary material and in Figs. S7-S10.

We used the expression of the two first principal components, PC1 and PC2, to model the variation of the inflammatory mediators across groups and time points using a GAM (see methods) (Fig. 8C-D and Tables S1–2). PC1 expression in the ipsilateral cortex peaks at 6 h after the trauma and steadily decreases up to the 30 days time point (Fig. 8C). On the other hand, PC1 expression on the contralateral cortex gradually decreases from time 0 to 30d after the trauma (Fig. 8C). In the hippocampus, PC1 expression slightly decreases in the contralateral side but does not change significantly in the ipsilateral side (Fig. 8C). PC2 expression (Fig. 8D) gradually increased from time 0 to 30 days after the trauma in the ipsilateral hippocampus (Fig. 8D) but did not vary significantly in the other three areas (Table S2). Finally, the model suggests that PC1 expression varies both across side and region, being higher in the ipsilateral side and cortex region, while PC2 expression only varies across regions, being higher in the hippocampus (Fig. 8C, D and S11).

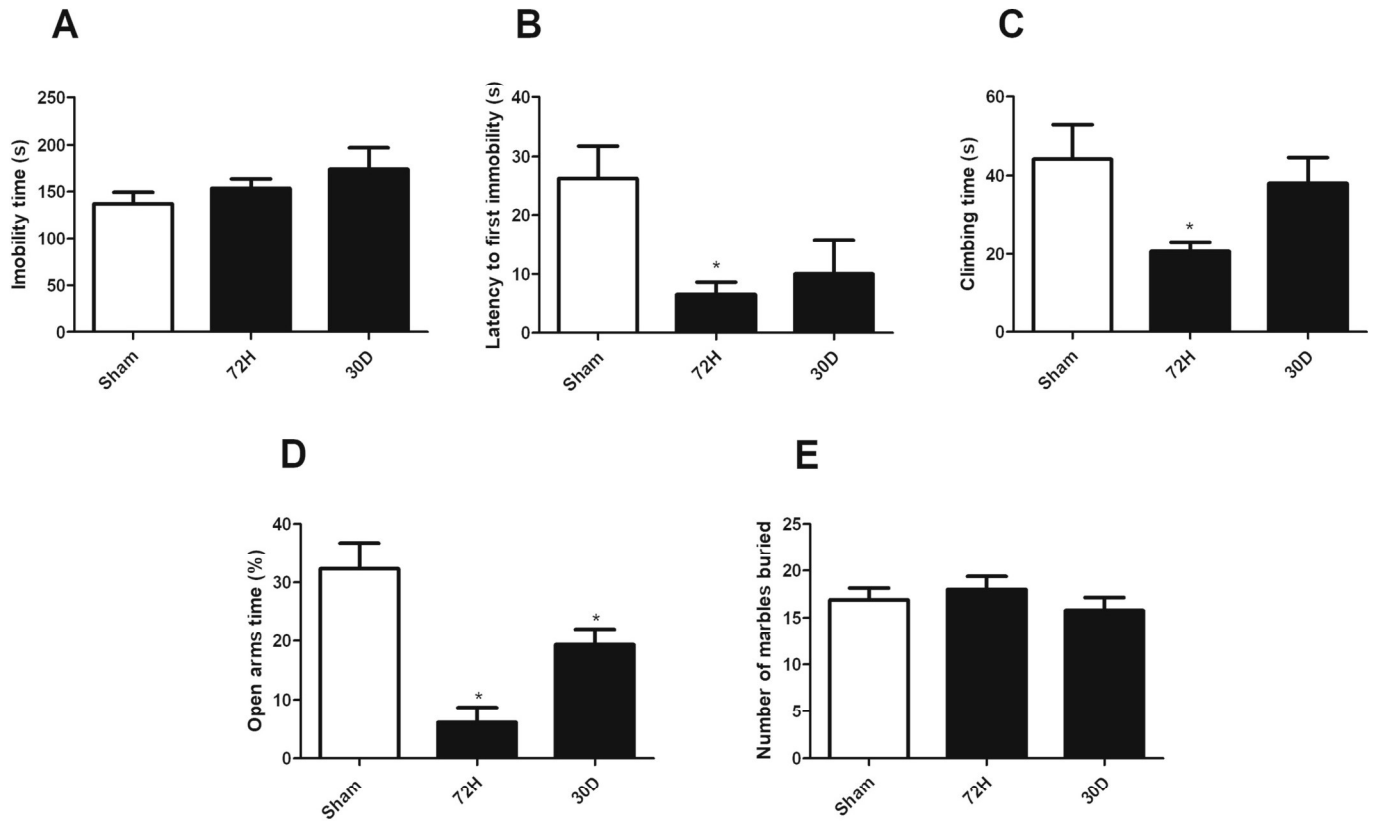

Fig. 3. Behavioral analysis after experimental mild TBI. The forced swim test revealed no significant difference in immobility time (A) but the latency to first immobility (B) and climbing (C) were decreased at 72 h after TBI. D - TBI-mice presented an anxiety-like behavior at 72 h and 30 days after injury in the Elevated plus maze test. E - The marble burying test suggested lack of compulsive-obsessive like-behavior in TBI-mice. The data shown is representative of two independent experiments with n = 10 mice per experimental group. *means significant difference between TBI and sham animals ($p < 0.05$).

3.7. Mild TBI decreases the CX3CL1/CX3CR1 signaling in the prefrontal cortex and hippocampus

Here, we found decreased levels of CX3CL1 in the ipsilateral hippocampus (Fig. 9F) at 6 and 24 h and in the ipsilateral prefrontal cortex (Fig. 9B) at 24 h after mild TBI. However, an increase of fractalkine was seen in the contralateral prefrontal cortex at 72 h and 30 days after injury (Fig. 9D). We also analyzed the mRNA expression of the receptor CX3CR1 in key brain areas associated with behavioral and cognition in animals after mild TBI. A significant decrease in the mRNA expression of CX3CR1 was found in both ipsilateral prefrontal cortex (Fig. 9A) and hippocampus (Fig. 9E) of TBI-mice at 72 h after injury compared with sham. No significant changes were observed in the contralateral side of the brain injury (Fig. 9C and G).

3.8. Mild TBI causes changes in oxidative stress parameters in the prefrontal cortex and hippocampus

A significant increase of TBARS levels was found in all time-points evaluated compared with sham animals in the prefrontal cortex and hippocampus, regardless of the side of the lesion (Fig. S12A-D).

TBI-mice displayed a significant decrease in iNOS and eNOS mRNA expression at 6 h after injury (Fig. 10A-B) as well as an increase in nNOs mRNA expression at 72 h (Fig. 10C) in the ipsilateral prefrontal cortex compared with sham. No significant changes were found in the contralateral prefrontal cortex (Fig. 10D-F).

In the ipsilateral hippocampus mTBI group did not change the mRNA expression of iNOS (Fig. 11A). However, a significant decrease in the eNOS mRNA expression was found at all time points compared with sham (Fig. 11 B). A significant reduction in the nNOS expression was also observed at 30 days post-TBI in the ipsilateral hippocampus (Fig. 11C).

In the contralateral hippocampus, TBI-mice displayed a significant decrease in the mRNA expression of iNOS and eNOS at 6 h, 72 h and 30 days after the trauma in comparison with sham (Fig. 11D-E). Interestingly, a significant reduction in the nNOS expression was also found at 72 h and 30 days, but not at 6 h post-TBI (Fig. 11F).

4. Discussion

Several clinical studies have shown that psychiatric symptoms, including anxiety and depression, and cognitive impairment are common sequelae in patients with mild TBI (de Freitas Cardoso et al., 2019; Theadom et al., 2018; Ma et al., 2019; Bloom et al., 2017; Rutherford et al., 1979). The biological mechanisms underlying these neuropsychiatric problems remain unclear and the use of different TBI-models in rodents has been explored to investigate the development of these symptoms following experimental mild TBI (Bader et al., 2020; McAteer et al., 2016; Rachmany et al., 2013; Shapira et al., 2007).

Herein, we employed a weight-drop model to investigate the underlying mechanisms of cognitive and behavioral impairment following mild TBI in mice. We carried out a careful kinetic analysis and provided original and robust evidence that the mild TBI can affect not only the ipsilateral but also the contralateral side of the lesion. We also provided evidence of secondary lesions such as increased inflammation and oxidative stress in key brain areas associated with behavioral and cognitive changes like prefrontal cortex and hippocampus.

It is worth highlighting that the weight-drop model does not require craniotomy, which prevents the procedure-related inflammatory response (Szczygielski et al., 2016). For skull exposure in the weight-drop model, a midline incision is performed which only requires basic anatomical knowledge and technical abilities, granting the model

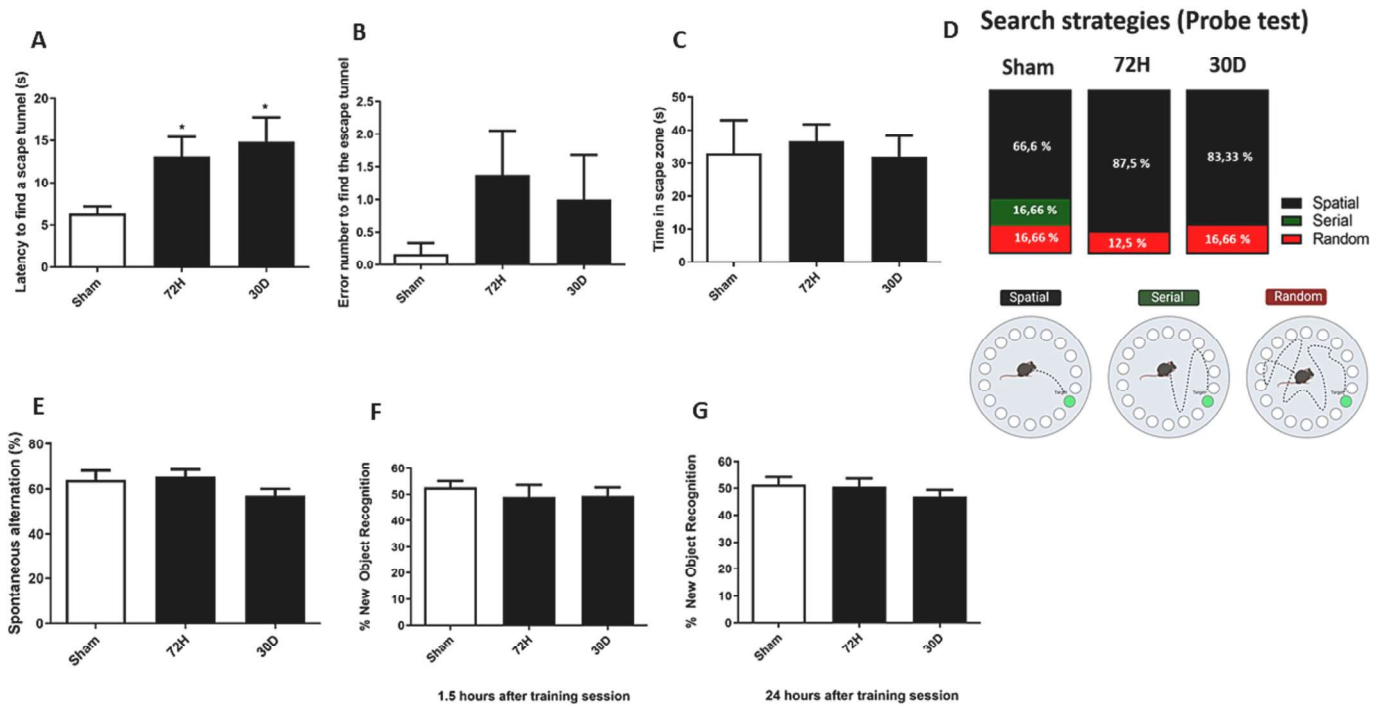

Fig. 4. Cognitive analysis after experimental mild TBI. A - The Barnes Maze test showed alteration in memory of TBI-group at 72 h and 30 days as revealed by a significant decrease in the latency to find a scape tunnel. B - No significant differences were found in the numbers of errors to find the escape tunnel and in the C- time in the scape zone area. D - Search strategies during the probe test - most animals used spatial strategy to find the scape tunnel. E - The Y-Maze test revealed no cognitive impairment in TBI-mice. F- No significant differences were found in the percentage of new object recognition at 1,5 h or G- 24 h after the training session. n = 10 per experimental group * means a significant difference between TBI and sham animals ($p < 0.05$).

reproducibility across laboratories. In addition, the weight-drop model allows weight adjustment for the assessment of mild, moderate or severe TBI (Flierl et al., 2009).

We demonstrated that after weight-drop mice develop anxiety- and depression-like behaviors alongside hippocampus-dependent spatial learning impairment. We also showed that these alterations were sustained up to later time points of investigation. From a pathophysiological perspective, our results suggested that early neurological symptoms (i.e. locomotor change and increased scores) were caused by the direct effect of the trauma, which leads to hemorrhage and BBB disruption, while later neuropsychiatric symptoms were region-dependent and possibly related to secondary inflammatory response and increase of lipid peroxidation.

Spontaneous recovery in the NSS along with maintenance of body weight and absence of mortality and skull fracture show that our standardized TBI model was able to generate an injury compatible with a mild TBI. Our results are consistent with others that showed progressive improvement (i.e., reduction) of NSS at three (Ji et al., 2017) and five (Sharma et al., 2012) days after controlled TBI in rodents. We also investigated locomotor changes following experimental mild TBI. Locomotor activity was impaired at 6 and 24 h after mild TBI, but recovered at 72 h and 30 days post-injury. No locomotor changes were found in C57BL/6 mice at 24 and 72 h after mild TBI in modified CCI model (Vu et al., 2018) or in Swiss mice 10 days after mild TBI induced by weight drop (Schwarzbold et al., 2010). Decreased locomotion after 24 days of injury in C57BL/6 mice was only seen after severe TBI (Zhao et al., 2012).

Depression and anxiety are the main psychiatric symptoms seen in patients after mild TBI (Ma et al., 2019; Vedantam et al., 2021). In this work, we investigated whether our model of mild TBI is suitable for reproducing these neuropsychiatric sequelae. Depression-like behavior was observed at 72 h after mild TBI. Tweedie et al. (2007) (Tweedie et al., 2007), and Shapira et al. (2007) (Shapira et al., 2007) also showed depression-like behavior at 72 and 24 h after mild TBI induced by weight drop in ICR and C57BL/6 mice, respectively. Milman et al. (2008), found depressive-like behavior at 7 days after mild injury, but not after 30 days of TBI (Milman et al., 2008), similar to our results.

We also observed anxiety-like behavior at 72 h and 30 days following mild TBI, consistent with findings in ICR mice (Rachmany et al., 2013). In another study, an anxiety-like behavior was seen in NMRI mice at 11 days after mild TBI resultant from weight drop (Kosari-Nasab et al., 2018), but not at 3 days after mild TBI, induced by CCI, in Sprague-Dawley rats (Hogg et al., 1998).

To date, a small number of experimental studies have examined compulsive-like behavior following mild TBI. We did not find alterations in this behavior after mild TBI, consistent with a study in which C57BL/6 mice did not present changes in compulsive-like behavior at 10 days after mild TBI induced by weight drop (Nichols et al., 2016). In contrast, increased marble burring behavior was seen in Wistar rats at 15 (Pandey et al., 2009) and 27 (Bhatt et al., 2017) days after TBI caused by weight drop. No differences in marble burying behavior at 7 days after mild TBI were seen in C57BL/6 mice after CCI (Tucker et al., 2017). Methodological differences including the TBI model employed, the time point analyzed, mice strain or use of another rodent model like rats, may explain the discrepancies in these findings.

Emerging data supports the role of mild TBI in the development of cognitive impairment in patients (de Freitas Cardoso et al., 2019; Sun et al., 2019; Dikmen et al., 2017). For instance, our group reported cognitive impairment, such as worse performance on immediate memory and learning tasks, in patients evaluated at 24 h after mild TBI (de Freitas Cardoso et al., 2019). A population-based cohort study showed cognitive symptoms at 4 years post-mild TBI (Theadom et al., 2018). Here, we detected sustained impairment of hippocampal-dependent spatial learning in TBI-mice. Our results are in accordance with previous studies that demonstrated significant impairment in learning and memory functions following closed head injury in mice (Yaka et al., 2007; Farr et al., 2020; Friedman-Levi et al., 2021; Ho et al., 2021).

We also investigated the role of primary and secondary injury

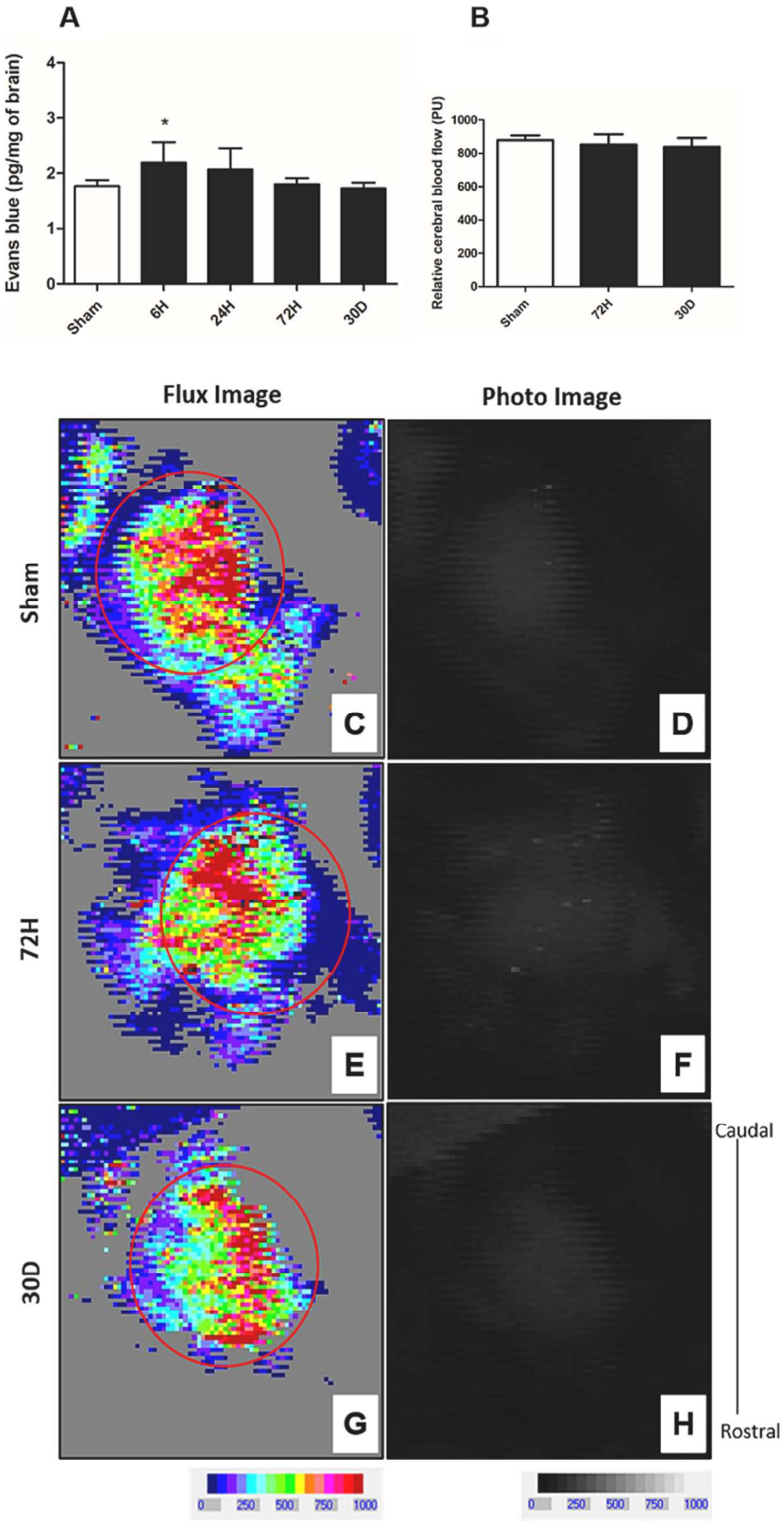

Fig. 5. Analysis of primary injuries following experimental mild-TBI. A – Evans blue marker revealed damage in the blood-brain barrier only at 6 h after trauma. B – Cerebral blood flow (CBF) performed in the right parietal side of the skull at 72 h (E,F) and 30 days (G,H) after TBI revealed no differences in relation to sham animals (C,D). The CBF was measured in perfusion units (PU) $n = 5$ per experimental group * means significant difference between TBI and sham animals ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

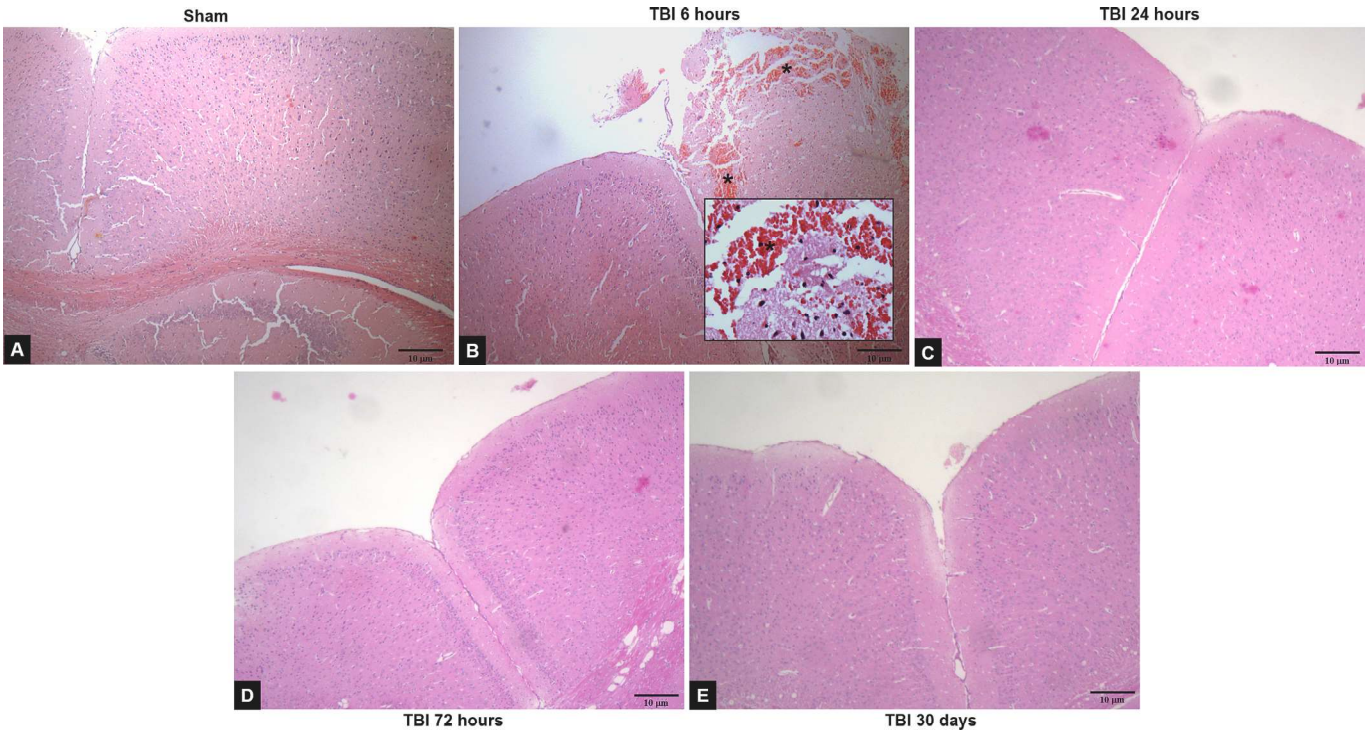

Fig. 6. Representative photomicrographs of H&*E*-stained brain sections. Frontal cortex from sham animal with normal histological tissue (A). Frontal cortex from TBI animal showing microhemorrhages (asterisks) (B), 6 h after TBI. Insert: hemorrhagic area in detail. No histopathological alterations were observed at 24 (C) and 72 (D) hours and 30 days (E) post-TBI. Scale bar: 10 mm. n = 5 per experimental group.

mechanisms in symptom emergence after TBI. Initially, TBI-mice presented locomotor alterations that seem to be related with the direct effect of the trauma that includes hemorrhage and BBB disruption, all of which were observed at 6 h after injury. Later, we found axonal damage, as indicated by a significant increase in APP immunoreactivity, in the ipsilateral hippocampus at 30 days post-TBI. Accordingly, axonal damage has been reported in mild closed head injury up to 90 days following TBI in rodents (Zhang et al., 2015; Yu et al., 2016).

