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PRISCILA MARQUES SOSA

ESTRATÉGIAS DE NEUROPROTEÇÃO EM DIFERENTES MODELOS DE ACIDENTE VASCULAR ENCEFÁLICO: AVALIAÇÃO DO DANO NEUROMOTOR E ESTRESSE OXIDATIVO ESTRIATAL

URUGUAIANA

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Dissertação apresentada ao Programa de Pós-Graduação em Bioquímica da Universidade Federal do Pampa (UNIPAMPA, RS) como requisito parcial para a obtenção de grau de Mestre em Bioquímica.

Orientador: Prof^a. Dr^a. Pâmela Billig Mello-Carpes

Co-orientador: Dr^a. Mauren Assis de Souza

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2016

Dedico à minha família, professores, colegas e amigos pelo apoio e exemplo.

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SUMÁRIO

LISTA DE FIGURAS

Figura 01: Figura representativa da cascata de eventos químicos que formam a fisiopatologia da lesão de isquemia-reperfusão.

Figura 02: Figura representativa da cascata de eventos químicos que formam a fisiopatologia da lesão de hemorragia intracerebral.

Figura 03: Figura representativa do estriado e suas conexões aferentes e eferentes.

Figura 04: Figura representativa do complexo multienzimático NADPH-oxidase e da atividade da apocinina.

LISTA DE ABREVIATURAS

AVE: Acidente Vascular Encefálico

BHE: Barreira Hemato-encefálica

Ca^{2+}: Cálcio

EROs: Espécies reativas de oxigênio

GP: Globo pálido

H_2O_2: peróxido de hidrogênio

HIC: Hemorragia Intracerebral

iNOS: Oxido nítrico sintase induzível

IVE: Insulto Vascular Encefálico

MMP: Metaloproteinases da matriz

MPO: Mieloperoxidase

Na^+: Sódio

NFkβ: Fator nuclear kappa beta

NMDA: N-metil D-Aspartato

O_2: Oxigênio molecular

O_2^-: Radical superóxido

OMS: Organização Mundial da Saúde

PARP: Enzima reparadora do DNA

PNM: Polimorfonuclear

SNC: Sistema Nervoso Central

SNc: Substância negra pars compacta

SNr: Substância negra reticulata

TNA α: Fator de necrose tumoral-alfa

RESUMO

Dissertação de Mestrado

Programa de Pós-Graduação em Bioquímica

Universidade Federal do Pampa, RS, Brasil

ESTRATÉGIAS DE NEUROPROTEÇÃO EM DIFERENTES MODELOS DE ACIDENTE VASCULAR ENCEFÁLICO: AVALIAÇÃO DO DANO NEUROMOTOR E ESTRESSE OXIDATIVO ESTRIATAL

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Co-orientador: Dr^a. Mauren Assis de Souza

Data e local da defesa: Uruguaiana, 15 de março de 2016.

O AVE é uma das principais causas de morte e incapacidade funcional em todo mundo, sendo dividido em dois subtipos: isquêmico, causado pela diminuição do fluxo sanguíneo; e hemorrágico, caracterizado pelo extravasamento de sangue nos tecidos encefálicos. Considerando a alta taxa de mortalidade e a gravidade das sequelas pós AVE, torna-se de extrema importância a busca por alvos terapêuticos que visem diminuir as sequelas causadas pelos quadros isquêmico e hemorrágico. Sendo assim, este estudo investigou os efeitos neuroprotetores do exercício físico (8 semanas previamente à lesão) em um quadro de AVE isquêmico (através da oclusão bilateral das artérias carótidas comuns) e os efeitos neuroprotetores da apocinina (posteriormente à lesão – 2, 6 e 24h – na dose 0,5mg/kg) em um quadro de AVE hemorrágico (através da infusão de colagenase no corpo estriado) em ratos Wistar. Para avaliar a função motora dos animais, foram utilizados os testes de Campo Aberto (CA), Rotarod (RR) e Escala de Déficit Neurológico (NDS), e, para avaliar o balanço redox estriatal, avaliamos a presença de EROs, TBARS (espécies reativas ao ácido tiobarbitúrico) e capacidade antioxidante total (FRAP). Nossos resultados mostraram que o exercício físico é uma estratégia parcialmente eficaz de proteção em um modelo de AVE isquêmico. No entanto, a apocinina não se mostrou uma estratégia de neuroproteção eficaz em um modelo de AVE hemorrágico. Estes resultados revelam a possibilidade da utilização do exercício físico como estratégia de neuroproteção. A apocinina, por sua vez, precisa ser melhor estudada em casos de AVE hemorrágico, considerando a investigação do seu mecanismo, doses e tempos de administração. .

10

ABSTRACT

Master Thesis

Graduate Program in Biochemistry

Federal University of Pampa

NEUROPROTECTION STRATEGIES IN DIFFERENT MODELS OF STROKE:
EVALUATION OF NEUROMOTOR DAMAGE AND STRIATAL OXIDATIVE STRESS

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Co-advisor: Mauren Assis de Souza, PhD

Place and date: Uruguaiana, March 15th, 2016.

The stroke is one of the leading causes of death and disability worldwide, and is divided into two subtypes: ischemic, caused by a decreased on blood flow; and hemorrhagic, characterized by leakage of blood in brain tissue. Considering the high mortality rate and severity of post stroke sequelae, it is extremely important to search for therapeutic targets aimed at reducing the consequences caused by ischemic and hemorrhagic frames. Thus, this study investigated the neuroprotective effects of physical exercise (8 weeks prior to injury) in an ischemic stroke modle (by bilateral occlusion of the common carotid arteries) and the neuroprotective effects of apocynin (after the injury - 2, 6 and 24 hours - at a dose 0.5 mg/kg) in a hemorrhagic stroke model (by collagenase infusion into the striatum) in Wistar rats. Open Field (OF), Rotarod (RR) and Neurologic Disabilities Scale (NDS) were used to evaluate the motor function of the animals. To the striatal redox balance evaluation we assessed the presence of ROS, TBARS (reactive species to thiobarbituric acid) and total antioxidant capacity (FRAP). Our results showed that physical exercise is a partially effective strategy to protect against ischemic stroke. However, apocynin was not an effective neuroprotective strategy in a experimental model of hemorrhagic stroke. These results show the possibility of using exercise as a neuroprotective strategy. The apocynin need to be better studied in cases of hemorrhagic stroke, whereas the investigation of its mechanism, dosages and times of administration.

Key-words: Stroke, ischemia-reperfusion injury, intracerebral hemorrhage, neuroprotection, exercise, apocynin.

APRESENTAÇÃO

Esta dissertação está dividida em três partes. A parte I é composta pela introdução, que apresenta uma breve revisão da literatura sobre a problemática do trabalho, bem como sua importância, culminando na apresentação dos objetivos desta dissertação. Na parte II são apresentados os materiais e métodos, resultados e discussão, no formato de dois artigos originais, o primeiro já publicado no periódico *Brazilian Journal of Medical and Biological Research,* e o segundo a ser submetido para apreciação no periódico *Neurochemistry International*. A parte III é composta pelos itens discussão, conclusões e perspectivas, que englobam os comentários e as interpretações relacionadas aos resultados dos dois artigos. As referências bibliográficas contemplam apenas as citações pertencentes aos itens introdução, discussão e conclusão, uma vez que as referências do artigo científico estão inseridas na parte II.

PARTE I

1. INTRODUÇÃO

1.1 Acidente vascular encefálico

O Acidente Vascular Encefálico (AVE), também conhecido como Insulto Vascular Encefálico (IVE), é a condição na qual ocorre diminuição ou perda da função de alguma região encefálica devido à interrupção do fluxo sanguíneo para o cérebro (Zuhaid et al., 2014). Esta interrupção do fluxo sanguíneo pode ser decorrente de uma isquemia ou hemorragia encefálica sendo assim, o AVE pode ser classificado como isquêmico ou hemorrágico (Liang et al., 2015).

Segundo a Organização Mundial da Saúde (OMS), o AVE é a segunda maior causa de morte em todo o mundo, sendo responsável por 11% do número total de mortes no ano (Farkouh et al., 2012). Nos pacientes que sobrevivem ao AVE, a área do cérebro afetada perde sua funcionalidade, parcial ou totalmente, podendo resultar em incapacidade funcional de um ou mais membros de um lado do corpo, falhas na compreensão ou pronúncia da fala, déficits visuais, entre outros (Extramiana e Maison-Blanche, 2015). Estima-se que aproximadamente 60% dos indivíduos que sofreram um AVE apresentam dificuldades motoras que permanecem após 6 meses ao evento, interferindo nas suas Atividades de Vida Diária (AVDs) e na prática de suas atividades profissionais (Chen et al., 2014). Além disso, gastos hospitalares e a grande necessidade de cuidados institucionais de longo prazo acarretam em grande custo para a sociedade (Go et al., 2014).

O tratamento clínico após um AVE segue sendo um desafio para os profissionais da saúde, pois as lesões causadas podem acarretar danos neurológicos permanentes (Allen e Bayraktutan, 2009). Considerando as limitações na reabilitação, bem como os custos envolvidos neste processo, observa-se uma grande necessidade de desenvolver e implementar estratégias de prevenção e reabilitação que sejam fundamentadas e específicas para cada tipo de lesão cerebral (Moretti et al., 2015). Os estudos que investigam a fisiopatologia do AVE tem apresentado grande evolução nos últimos anos, especialmente em virtude do desenvolvimento de modelos experimentais, os quais possibilitam um maior entendimento dos fenômenos neurobiológicos envolvidos (Schimidt et al., 2014). Sabe-se que existem grandes diferenças fisiopatológicas entre insultos isquêmicos e hemorrágicos (Mestriner et al., 2013), o que tem motivado estudos cada vez mais específicos para permitir o melhor entendimento de cada um dos subtipos de AVE (Suto et al., 2011).