Apart from reversible symptoms (e.g., locomotor activity) related to changes at the primary injury site, TBI-mice showed neuropsychiatric symptoms and cognitive impairment at 72 h and 30 days. These behavioral and cognitive deficits seem to be related to increased lipid peroxidation, and changes in inflammatory mediator levels and in the mRNA expression of isoforms of NOS (nNOS, iNOS and eNOS), which were region-dependent. Our results suggest that the main primary effect of the trauma is associated with an inflammatory peak (PC1 increase) that occurs in the ipsilateral cortex. In line with our results, Tweedie et al. (2020) evaluated different inflammatory mediators in the ipsilateral cortex of CD-1 mice after weight-drop. The author's detected changes in the levels of several proteins between 8 h and 30 days after mild injury, including IL-6 and IL-10, cytokines present in the PC1 (Tweedie et al., 2020). Other pivotal studies also provided consistent evidence of the involvement of inflammation in TBI pathophysiology using closed-head injury models (Tweedie et al., 2013; Tweedie et al., 2016; Israelsson et al., 2008; Israelsson et al., 2009; Baratz et al., 2011; Israelsson et al., 2015). Our study follows up on this earlier research by addressing not only inflammatory but also neurotrophic mediators in different brain areas and in both ipsilateral and contralateral sides of the lesion after a single mild TBI. Accordingly, the PC1 decrease seen over time in the contralateral cortex and hippocampus could indicate a compensatory anti-inflammatory response to the sharp inflammatory increase in the ipsilateral cortex. The PC2 results reveal a secondary, potentially neuroprotective response of the ipsilateral hippocampus, which indicates a delayed involvement of this area in the process of lesion repair. Although the PC1 and PC2 analysis did not give us a specific therapeutic target, it provides meaningful information regarding temporal and spatial differences in the inflammatory and neuroprotective profiles after TBI. A better comprehension of the dynamic of TBI-associated short and long-term pathological processes may open new venues for the identification and development of successful therapies focused on TBI secondary injuries.

In line with our findings, preclinical and clinical evidence has suggested that TBI-associated inflammatory responses are not restricted to the site of injury, also affecting other areas of the brain like the hippocampus. The progression of post-traumatic brain inflammation seems to occur in both acute and chronic stages and may play a significant role in TBI-related neuropsychiatric outcomes (Johnson et al., 2013; Ramlackhansingh et al., 2011; Folkersma et al., 2011; Ertürk et al., 2016). The pathophysiology of this 'remote' inflammation remains to be understood, but might involve enhanced brain inflammatory response (elicited by the trauma) followed by the impairment of immune regulatory mechanisms. Therapeutic strategies targeting this extended brain inflammatory response might be promising in preventing or minimizing TBI behavioral and cognitive sequelae (Shi et al., 2019).

IL-1β is a proinflammatory cytokine that is involved in long-term potentiation (Barrientos et al., 2002), while its exacerbated activity can cause impairment in hippocampal-dependent memory (Goshen et al., 2007). We found an increase in IL-1β levels in the ipsilateral hippocampus at 72 h after TBI, the same time point we detected impairment in hippocampal-dependent spatial learning, which was sustained up to 30 days after injury. These results suggest that IL-1β could play a role in the cognitive damage following mild TBI. Similar findings were reported in Swiss mice subjected to the CCI model that presented increased IL-1β levels in both ipsilateral and contralateral hippocampus at 6 and 24 h after mild TBI. These animals also exhibited cognitive damage in the Barnes maze 3 months after injury (Taib et al., 2017). Of note, a recent clinical study showed that an increase in serum IL-1β within 1 week of mild TBI was associated with worsening working memory in patients 1 month after the traumatic event (Sun et al., 2019).

Resident microglia and astrocytes seem to play pivotal roles in

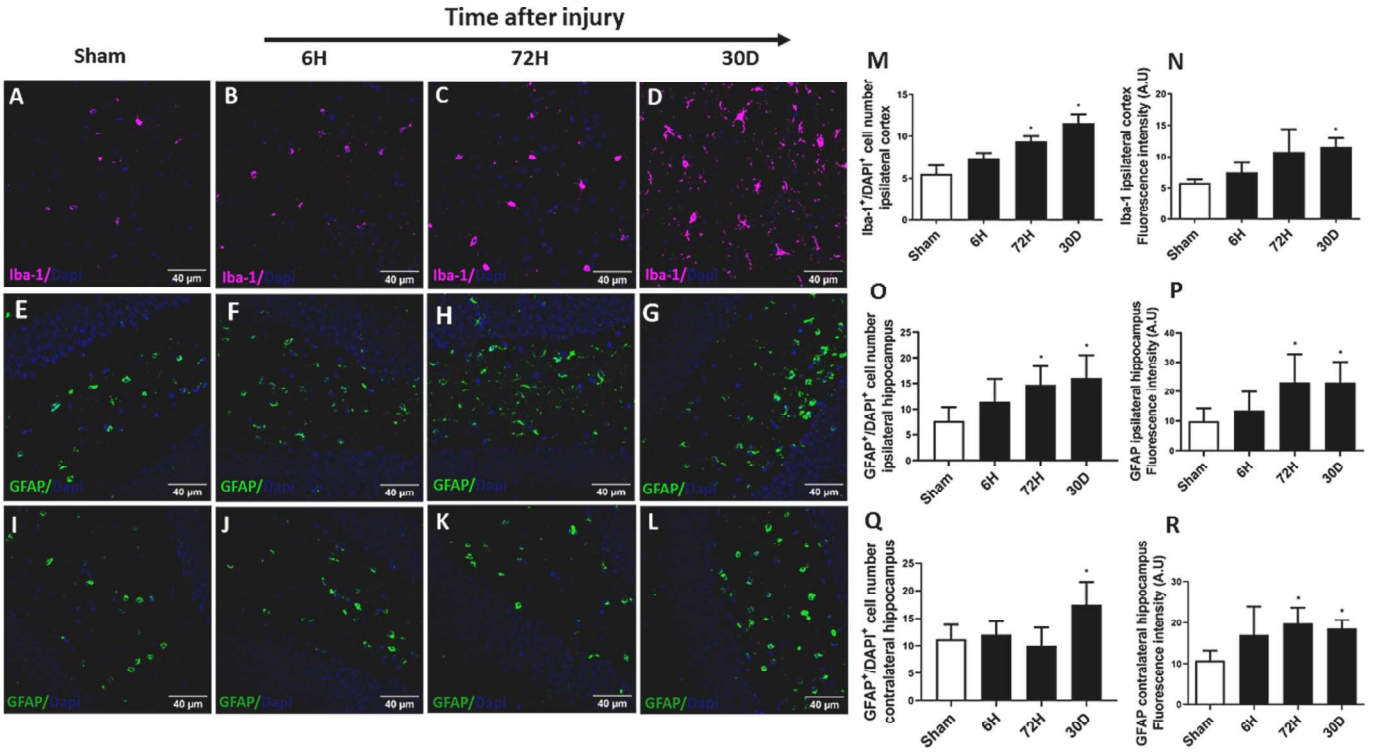

Fig. 7. Glial activation after experimental mild TBI. Analysis of immunoreactivity of Iba-1 (pink) in the ipsilateral cortex in the sham group (A), 6 h (B), 72 h (C), and 30 days (D) after mild TBI. Analysis of immunoreactivity of GFAP (green) in the ipsilateral hippocampus in the sham group (E), 6 h (F), 72 h (G), and 30 days (H) after mild TBI. Analysis of immunoreactivity of GFAP (green) in the contralateral hippocampus in the sham group (I), 6 h (J), 72 h (K), and 30 days (L) after mild TBI. The nuclear marker DAPI is in blue. M - Quantification of Iba-1+/DAPI+ cells in the ipsilateral cortex of mice submitted to the mild TBI. O - Quantification of GFAP+/DAPI+ cells in the contralateral cortex of mice submitted to the mild TBI. Q - Quantification of GFAP/DAPI+ cells in the contralateral hippocampus of mice submitted to the mild TBI. N – Fluorescence intensity of Iba-1 in the ipsilateral cortex. P - Fluorescence intensity of GFAP in the ipsilateral hippocampus. R - Fluorescence intensity of GFAP in the contralateral hippocampus. n = 5 per experimental group * means a significant difference between the sham group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

inflammatory responses secondary to a TBI. Microglial activation results in the release of several inflammatory mediators, including cytokines like IL-1β, which has been associated with TBI behavioral and cognitive outcomes (Taib et al., 2017; Bruhns et al., 2022). Activation of glial cells has been reported in clinical (Johnson et al., 2013) and experimental studies (Bader et al., 2020; Liu et al., 2013) of mild TBI, supporting our findings. For instance, increased expression of Iba-1 and GFAP in the cerebral cortex and hippocampus was described during the acute and sub-acute phases of weight drop mild TBI model in rats and mice (Bruhns et al., 2022; Ponomarenko et al., 2021). Herein, we found enhanced GFAP positive cells only in the contralateral side of the lesion at 30 days after TBI. A potential explanation may rely on the evaluated time points, i.e. on the time window between the acute (72 h) and chronic (30 days) phases. We would expect earlier increase of GFAP expression in the ipsilateral side of the lesion. Future studies employing the weight drop model to induce mild TBI in mice have to assess GFAP expression in the ipsilateral cortex between 1 and 3 weeks after the traumatic event, as previously described in CCI model in rodents (Bruhns et al., 2022; Drieu et al., 2022).

It has been reported that microglial activation can be regulated by CX3CL1/CX3CR1 signaling. The CX3CL1/CX3CR1 pathway mediates the communication between neurons and microglia, contributing to CNS homeostasis. Microglial activation has been reported following disruptions in the CX3CL1/CX3CR1 axis (Cardona et al., 2006; Zanier et al., 2016). However, the role of this axis in response to a TBI remains unclear. Longitudinal changes in CX3CL1 levels in the prefrontal cortex and hippocampus suggest that the neuroprotective vs. neurotoxic role of microglia after TBI is time-dependent, which is supported by other studies (Zanier et al., 2016; Febinger et al., 2015). CX3CR1 deletion has neuroprotective effects in acute mild TBI induced by CCI, with CX3CR1−/− mice displaying less motor deficits and neuronal loss compared with WT mice at 24 h and 15 days after TBI. Nevertheless, the CX3CR1−/− mice exhibited greater cognitive dysfunction and increased neuronal death than WT at 30 days after CCI-induced mild TBI (Szczygielski et al., 2016). In sum, the CX3CR1−/− mice presented less sensorimotor deficits and cellular damage in the early phase after CCI-injury, which was not sustained after 5 weeks of TBI (Zanier et al., 2016). Our findings showing significant reductions in the levels of CX3CL1 and in the mRNA expression of CX3CR1 in the ipsilateral side of the lesion add evidence to the emerging studies regarding the potential role of CX3CL1/CX3CR1 signaling in mild TBI pathophysiology. Moreover, this pathway might be further investigated as a potential therapeutic target for prevent or minimize mild TBI outcomes.

In parallel with brain inflammation we detected increased lipid peroxidation in both hemispheres of the prefrontal cortex and the hippocampus at 6, 24 and 72 h and also 30 days after injury. In line with our results, Tweedie et al. (2016) found increased TBARS levels in the ipsilateral hippocampus at 14 days after mild TBI resultant from weight drop in ICR mice (Tweedie et al., 2016). TBARS levels in our animals peaked at 72 h and remained elevated up to 30 days in the prefrontal cortex and hippocampus. This finding suggests a potential role of oxidative stress in the manifestation of cognitive and behavioral changes during the acute and subacute phases of mild TBI.

In addition to lipid peroxidation, we investigated the mRNA expression of three different isoforms of NOS, the iNOS, eNOS and nNOS. The role of NOS isoforms in mild TBI remains to be fully clarified. In line with our findings of nNOS expression in the ipsilateral prefrontal cortex, a previous study demonstrated higher nNOS production in this

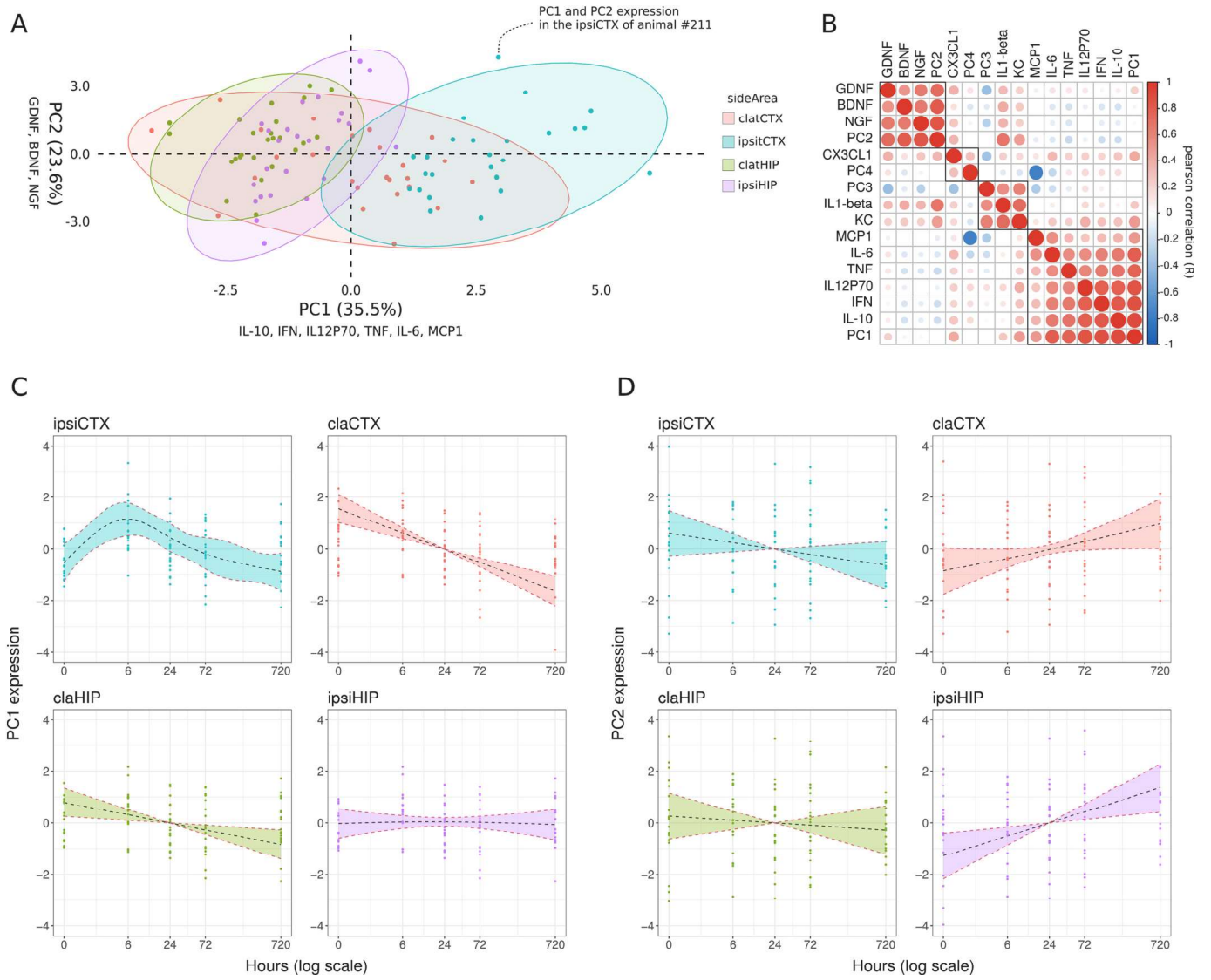

Fig. 8. Principal components analysis (PCA) and generalized additive model (GAM) of the expression of inflammatory mediators in the brain of TBI mice. A. PCA of the expression measures of inflammatory mediators in all areas, sides and time points. Each dot represents the expression of PC1 and PC2 in a brain region and side relative to trauma (colors) in one of the 25 analyzed animals. There is a total of 100 dots, four for each animal. Shaded ellipses group samples from the same side and brain region (side Area). PC1 expression represents 35.5% of the variance of the data and PC2 corresponds to 23.6% of the variance. B. Correlation matrix of the expression of all inflammatory mediators and the expression of the four first principal components in the PCA. PC1 expression clusters with the expression of the inflammatory cytokines IL-10, IFN, IL12P70, TNF, IL-6 and MCP1. PC2 expression clusters with the expression of the neurotrophic factors GDNF, BDNF and NGF. Higher correlation is observed between mediators grouped in the same PC, as expected. C. Generalized additive model (GAM) of PC1 expression across time after TBI in the ipsilateral (ipsi) and contralateral (clat) cortex (CTX) and hippocampus (HIP). D. Generalized additive model (GAM) of PC2 expression across time after TBI in the ipsilateral (ipsi) and contralateral (clat) cortex (CTX) and hippocampus (HIP). A total of 5 mice per group was used.

brain area at 1 and 7 days following a mild TBI induced by weight drop in rats (Ponomarenko et al., 2022). There is evidence that in response to a CNS trauma, nNOS contributes to NMDA receptor-mediated excitotoxicity and also induces DNA damage by enhancing the production of NO and its highly reactive metabolite peroxynitrite (Pieper et al., 2000; Hortobágyi et al., 2003). On the other hand, other studies have pointed out a protective role of the NOS isozymes eNOS and iNOS in response to a brain injury (Bayir et al., 2005; Garry et al., 2014; Lenz et al., 2021). Genetic deletion and pharmacological inhibition of iNOS were associated with poor behavioral and cognitive outcomes alongside increased neuronal loss in rodent models of CCI (Bayir et al., 2005; Garry et al., 2014). However, the data regarding the role of iNOS in the TBI context is conflicting as other studies support a deleterious effect of iNOS after a brain trauma (Garry et al., 2014). Endothelial production of NO by eNOS has often been considered neuroprotective following brain injuries. The eNOS-derived NO protective effect seems to rely on its capability of maintaining microvascular stability and vascular tone (Garry et al., 2014; Lenz et al., 2021). For instance, eNOS knockout mice displayed a significant decrease in the CBF at 24 h after TBI when compared with controls (Lundblad et al., 2009). TBI patients with genetic variants of eNOS, resulting in decreased endogenous eNOS levels, also presented disruptions in the CBF and higher mortality compared with those patients with alleles that did not affect eNOS levels (Robertson et al., 2011). Altogether these studies corroborate our findings suggesting that alterations in the NOS isoforms contribute to mild TBI-related behavioral and cognitive outcomes.