1.2 Tipos de acidente vascular encefálico

O AVE é caracterizado pela rápida disfunção neurológica decorrente da obstrução ou do rompimento de vasos sanguíneos encefálicos (Liang et al., 2015). No AVE isquêmico (isquemia cerebral), o tipo mais comum de AVE, que a corresponde a 85-90% de todos os casos (Xavier et al., 2012), a redução do fluxo sanguíneo está relacionada à obstrução do fluxo sanguíneo pela oclusão de uma artéria que supre determinada região encefálica (Yu et al., 2013), causando danos metabólicos e funcionais a esta região (Mestriner et al., 2013). A isquemia encefálica é causada por inúmeros fatores, tais como a idade avançada, hipertensão arterial, interrupção transitória do fluxo sanguíneo de uma artéria e parada cardíaca (Donnan et al., 2014). Geralmente resulta em morte neuronal, afetando regiões do cérebro que são mais vulneráveis a baixos níveis de oxigênio e glicose, incluindo a região CA1 do hipocampo e corpo estriado (Ohk et al., 2012). Dentre os mecanismos característicos da morte celular por isquemia, a necrose e a apoptose parecem atuar de modo importante (Heo et al., 2013; Zhang et al., 2014; Chen et al., 2014).

Durante um evento isquêmico há uma série de mecanismos patogênicos envolvidos na cascata isquêmica que podem contribuir para estes desfechos. Tais mecanismos incluem: a falha energética (falha no fornecimento de glicose) (Abramov et al., 2007), a elevação dos níveis intracelulares de Ca^{++} (Li et al., 2014), a excitotoxicidade (Allen e Bayraktutan, 2009; Angel et al., 2016; Taniguchi et al., 2000), a geração de radicais livres (Allen e Bayraktutan, 2009; Sosa et al., 2015), a disfunção da barreira hemato-encefálica, e a inflamação. Além disso, a reperfusão sanguínea que ocorre após isquemia focal encefálica exacerba o edema na região encefálica afetada (Allen e Bayraktutan, 2009). O edema isquêmico é possivelmente iniciado por influxo de Na^+ associado à falha de energia (Lai et al., 2014). As condições de osmolaridade mais elevadas induzem o influxo de água para as células, resultando em edema iônico (Liang et al., 2007). Esta fase do edema pode durar várias horas antes do vazamento de grandes volumes de água no cérebro, o que reduz ainda mais o fluxo de sangue para os neurônios, causando morte neuronal irreversível (Spatz, 2010). A figura 1 resume os principais eventos neuroquímicos envolvidos no AVE isquêmico.

Figura 1: Figura representativa da cascata de eventos químicos que formam a fisiopatologia da lesão de isquemia-reperfusão. (Ca2+: Cálcio; Na+: Sódio; PARP: enzima reparadora do DNA; iNOS: Oxido nítrico sintase induzível; EROs: Espécies reativas de oxigênio; Ác: Ácido). Adaptado de (Smith, 2004).

O AVE hemorrágico, por sua vez, é causado por sangramento de um ou mais vasos sanguíneos do encéfalo que atinge diretamente o parênquima cerebral ou o espaço subaracnóide (Liang et al., 2015). Está associado à alta morbidade, mortalidade, invalidez e taxa de recorrência (Xi e Keep, 2012; Zhou et al., 2014) e corresponde a aproximadamente 15% dos casos de AVE (Balami et al., 2011).

O extravasamento sanguíneo gera, além do edema, apoptose e/ou necrose e inflamação (Xiong et al., 2015). Além disso, o hematoma pode levar a danos morfológicos, causados pela deformação mecânica nos tecidos, o que leva ao afastamento de células gliais (Titova et al, 2007), despolarização da membrana, liberação de neurotransmissores e disfunção mitocondrial (Qin et al., 2015). De modo adicional, uma cascata secundária é iniciada pelos produtos da coagulação e do metabolismo da hemoglobina, em particular da trombina, que é capaz de ativar células microgliais cerca de 4 horas após o início do evento hemorrágico. Por sua vez, a microglia ativada libera mediadores que levam à disfunção da barreira hematoencefálica, ao surgimento de edema vasogênico, e à apoptose neural e glial (Qin et al., 2015).

Além destes fatores, uma série de outros mecanismos contribuem para o desenvolvimento de danos teciduais após a hemorragia intracerebral, o que inclui a ativação do sistema complemento (componente da resposta imunológica particularmente importante no

edema hemorrágico) (Titova et al., 2007), a ativação de metaloproteinases de matriz (uma família de endopeptidases que degradam a matriz extracelular) (Xi e Keep, 2012), e a produção de espécies reativas de oxigênio (proveniente da hemoglobinólise, capazes de aumentar os efeitos danosos do estresse oxidativo) (Xi e Keep, 2012; Ziai, 2013; Xiong et al., 2015). A figura 2 resume os principais eventos neuroquímicos envolvidos no AVE hemorrágico.
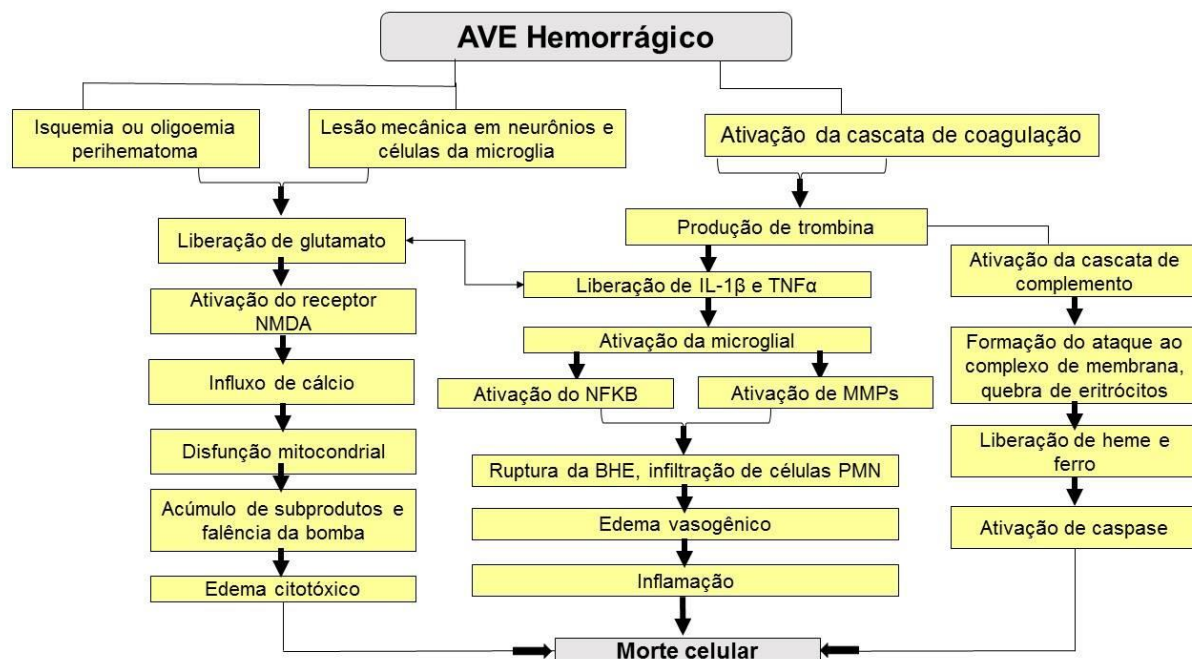

Figura 2: Figura representativa da cascata de eventos químicos que formam a fisiopatologia da lesão de hemorragia intracerebral. (NMDA: N-metil D-Aspartato; TNA α: fator de necrose tumoral-alfa; IL-1 β: Interleucina 1 beta; NFk β: Fator nuclear kappa beta; MMP: metaloproteinases da matriz; BHE: Barreira hemato envefálica; PNM: polimorfonuclear). Adaptado de (Brunswick et al., 2012)

1.3 O papel do estriado

O estriado ou corpo estriado é uma das estruturas cerebrais mais afetadas por doenças neurodegenerativas como Alzheimer, Parkinson e AVE (Li et al., 2014; Lin et al., 2015).

O corpo estriado é o módulo de entrada para os núcleos da base, fazendo ligação com o córtex sensório-motor e o cerebelo, um circuito neuronal necessário para o controle motor voluntário (Baez-Mendoza e Schultz, 2013). O corpo estriado é o núcleo que recebe as aferências provenientes de diferentes regiões corticais. Partindo do estriado, axônios são projetados ao globo pálido, de onde se direcionam ao tálamo, que atua processando

informações e enviando-as ao corpo estriado, globo pálido e núcleo subtalâmico (Baez-Mendoza e Schultz, 2013). As informações retornam ao córtex frontal, possibilitando o controle dos movimentos e também de outras funções não motoras (Pawlowsky et al., 2013), como ilustrado na figura 3.
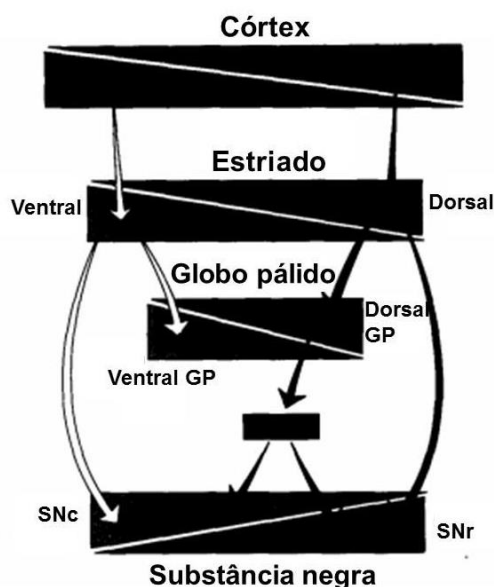

Figura 3: Figura representativa do estriado e suas conecções aferentes e eferentes. (SNc: substância negra pars compacta; SNr: substância negra reticulata; GP: Globo pálido). Adaptado de (Gerfen, 1992).