We are aware of the limitations of our study. We measured the levels of neurotrophic factors and inflammatory mediators in two brain regions, the prefrontal cortex and the hippocampus. The assessment of other biological sources, especially blood, could provide meaningful

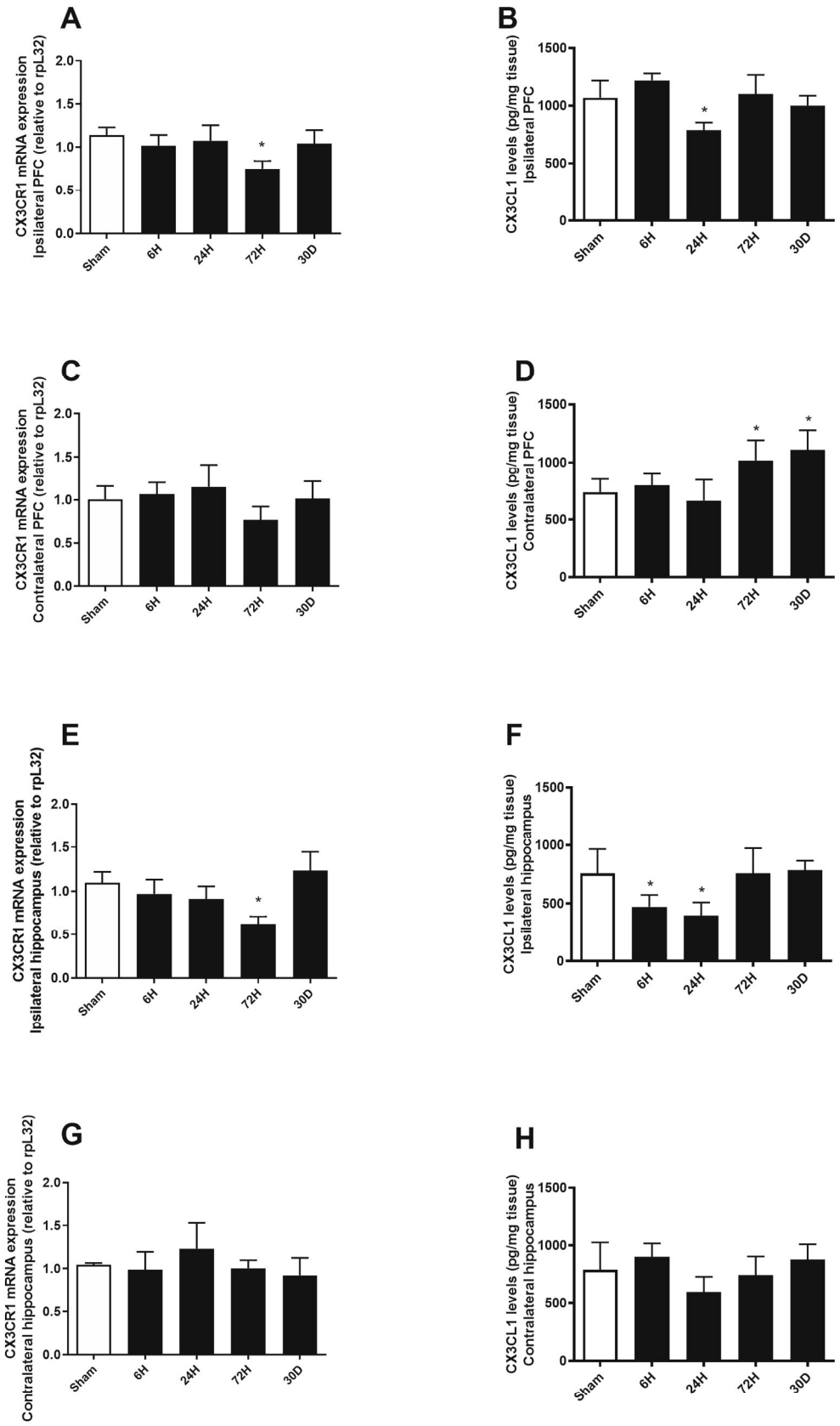

Fig. 9. Effects of experimental mild-TBI in CX3CR1 mRNA levels and in CX3CL1 levels. A decrease in CX3CR1 mRNA expression was found at 72 h in the ipsilateral prefrontal cortex and ipsilateral hippocampus (results are presented as normalized values relative to sham group). A decrease of CX3CL1 levels was found at 24 h in the ipsilateral prefrontal cortex and at 6 and 24 h in ipsilateral hippocampus. Also was found an increase of CX3CL1 levels at 72 h and 30 days after TBI. The analyzes were performed at 6, 24 and 72 h and 30 days following TBI in ipsilateral prefrontal cortex (A,B), contralateral prefrontal cortex (C,D), ipsilateral hippocampus (E,F) and contralateral hippocampus (G,H). n = 5 per experimental group * means a significant difference between trauma and sham group ($p < 0.05$).

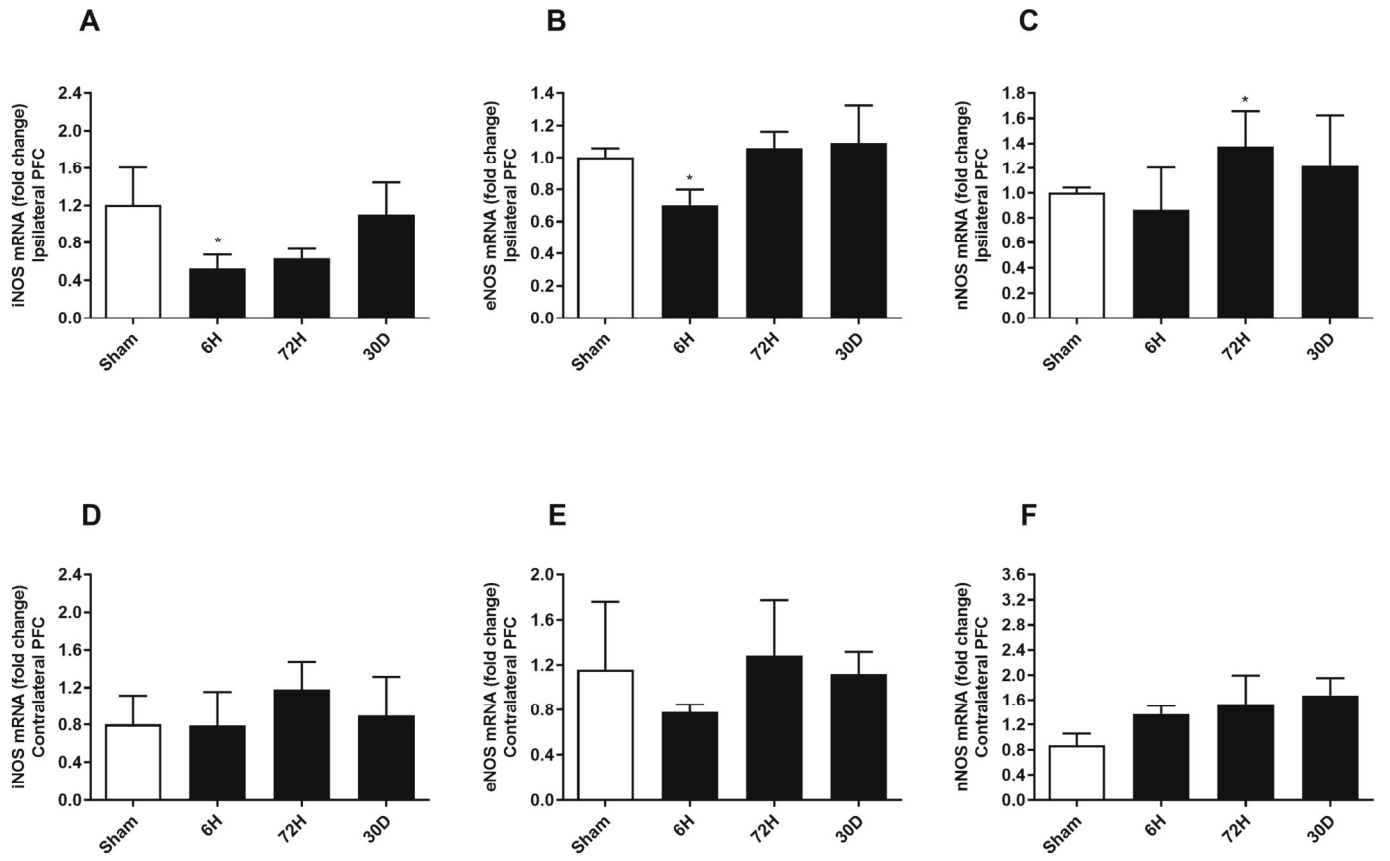

Fig. 10. Expression of nitric oxide synthases in the prefrontal cortex of mice after mild-TBI. The analysis of expression of iNOS (A and D), eNOS (B and E), and nNOS (C and F) in the ipsilateral and contralateral hemispheres at 6 and 72 h and 30 days after mild TBI. n = 5 per experimental group * means a significant difference between the sham group.

information and insights regarding human studies. Cognitive, behavior and immunological analysis on time points between the acute (up to 72 h) and the chronic (up to 30 days) stages as well as on later time points would also be valuable to improve the understanding of the neurobiological basis of mild TBI long-term outcomes. A systematic characterization of TBI-associated hypoxia and oxidative stress including the evaluation of brain levels of mitochondrial reactive oxygen species (ROS) as well as of calcium proteases, phospholipases and glucose needs to be carried out in this model. Further studies are also necessary to better characterize axonal injury and neuronal damage in this model of mild TBI, including the expression of other markers of axonal injury like NFL, MAP-2 and of neuronal maturity such as NeuN. Additionally, stereology analysis of APP immunostaining would add valuable information regarding axonal damage in this TBI model. Correlation analyses involving behavioral/cognitive data and histopathological and inflammatory mediators should also be performed in future studies.

A better comprehension of the mechanisms underlying mild TBI pathophysiology may open new venues for the development of potential therapeutic targets focused in preventing and/or minimizing the TBI-related behavioral and cognitive changes. In this scenario, the weight-drop model developed and characterized here was able to reproduce clinical, behavioral, and cognitive changes observed in patients. This model may become a useful tool for the investigation of mild TBI outcomes. We provide unequivocal evidence that neuroinflammation plays an important role in the acute phase of mild TBI, and that mild TBI affects not only the ipsilateral side of the lesion, but also the contralateral side in a region and time-dependent manner.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2023.578242.

Ethics approval and consent to participate

All animal studies were approved by the Ethics Committee on Animal Experimentation of UFMG (under the protocol 250/2017).

Consent for publication

Not Applicable.

Funding

This article received financial support from 2016 NARSAD Young Investigator Grant from Brain & Behavior Research Foundation (grant #25414), 2019 “For Women in Science”- L’Oreal Brazil-UNESCO- Brazilian Academy of Science (ABC), FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Brazil), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

Authors’ contributions

CAM, BSO, TLD, JLVMB, GMFF, TMC, VF, CHA, LKSA, ELMV, PRC, RNF, LMK, WNS, BCC, HBF, GRF, CG, LSB, ACSS, ECBT, MAR, ALT, ASM conducted experiments, contributed to writing and editing the manuscript and/or analyzed data. All authors contributed to research design and reviewed the final manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

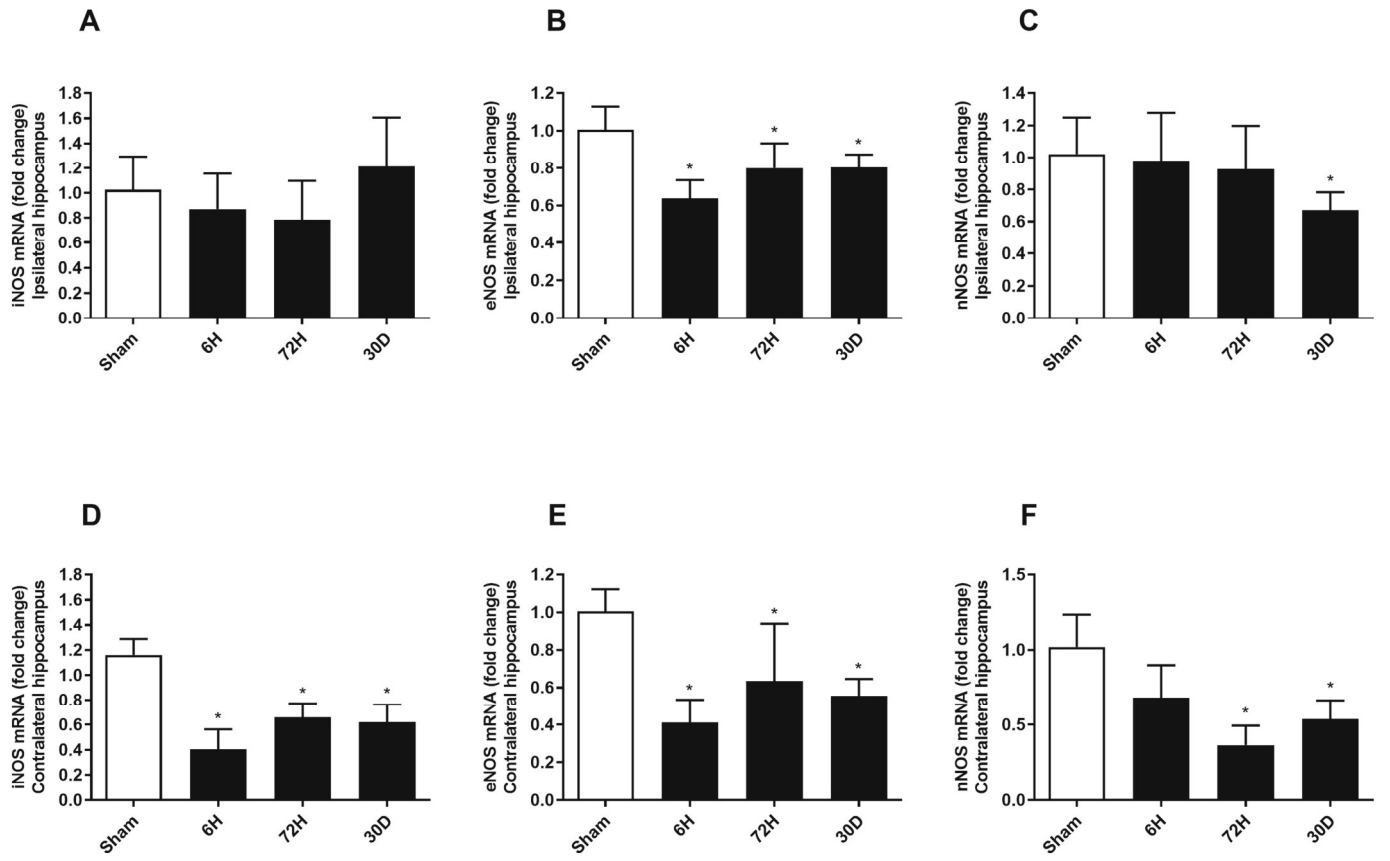

Fig. 11. Expression of nitric oxide synthases in the hippocampus of mice after mild-TBI. The analysis of expression of iNOS (A and D), eNOS (B and E), and nNOS (C and F) in the ipsilateral and contralateral hemispheres at 6 and 72 h and 30 days after mild TBI. n = 5 per experimental group * means a significant difference between the sham group.

Data availability

The data that support the findings of this study are available on request from the corresponding author, ASM.

Acknowledgements

This article received financial support from 2016 NARSAD Young Investigator Grant from Brain & Behavior Research Foundation (grant #25414), 2019 "For Women in Science"- L'Oreal Brazil-UNESCO- Brazilian Academy of Science (ABC), FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Brazil), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

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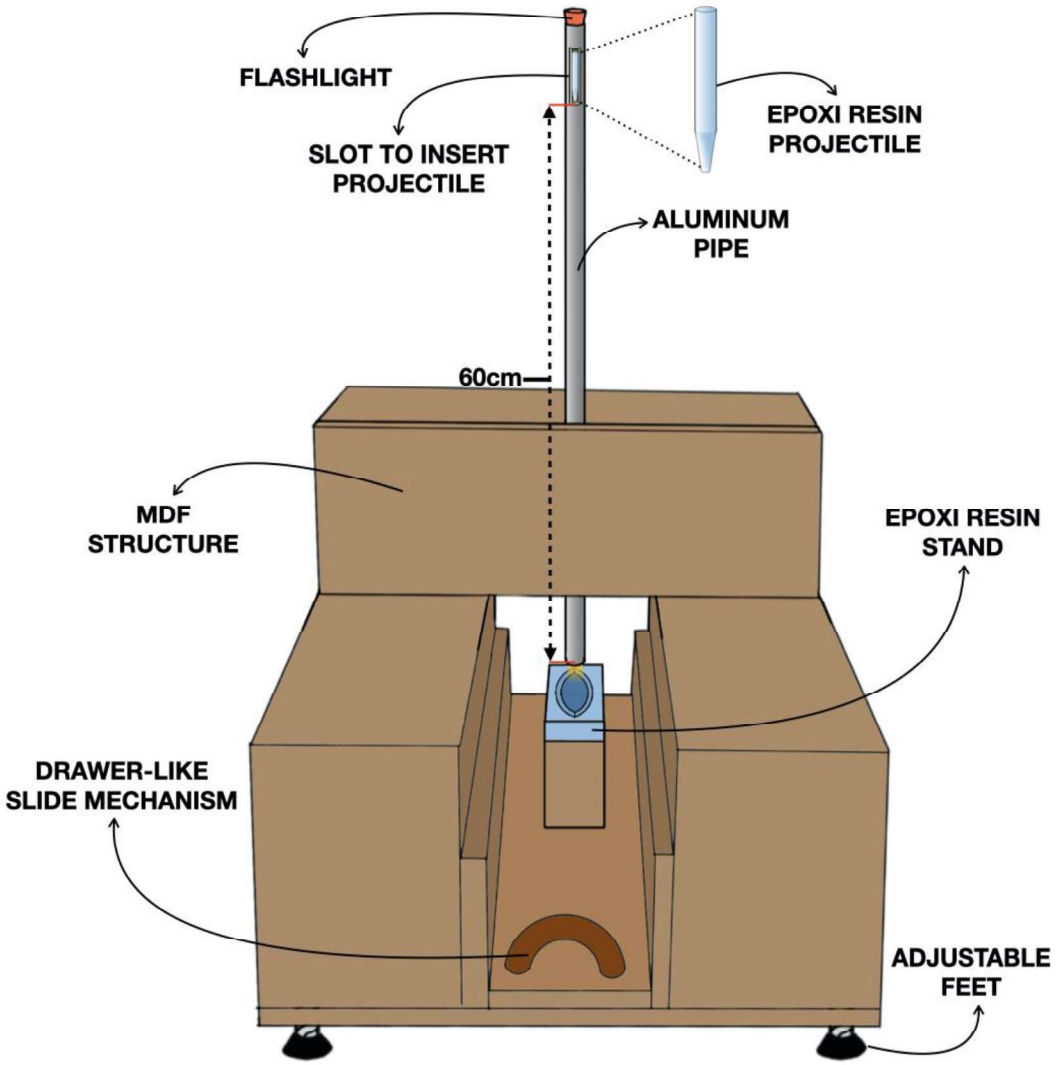

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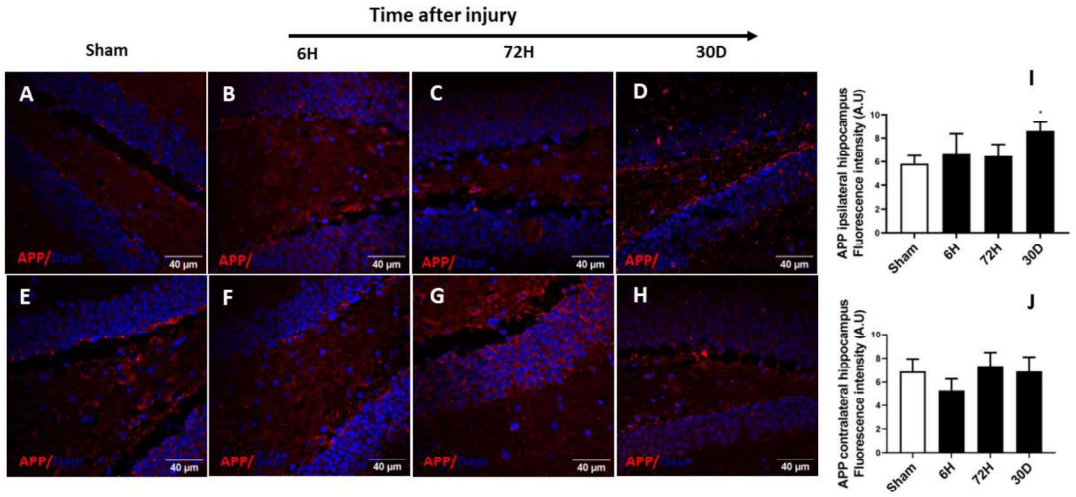

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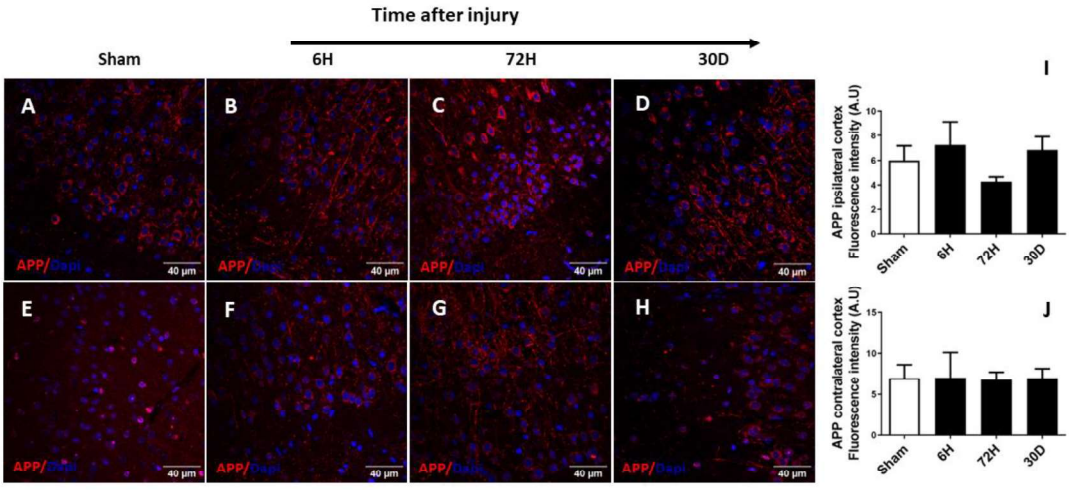

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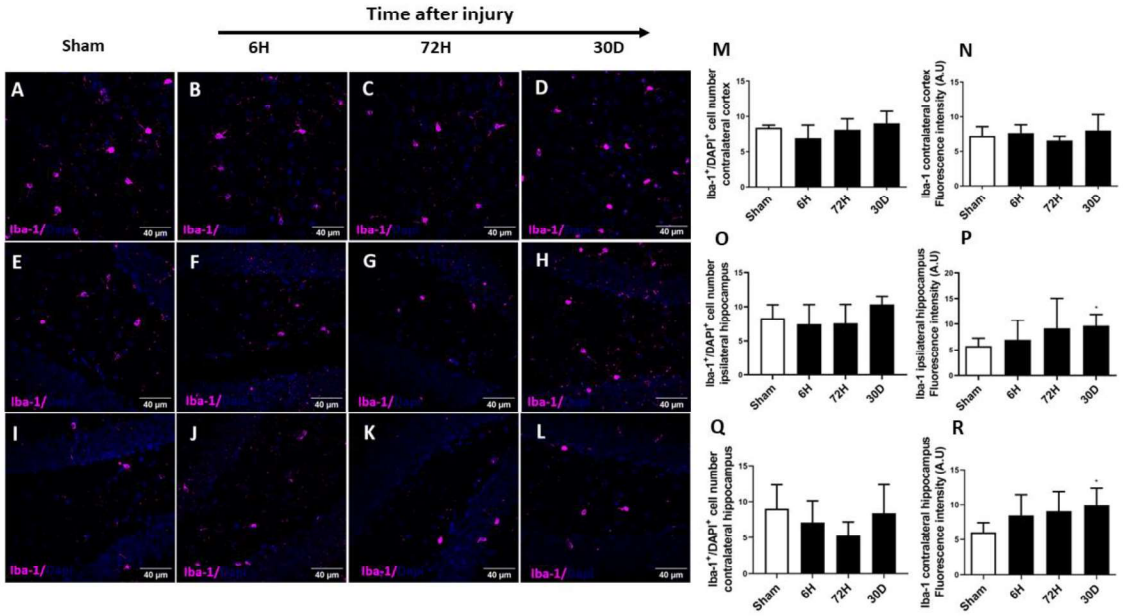

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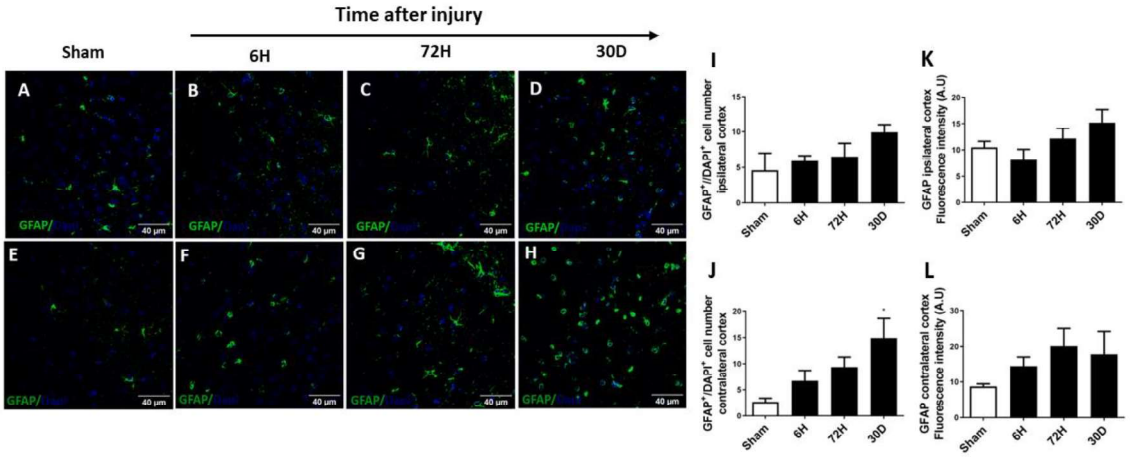

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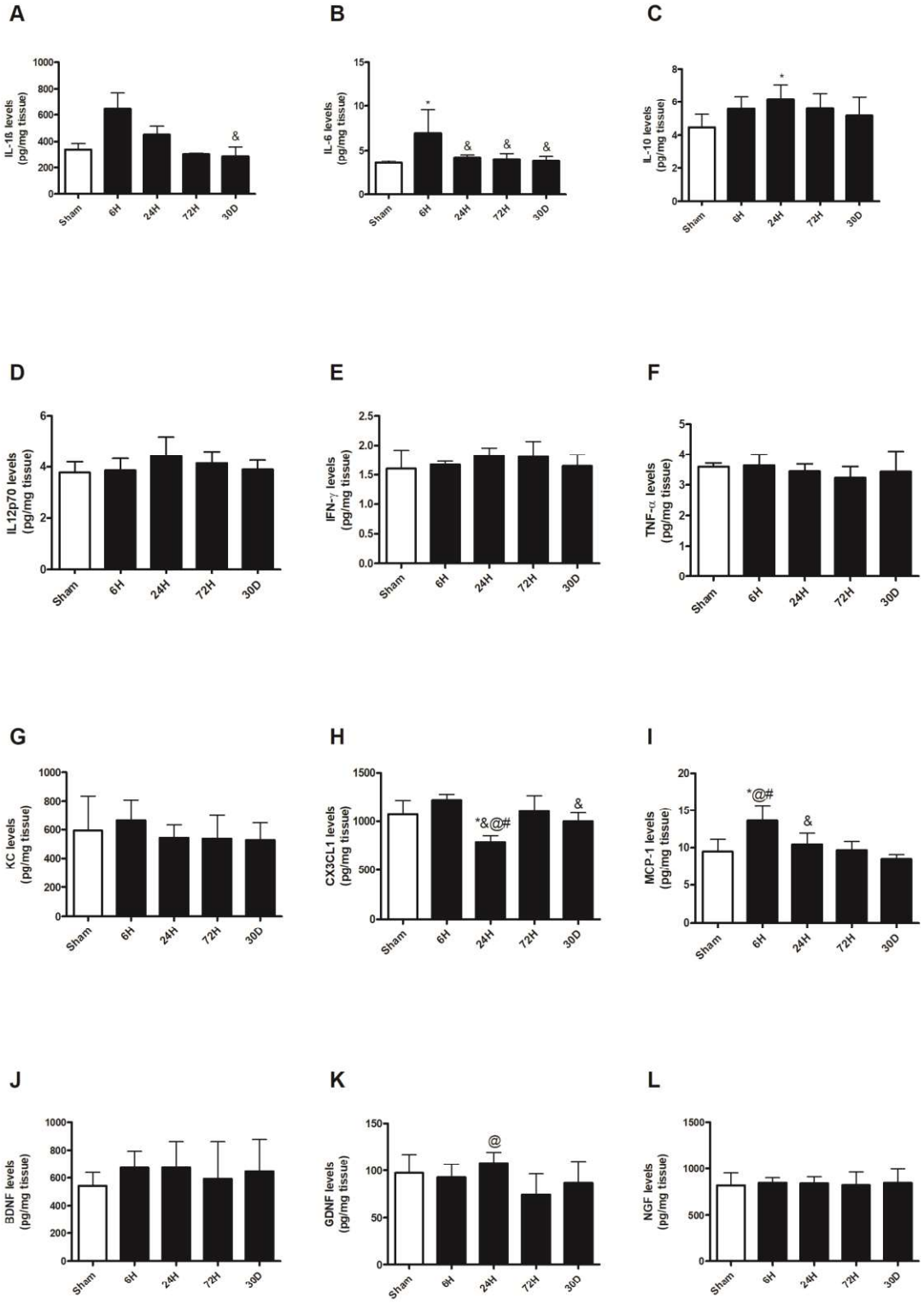

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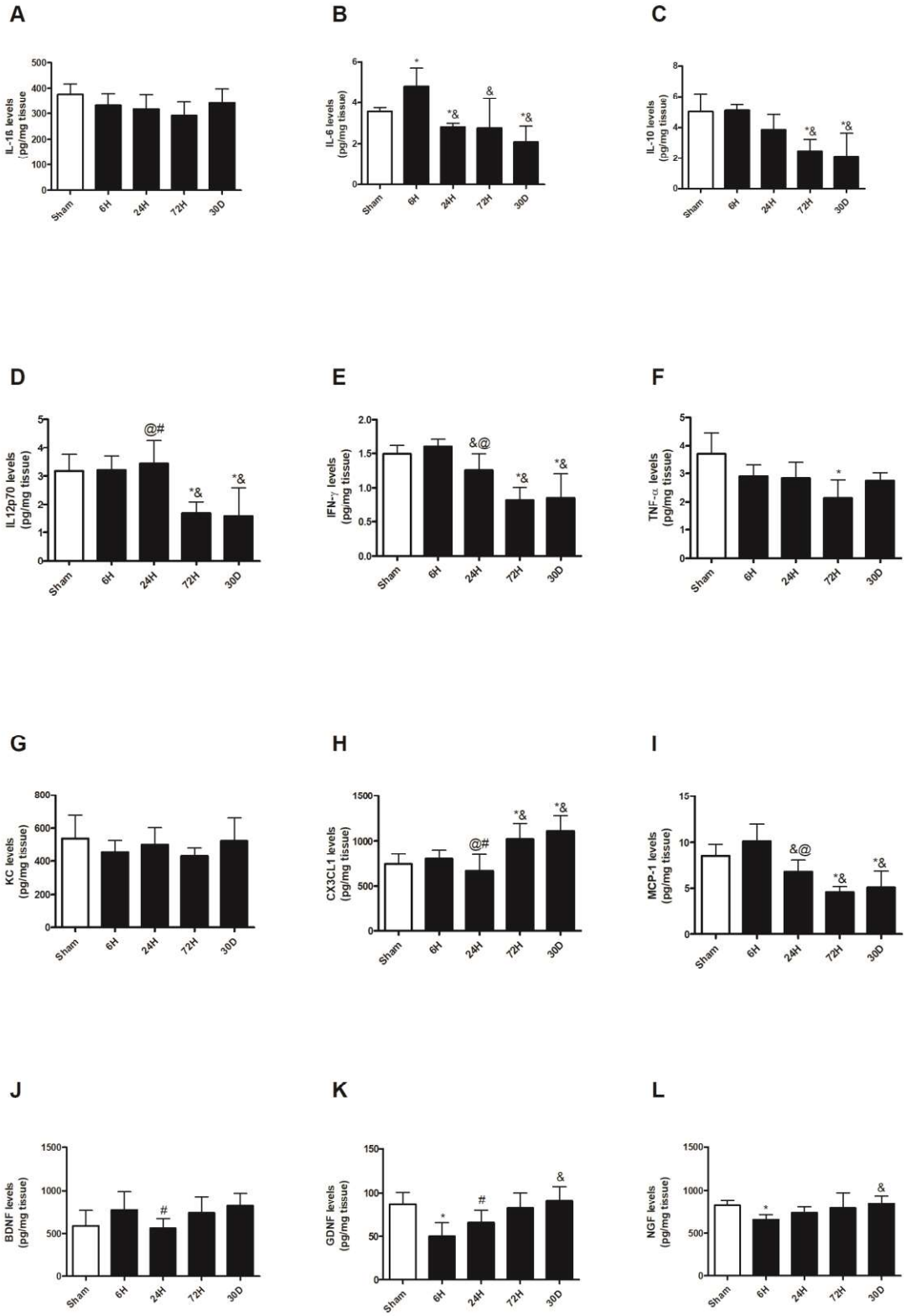

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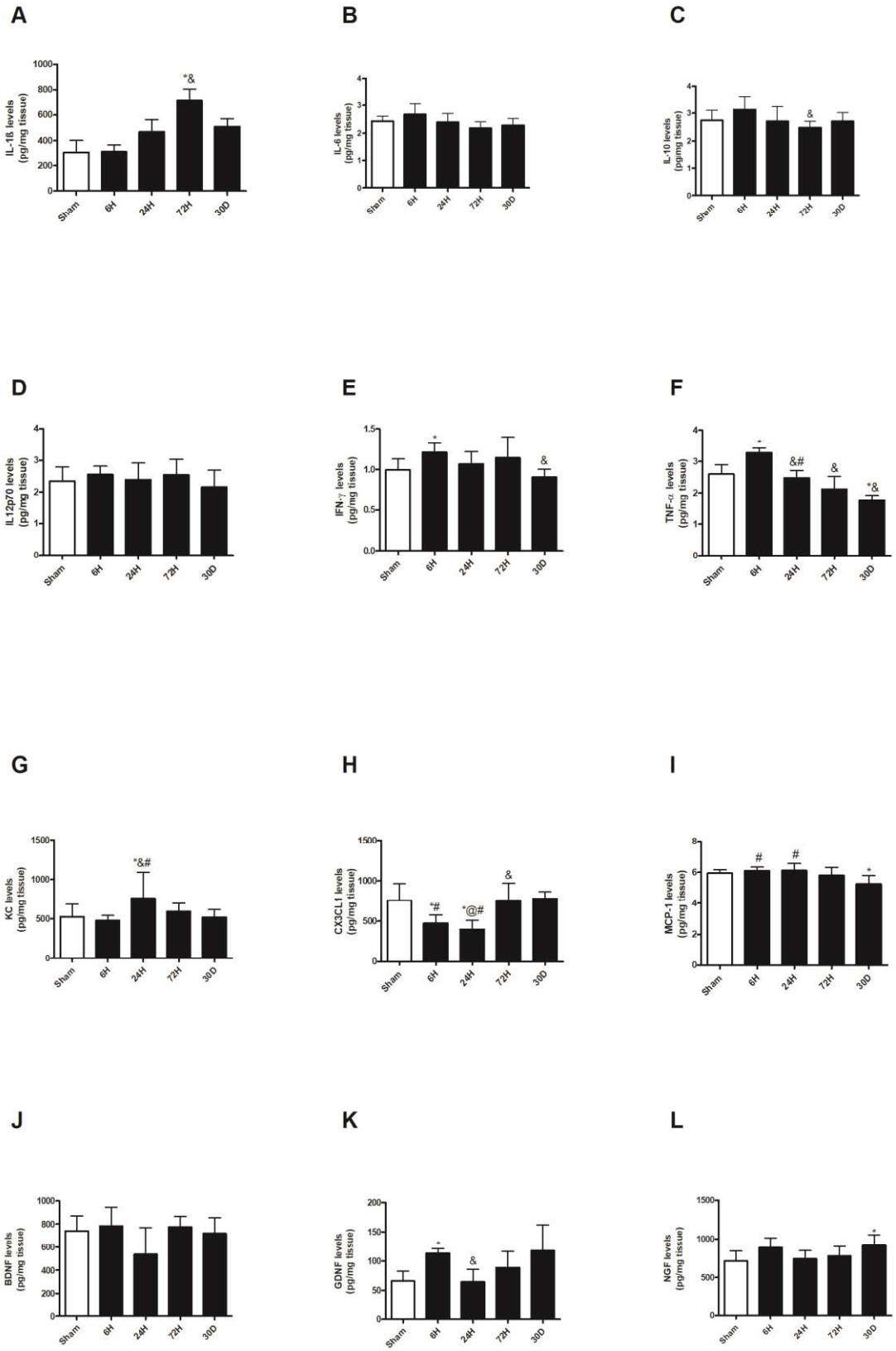

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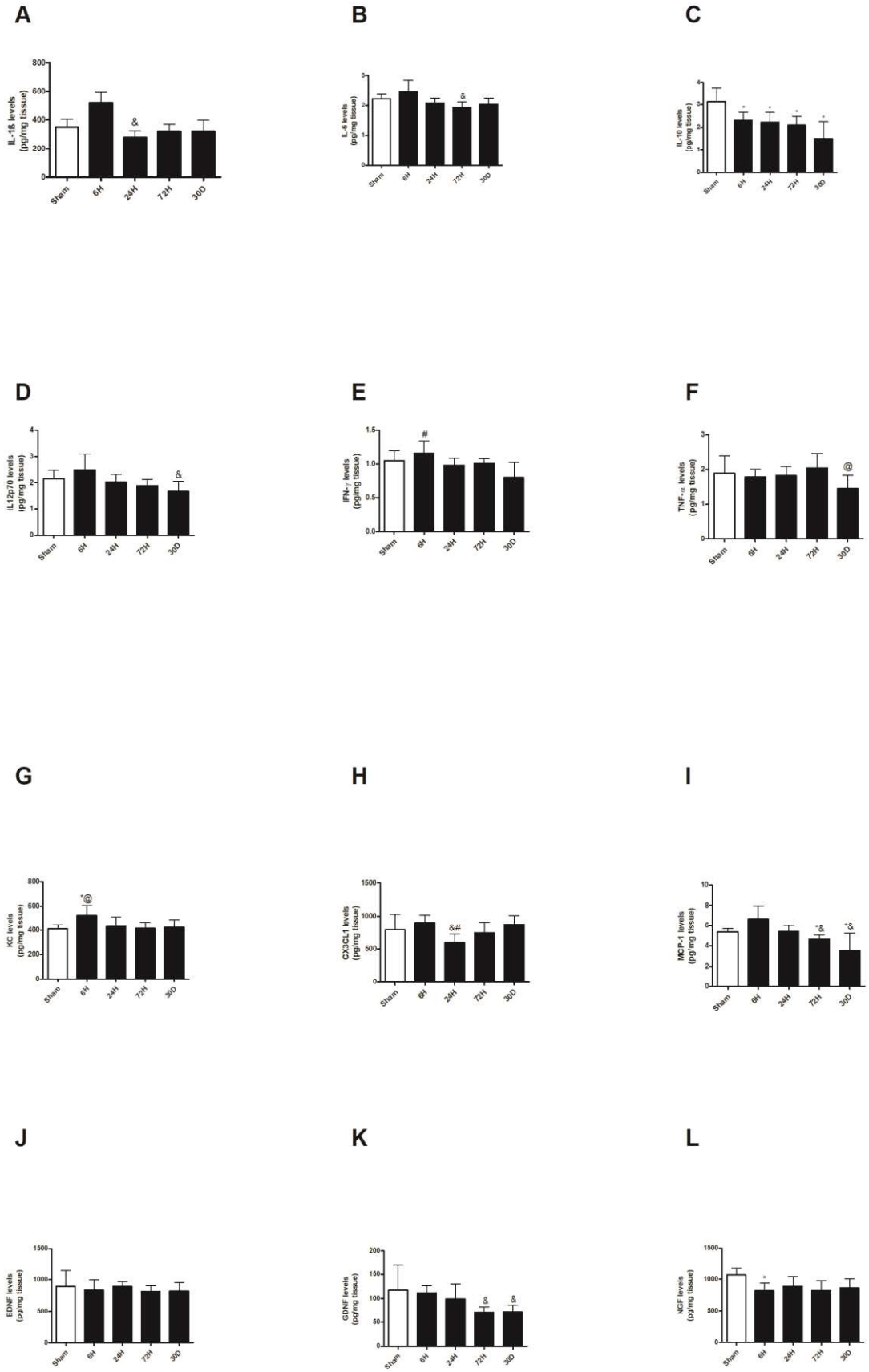

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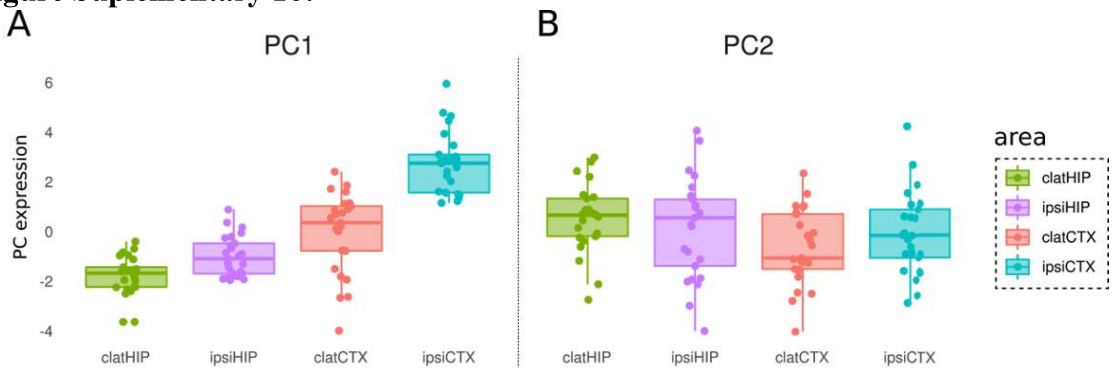

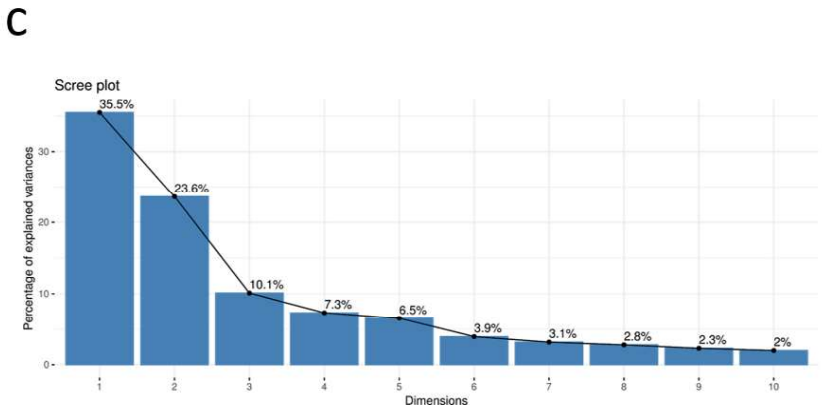

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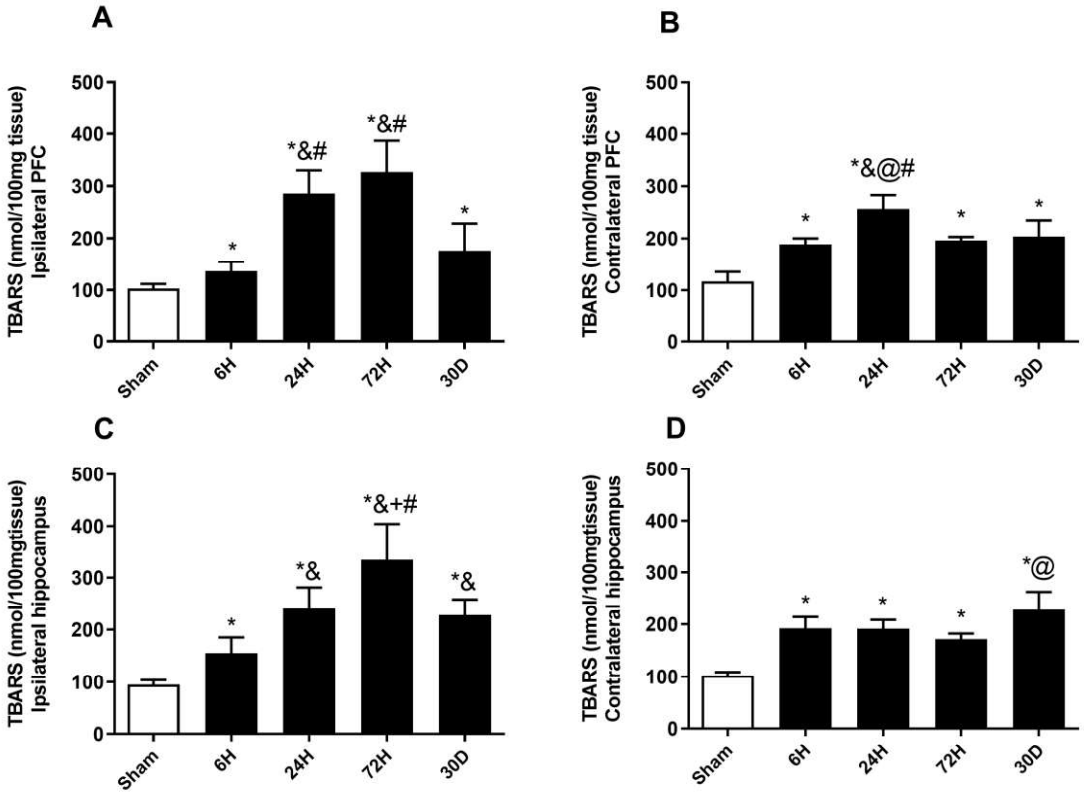

6.3- ARTIGO CIENTÍFICO III submetido na revista *Neuroscience Bulletin*

Título: ***Involvement of Renin-Angiotensin System (RAS) components in Mild Traumatic Brain Injury***

Involvement of Renin-Angiotensin System (RAS) components in Mild Traumatic Brain Injury

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Abstract

Mild traumatic brain injury (TBI) is one of the most common types of head injuries worldwide. Evidence suggests that RAS modulation possibly plays a role in mild TBI pathophysiology. In the present study, we aimed to investigate the levels of components from both the classical and counter-regulatory axis of RAS in a mild TBI animal model and investigate the effect of treatment with perindopril, telmisartan, and AVE0991 on locomotor, behavioral, and cognitive changes following mild TBI. TBI mice displayed an increase in ACE/Ang II/AT_1R axis ipsilaterally and contralaterally in the hippocampus acutely and at a later time-point (30 days). Additionally, Ang-(1-7) levels increased and Mas receptor levels decreased in both hippocampus at 30 days after injury. TBI-mice also presented lower AT_1 levels and higher Ang II levels in the contralateral prefrontal cortex following mild TBI. We also found that treatment with perindopril prevented impairment in locomotor activity in animals subjected to mild TBI. Our results demonstrate that molecules of classical and alternative RAS axes undergo alterations in key brain areas in mild TBI and that treatment with perindopril may have a role in improving locomotor changes in mice submitted to mild TBI.