Assim, os neurônios do estriado apresentam atividade relacionada com a preparação/planejamento, iniciação e execução de movimentos voluntários (Baez-Mendoza e Schultz, 2013). Diversos estudos sugerem a participação de forma efetiva do estriado na execução das tarefas motoras (Sosa et al., 2015), na orientação espacial e percepção sensório-motora (Zuhaid et al., 2014; Baez-Mendoza e Schultz, 2013) e também na "escolha e filtragem" dos movimentos que compõe a atividade motora (Pawlowsky et al., 2013).

Considerando que o acesso destas estruturas cerebrais no homem é bastante delicado, para melhor descrever e estudar a sua fisiopatologia e relacioná-la com o quadro clínico dos diferentes tipos de AVE faz-se necessário a utilização de modelos animais. Os modelos animais permitem a análise do tecido cerebral com intuito de elucidar as alterações causadas em relação à presença de moléculas agressoras e protetoras que podem estar presente após um quadro de AVE, bem como algumas alterações morfológicas cerebrais, que só podem ser vistas a partir da dissecação do tecido (Wakisaka et al., 2008).

Os modelos de pesquisa animal que melhor mimetizam a HIC utilizam a injeção autóloga de sangue nas estruturas cerebrais ou a infusão de colagenase (substância que

degrada a lâmina basal dos vasos, causando sangramento) (Kirkman et al., 2011). Para pesquisas envolvendo a isquemia cerebral, os modelos mais utilizados são a oclusão da Artéria Cerebral Média (ACM) (Titova et al., 2011), ou das Artérias Carótidas Comuns (ACC) (Schimidt et al., 2014; Sosa et al., 2015; Xiong et al., 2015), e as duas técnicas podem ser feitas de modo a proporcionar ou não a reperfusão sanguínea, de modo que a oclusão destas artérias resulta na redução ou interrupção do fluxo sanguíneo nas regiões do córtex e estriado, sendo que a severidade do evento isquêmico depende da duração do insulto (Durukan e Tatlisumak, 2007).

1.3 Disfunções motoras associadas ao estresse oxidativo

O estresse oxidativo está presente na patogênese de diversas doenças que envolvem a neurodegeneração, incluindo a isquemia (Cechetti et al., 2012) e a hemorragia cerebral (Kim et al., 2013). Este refere-se a um distúrbio celular causado pelo desequilíbrio entre a produção de agentes pró-oxidantes e antioxidantes, levando a reações deletérias em diversas organelas celulares (Allen e Bayraktutan, 2009). Essas reações vão desde a lipoperoxidação nas membranas celulares até a inativação de enzimas essenciais (Cechetti et al., 2012), incluindo danos ao DNA e RNA (Alen et al., 2009).

O encéfalo apresenta maior suscetibilidade ao estresse oxidativo do que outros tecidos, isso porque apresenta grande concentração de ácidos graxos poliinsaturados, uma alta atividade metabólica, baixa atividade antioxidante e pouca capacidade de reparo de células neuronais (Javed et al., 2011). Durante um evento isquêmico ocorre um aumento do influxo de Ca^{++} e falência no fornecimento de energia; estes dois fatores promovem a liberação de ácidos graxos livres que apresentam diversos efeitos danosos às células encefálicas, incluindo a lipoperoxidação (Chen et al., 2014). O aumento da concentração de Ca^{++} no interior das células nervosas também é responsável pelo aumento da síntese de NO (óxido nítrico, do inglês *nitric oxide),* que atua dilatando os vasos, proporcionando maior oferta de oxigênio e glicose. Em contrapartida o NO também é capaz de reagir com o radical superóxido, o que causa dano nas proteínas, lipídeos de membrana e DNA (Sarkaki et al., 2013).

O estresse oxidativo também apresenta papel importante nos danos causados pelo AVE hemorrágico (Aronowski e Zhao, 2011). Evidências experimentais relacionam a toxicidade da hemoglobina e o estresse oxidativo com a lesão cerebral secundária (Allen e Bayraktutan, 2009), um dos fatores responsáveis pela deterioração neurológica após a HIC (Chen et al., 2014). A forma oxidada do grupamento heme (hemina) acumula-se nos

hematomas causados pelo sangramento, levando a um quadro de citotoxicidade (Chen-Roetling et al., 2014).

Considerando que em ambos os tipos de AVE o estresse oxidativo parece ter um papel importante na fisiopatologia, ele pode também representar um possível alvo terapêutico, tendo em vista a grande contribuição do desbalanço redox nas alterações morfofuncionais do tecido cerebral após um AVE (Liang et al., 2016). Sendo assim, a investigação dos efeitos de estratégias relacionadas à diminuição ou prevenção da formação de espécies reativas nos diferentes tipos de AVE é de grande importância.

1.4 Estratégias de neuroproteção

Sendo o AVE uma das principais causas de morte em todo o mundo (Park et al., 2013), é importante que novas pesquisas que visem a busca por estratégias de neuroproteção eficazes e condizentes com a especificidade de cada tipo de AVE sejam estimuladas.

Neuroproteção refere-se a qualquer estratégia que tem como alvo diretamente o parênquima cerebral, buscando antagonizar os eventos, a nível molecular e celular, responsáveis pelo dano, permitindo que as células do cérebro sobrevivam às condições críticas impostas pela injúria (Moretti et al., 2015). Devido à complexidade dos danos causados pelo AVE inúmeros alvos moleculares têm sido analisados com intuito de atenua-los. Dentre estes alvos podemos citar: a excitotoxicidade, o influxo de cálcio, as espécies reativas de oxigênio, a produção de o óxido nítrico, as reações inflamatórias e a apoptose (Minnerup et al., 2012; Sutherland et al., 2012). Ao longo das últimas décadas, produziram-se estudos que buscaram elucidar os potenciais efeitos de agentes neuroprotetores em diversos modelos experimentais de AVE *in vivo* e *in vitro*. Estes estudos têm colaborado para elucidar a viabilidade de drogas experimentalmente eficazes e outras estratégias como o exercício físico e a hipotermia (Moretti et al., 2015).

Embora a prevenção dos AVEs deva ser discutida ativamente, principalmente no que diz respeito ao combate dos fatores de risco, os enormes custos socioeconômicos de tratamento agudo e a longo prazo causados pelo AVE trazem a necessidade de avanço no que diz respeito à neuroproteção (O'donnell et al., 2010). A complexidade das vias da cascata isquêmica e da hemorrágica sugere a busca por estratégias específicas, baseadas na fisiopatologia de cada doeça.

1.4.1 Exercício físico como estratégia de neuroproteção

Diversas evidências mostram que exercício físico, independente da modalidade, traz inúmeros benefícios para a saúde do sistema nervoso de humanos (Notarius et al., 2015), e animais (Schimidt et al., 2014; Sosa et al., 2015; Cechetti et al., 2012). Estudos comprovam melhoras no aprendizado, na memória e na plasticidade do sistema nervoso como resposta ao exercício (Flores et al., 2014); , sendo este capaz de aumentar a angiogênese cerebral (Allen et al., 2009) e atenuar o declínio mental decorrente do envelhecimento (Flores et al., 2014).

Também foram observados efeitos protetores do exercício no sistema nervoso, aumentando a resistência a lesões e facilitando a recuperação funcional pós-lesão (Cechetti et al., 2012). O exercício protege o sistema nervoso minimizando a lesão por falta de glicose e oxigênio (Cechetti et al., 2012; Austin et al., 2014), além de atenuar respostas neurais ao estresse, possivelmente contribuindo para a redução de patologias clínicas como hipertensão, insuficiência cardíaca, estresse oxidativo e imunodepressão (Arrick et al., 2014). A falta do exercício físico, por sua vez, está relacionada a transtornos de humor, imunossupressão, piora do perfil lipídico, glicêmico e da qualidade do sono (Wilmore, 2001).

A adaptação que o exercício físico causa no sistema nervoso tem implicações na prevenção e tratamento da obesidade, câncer, depressão (Dishman et al., 2006), declínio cognitivo associado ao envelhecimento (Flores et al., 2014) e distúrbios neurológicos como doença de Parkinson, doença de Alzheimer (Li et al., 2015), acidente vascular isquêmico (Sosa et al., 2015; Schimidt et al., 2014) e lesões medulares ou encefálicas (Dishman et al., 2006). Embora tenhamos certa clareza sobre os benefícios do exercício físico à saúde cerebral como um todo, os mecanismos envolvidos na neuroproteção induzida pelo exercício parecem ser múltiplos e complexos, e não estão ainda completamente elucidados. Além disso, aspectos como intensidade, modalidade e duração parecem ser determinantes importantes do efeito observado. Assim, para que seja possível associar o exercício físico à neuroproteção em diferentes tipos de doenças, em especial as cerebrovasculares, estudos adicionais ainda são necessários.