Keywords: mild traumatic brain injury, renin-angiotensin system, weight-drop model, angiotensin II, perindopril, telmisartan, AVE0991.

1. Introduction

Traumatic brain injury (TBI) is considered one of the main causes of disability and mortality worldwide, affecting about 1.7 million individuals in the USA annually (Faul et al., 2010). Approximately 70% of these cases are of mild TBI – a TBI severity associated with the development of anxiety, depression, sleep disturbances, and cognitive impairment (Feigin et al., 2013; Tabish and Syed, 2012; Yates et al., 2006; Cardoso et al., 2019; Ma et al., 2019; Ouellet et al., 2018; Theadom et al., 2018). Such outcomes negatively affect the quality of life of both survivors and their families (Ma et al., 2019; Theadom et al., 2018).

The pathophysiology of TBI is highly complex. Initially, TBI injures structures of the central nervous system (CNS), sometimes promoting intracranial hemorrhage (Anderson et al., 2020), diffuse axonal injury (Graham et al., 2020), and increasing the blood-brain barrier (BBB) permeability (Yoen et al., 2021). This initial injury is known as the primary lesion. The secondary injury that follows the primary injury involves molecular mechanisms that may persist for years and result in inflammation (Ebert et al., 2019) and oxidative stress (Mojtahedzadeh et al., 2014). Current research investigates how to modulate this secondary response, as it is associated with neuropsychiatric symptoms and cognitive impairment (Sun et al., 2019; Vedantam et al., 2020; Haber et al., 2013; Katz et al., 2015). Many systems are involved in this secondary response, including possibly the Renin-Angiotensin System (RAS).

Clinical and experimental evidence suggest that RAS plays a pivotal role in injury secondary to TBI (Kehoe et al., 2008; Villapol et al., 2015). Although RAS is known for maintaining electrolytic homeostasis and cardiovascular control, evidence demonstrates that severe TBI induced higher ACE activity (an enzyme that catalyzes the conversion of angiotensinogen to angiotensin) and higher mortality rate (Kehoe et al., 2008). Also, the administration of angiotensin-(1-7), a molecule that belongs to the anti-inflammatory counter-regulatory RAS system axis, not only improved severe TBI-induced motor deficit as well as it decreased astrogliosis and neuronal loss (Janatpour et al., 2019). However, a limited number of studies explored RAS involvement in TBI secondary injury.

In the present study, we aimed to investigate the modulation of RAS molecules in the pre-frontal cortex and hippocampus in a mild TBI model and the effect of treatment with perindopril, telmisartan, and AVE0991 on locomotor, behavioral, and cognitive changes in animals submitted to the mild TBI.

2. Material and Methods

Animal care

Male C57BL/6 mice, aged 10-12 weeks were obtained from the Bioterism Center at the Federal University of Minas Gerais (UFMG), Belo Horizonte, Brazil. The study was conducted according to the Ethics Committee on Animal Experimentation of UFMG (under protocol 250/2017). The animals were conditioned in groups of five mice *per* cage, in a controlled room with a 12-hour light-dark cycle, and water and food *ad libitum*. A total of five mice were used for each group.

Weight-drop mild TBI

Mice were submitted to the weight-drop model as described in Flierl et al., 2009. Briefly, under xylazine/ketamine anesthesia, mice were submitted to the midline incision

and a weight of 15 grams was dropped by a guide tube (60 centimeters) in the right parietal side of the skull. After the mild injury, the mice were sutured and returned to cages for recovery. The sham group was submitted to the same procedures except for the weight-drop TBI.

Measurement of RAS components

Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine and euthanized at 24 and 72 hours, and 30 days after mild TBI. The brains were dissected for isolation of the ipsilateral (right) and contralateral (left) hemispheres of the hippocampus and prefrontal cortex. Then, tissues were used to assess levels of ACE1, Ang II, ACE2, and Ang-(1-7) by Enzyme-linked immunosorbent (ELISA), according to the manufacturer procedures (MyBioSource, San Diego, CA, USA). Also, AT_1, Mas, and AT_2 levels were measured via real-time PCR in these same tissues. The primer sequences used were AT_1: 5'-GGT GGG AAT ATT GGA AAC AG-3' (forward) and 5'-AAG AAG AAA AGC ACA ATC GCC-3' (reverse); AT_2: 5'-GCT GAG TAA GCT GAT TTA TG-3' (forward) and 5'TTA AGA CAC AAA GGT GTC CA-3' (reverse); Mas receptor: 5'-CCC ACC CAT TCC CAT AGT GC-3' (forward) and 5'-CCG AGA GGA GAG ATG CTC ATG-3' (reverse); endogenous control S26: 5'-CGA TTC CTG ACA ACC TTG CTA TG-3' (forward) and 5'-CGT GCT TCC CAA GCT CTA TGT-3' (reverse).

Drug treatment

RAS modulators were administered via oral gavage 1, 3, 24, 48, and 72 hours after injury (Figure 1). Telmisartan (10 mg/kg) and perindopril (5 mg/kg) were dissolved in polyethylene glycol and AVE0991 (3 mg/kg) was dissolved in saline. Sham mice received oral gavage with saline.

Open field test

The open field test was performed to investigate the effect of RAS modulators on locomotor activity in mice submitted to mild TBI. The test was performed 6 hours after injury (Miranda et al., 2017) using the PhenoTyper system (Noldus Information Technology, Leesburg, VA, USA). Mice were placed in an opaque arena (30 cm x 30 cm x 35 cm) and allowed to freely explore for 30 min. The total distance traveled was analyzed using the Ethovision XT software (Noldus Information Technology, Leesburg, VA, USA).

Elevated plus maze test

The elevated plus maze test was performed to assess the effect of RAS modulators on anxiety-like behavior in mice submitted to mild TBI. Mice were placed in a maze composed of 2 open arms (30cm x 7 cm x 0.25 cm) and 2 closed arms (30cm x 7 cm x 15 cm), elevated at 30 cm above the floor. The test was performed 72 hours after injury for 5 minutes and analyzed using the Ethovision XT software (Noldus Information Technology, Leesburg, VA, USA). The time spent in each arm was recorded. The percentage of time spent in the maze open arm was used to measure the anxiety-like behavior (Fernandes et al., 2020).

Barnes Maze test

Barnes Maze test was performed to measure the effect of RAS modulators on hippocampal-dependent spatial memory in mice submitted to mild TBI. The Barnes Maze test was performed 72 hours after injury. The protocol used was adapted from Pitts et al., 2018 and the test was conducted in a circular maze (90 cm diameter) with 20 holes and used 3 visual cues (triangle, circle, and square), an escape tunnel, and a black box. The test consisted of an adaptation phase where mice were placed in the escape tunnel for 1 minute and then in the center of the maze for 5 minutes or until they returned to the escape

tunnel. The test also included a training phase that started 1 hour after the habituation phase. Training was performed with 2 daily trials, for 9 days, with 1-hour intervals between trials. In each trial, mice were contained in a black box for 15 seconds and placed in one of the maze's quadrants. Mice were then released to explore the maze and find the escape tunnel in 3 minutes. Mice that did not locate the escape tunnel in 3 minutes were conducted back to and maintained in the tunnel for 15 seconds to learn the location. The probe trial test was performed 24 hours after the last training. Mice were placed in a maze to explore freely for 1 minute. The time for the mice to find the escape tunnel (latency) and the escape zone exploration time in the probe test were assessed and used as a measure of hippocampus-dependent spatial memory (Pitts, 2018).

Statistics analysis

We used the software GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Normal distribution was analyzed using the Shapiro-Wilk test. For normally-distributed variables, we performed an analysis of variance (ANOVA). Comparisons between non-parametrical data were made by the Kruskal-Wallis test. Comparisons between the two groups were made via unpaired t-tests. The significance value was set at $p < 0.05$.

3. Results

Mild TBI induces changes in RAS peptides and enzymes in the hippocampus

The ipsilateral hippocampus displayed an increase in Ang II levels at 72 hours (Figure 2A). The contralateral hippocampus displayed the same increase in 24 hours (Figure 3A). Additionally, the ipsilateral hippocampus exhibited an increase in ACE1 levels 30 days post-mild TBI (Figure 2B). These molecules all belong to the inflammatory classical RAS axis.

One component of the RAS counter-regulatory, anti-inflammatory axis also oscillated. Both hippocampal hemispheres displayed an increase in Ang-(1-7) levels 30 days post-injury (figure 2C, 3C). The calculated Ang-(1-7)/AngII and ACE2/ACE1 ratios, used to detect shifts in RAS axis predominance, displayed no significant differences (Figures 2E-2F, and 3E-F).

Mild TBI induces changes in RAS peptides and enzymes in the prefrontal cortex

We did not detect any significant differences in the RAS component levels in the ipsilateral prefrontal cortex at any of the time-points (Figure 4). However, we observed that AngII levels increased in the contralateral prefrontal cortex 30 days after injury (Figure 5A). Interestingly, the Ang-(1-7)/AngII and ACE2/ACE1 ratios decreased 24 hours and 30 days after the injury, respectively, in the ipsilateral cortex (Figure 4E). This pattern was surprisingly not observed in the contralateral prefrontal cortex (Figure 5F).

Mild TBI induces changes in RAS receptors in the hippocampus

Levels of the AT_1 receptor increased at 6, 24, and 72 hours in the ipsilateral hippocampus (Figure 6A). In the contralateral hippocampus, we only observed an increase of this receptor 24 hours post-trauma (Figure 6B).

The Mas receptor, which belongs to the counter-regulatory axis of RAS, decreased ipsilaterally 24 hours, 72 hours, and 30 days post-injury (Figure 6C), as well as 30 days contralaterally (Figure 6D). We detected an increase in AT_2 levels contralaterally in the hippocampus 24 hours post-injury (Figure 6F).

Mild TBI induces changes in RAS receptors in the prefrontal cortex

The AT_1 levels increased 24 and 72 hours after the trauma in the ipsilateral prefrontal cortex (Figure 7A). However, in the contralateral hemisphere, the levels of this

receptor decreased 6 hours and 30 days after mild TBI (Figure 7B). Mas levels decreased between 24 and 72 hours in the ipsilateral prefrontal cortex (Figure 7C). Levels of the AT_2 receptor increased 24 hours, 72 hours, and 30 days ipsilaterally post mild TBI (Figure 7E), while it only increased contralaterally at the 72-hour time-point (Figure 7F).

Perindopril prevents locomotor damage 6 hours after TBI

The open field test revealed locomotor impairment in the TBI+SAL group in comparison with the Sham+SAL group 6 hours after TBI. This locomotor damage was prevented by treatment with perindopril. Treatment with Telmisartan and AVE0991 did not prevent locomotor damage (Figure 8).

Treatment with perindopril, telmisartan, and AVE 0991 did not prevent anxiety-like behavior

The elevated plus maze test showed a decrease in the proportion of time spent in the open arms of the TBI+SAL group, in comparison with the Sham+SAL group (Figure 9A), which indicates anxiety-like behavior, 72 hours after TBI. Treatment with perindopril, telmisartan, and AVE 0991 did not prevent anxiety-like behavior (Figure 9A). There was no difference in the proportion of entries in the open arm (Figure 9B).

In the Barnes Maze test, the Sham+SAL group did not show a significant decrease in the time to find the escape tunnel, in relation to the TBI+SAL group (Figure 10A). The Sham+SAL group also did not show a significant increase in the time spent exploring the escape zone, in relation to the TBI+SAL group (Figure 10B), which indicates that this test did not work.

4. Discussion

In the present study, we investigated the modulation of RAS components in a mild TBI animal model at different time-points in two specific brain locations, the prefrontal cortex and the hippocampus, ipsilaterally and contralaterally, and the effect of treatment with Perindopril, Telmisartan, and AVE0991 in behavioral and cognitive alterations in mice submitted to the mild TBI. We observed that mild TBI induced an increase in AngII, Ang-(1-7), and AT_1r levels and a decrease in MasR in both hemispheres of the hippocampus. We also found that perindopril prevents damage to the locomotor activity of mice subjected to TBI. To the best of our knowledge, we are the first group to demonstrate a direct association between mild TBI and RAS modulation in an experimental study.

AT_1 binds to the Ang II peptide and regulates the expression of growth factors and inflammatory mediators that induce apoptosis, oxidative stress, and inflammation (Abdul-Muneer et al., 2018; Kırça and Yeşilkaya, 2018). We observed that mild TBI increased the ACE/AngII/AT_1 axis in both hippocampal hemispheres, possibly as an inflammatory response to the injury. The Ang-(1-7) molecule – which belongs to the counter-regulatory (anti-inflammatory) RAS axis – significantly increased 30 days post-injury, potentially to counteract the inflammation induced by molecules of the classical axis. Ang-(1-7)'s main receptor, Mas, decrease may underlie a negative feedback response to the abnormal increase in Ang-(1-7). The role of these molecules in mild TBI remains unexplored, although evidence linking abnormal expression of RAS components and mild TBI has increased.

Our findings indicate that modulation of the RAS classical axis components differed between both hemispheres in the prefrontal cortex after mild TBI. AT_2 levels, for example, increased ipsilaterally and contralaterally in the prefrontal cortex, demonstrating a potential modulating role of this receptor in mild TBI. Accordingly,

Timaru-Kast et al., 2019, found an increase in AT_2 mRNA levels in brain tissue at 24 hours in a controlled-cortical impact (CCI) TBI model – also demonstrating AT_2's potential involvement in TBI (Timaru-Kast et al., 2019). Although mechanisms around AT_2 remain not well understood, evidence suggests the AT_2 receptor induces vasodilation and tissue repair (Carey, 2005; Kangussu et al., 2020), which can potentially explain our findings. Although AT_2 also belongs to the classical axis, we know that AT_2 counteracts the effects of the AT_1 receptor, which was also observed to be differentially expressed in our mild TBI model.

The use of ACE inhibitors – which inhibit the cleavage of Ang I to Ang II – has been underexplored in brain trauma. Here, treatment with perindopril prevented impairment in locomotor activity in animals subjected to mild TBI. A study that used other ACE inhibitors, captopril, and enalapril, in the treatment of Sprague-Dawley rats subjected to moderate TBI showed that these medications exacerbated motor deficits 7 days after the injury (Harford-Wright et al., 2010). In patients with severe TBI, the use of ACE inhibitors was associated with an increase in mortality rates (Catapano et al., 2019). The molecular mechanisms that underlie these discrepant results remain obscure. Although in this study we did not find protection in TBI-sequelae with the use of telmisartan, there is some evidence in the literature that treatment with an AT1 antagonist may be promising (Villappol et al., 2015; Xiong et al., 2020). For instance, the intraperitoneal administration of 0.1 mg/kg of candesartan, an AT_1 antagonist decreased oxidative stress, and microglial and astrocyte activation at 6 hours after moderate TBI in mice (Villapol et al., 2015). Also, it reduced neutrophilic infiltration and apoptosis in the cortex at 3 days after injury (Villapol et al., 2015). Additionally, losartan, another AT_1 antagonist, reduced brain edema, permeability in the blood-brain barrier, and apoptosis

in comparison with the vehicle-group in mice cortex 72 hours after TBI (Xiong et al., 2020).

In the present study, we provide direct evidence that mild TBI upregulates Ang II, Ang-(1-7), and ACE as well as downregulates the Mas receptors in the hippocampus and prefrontal cortex in an animal model. Although our findings are remarkably novel, we understand that this study bears limitations. For example, we only measured enzyme concentrations rather than activity – which limits our interpretation of the role of RAS in mild TBI. The present findings shed some light on the role of RAS molecules induced-TBI and the potential of perindopril as treatment for TBI locomotor outcomes. Future work should focus on the potential of RAS molecules modulation in TBI outcomes.

Conflict of interest

The authors declare no conflict of interest.

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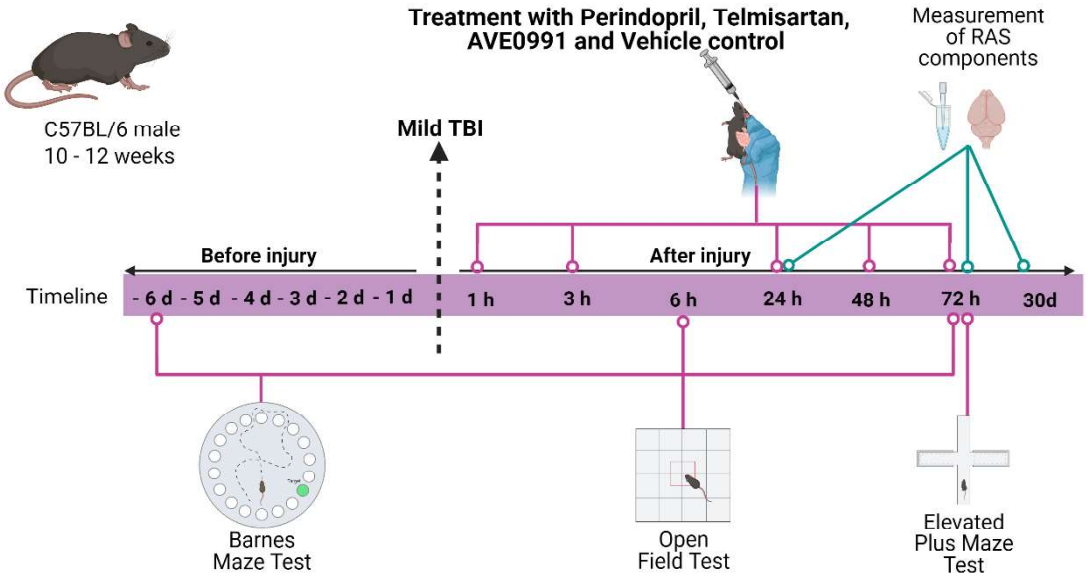

Figure 1. Experimental Design.

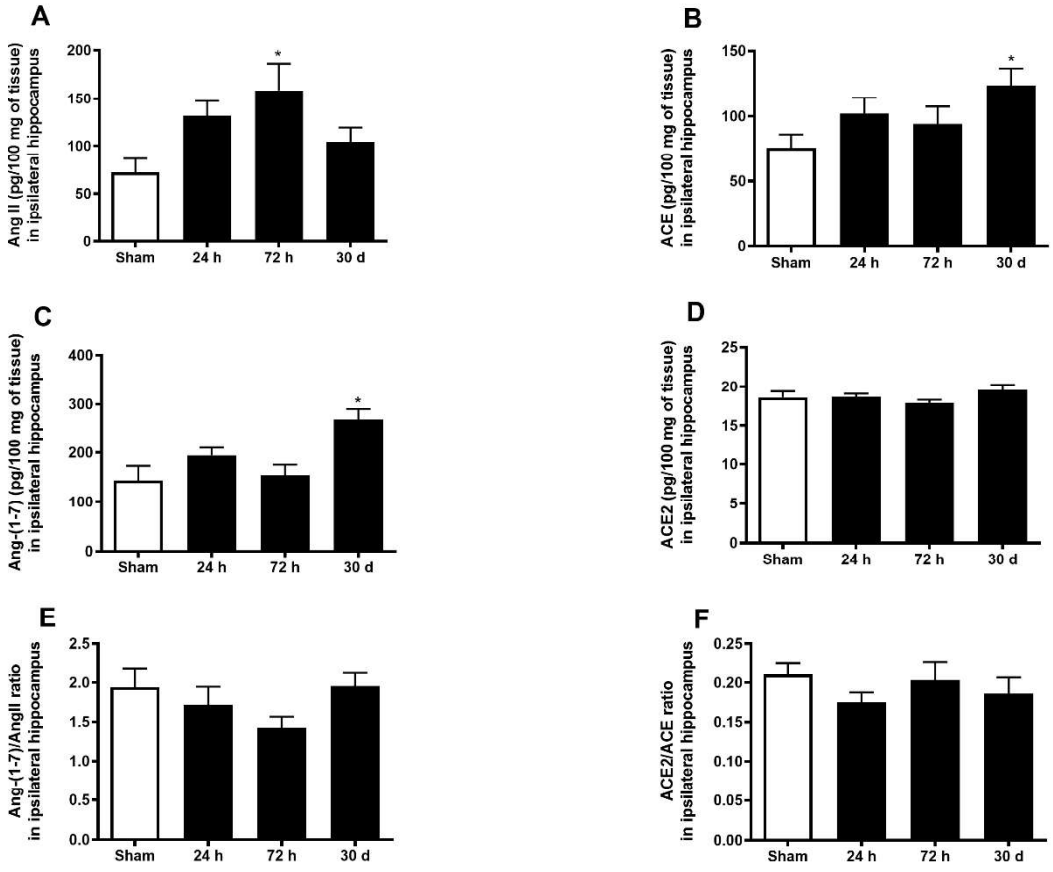

Figure 2. RAS peptides and enzymes in the ipsilateral hippocampus of mice after mild-TBI. The analysis was performed with an assessment of Ang II (A), ACE (B), Ang-(1-7) (C), ACE2 (D), Ang-(1-7)/AngII ratio (E), and ACE2/ACE ratio. n = 5 per experimental group * means significant difference between sham group ($p < 0.05$).