1.4.2 Apocinina como estratégia de neuroproteção

Extratos vegetais e fitoquímicos purificados representam outra alternativa frequentemente testada para possíveis atividades farmacológicas que envolvem reações bioquímicas, tais como ensaios de oxidação (Wong et al., 2015).

A apocinina (acetovanilona) é um fitoquímico isolado a partir da planta medicinal *Picrorhiza kurroa*, (Engels et al., 1992) e tem sido utilizada como parte da medicina tradicional na Ásia (Wong et al., 2015). Há uma gama de características terapêuticas relacionadas à apocinina, por exemplo, capacidade antioxidante, anti-inflamatória, e a proteção da integridade vascular encefálica (Schreurs et al., 2014; Heumüller et al., 2008). Várias destas ações são atribuídas à capacidade de inibição da atividade da NADPH-oxidase (Wong et al., 2015). A NADPH oxidase (NOX) é um complexo multienzimático ligado à membrana celular, que, quando ativado é capaz de produzir ânion superóxido, a principal fonte de EROs, dedicando-se exclusivamente à sua produção. As isoformas NOX atuam na formação de radical superóxido, que é subsequentemente convertido em H_2O_2 (Moretti et al., 2015). Um estudo prévio identificou que os leucócitos são as células capazes de mediar o efeito inibitório da apocinina (Heumuller et al., 2008). Sugere-se que a mieloperoxidase (MPO), que é seletivamente expressa em leucócitos, é necessária para a ocorrência do efeito inibitório desta substância (Wong et al., 2015) (Figura 4).
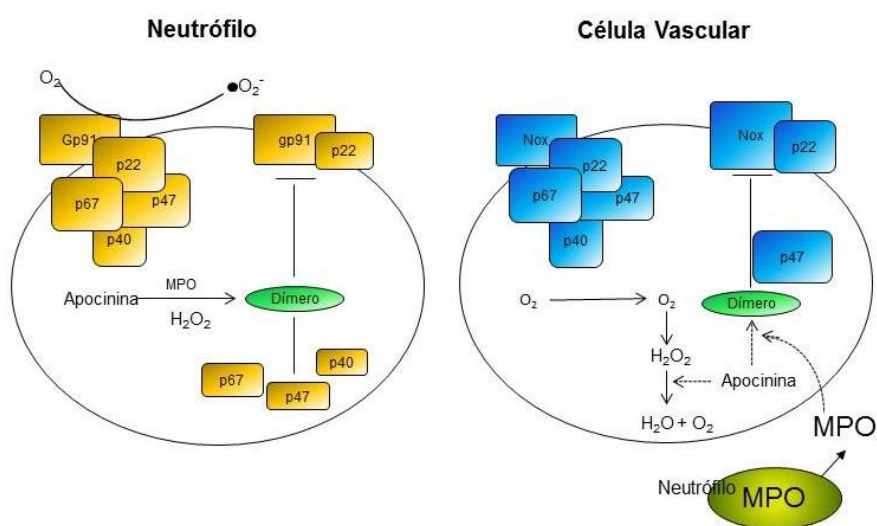

Figura 4: Figura representativa do complexo multienzimático NADPH-oxidase e da atividade da apocinina. (O_2: oxigênio molecular; O_2^-: radical superóxido; H_2O_2: peróxido de hidrogênio; MPO: mieloperoxidase). Adaptado de Touyz, 2008.

Em células desprovidas de MPO, a formação de dímeros de apocinina não ocorre mesmo após superexpressão das isoformas NOX. Quando MPO humana é adicionada, os dímeros são identificados nas células com superexpressão de isoformas Nox ou nas células coincubadas com H_2O_2 (Touyz, 2008). Entretanto, outras peroxidases que não a MPO podem influenciar a atividade da apocinina, sendo possível que células vasculares possuam enzimas peroxidativas capazes de ativar este inibidor. Tal fato é reforçado por achados em células endoteliais, nas quais dímeros de apocinina foram identificados, com a apocinina inibindo de forma concentração-dependente a atividade da NADPH oxidase, a formação de espécies reativas de oxigênio e nitrogênio e a proliferação celular (Wong et al., 2015).

Estudos prévios relatam efeitos protetores da apocinina após AVE isquêmico (Tang et al., 2011) e traumatismo crânio-encefálico (TCE) (Ferreira et al., 2013). Ainda, a apocinina está relacionada com a preservação da permeabilidade da barreira hematoencefálica (Tang et al., 2008), diminuição do volume de lesão cerebral (Zhang et al., 2015), e atenuação dos danos neuromotores causados pelo TCE (Ferreira et al., 2013) e isquemia cerebral (Zhang et al., 2015), efeitos que poderiam ser benéficos para a recuperação pós-AVE hemorrágico.

Embora os efeitos da apocinina estejam bem descritos na literatura, faltam informações sobre os seus efeitos especificamente em quadros de AVE hemorrágico, além de evidências concisas em relação às melhores vias de administração e doses para este fármaco. Sendo assim, novos estudos são necessários.

2. OBJETIVOS

2.1 Objetivo Geral

Investigar possíveis estratégias de neuroproteção em diferentes modelos de acidente vascular encefálico (isquêmico e hemorrágico), considerando a manutenção das funções motoras e do equilíbrio oxidativo estriatal.

2.2 Objetivos específicos

- Verificar os efeitos da isquemia-reperfusão cerebral sobre a função motora de ratos Wistar;
- Verificar os efeitos da isquemia-reperfusão cerebral sobre o equilíbrio oxidativo em tecido neural envolvido na função motora (estriado) de ratos Wistar;
- Verificar os efeitos neuroprotetores do exercício físico sobre a função motora e equilíbrio oxidativo estriatal em um modelo de isquemia-reperfusão cerebral em ratos Wistar;
- Verificar os efeitos da hemorragia intracerebral sobre a função motora de ratos Wistar;
- Verificar os efeitos da hemorragia intracerebral cerebral sobre o equilíbrio oxidativo em tecido neural envolvido na função motora (estriado) de ratos Wistar;
- Verificar os efeitos neuroprotetores do tratamento com apocinina sobre a função motora e equilíbrio oxidativo estriatal em um modelo de hemorragia intracerebral em ratos Wistar.

PARTE II

Os resultados desta dissertação estão organizados em dois artigos científicos. O primeiro artigo, intitulado "*Physical exercise prevents motor disorders and striatal oxidative imbalance after cerebral ischemia-reperfusion*", publicado na revista *Brazilian Journal of Medical and Biological Research,* investiga a neuroproteção induzida pelo exercício físico em um modelo de AVE isquêmico (isquemia-reperfusão cerebral).

O segundo artigo que compõe esta dissertação, intitulado "*Apocynin administration do not protect against oxidative damage and neuromotor deficit induced by hemorrhagic stroke in rats*" a ser submetido à revista *Neurochemistry International*, investiga a proposta da administração da apocinina em uma curta janela temporal após AVE hemorrágico (hemorragia intracerebral) com a finalidade de promover neuroproteção. Ambos os artigos avaliam os danos motores através de testes motores comportamentais, bem como o equilíbrio oxidativo estriatal.

Artigo 1

Physical exercise prevents motor disorders and striatal oxidative imbalance after cerebral ischemia-reperfusion

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Abstract

Stroke is the third most common cause of death worldwide, and most stroke survivors present some functional impairment. We assessed the striatal oxidative balance and motor alterations resulting from stroke in a rat model to investigate the neuroprotective role of physical exercise. Forty male Wistar rats were assigned to 4 groups: a) control, b) ischemia, c) physical exercise, and d) physical exercise and ischemia. Physical exercise was conducted using a treadmill for 8 weeks. Ischemia-reperfusion surgery involved transient bilateral occlusion of the common carotid arteries for 30 min. Neuromotor performance (open-field and rotarod performance tests) and pain sensitivity were evaluated beginning at 24 h after the surgery. Rats were euthanized and the corpora striata was removed for assay of reactive oxygen species, lipoperoxidation activity, and antioxidant markers. Ischemia-reperfusion caused changes in motor activity. The ischemia-induced alterations observed in the open-field test were fully reversed, and those observed in the rotarod test were partially reversed, by physical exercise. Pain sensitivity was similar among all groups. Levels of reactive oxygen species and lipoperoxidation increased after ischemia; physical exercise decreased reactive oxygen species levels. None of the treatments altered the levels of antioxidant markers. In summary, ischemia-reperfusion resulted in motor impairment and altered striatal oxidative balance in this animal model, but those changes were moderated by physical exercise.

Key words: Stroke; Striatum; Locomotion; Oxidative stress; Antioxidants; Running

Introduction

Stroke is the third most common cause of death worldwide (1). Up to 20% of stroke survivors require long-term institutional care, and 15–30% are unable to perform daily life or work activities (2). Stroke events result from suppression of blood flow to the brain, which decreases oxygen and glucose delivery to brain tissue (3). This deprivation may result from disruption of a blood vessel, leading to hemorrhagic stroke, or from interruption of blood flow, leading to ischemic stroke (4). Most stroke events (85–90%) are ischemic in origin (5). In an ischemic event, blood reperfusion leads to tissue damage (6). Such damage has deleterious effects on important cellular structures including the basal membrane and mitochondria (7).