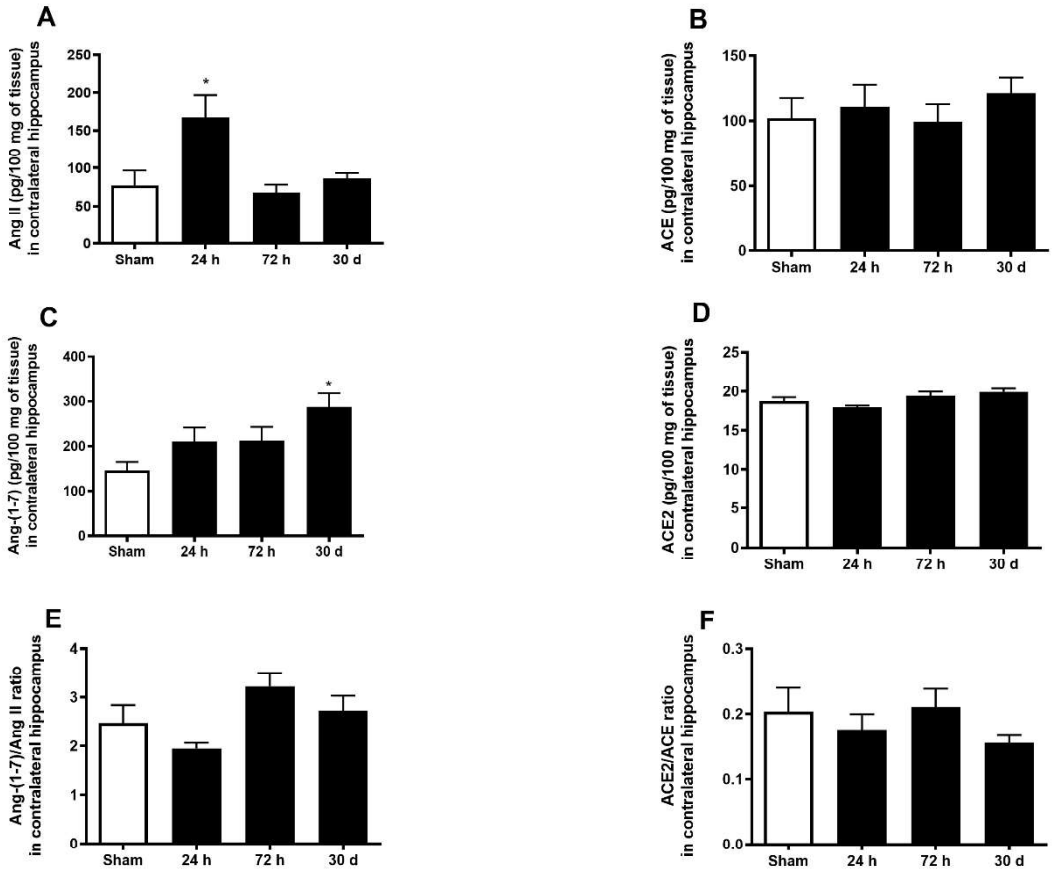

Figure 3. RAS peptides and enzymes in the contralateral hippocampus of mice after mild-TBI. The analysis was performed with an assessment of Ang II (A), ACE (B), Ang-(1-7) (C), ACE2 (D), Ang-(1-7)/AngII ratio (E), and ACE2/ACE ratio. n = 5 per experimental group * means significant difference between sham group ($p < 0.05$).

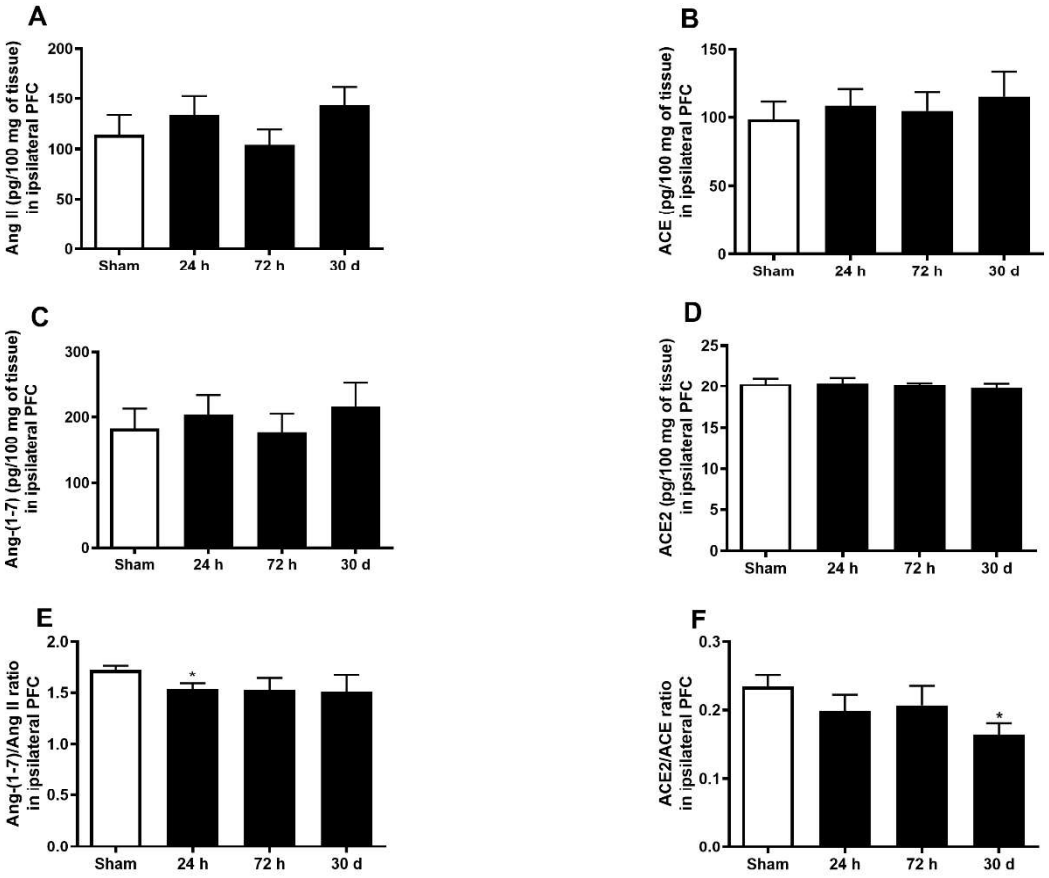

Figure 4. RAS peptides and enzymes in the ipsilateral prefrontal cortex of mice after mild-TBI. The analysis was performed with an assessment of Ang II (A), ACE (B), Ang-(1-7) (C), ACE2 (D), Ang-(1-7)/AngII ratio (E), and ACE2/ACE ratio. n = 5 per experimental group * means significant difference between sham group ($p < 0.05$).

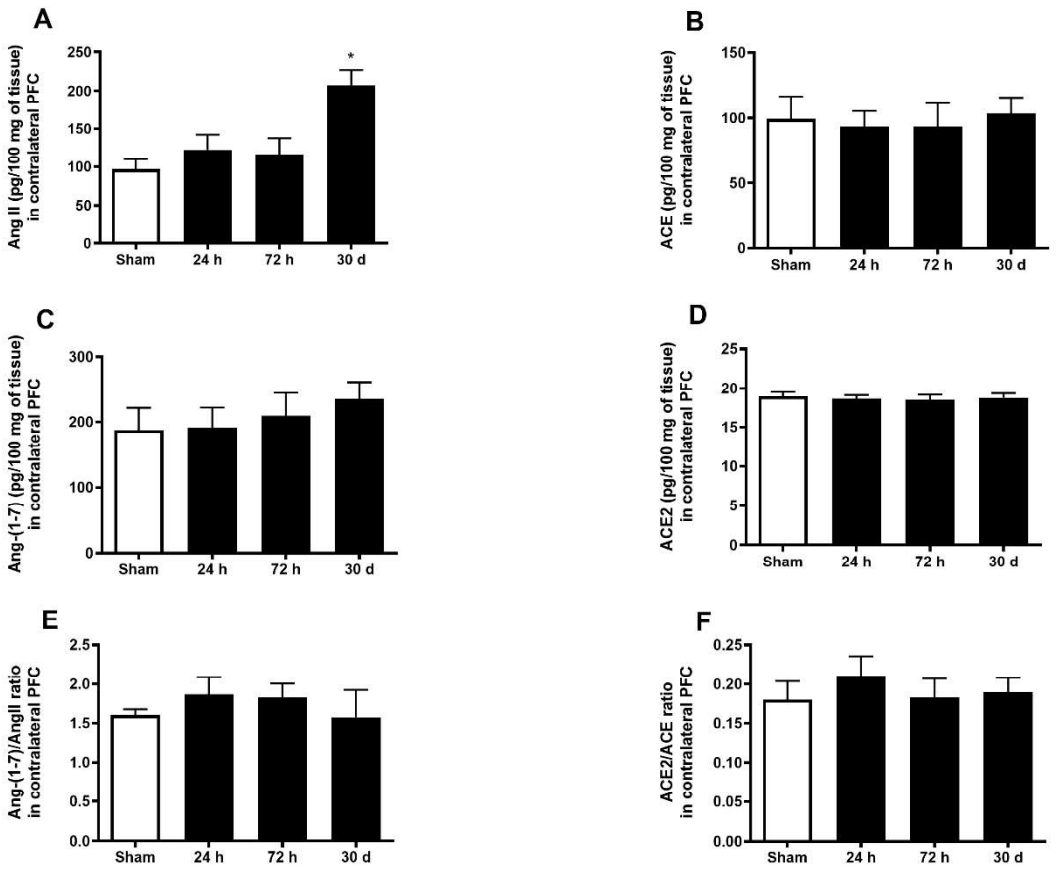

Figure 5. RAS peptides and enzymes in the contralateral prefrontal cortex of mice after mild-TBI. The analysis was performed with an assessment of Ang II (A), ACE (B), Ang-(1-7) (C), ACE2 (D), Ang-(1-7)/AngII ratio (E), and ACE2/ACE ratio. n = 5 per experimental group * means significant difference between sham group ($p < 0.05$).

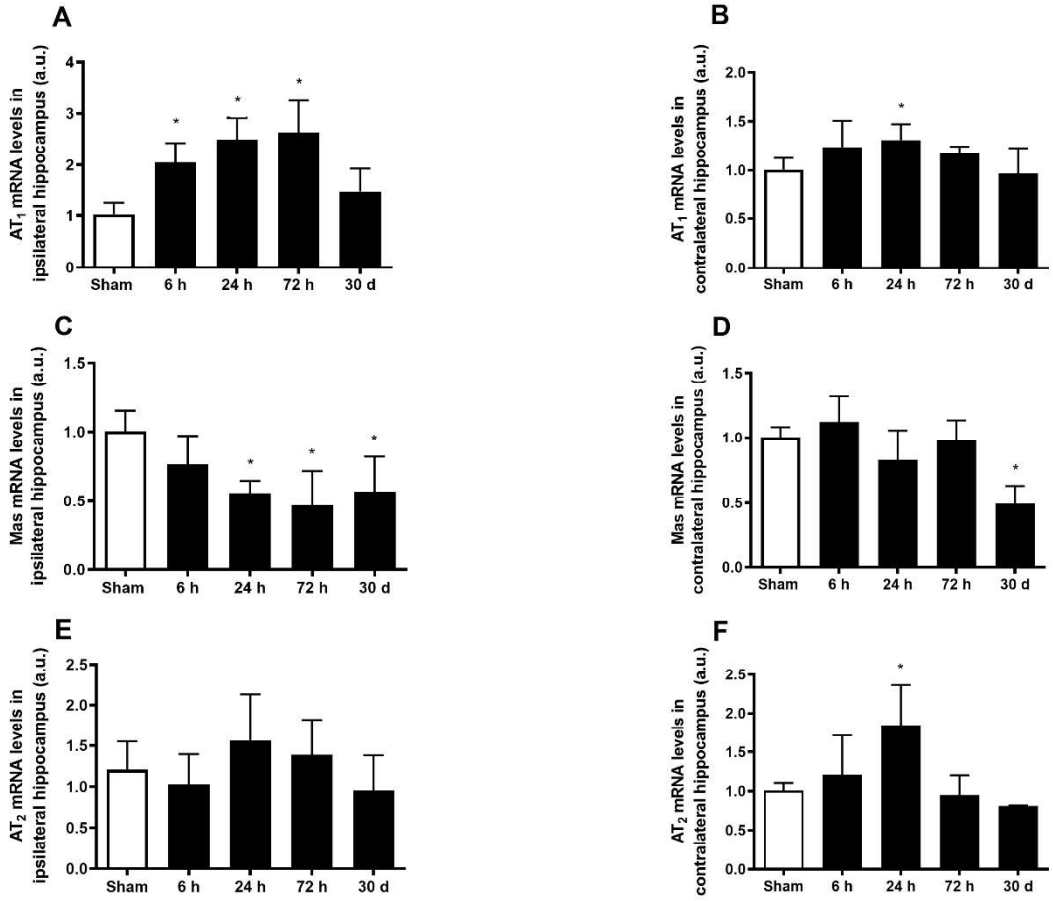

Figure 6. RAS receptors in the ipsilateral and contralateral hippocampus of mice after mild TBI. The analysis was performed with an assessment of AT_1 (A-B), Mas (C-D), and AT_2 (E-F). n = 5 per experimental group * means significant difference between sham group ($p < 0.05$).

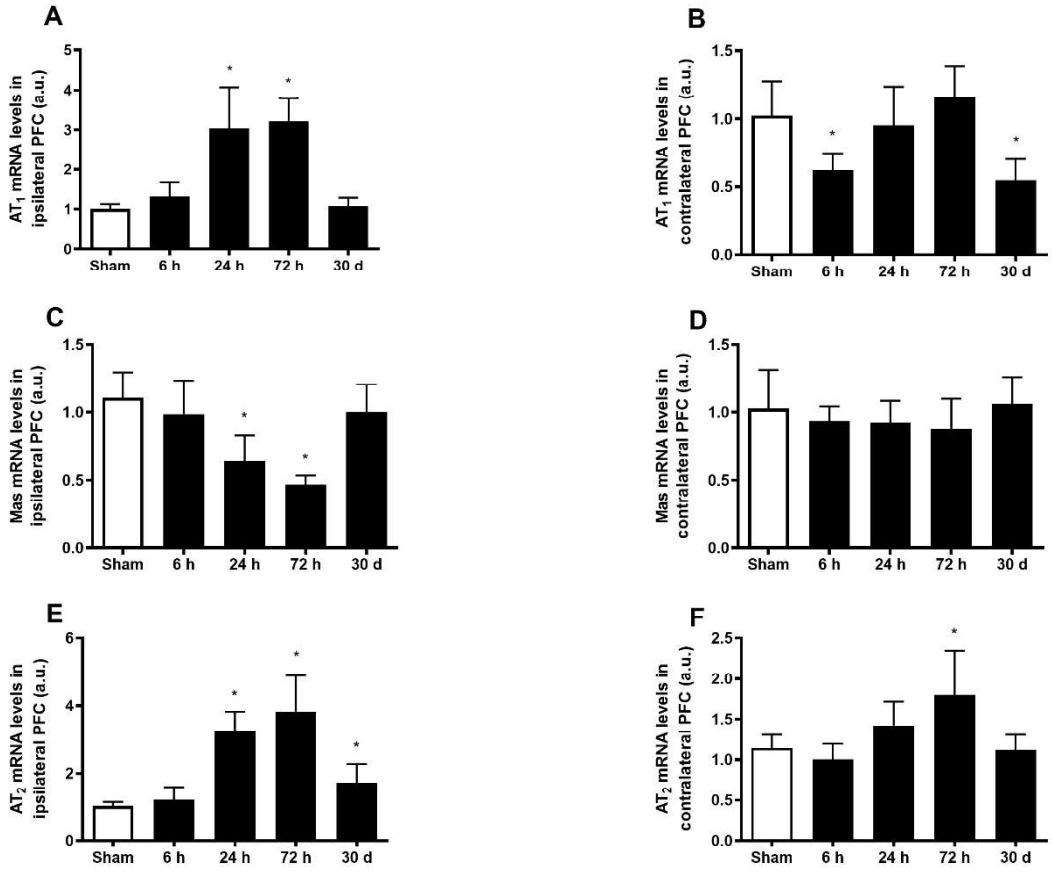

Figure 7. RAS receptors in the ipsilateral and contralateral prefrontal cortex of mice after mild TBI. The analysis was performed with an assessment of AT_1 (A-B), Mas (C-D), and AT_2 (E-F). n = 5 per experimental group * means significant difference between sham group ($p < 0.05$).

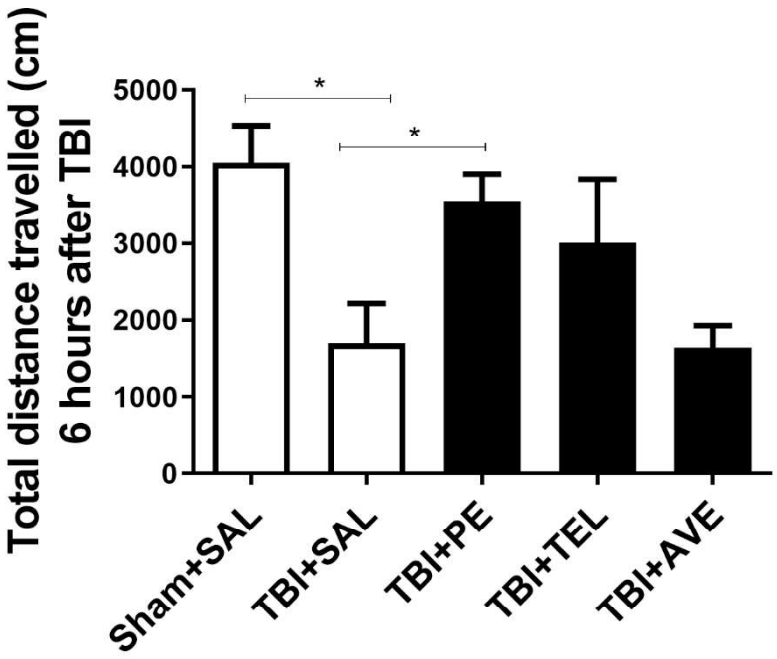

Figure 8. Evaluation of the effect of treatment with Perindopril, Telmisartan, and AVE0991 on locomotor activity in mice submitted to mild TBI. n = 5 per experimental group * means significant difference between the highlighted groups ($p < 0.05$).

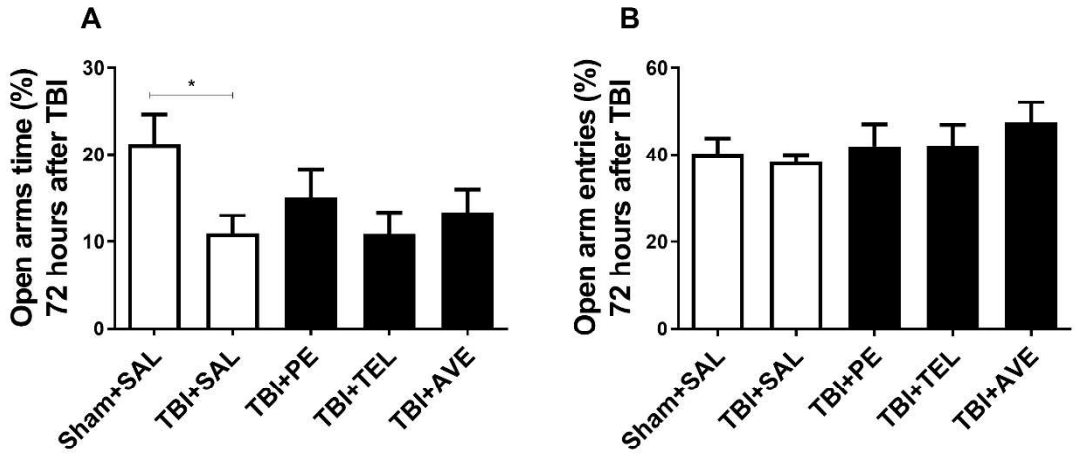

Figure 9. Effect of treatment with Perindopril, Telmisartan, and AVE0991 on anxiety-like behavior in mice submitted to mild TBI. (A) The percentage of open arms time, 72 hours after TBI. (B) The percentage of open arm entries, 72 hours after TBI. n = 5 per experimental group * means a significant difference between the highlighted groups ($p < 0.05$).

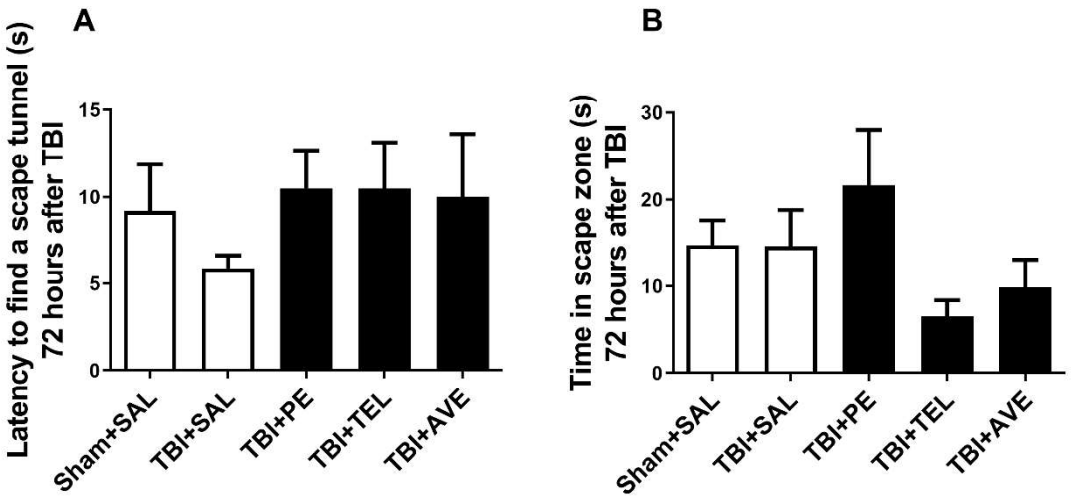

Figure 10. Effect of treatment with Perindopril, Telmisartan, and AVE0991 on hippocampal-dependent spatial memory in mice submitted to mild TBI. (A) Latency to find a scape tunnel, 72 hours after TBI. (B) Time spent in scape zone, 72 hours after TBI. n = 5 per experimental group * means a significant difference between the highlighted groups ($p < 0.05$).

7.4 - ARTIGO CIENTÍFICO IV

Título: ***Striatal Renin-Angiotensin System and Oxidative Stress activation is associated with motor learning impairment after experimental mild traumatic brain injury***

Striatal Renin-Angiotensin System and Oxidative Stress activation are associated with motor learning impairment after experimental mild traumatic brain injury

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Abstract

Traumatic brain injury (TBI) is a cerebral lesion caused by a mechanical force against the skull. TBI is the leading cause of disability and death among young adults worldwide and, mild TBI accounts for more than 70 % of TBI cases. There is evidence that inflammation and oxidative stress might be involved in secondary lesions associated with mild TBI. However, the role of these events in motor learning changes secondary to mild TBI remains to be further explored. We hypothesized that inflammation and oxidative stress could have a role in motor learning impairment secondary to experimental mild TBI. To confirm this hypothesis, we subjected the animals to the weight drop model and investigated motor learning and changes in RAS components, as well as oxidative damage and inflammatory response after injury. Mice showed a decrease in the motor learning index after TBI, revealing impairment in motor learning. TBI-mice presented an increase in the AT1 receptor, which belongs to the classical RAS axis, while there was a decrease in the levels of the Mas receptor, which belongs to the counterregulatory axis of the RAS. There was also an increase in AT2 receptor levels. Along with these changes in RAS components, we found an increase in TBRAS levels, which indicates an increase in oxidative stress, and also changes in the levels of GDNF, CX3CL1, and TNF alpha, indicating that the inflammatory response is also active. Our results suggest that inflammatory processes and oxidative stress in the *striatum* can play a role in motor learning impairment after acute mild TBI.

Key-words: mild traumatic brain injury, renin-angiotensin system, oxidative stress

1. Introduction

Traumatic brain injury (TBI) is a well-known cause of disability and mortality worldwide, with an occurrence estimate of 1.7 million new cases annually in the USA (Faul et al., 2010). Mild TBI represents more than 70% of TBI cases and can induce behavioral alterations, cognitive damage, and sleep disturbances which can persist for years after the primary insult (Cardoso et al., 2019; Feigin et al., 2013; Lecuyer Giguere et al., 2020). Previous work has only focused on motor alterations after injury (Sosnoff et al., 2008). However, the mechanisms that underlie the motor learning after mild TBI still unclear.

The *striatum*, which is an important component of basal ganglia, has connections with the sensorimotor cortex and is related to motor control, learning, and cognitive function (Cataldi et al., 2021; Mello and Villares, 1997). Previous studies showed that TBI induces damage in motor function associated with changes in striatal oxidative stress at 24 hours (Huang et al., 2018) and 6 days (Harmon et al., 2017) after injury.

The Renin-Angiotensin system has receptors in the brain key areas, including *striatum* (Häuser et al., 1998), and has been implicated with TBI outcomes (Janatpour et al., 2019; Khaksari et al., 2018). Angiotensin II is the main component of the system and acts through its AT1 receptor, promoting neuroinflammation and oxidative stress. On the other hand, Angiotensin 1-7, acts mediated by the Mas receptor and has effects opposite to the AT1 receptor, which includes anti-inflammatory and anti-oxidative activation (Guo et al., 2001; Santos et al., 2003). The pharmacology blockade of the AT1 receptor as a therapeutic strategy for TBI sequelae found a reduction in behavioral changes, a decrease in oxidative stress and neuroinflammation, and an improvement in neurologic and motor function (Timaru-Kast et al., 2012; Villapol et al., 2015; Xiong et al., 2020). Here we

investigate the role of striatal RAS and oxidative stress activation on motor learning impaired following mild TBI.

2. Material and Methods

Animal care

Male C57BL/6 mice aged 10-12 weeks and obtained in Bioterism Center at Federal University of Minas Gerais (UFMG) were used in this study. These animals were maintained in a controlled temperature room (21-24 °C), with 12-hour light-dark cycle and food and water ad libitum. All procedures were approved by the Ethics Committee on Animal Experimentation of UFMG (under protocol 250/2017).

Mild TBI Injury

Mild TBI was induced using a weight-drop model based on the model described by Flierl et al., 2009. Briefly, mice were anesthetized with ketamine/xylazine. Then, animals were placed in a prone position and submitted to the midline incision. After the skull was exposed, the animals were placed in the weight drop model platform and the 15 grams weight falls on the right parietal side. Each animal suffered a single mild injury during this study. Next, the animals returned to the cage for recovery. The sham group was submitted to all procedures of the TBI group, except the trauma.

Accelerating rotarod test

The rotarod (Insight, Ribeirao Preto, Brazil) was performed to evaluate motor skill learning after mild TBI as described in Kao et al., 2015. The test was performed during 5 days, with the first and second days being performed before the TBI. The third day was performed 24 hours after injury, the fourth day was performed 48 hours after the injury and the fifth day was performed 72 hours after the injury. On the first day of the test,

animals were habituated with a constant speed of 4 rpm for 30 seconds before the first trial. This step occurs only on the first day of the test. Thirty minutes after this habituation time, the animals were trained for 5 minutes at an accelerating speed (4-40 rpm) in three trials daily with an intertrial interval of 30 minutes during 5 consecutive days. The falling latency was noted and a median of three trials was used for statistical analysis by two-way analysis variance (ANOVA) followed by Sidak's multiple comparisons test. The motor learning performance was obtained for the percentage of learned skills in two consecutive days through the motor learning index formula: $(T_n - T_n - 1)/T_{n-1} \times 100$ %, where T_n is the falling latency on the n^{th} testing day. The differences between TBI and sham groups were assessed by impaired test T. The level of significance was set at $p < 0.05$. Mice were habituated in the test room for at least 30 minutes before measurements were initiated (n = 10 animals per group).

Measurement of RAS receptors mRNA expression, TBARS levels, nitric oxide synthases, and inflammatory mediators

After motor evaluation, mice were anesthetized with an intraperitoneal injection of ketamine/xylazine and euthanized. The ipsilateral (right) and contralateral (left) *striatum* were dissected and stored at -70° for the later measurement.

Striatal mRNA expression of RAS receptors and nitric oxide synthases

The RAS receptors mRNA expression and the isoforms of the enzyme nitric oxide synthase (NOS), known as the isozymes neuronal ‘n’NOS, inducible ‘i’NOS, and endothelial ‘e’NOS, were assessed using a total RNA isolation according to the manufacturer protocol (Invitrogen, Life Technologies, USA/ Biorad, USA). qRT-PCR was performed using SYBR Green reagent (Applied Biosystems, NY, USA). Total RNA was obtained using the Trizol reagent (Life Technologies, ThermoFisher Scientific, MA,

USA) following manufacturer's instructions. Primer's sequence (sense and antisense) of AT_1: (GGTGGGAATATTGGAAACAG) and (AAGAAGAAAAGCACAATCGCC); AT_2: (GCTGAGTAAGCTGAT TTATG) and (TTAAGACACAAAGGTGTCCA); Mas receptor: (CCCACCCATTCCCATAGTGC) and (CCGAGAGGAGAGATGCTCATG); endogenous control S26: (CGATTCCTGACAACCTTGCTATG) and (CGTGCTTCCCAAGCTCTATGT) and nNos: (CTGGTGAAGGAACGGGTCAG) and CCGATCATTGACGGCGAGAAT); eNos: (CTCTACCGGGACGAGGTACT) and (GAGGTCTTGCACGTAGGTCT); iNos: (GCTTGCCCCAACAGGAGAAG) and (GCTGCCCGGAAGGTTTGTAC) and Rpl32 (GCTGCCATCTGTTTTACGG) and (TGACTGGTGCCTGATGAACT).

Analyses of striatal Lipid Peroxidation

The analysis of striatal lipid peroxidation was performed by malondialdehyde (MDA) content by thiobarbituric acid reactive substances (TBARS) levels as described in Mihara and Uchiyama (1978).

Analyses of Cytokines and Neurotrophic Factors in striatum

The measurement of levels of brain-derived neurotrophic factor (BDNF), glial cell line-derived growth factor (GDNF), nerve growth factor (NGF), C-X3-C motif chemokine ligand 1 (CX3CL1), interferon-gamma (IFN-γ), tumor necrosis factor (TNF-α), interleukin 1 beta (IL-1β), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 10 (IL-10) and interleukin 12p70 (IL-12p70), by Enzyme-linked immunosorbent (ELISA), according to the manufacturer protocol (R&D Systems, Minneapolis, MN, USA). Results are expressed as picogram (pg) per 100 mg of tissue.

3. Results

TBI-mice presented a decrease in falling latency time (Figure 1A) and a reduction in motor learning index over the Rotarod test in comparison with sham (Figure 1B). These results indicate that mild TBI induces impairment in motor learning over 72 hours after injury. TBI-mice also presented a decrease in motor learning index from day 3 to day 4 in comparison with sham (Figure 1B).

Along with the motor learning impaired, we showed an increase in AT1 receptor levels at 24 and 72 hours in the ipsilateral *striatum* after injury (Figure 2A). The Mas receptor levels were decreased at 24 and 72 hours in the ipsilateral (Figure 2C) and at 24 hours in the contralateral *striatum* (Figure 2D). Also, we found higher AT2 receptor levels at 24 and 72 hours in the ipsilateral *striatum* after mild TBI (Figure 2E).

Based on the role of AT1 receptor in induces oxidative stress and inflammatory response (Abdul-Muneer et al., 2018; Kirça and Yeşilkaya, 2018) we measured the levels of lipid peroxidation and the expression of SOD enzymes, as well as the levels of cytokines, chemokines and neurotrophic factors associated with motor learning damage following mild TBI. We found an increase of thiobarbituric acid reactive substances (TBARS) levels in both hemispheres of the *striatum* at 24 and 72 hours after mild TBI (Figure 3). No differences are found in the expression of SOD enzymes (Figure S1). TBI-mice also displayed a significant decrease in GDNF levels (Figure S2B) and an increase in CX3CL1 levels (Figure S2D) in the ipsilateral *striatum* at 72 hours after TBI. In the contralateral *striatum*, we found an increase in TNF-α levels (Figure S3F) after 72 hours after TBI.

4. Discussion

This is the first study investigating the role of striatal RAS and oxidative stress activation on motor learning impaired induced by mild TBI. TBI-mice exhibited motor learning impairment in at least 3 days after mild TBI. This damage was accompanied by the higher AT_1 receptor levels and a decrease in Mas receptor levels in the ipsilateral *striatum* over time. Also, TBI-mice showed markedly enhance in striatal oxidative stress in the first hours after injury. These results suggest an involvement between the striatal overexpression of AT_1 receptor, increase in oxidative stress, and impaired motor learning after mild TBI in our animals.

Although our results are the first to investigate the role of striatal AT_1 receptor activation in motor learning impaired, there is evidence that supports this involvement. Studies that used the pharmacology blockade of AT_1 receptors, showed decrease in oxidative stress and improve in motor function after TBI (Villapol et al., 2012; Villapol et al., 2015). For instance, Khaksari et. (2018) using weight-drop model showed that Wistar rats treated with candesartan (0,3 mg/kg) at 30 minutes after injury displayed decreased cerebral oxidative stress at 5 hours and recovered the motor performance at 24 hours after moderate TBI.

Our findings indicate an association between the increase in striatal oxidative stress parameters and motor learning impairment. In line with our results, Huang et (2018) found an increase in oxidative stress in *striatum* associated with motor coordination and sensory-motor impairment at 24 hours after TBI in Sprague-Dawley rats submitted to the CCI model. Also, a study with Long Evans rats submitted to the CCI model showed an increase in superoxide dismutase 2 (SOD2) levels at 24 hours and uncoupling protein 2 (UCP2) highest levels at 3 and 6 days in ipsilateral striatum. Both SOD2 and UCP2 are compounds that act in protection against oxidative stress, reducing ROS. The increase in

the expression of these molecules was associated with unilateral forelimb motor deficit after 24 hours and 6 days after severe TBI (Harmon et al., 2017).

To our knowledge, this is the first study to show that mild TBI also induces downregulation of the striatal Mas receptor at 24 and 72 hours. These data suggest activation of the ACE2/Ang-(1-7)/Mas receptor axis in the *striatum* after mild TBI. Evidence from other cerebrovascular diseases supports these findings. For instance, a study showed a decrease in cerebral oxidative stress in Sprague-Dawley rats treated with Ang-(1-7) 24 hours after permanent middle cerebral artery occlusion (pMCAO) in the cerebral ischemia model. This result was reversed with the use of A-779, a Mas receptor antagonist (Jiang et al., 2012).

The evidence from this study suggest that striatal RAS and oxidative stress activation acts secondary to motor learning impairment TBI-associated. A limitation of our study is the lack of analyses of the RAS peptides for a complete understanding of the role of striatal RAS in motor learning TBI outcomes. Future experimental research should focus on RAS modulation to improve motor learning impairment induced by mild TBI.

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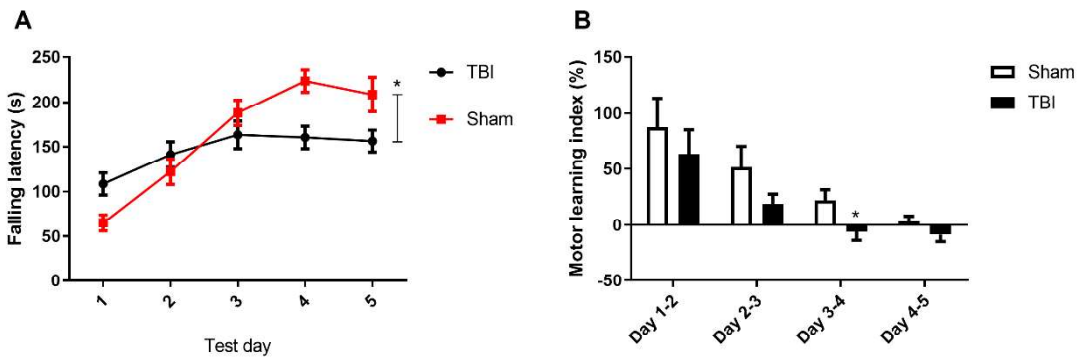


Figure 1. Evaluation of motor learning in Rotarod test after experimental mild TBI. A - TBI-mice show an impairment in motor coordination in comparison with sham. N = 10 per experimental group * means a significant difference between TBI and sham animals ($p < 0.05$); B - TBI-mice presented a decrease in motor learning deficit overtime. * Means a significant difference between TBI and sham animals ($p < 0.05$); unpaired t test.

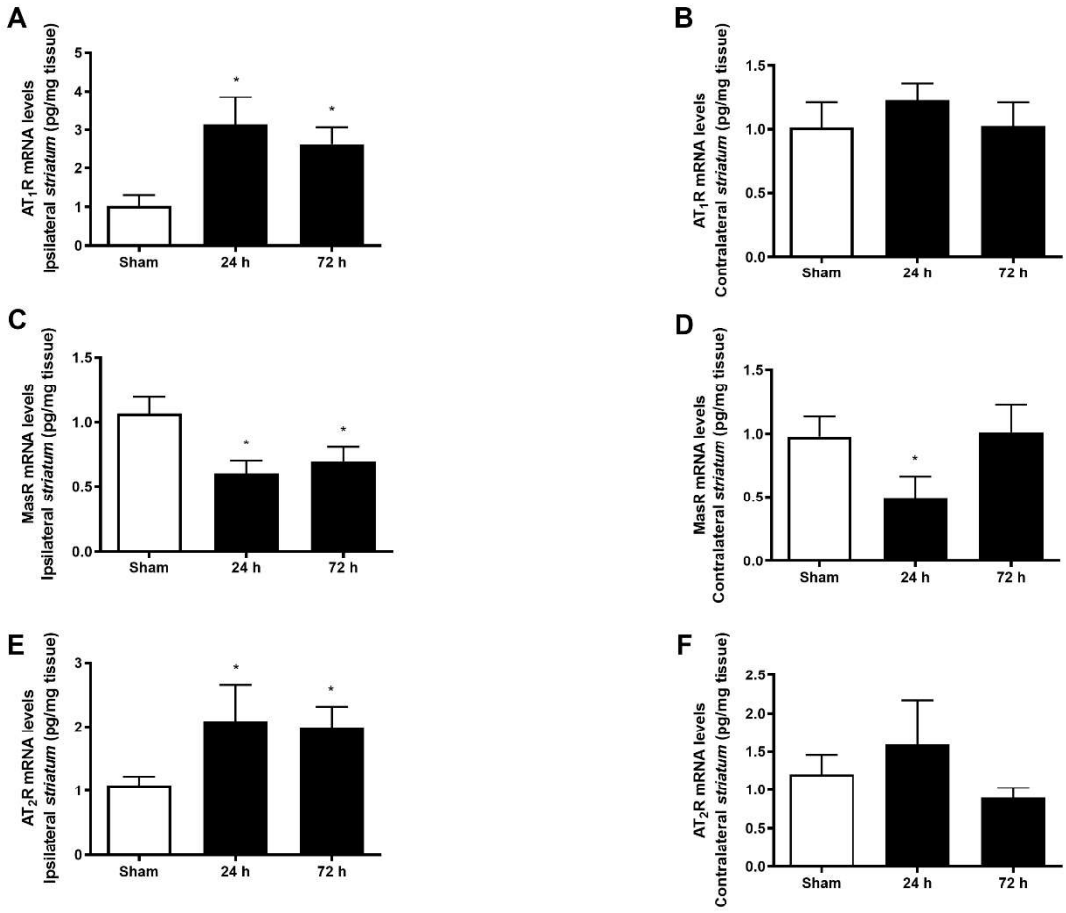


Figure 2. Effects of experimental mild-TBI in RAS receptors in *striatum*. The analysis was performed with an assessment of ipsilateral AT_1R (A), contralateral AT_1R (B), ipsilateral Mas receptor (C), contralateral Mas receptor(D), ipsilateral AT_2R (E) and contralateral AT_2R (F) in mice after mild-TBI. N = 4-5 per experimental group * means a significant difference between sham group ($p < 0.05$).