Evidence suggests that reperfusion injury results from oxidative stress (8) characterized by increased levels of reactive oxygen species (ROS) that induce neuronal damage due to lipid peroxidation (6). Under conditions of oxidative stress, cells are unable to balance the deleterious effects of ROS through antioxidant mechanisms (8). Some brain regions, including the striatum, appear to be particularly susceptible to oxidative damage due to ROS levels (9). The striatum plays an important role in the control of voluntary movements (10) and contains a high concentration of dopaminergic receptors, which are responsible for motor activation (11). Further, dopaminergic receptors are highly susceptible to ischemic damage (12). Therefore, in models of transient ischemia-reperfusion, rats can present motor impairments that may be explained by striatal damage resulting from oxidative stress and by neuronal death (13).

The high incidence of stroke, the disabilities observed among survivors (14), and the costs of currently available treatments have promoted efforts to improve post-stroke recovery and to prevent insults to the central nervous system. One interesting strategy is physical exercise, which is easy to offer to patients and does not involve high costs. It might thus become an important public health strategy. Previous reports suggest that physical exercise may be an effective neuroprotective strategy. Aerobic exercise ameliorates memory impairment after cerebral ischemia (12,15,16), reduces cognitive deficits related to aging (17), delays neurodegeneration in Alzheimer's disease models (18), and facilitates functional recovery after stroke (5). The mechanisms involved in these effects include the increase of antioxidant defenses in the hippocampus, promotion of neuronal resistance to oxidative stress (13), upregulation of BDNF (brain-derived neurotrophic factor) and VEGF (vascular endothelial growth factor) (19), and the prevention of neuronal death (1). In addition, acute exercise improves motor memory and skill acquisition (20).

Considering the results of previous studies, we assessed the neuroprotective role of physical exercise on the oxidative imbalance and motor impairments resulting from ischemia-reperfusion. Invasive experimental protocols cannot be conducted in humans, which makes animal experimentation important in advancing the understanding of behavioral and biochemical parameters associated with oxidative stress and allows dissection of brain structures. Thus, we used a rat ischemia-reperfusion model in the experiments described below.

Material and Methods

### Animals and experimental groups

Forty male Wistar rats were purchased from the Central Vivarium of the Universidade Federal de Santa Maria (RS, Brazil) and housed 3 per cage under controlled light and environmental conditions (12-h light/dark cycle at $23 \pm 2°C$ and $50 \pm 10\%$ humidity) with food and water ad libitum. All experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH, 1996) and the Animal Care and Use Committee (IRB #0132012) of the Universidade Federal do Pampa. The weight of each rat and the liquid ingested in each cage were measured daily. At the age of 2 months, rats were randomly assigned to one of four experimental groups. A control group (SHAM) was subjected to sham surgery without occlusion of the common carotid arteries. An ischemia-reperfusion group (ISCH) was subjected to surgery to produce temporary bilateral occlusion of the common carotid arteries. An exercise group (EXERC) performed physical exercise before sham surgery. An exercise and ischemia-reperfusion group (EXERC-ISCH) performed physical exercise before ischemia-reperfusion surgery.

Rats were subjected to motor function testing beginning at 24 h after surgery, and 8 days after surgery. Rats were euthanized to collect brain tissue for evaluation. Figure 1 illustrates the experimental design of the study.

Physical exercise protocol

The physical exercise routine consisted of an 8-week protocol of running on a motorized treadmill built for rodents (Insight Ltda., Brazil). Running was performed at an intensity of 60–70% maximal oxygen uptake (VO_2), i.e., a treadmill belt velocity of 9–13 m/min, for 30 min. Sessions were conducted 5 days each week at approximately the same time of day during the light time period (21). In the week before the experimental intervention, rats performed daily treadmill running for 10 min to habituate before performing the first VO_2 test. An indirect VO_2 running test was performed to determine the individual exercise intensity beginning at a low velocity and increasing by 5 m/min every 3 min until the rat was unable to run. Time to fatigue (min) and the work volume (m/min) were considered as indirect measures of maximum VO_2 uptake (16,21). During the fourth week of exercise, an additional indirect VO_2 running test was conducted to adjust the exercise intensity for each rat.
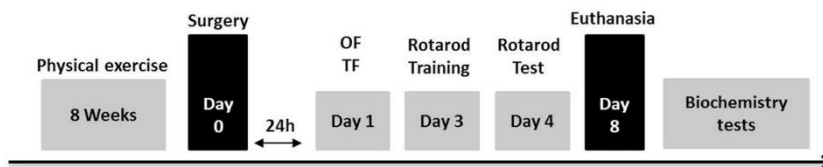
Figure 1. Rats in the exercise (EXERC) and exercise and ischemia-reperfusion groups (EXERC-ISCH) were subjected to 8 weeks of physical exercise on a treadmill for rodents. On day 0, rats from all groups underwent surgery with or without occlusion of the carotid arteries. Twenty-four hours after surgery rats were given open-field (OF) and tail-flick tests (TF). On the third day, rats were trained in the rotarod test and on the fourth day they were given the rotarod test. To avoid changes in brain markers resulting from stress due to the rotarod test, rats were euthanized and brain tissue was collected a few days later, on the eighth day post-surgery. Bilateral striatum tissue was removed and used in subsequent biochemical assays.

Ischemia-reperfusion surgery

After 8 weeks of intervention, rats were subjected to the ischemia-reperfusion or sham surgery procedures. The surgery was performed in the morning, under ketamine and xylazine anesthesia, 75 and 10 mg/kg, respectively, given intraperitoneally. Rats were placed on a heating pad, the neck was shaved, and a midline incision was performed. The muscles and trachea were separated; the common carotid arteries were freed from the adventitial sheath and the vagus nerve, and carefully separated prior to occlusion (22). The temporary occlusion of the common carotid arteries lasted 30 min and was performed using a vascular clip. When restoration of blood flow in the carotid arteries was confirmed by careful observation by an experienced researcher, the neck skin incision was then closed and sutured. Body temperature was maintained during surgery, and until the rat awoke, using a heating pad. After awakening, rats were returned to their cages. Sham-operated rats underwent the same surgical procedure without application of the vascular clip.

Neuromotor tasks

Open-field test. To analyze exploratory behavior, each rat was placed in the left quadrant of a $50 \times 50 \times 39$ cm open-field arena consisting of a wooden panel painted white and a front wall of transparent glass. Black lines were drawn on the floor to divide it into 12 equal quadrants. The number of crossings and rearings, as measures for locomotor and exploratory activity, respectively, were monitored for 5 min (23).

Rotarod test. Rats were first trained to walk on the rotarod (Insight), which was $5 \times 8 \times 20$ cm in diameter, width and height, respectively, at a constant rotational speed of 16 rpm for 1 min. During training, rats were placed back on the rod each time they fell off until the session was completed. At 24 h after training, rats were tested on the rotarod at a constant speed of 20 rpm. Each test consisted of 5 trials lasting 60 s each. The time at which the rat fell off the rotarod and the number of falls were recorded. Rats that fell more than five times were excluded from the experiment and were returned to their cages (24).

Nociception evaluation. Nociception was measured using the tail-flick test (25), in which pain was induced by applying an infrared light to the rat's tail 5 cm from the tip. Reaction time (tail-flick latency) was measured as the interval between placing the tail on the infrared light source and its voluntary withdrawal (25).

Striatum oxidative status assessment. For tissue preparation, rats were euthanized 24 h after the behavioral experiments were completed. The brain was removed, and the striatum was quickly dissected and homogenized in 50 mM Tris HCl, pH 7.4 (1/10, w/v). The tissue samples were centrifuged at 2400 g for 20 min, and supernatants (S1) were used for subsequent assays.

ROS. ROS content was assayed spectrofluorimetrically (Shimadzu model RF-5301PC, Japan) using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as a probe. S1 samples were incubated in the dark with 5 μL DCFH-DA (1 mM) and intracellular ROS were detected by the oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF). DCF fluorescence intensity was recorded at 520 nm (480 nm excitation) 30 min after the addition of DCFH-DA to the medium. Results are reported as AU (arbitrary units).

Lipoperoxidation assay. Lipoperoxidation activity was assayed by the formation of thiobarbituric acid reactive substance (TBARS) (26). One aliquot of S1 was incubated with a 0.8% thiobarbituric acid solution in acetic acid buffer (pH 3.2) and 8% sodium dodecyl sulfate at 95°C for 2 h, and the color reaction was measured at 532 nm. Results are reported as nmol of malondialdehyde (MDA) per mg protein.

Antioxidant markers. Catalase (CAT) activity was determined spectrophotometrically at 240 nm (27) by monitoring H_2O_2 consumption in the presence of a 20 μL sample (S1). Enzyme activity is reported in units (1 U=1 μmol H_2O_2 decomposed/min, at pH 7 and 25°C). Glutathione (GSH) levels were determined fluorometrically (28). An aliquot of the homogenized sample was mixed (1:1) with perchloric acid ($HClO_4$) and centrifuged at 3000 g for 10 min. After centrifugation, the protein pellet was discarded and free-SH groups were determined in the clear supernatant. An aliquot of supernatant was incubated with ortho-phthalaldehyde, and fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. Results are reported as nmol/g of tissue. Superoxide dismutase (SOD) activity was measured as previously described (29) by inhibition of the auto-oxidation of epinephrine to adrenochrome. The color reaction was monitored at 480 nm. One enzymatic unit (1 IU) was defined as the amount of enzyme necessary to inhibit the auto-oxidation rate by 50% at 26°C.

Statistical analysis

The normality of the data distributions was verified using the Shapiro-Wilk test. Open-field and rotarod test results were compared between groups using the Kruskal-Wallis and Dunn's *post hoc* tests. The Mann-Whitney test was used for further comparisons between pairs of groups. One-way analysis of variance (ANOVA) and independent *t*-tests were used to compare between-group differences in tail flick, ROS, TBARS, CAT, GSH, and SOD data. In all cases, statistical significance was set at $P < 0.05$.