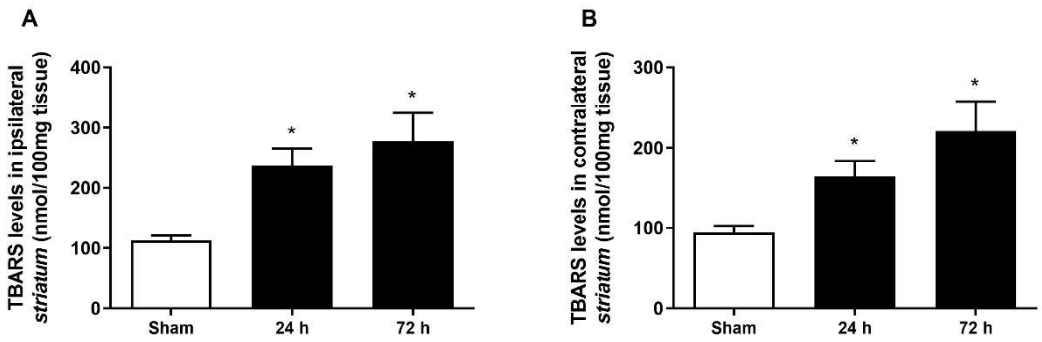
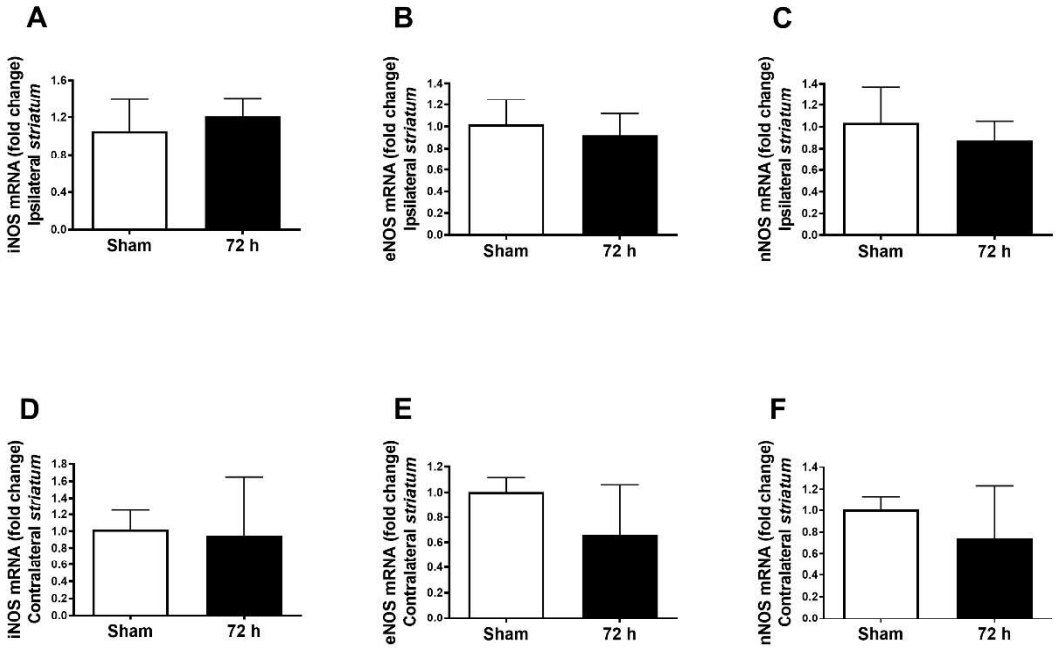
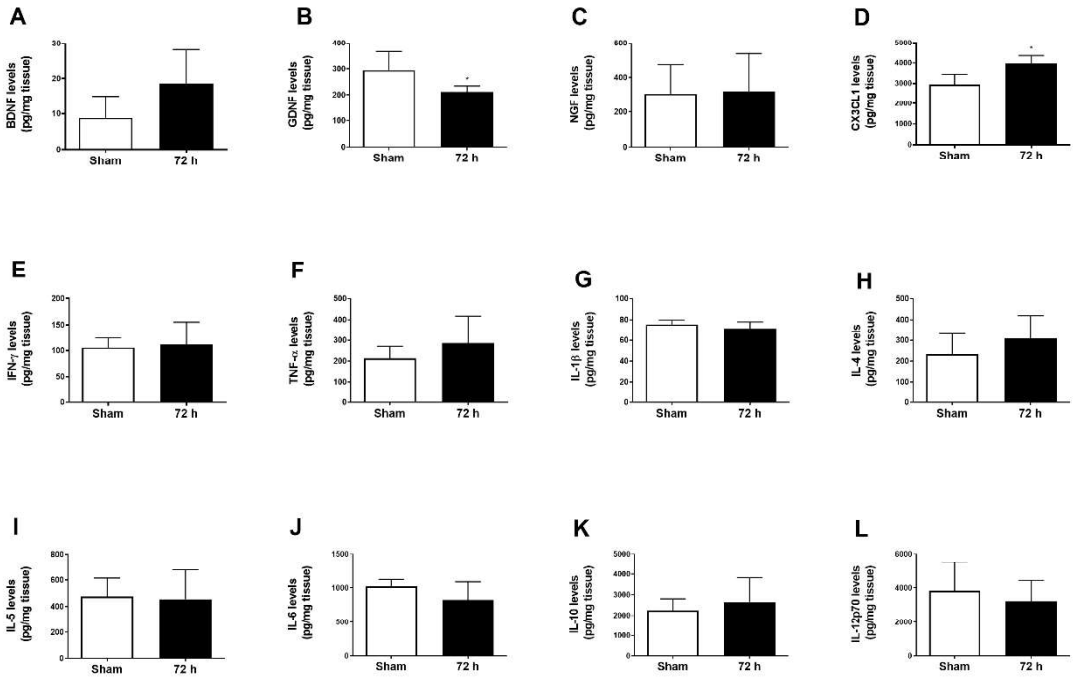


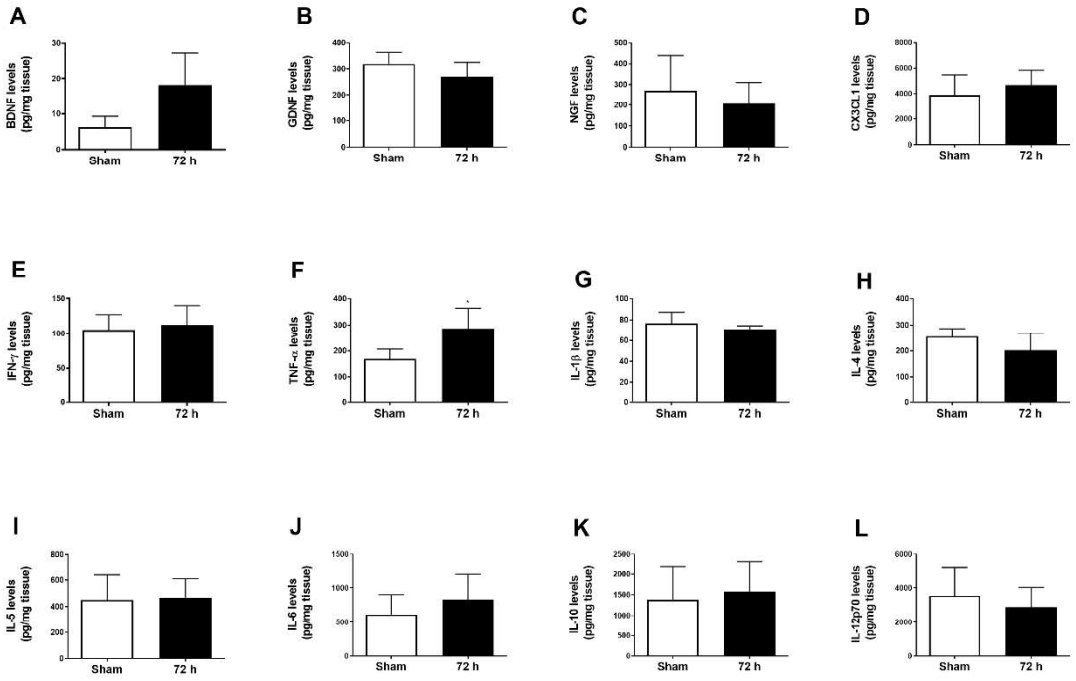
Figure 3. Effects of experimental mild-TBI in oxidative stress parameters in *striatum*. The marker TBARS revealed an increase of lipid peroxidation levels at 24 and 72 hours following TBI. The analysis was performed in ipsilateral striatum (A) and contralateral striatum (B). n = 5 per experimental group * means a significant difference between sham group.



Supplementary figure S1. Expression of nitric oxide synthases in the *striatum* of mice after mild-TBI. The analysis of expression of iNOS (A and D), eNOS (B and E), and nNOS (C and F) in the ipsilateral and contralateral *striatum* at 72 hours after mild TBI. n = 4-5 per experimental group * means a significant difference between the sham group.



Supplementary figure S2. Inflammatory mediators in ipsilateral *striatum* of mice after mild-TBI. The analysis was performed with an assessment of BDNF (A), GDNF (B), NGF (C), CX3CL1 (D), IFN-γ (E), TNF-α (F), IL-1β (G), IL-4 (H), IL-5 (I), IL-6 (J), IL-10 (K) and IL-12p70 (L). n = 4-6 per experimental group * means significant difference between TBI-72 hours group ($p < 0.05$).



Supplementary figure S3. Inflammatory mediators in contralateral *striatum* of mice after mild-TBI. The analysis was performed with an assessment of BDNF (A), GDNF (B), NGF (C), CX3CL1 (D), IFN-γ (E), TNF-α (F), IL-1β (G), IL-4 (H), IL-5 (I), IL-6 (J), IL-10 (K) and IL-12p70 (L). n = 4-6 per experimental group * means significant difference between TBI-72 hours group ($p < 0.05$).

6.5 – Resultados produzidos durante o doutorado que não foram incluídos nos artigos

Modelo de TCE leve X Animais Apo E-/-

Em parceria com a professora Jacqueline Isaura Alvarez-Leite do departamento de Bioquímica e Imunologia da UFMG e com o dr. Weslley Fernandes Braga nós obtemos animais *knockout* para a apolipoproteína E com o objetivo de investigar se a ausência desse gene poderia ser capaz de proteger contra o surgimento de alterações comportamentais após o TCE leve. Com o início da pandemia Covid-19 esse projeto foi paralisado.

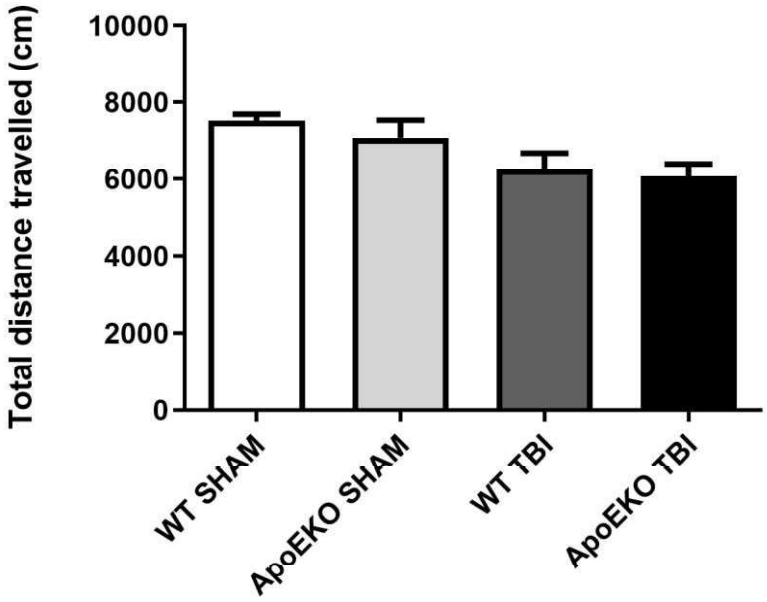


Figura 7: Teste de Campo Aberto – O teste de campo aberto foi realizado 72 horas após o TCE. Não houve diferença significativa na distância total percorrida, indicando que não há alteração locomotora em nenhum dos grupos ($p < 0.05$).

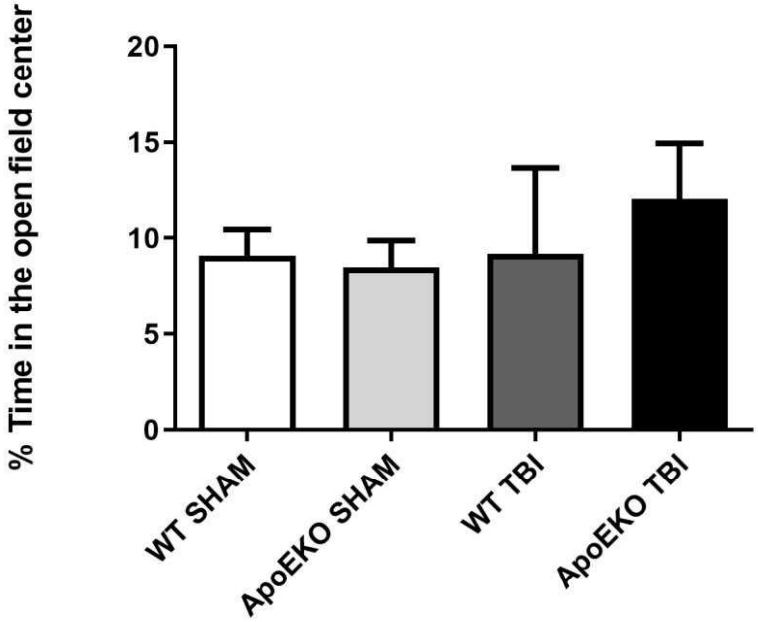


Figura 8: Teste de Campo Aberto – O teste de campo aberto foi realizado 72 horas após o TCE. Não houve diferença significativa na proporção do tempo no centro do aparelho, indicando que não há indícios de comportamento do tipo ansioso utilizando esse teste em nenhum dos grupos ($p < 0.05$).

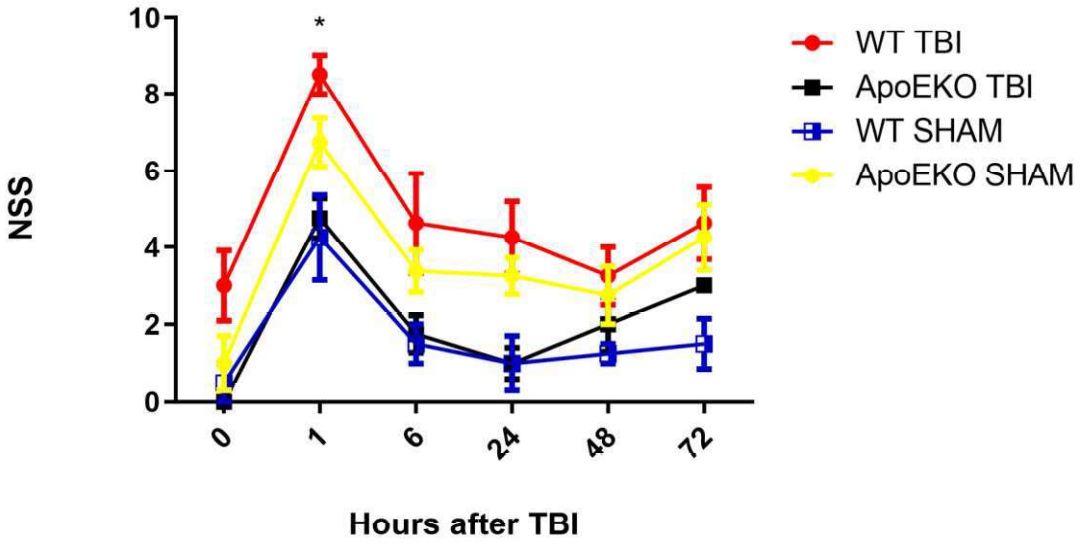


Figura 9: Escore neurológico de gravidade – A escore neurológico foi realizado ao longo de 72 horas após o TCE. Nas primeiras horas os animais WT-TBI apresentaram uma pontuação maior em relação aos animais dos grupos ApoEKO-TBI e WT-Sham, indicando que a ausência do gene APOE poderia estar protegendo os animais que sofreram TCE de apresentar um comprometimento neurológico 1 hora após a lesão. ($p < 0.05$).

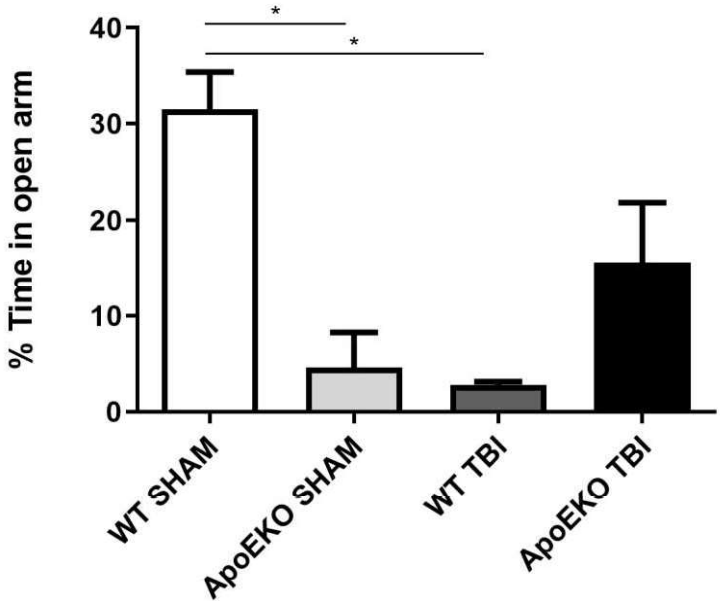


Figura 10: Teste do Labirinto em cruz elevado – O teste do labirinto em cruz elevado foi realizado 72 horas após o TCE. Houve diferença significativa entre os grupos WT-Sham e WT-TBI, o que era esperado e indicando que o teste realizado funcionou. Entretanto, não houve diferença significativa entre os animais ApoEKO, o que pode indicar que seja necessário repetir esse teste ($p < 0.05$).

7 – CONSIDERAÇÕES FINAIS

Ao longo desta tese, buscamos investigar o papel do sistema renina-angiotensina no modelo experimental de TCE leve. O presente estudo forneceu evidências de que o modelo padronizado foi capaz de reproduzir em camundongos alterações comportamentais e cognitivas vistas na clínica. Essas alterações foram associadas a um aumento da inflamação, estresse oxidativo e ativação de células da glia no córtex pré-frontal e hipocampo de camundongos. Provamos também que essas alterações são região-dependente e que mudam ao longo do tempo depois da lesão. Também fornecemos evidências que o SRA tem envolvimento na fisiopatologia do TCE ao mostrar alterações nos níveis de seus componentes no córtex-pré frontal e hipocampo de camundongos. Além de mostrarmos que o perindopril melhora danos locomotores nas primeiras horas após a lesão. Também observamos que o TCE leve induz alteração nos componentes do SRA no estriado, aumento do estresse oxidativo e mudança nos níveis de mediadores inflamatórios associadas a danos na aprendizagem motora.

Além disso, ao longo deste trabalho, foram identificadas algumas limitações como não mensuramos outros parâmetros mais diretos para determinação do estresse oxidativo e também não medimos a atividade das enzimas do SRA. Além disso, não tivemos tempo hábil para repetir o tratamento com os moduladores para tentar observar o efeito de telmisartan e AVE0991 e nem realizar um tratamento com os moduladores do SRA voltados para tentarmos testar se os moduladores possuem alguma proteção frente ao dano na aprendizagem motora que foi observado. Essas limitações sugerem oportunidades para investigações futuras do nosso grupo.

8 – CONCLUSÃO

O presente estudo mostrou que o modelo de queda de peso foi capaz de causar um TCE em camundongos, resultando em alterações locomotoras, cognitivas e comportamentais, tanto na fase aguda como subcrônica da lesão. Essas alterações clínicas foram associadas a danos na barreira hematoencefálica, aumento da resposta inflamatória e no estresse oxidativo no córtex pré-frontal e hipocampo. No presente estudo, nossos animais apresentaram alterações nos níveis de componentes do sistema renina-angiotensina em várias regiões do cérebro e que um dos moduladores desse sistema, o Perindopril, foi capaz de prevenir danos locomotores nos animais submetidos ao TCE. A nossa lesão também foi capaz de causar danos na aprendizagem motora, associadas ao aumento dos níveis dos componentes do eixo clássico do SRA e também ao aumento da inflamação e do estresse oxidativo no estriado. Esses resultados mostram que o modelo de TCE padronizado foi capaz de gerar uma lesão compatível com um TCE leve, além de que, sugerem que processos neuroinflamatórios podem ter envolvimento nas alterações de comportamento em camundongos submetidos ao modelo experimental de TCE leve.

9 - REFERÊNCIAS

PRODUÇÃO BIBLIOGRÁFICA E OUTRAS ATIVIDADES

- Artigos publicados durante o doutorado e que se relacionam com o objeto de estudo da Tese

Artigo de Revisão

> MARZANO, L. ; CASTRO, F. L. M. ; **MACHADO, CAROLINE AMARAL** ; BARROS, J. L. V. M. ; CORDEIRO, T. M. ; Simões e Silva, Ana Cristina ; MIRANDA, Amanda. S. ; TEIXEIRA, A. L. ; Miranda, Aline S. . Potential Role of Adult Hippocampal Neurogenesis in Traumatic Brain Injury. CURRENT MEDICINAL CHEMISTRY, v. 28, p. 1-15, 2021 (Anexo 1)

Artigo Original

> DE BARROS, JOÃO LUÍS VIEIRA MONTEIRO ; CARDOSO, MAÍRA GLÓRIA ; **MACHADO, CAROLINE AMARAL** ; VIEIRA, ÉRICA LEANDRO MARCIANO ; FALEIRO, RODRIGO MOREIRA ; PEDROSO, VINÍCIUS SOUSA PIETRA ; Simões e Silva, Ana Cristina ; DE SOUZA, LEONARDO CRUZ ; TEIXEIRA, ANTÔNIO LÚCIO ; DE MIRANDA, ALINE SILVA . The potential role of renin-angiotensin system in mild traumatic brain injury. Neurological Sciences, v. 22, p. 1-10, 2022.

- Artigos publicados durante o doutorado e que não se relacionam com o objeto de estudo da Tese

VALADÃO, PRISCILA APARECIDA COSTA ; OLIVEIRA, BRUNA DA SILVA ; **MACHADO, CAROLINE AMARAL** ; FERNANDES, HELIANA DE BARROS ; MACHADO, THATIANE CRISTINA ; SOARES, KÍVIA SANTOS ; TEIXEIRA, ANTONIO LUCIO ; GUATIMOSIM, CRISTINA ; MIRANDA, ALINE SILVA DE . Investigating the Involvement of Cytokines and Neurotrophic Factors in the Advanced Stages of Huntington?s Disease: A BACHD Study. AUSTIN ALZHEIMER'S AND PARKINSON'S DISEASE, v. 6, p. 1, 2023.

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MAURO M. ; COSTA, VIVIAN V. ; Miranda, Aline S. ; GUATIMOSIM, CRISTINA . Neuromuscular defects after infection with a beta coronavirus in mice. NEUROCHEMISTRY INTERNATIONAL, v. 169, p. 105567, 2023.

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- Prêmios

2023 - ISN Travel Awards to attend ISN-ESN Meeting in Porto - Portugal, International Society for Neurochemistry.

2022 - ISN Travel Awards to attend ISN-APSN Meeting in Honolulu - USA, International Society for Neurochemistry.

2021 - NNS Trainee & Diversity Award, National Neurotrauma Society.

2021 - TOP Finalist Trainee Poster Competition, National Neurotrauma Society.

- Organização de Eventos

8º Simpósio de Integração dos Programas de Pós-Graduação em Biologia Celular da UFMG, UFV, UFU e UFSJ. 2023

VIII Curso de Férias do PPGBIOCEL - COVID-19: Do vírus a pandemia, o que sabemos? 2020.

1º Semana de Divulgação científica - Canal Fala aí Cientista. 2020.

Aula Magna da Pós-Graduação do ICB: Inteligência Artificial Impactos e desafios. 2019.

- Atividades Representativas e de administração

2019 – 2023 Membro Discente do Colegiado do Programa de Pós-Graduação em Biologia Celular da UFMG
Auxiliando na coordenação didática e administrativa do Curso e na tomada de decisões.

2021 – 2023 Estudante membro do Comitê de Gestão Orçamentária do Programa de Excelência Acadêmica – PROEX

Elaboração de propostas de utilização do recurso; monitorando a aplicação de recursos cedido pela Agência CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), indicar usos e debater sobre as melhores formas de gestão dos recursos, auxiliando o colegiado de Programa de Pós-Graduação em Biologia Celular da UFMG para tomar as melhores decisões.

2021 – 2023 Aluno Membro da Comissão de Acompanhamento Acadêmico (CAA) do Programa de Pós-Graduação em Biologia Celular na UFMG

Mediação e resolução de conflitos em casos especiais envolvendo relações estudante-docentes; acompanhamento de prazos de relatórios de atividades, dissertações e teses de estudantes de Pós-graduação.