Results

Neuromotor results

Results of the open-field test (P=0.001 for crossings; P=0.01 for rearings; Kruskal-Wallis) and the rotarod test (P=0.03; Kruskal-Wallis) revealed significant differences between the groups. Neuromotor deficits were observed in the rats subjected to ischemia-reperfusion surgery. In

the open-field test, impaired performance of crossings (P=0.048; Figure 2A) and rearings (P=0.024; Figure 2B) were observed in the ischemia-reperfusion group compared with the sham group. Crossings (P=0.260; Figure 2A) and rearings (P=0.480; Figure 2B) were similar in the physical exercise and sham groups. Physical exercise minimized the deficits resulting from ischemia-reperfusion, as shown by the crossings (P=0.003; Figure 2A) and rearings (P=0.004; Figure 2B) data.

Rotarod test performance was impaired in the ischemia-reperfusion group compared with the sham group as shown by the number of falls (P=0.034; Figure 2C) and the times at which the rats fell off the rotarod (P=0.038; Figure 2D). Exercise *per se* did not improve performance on the rotarod test, as the number of falls (P=0.700; Figure 2C) and the times at which the rats fell off the rotarod observed in the physical exercise and sham groups were similar (P=0.650; Figure 2D). Exercise did not decrease the number of falls among rats in the ischemia-reperfusion group (P=0.140; Figure 2C), but it significantly increased the latency to the first fall (P=0.020; Figure 2D).

Nociception

Pain sensitivity was similar among rats in the four experimental groups (P=0.800; one-way ANOVA; Figure 3).

### Oxidative status of the striatum

Increased oxidative stress status in the striatum was observed, as shown by the increase in ROS levels without any change in antioxidant markers after ischemia-reperfusion. Physical exercise partially reversed this condition.
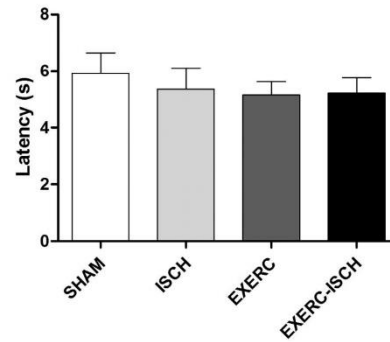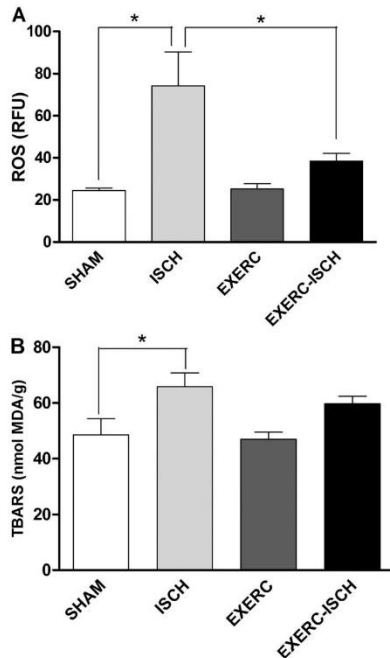

Figure 3. Transient global ischemia-reperfusion did not alter pain sensitivity measured by the tail-flick test. Data are reported as means ± SD for n=10 rats/group. SHAM: rats submitted to surgery without arterial occlusion; ISCH: rats submitted to ischemia-reperfusion surgery; EXERC: rats submitted to physical exercise before surgery without arterial occlusion; EXERC-ISCH: rats submitted to physical exercise before ischemia-reperfusion surgery.

Ischemia-reperfusion increased ROS (P=0.020; Figure 4A) and TBARS (P=0.040; Figure 4B) in the striatum, and physical exercise reduced the increase in ROS levels (P=0.090; Figure 4A) but not the increase in TBARS (P=0.250; Figure 4B).

No significant differences in the activities of the antioxidant markers that were assayed were observed among the groups (CAT, P=0.390; GSH, P=0.700; SOD, P=0.340; one-way ANOVA; Figure 5).
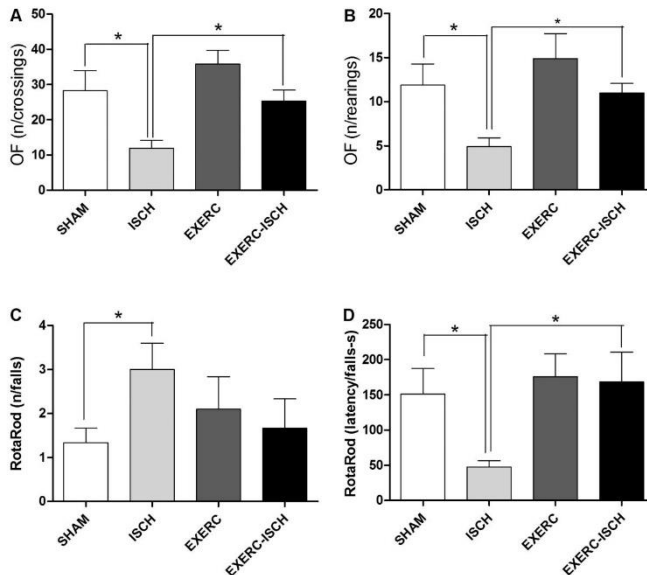
Figure 2. Transient global ischemia-reperfusion led to motor alterations and physical exercise prevented such alterations. *A* and *B*, Results of the open-field (OF) test. The number of crossings are shown in *A* and the number of rearings are shown in *B*. *C* and *D*, Results of the rotarod test. The number of falls are shown in *C* and the latency of the first fall (in seconds) is shown in *D*. Data are reported as means ± SD for n=10 rats/group. SHAM: rats submitted to surgery without arterial occlusion; ISCH: rats submitted to ischemia-reperfusion surgery; EXERC: rats submitted to physical exercise before surgery without arterial occlusion; EXERC-ISCH: rats submitted to physical exercise before ischemia-reperfusion surgery. *P < 0.05 (Kruskal-Wallis test followed by Mann-Whitney test).

Discussion

Figure 4. Transient global ischemia-reperfusion promotes an increase of reactive oxygen species and oxidative damage (lipoperoxidation) in the striatum, and physical exercise partially prevents these alterations. *A*, Reactive oxygen species (ROS) levels in the striatum and *B*, lipoperoxidation assessed by TBARS. Data are reported as means ± SD. SHAM: rats submitted to surgery without arterial occlusion; ISCH: rats submitted to ischemia-reperfusion surgery; EXERC: rats submitted to physical exercise before surgery without arterial occlusion; EXERC-ISCH: rats submitted to physical exercise before ischemia-reperfusion surgery. *P < 0.05 (one-way ANOVA; *t*-test).

We assessed the neuroprotective role of physical exercise on striatal oxidative balance and motor impairments resulting from ischemia-reperfusion injury in rats. Our results indicated that ischemia-reperfusion led to significant neuromotor impairments without changing nociception. Animals subjected to ischemia-reperfusion also experienced oxidative stress resulting in oxidative imbalance in the striatum. The damage observed in the striatum is consistent with the neuromotor deficits observed in the ischemia-reperfusion group (9). In this study, neuromotor impairment was demonstrated by the results of the rotarod and open-field tests.

Among our main findings is the capability of physical exercise to protect, although partially, against impairments resulting from an ischemia-reperfusion insult. Physical exercise reversed the impairments in the rotarod test (i.e., time to the first fall) and open-field test (i.e., crossings and rearings) performance observed in the ischemia-reperfusion group. Ischemia-reperfusion is known to induce the deficits in motor development and balance that are measured by the rotarod test (30) and has also been associated with lower neuronal density and area in the striatum after the ischemia-reperfusion injury (30). A neuroprotective role of exercise performed for 14 days before ischemia has been reported in rats, with similar effects in rats trained after ischemia (5).

Nociception was not affected by the ischemic event, which may be explained by the fact that nociception does not depend on striatal activity, but by nociception-specific cortical regions, areas were not evaluated in this study. Those cortical regions are also known to be less sensitive to ischemic events than the hippocampus and striatum are (31). The lack of change in nociception supports a model in which the motor impairments we observed resulted from damage to the striatum.

The motor impairments observed here most likely resulted from the ischemic event that the rats experienced (16). Neuronal degeneration induced by ischemia-reperfusion
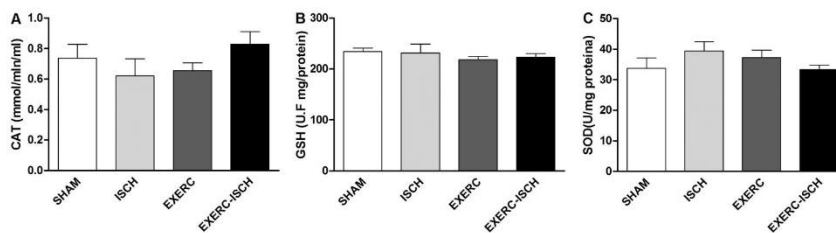
Figure 5. Transient global ischemia-reperfusion and physical exercise did not alter antioxidant markers. *A*, catalase activity (CAT), *B*, glutathione (GSH) levels, and *C*, superoxide dismutase activity (SOD) in the four groups. Data are reported as means ± SD. SHAM: rats submitted to surgery without arterial occlusion; ISCH: rats submitted to ischemia-reperfusion surgery; EXERC: rats submitted to physical exercise before surgery without arterial occlusion; EXERC-ISCH: rats submitted to physical exercise before ischemia-reperfusion surgery. There were no significant differences among groups (one-way ANOVA; *t*-test).

31

is associated with conditions of oxidative stress resulting from high levels of fatty acids in the brain (6). The striatum is one of the brain regions most affected by oxidative stress in ischemia-reperfusion and its relatively high densities of GABA receptors and glutamatergic neurons may be related to this neurotoxicity (12). Oxidative stress plays a major role in various pathological conditions, and it may occur in the striatum during aging (17), chronic unpredictable stress situations (32), neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (18,33), and also after strokes (16). Oxidative imbalance in the striatum is related to the loss of dopaminergic neurons and neurotoxicity (12,34) and to damage to DNA/RNA, lipids and proteins, resulting in altered cellular and molecular function and increased cell death (6,8,35,36).

The motor impairments observed here appear to be related to increased levels of ROS and lipoperoxidation in the striatum, leading to oxidative imbalance in this brain region (34). We found that exercise was effective for avoiding motor impairments and that it decreased ROS levels but not lipoperoxidation activity. A recent evaluation of the neuroprotective role of exercise in ischemia-reperfusion injury reported similar results for lipoperoxidation (16). The effects of exercise may be mediated by mitochondrial biogenesis; edema reduction, which would improve blood flow in the ischemic region; and the attenuation of acute neurotoxicity, which would facilitate the reorganization of the injured brain tissue (12,13,37,38).

As expected, levels of antioxidant enzymes (CAT and SOD) and GSH, a key non-enzymatic antioxidant, were not changed by exercise (37). Cechetti et al. (16) reported similar results in the cortex and hippocampus.

Understanding the mechanisms of brain injury, the brain's defense responses, and the adaptations in response to long-term exercise is important for improving the strategies for rehabilitation after ischemic events. Our research supports a model in which physical exercise reverses deficits in locomotor behavior and striatal oxidative balance but does not improve antioxidant status. We found that a relatively short period of physical conditioning benefited animals subjected to ischemia-reperfusion surgery.

In summary, our results demonstrate that physical exercise performed during 8 weeks before ischemia-reperfusion was effective to avoid or minimize motor deficits and oxidative stress conditions in the striatum. In this animal model, exercise was neuroprotective, attenuating the severity of ischemia-reperfusion sequelae.

Acknowledgments

References

1. Park S, Kim da S, Kang S. Exercise training attenuates cerebral ischemic hyperglycemia by improving hepatic insulin signaling and beta-cell survival. *Life Sci* 2013; 93: 153-160, doi: 10.1016/j.lfs.2013.06.005.

2. Chen S, Yin ZJ, Jiang C, Ma ZQ, Fu Q, Qu R, et al. Asiaticoside attenuates memory impairment induced by transient cerebral ischemia-reperfusion in mice through anti-inflammatory mechanism. *Pharmacol Biochem Behav* 2014; 122: 7-15, doi: 10.1016/j.pbb.2014.03.004.

3. Zamani M, Katebi M, Mehdizadeh M, Kafami L, Soleimani M. Combination therapy with A1 receptor agonist and vitamin C improved working memory in a mouse model of global ischemia-reperfusion. *Basic Clin Neurosci* 2013; 4: 111-116.

4. Mestriner RG, Miguel PM, Bagatini PB, Saur L, Boisserand LS, Baptista PP, et al. Behavior outcome after ischemic and hemorrhagic stroke, with similar brain damage, in rats. *Behav Brain Res* 2013; 244: 82-89, doi: 10.1016/j.bbr.2013.02.001.

5. Heo M, Kim E. Beneficial effects of antecedent exercise training on limb motor function and calpain expression in a rat model of stroke. *J Phys Ther Sci* 2013; 25: 943-946, doi: 10.1589/jpts.25.943.

6. Allen CL, Bayraktutan U. Oxidative stress and its role in the pathogenesis of ischaemic stroke. *Int J Stroke* 2009; 4: 461-470, doi: 10.1111/j.1747-4949.2009.00387.x.

7. Gulati P, Singh N. Neuroprotective effect of tadalafil, a PDE-5 inhibitor, and its modulation by L-NAME in mouse model of ischemia-reperfusion injury. *J Surg Res* 2014; 186: 475-483, doi: 10.1016/j.jss.2013.08.005.

8. Abramov AY, Scorziello A, Duchen MR. Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation. *J Neurosci* 2007; 27: 1129-1138, doi: 10.1523/JNEUROSCI.4468-06.2007.

9. Li PF, Zhan HQ, Li SY, Liu RL, Yan FL, Cui TZ, et al. Lactuside B decreases aquaporin-4 and caspase-3 mRNA expression in the hippocampus and striatum following cerebral ischaemia-reperfusion injury in rats. *Exp Ther Med* 2014; 7: 675-680.

10. Baez-Mendoza R, Schultz W. The role of the striatum in social behavior. *Front Neurosci* 2013; 7: 233.

11. Teixeira AM, Reckziegel P, Muller L, Pereira RP, Roos DH, Rocha JB, et al. Intense exercise potentiates oxidative stress in striatum of reserpine-treated animals. *Pharmacol Biochem Behav* 2009; 92: 231-235, doi: 10.1016/j.pbb.2008.11.015.

12. Park CY, Lee SH, Kim BK, Shin MS, Kim CJ, Kim H. Treadmill exercise ameliorates impairment of spatial learning ability through enhancing dopamine expression in hypoxic ischemia brain injury in neonatal rats. *J Exerc Rehabil* 2013; 9: 406-412, doi: 10.12965/jer.130053.

13. Lu F, Nakamura T, Toyoshima T, Liu Y, Hirooka K, Kawai N, et al. Edaravone, a free radical scavenger, attenuates behavioral deficits following transient forebrain ischemia by

inhibiting oxidative damage in gerbils. *Neurosci Lett* 2012; 506: 28-32, doi: 10.1016/j.neulet.2011.10.041.

14. Zheng G, Chen B, Fang Q, Yi H, Lin Q, Chen L, et al. Primary prevention for risk factors of ischemic stroke with Baduanjin exercise intervention in the community elder population: study protocol for a randomized controlled trial. *Trials* 2014; 15: 113, doi: 10.1186/1745-6215-15-113.

15. Schimidt HL, Vieira A, Altermann C, Martins A, Sosa P, Santos FW, et al. Memory deficits and oxidative stress in cerebral ischemia-reperfusion: neuroprotective role of physical exercise and green tea supplementation. *Neurobiol Learn Mem* 2014; 114: 242-250, doi: 10.1016/j.nlm.2014.07.005.

16. Cechetti F, Worm PV, Elsner VR, Bertoldi K, Sanches E, Ben J, et al. Forced treadmill exercise prevents oxidative stress and memory deficits following chronic cerebral hypoperfusion in the rat. *Neurobiol Learn Mem* 2012; 97: 90-96, doi: 10.1016/j.nlm.2011.09.008.

17. Flores MF, Martins A, Schimidt HL, Santos FW, Izquierdo I, Mello-Carpes PB, et al. Effects of green tea and physical exercise on memory impairments associated with aging. *Neurochem Int* 2014; 78: 53-60, doi: 10.1016/j.neuint.2014.08.008.

18. Lin TW, Shih YH, Chen SJ, Lien CH, Chang CY, Huang TY, et al. Running exercise delays neurodegeneration in amygdala and hippocampus of Alzheimer's disease (APP/PS1) transgenic mice. *Neurobiol Learn Mem* 2015; 118: 189-197, doi: 10.1016/j.nlm.2014.12.005.

19. Uysal N, Kiray M, Sisman AR, Camsari UM, Gencoglu C, Baykara B, et al. Effects of voluntary and involuntary exercise on cognitive functions, and VEGF and BDNF levels in adolescent rats. *Biotech Histochem* 2015; 90: 55-68, doi: 10.3109/10520295.2014.946968.

20. Skriver K, Roig M, Lundbye-Jensen J, Pingel J, Helge JW, Kiens B, et al. Acute exercise improves motor memory: exploring potential biomarkers. *Neurobiol Learn Mem* 2014; 116: 46-58, doi: 10.1016/j.nlm.2014.08.004.

21. Brooks GA, White TP. Determination of metabolic and heart rate responses of rats to treadmill exercise. *J Appl Physiol Respir Environ Exerc Physiol* 1978; 45: 1009-1015.

22. Collino M, Aragno M, Mastrocola R, Gallicchio M, Rosa AC, Dianzani C, et al. Modulation of the oxidative stress and inflammatory response by PPAR-gamma agonists in the hippocampus of rats exposed to cerebral ischemia/reperfusion. *Eur J Pharmacol* 2006; 530: 70-80, doi: 10.1016/j.ejphar.2005.11.049.

23. Bonini JS, Bevilaqua LR, Zinn CG, Kerr DS, Medina JH, Izquierdo I, et al. Angiotensin II disrupts inhibitory avoidance memory retrieval. *Horm Behav* 2006; 50: 308-313, doi: 10.1016/j.yhbeh.2006.03.016.

24. de Castro BM, Pereira GS, Magalhaes V, Rossato JI, De Jaeger X, Martins-Silva C, et al. Reduced expression of the vesicular acetylcholine transporter causes learning deficits in mice. *Genes Brain Behav* 2009; 8: 23-35, doi: 10.1111/j.1601-183X.2008.00439.x.

25. Tjølsen A, Lund A, Berge OG, Hole K. An improved method for tail-flick testing with adjustment for tail-skin temperature. *J Neurosci Methods* 1989; 26: 259-265, doi: 10.1016/0165-0270(89)90124-6.

26. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; 95: 351-358, doi: 10.1016/0003-2697(79)90738-3.

27. Aebi H. Catalase *in vitro*. *Methods Enzymol* 1984; 105: 121-126.

28. Hissin PJ, Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem* 1976; 74: 214-226, doi: 10.1016/0003-2697(76)90326-2.

29. Misra KB. Reliability optimization of a series-parallel system. *IEEE Trans Reliability* 1972; 21: 230-238, doi: 10.1109/TR.1972.5216000.

30. de Araujo FL, Bertolino G, Goncalves RB, Marini LC, Coimbra NC, de Araujo JE. Neuropathology and behavioral impairments after three types of global ischemia surgery in Meriones unguiculatus: evidence in motor cortex, hippocampal CA1 region and the neostriatum. *J Neurol Sci* 2012; 312: 73-78, doi: 10.1016/j.jns.2011.08.019.

31. Pereira LO, Nabinger PM, Strapasson AC, Nardin P, Goncalves CA, Siqueira IR, et al. Long-term effects of environmental stimulation following hypoxia-ischemia on the oxidative state and BDNF levels in rat hippocampus and frontal cortex. *Brain Res* 2009; 1247: 188-195, doi: 10.1016/j.brainres.2008.10.017.

32. Che Y, Zhou Z, Shu Y, Zhai C, Zhu Y, Gong S, et al. Chronic unpredictable stress impairs endogenous antioxidant defense in rat brain. *Neurosci Lett* 2015; 584: 208-213, doi: 10.1016/j.neulet.2014.10.031.

33. Le Douaron G, Schmidt F, Amar M, Kadar H, Debortoli L, Latini A, et al. Neuroprotective effects of a brain permeant 6-aminoquinoxaline derivative in cell culture conditions that model the loss of dopaminergic neurons in Parkinson disease. *Eur J Med Chem* 2015; 89: 467-479, doi: 10.1016/j.ejmech.2014.10.067.

34. Masoud ST, Vecchio LM, Bergeron Y, Hossain MM, Nguyen LT, Bermejo MK, et al. Increased expression of the dopamine transporter leads to loss of dopamine neurons, oxidative stress and l-DOPA reversible motor deficits. *Neurobiol Dis* 2015; 74: 66-75, doi: 10.1016/j.nbd.2014.10.016.

35. Colamartino M, Santoro M, Duranti G, Sabatini S, Ceci R, Testa A, et al. Evaluation of levodopa and carbidopa antioxidant activity in normal human lymphocytes in vitro: implication for oxidative stress in Parkinson's disease. *Neurotox Res* 2015; 27: 106-117, doi: 10.1007/s12640-014-9495-7.

36. Lalith Kumar V, Muralidhara. Ameliorative effects of ferulic Acid against lead acetate-induced oxidative stress, mitochondrial dysfunctions and toxicity in prepubertal rat brain. *Neurochem Res* 2014; 39: 2501-2515, doi: 10.1007/s11064-014-1451-7.

37. Marques-Aleixo I, Oliveira PJ, Moreira PI, Magalhaes J, Ascensao A. Physical exercise as a possible strategy for brain protection: evidence from mitochondrial-mediated mechanisms. *Prog Neurobiol* 2012; 99: 149-162, doi: 10.1016/j.pneurobio.2012.08.002.

38. Wang Z, Myers KG, Guo Y, Ocampo MA, Pang RD, Jakowec MW, et al. Functional reorganization of motor and limbic circuits after exercise training in a rat model of bilateral parkinsonism. *PLoS One* 2013; 8: e80058, doi: 10.1371/journal.pone.0080058.

33

Artigo2

APOCYIN ADMINISTRATION DO NOT PROTECT AGAINST STRIATAL OXIDATIVE STRESS AND NEUROMOTOR DEFICITS INDUCED BY HEMORRHAGIC STROKE IN RATS

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Highligths

- **ICH causes neuromotor impairment and oxidative stress**
- **Apocynin treatment did not improve neuromotor impairment**
- **Apocynin treatment did not protect against oxidative damage**

Abstract

Intracerebral hemorrhage (ICH) is the most severe form of stroke, may lead to permanent disturbances engines and even death. Evidence suggests that after an ICH, there is an increased production of reactive oxygen species, leading to oxidative stress. The striatum, a brain region greatly affected by bleeding events, is very sensitive to redox imbalance. Considering that the production of reactive oxygen species is linked to overactivation of the enzyme complex NADPH oxidase, present in cell membranes, the aim of this study was to evaluate the effects of apocynin (0.5mg/kg, 2, 6, and 24 hours after ICH, i.p.), a drug capable of inhibiting the activity of NADPH oxidase, on the neuromotor activity and on striatal oxidative balance in rats. Our results suggest that the apocynin is not an effective strategy of neuroprotection on hemorrhagic stroke, at least on the doses used here. Further studies are necessary since the effectiveness of apocynin to promote neuroproection on hemorrhagic stroke is not discarded and different doses and routes of administration should be tested.

Key-Words: hemorrhagic stroke, intracerebral hemorrage, NADPH-oxidase and apocynin.

Introduction

Intracerebral hemorrhage (ICH) is a devastating form of stroke, caused by the rupture of an intraparenchymal blood vessel (Wang et al., 2006a). This event accounts for around 15% of all strokes and is much more devastating than the ischemic stroke (IS), considering that around 80% of survivors remain with neurological impairment (Mayer and Rincon, 2005).

Primary injury caused by ICH is mainly due to mechanical trauma related to the blood spilling into the brain (Balami and Buchan, 2012; Wang et al., 2006a). The molecular mechanisms of secondary damage after ICH have not been well-established, but they may represent therapeutic targets to prevent further brain injury. As a result of hemorrhage, the extracellular spaces of the brain becomes exposed to hemoglobin and its breakdown products (iron, biliverdin, and carbon monoxide), which subsequently activates cytotoxic, oxidative and inflammatory pathways (Aronowski and Zhao, 2011; Xi and Keep, 2012)

The evidence that oxidative stress, via the generation of ROS, contributes to secondary brain injury induced by ICH is increasing (Hackett et al., 2015; Nakamura et al., 2004; Wu et al., 2002). Activated enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) is a multi-subunit enzyme complex that contributes to brain injury related to oxidative stress. Although there are more evidences from the role of NADPH oxidase on the brain damage after IS (Kahles and Brandes, 2013; Kahles et al., 2007; Walder et al., 1997), suggesting that the reduction of NOX mediated oxidative stress may have potential value in prevention of cerebral ischemic-reperfusion (I-R) injury, recent research also point the role of NOX on the damage after an ICH event (Tang et al, 2005).

Apocynin is a NOX inhibitor isolated from the medicinal plant *Picrorhiza kurroa*. It has been reported that apocynin reduces the histologic injury following global (Wang et al., 2006a) and focal (Tang et al., 2007) cerebral ischemia, epilepsy and trauma. Although the effect of inhibition of NADPH oxidase presents neuroprotection in some models of injury (Ferreira et al., 2013) the effects of NADPH oxidase inhibition on ICH are still controversial (Titova et al., 2007, Tang et al., 2005). So, considering that (i) despite the different physiopathology of ischemic and hemorrhagic stroke NADPH oxidase contribute to acute brain injury in both (Liang et al., 2016); and, (ii) that an effective therapy for ICH induced secondary brain injury is currently not available (Wang et al., 2010), in the present study we investigated whether apocynin is able to protect against brain injury in a hemorrhagic stroke (ICH) rat model.

Material and methods

Animals

The sample consisted of 40 male Wistar rats (250-350 g) obtained from the Central Animal Laboratory of the Federal University of Pelotas (UFPEL). The rats were housed in boxes (5 per box) and kept in a temperature-controlled room ($24°C \pm 1$), with light/dark cycle of 12 hours and food and water *ad libitum*.

We certify that all the experiments were performed in accordance with the standards of the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1996) and the experiments were previously approved by the Animal Use Ethics Committee of the Federal University of Pampa (protocol #032/2014).

Drugs and reagents

NADPH, 2', 7'- dichlorofluorescein diacetate (DCFH-DA), GSH reagents and apocynin were purchased from Sigma (St. Louis, MO, USA). Other reagents used in this study were of analytical grades and obtained from standard commercial supplier.

Experimental design

Rats were initially divided into 4 groups:

- G1: Sham treated with saline (n=10): The rats were submitted to ICH as described below except by the injection of collagenase, that was replaced by saline. After, they received 0.5 ml of saline (i.p.) at the same times that the other animals received apocynin.

- G2: ICH treated with saline (n=10): G2 rats were submitted to ICH as described below. After, received 0.5 ml of saline (i.p.) at the same times that the other animals received apocynin.

- G3: Sham treated with apocynin (n=10): The rats were submitted to ICH as described below except by the injection of collagenase, that was replaced by saline. After, were treated with apocynin (i.p.) starting from two hours after ICH (Titova et al., 2007) in a concentration of 0.5 mg/kg in 0.5 ml (Ferreira et al., 2013). Subsequent doses are administrated 6 and 24 hours after injury (Abdul-Muneer et al., 2013).

- G4: ICH treated with apocynin (n=10):G4 rats were submitted to ICH as described below and received apocynin i.p. in the same way of G3.

The doses of apocynin (0.5 mg/kg) or saline were administered intraperitoneally (i.p.) 2h, 6h and 24h after the ICH (Ferreira et al., 2013). Neuromotor tests (Open Field–OF, Rotarod - RR and Neurological Deficit Score - NDS) were made before ICH induction to have a baseline, and 24 hours and 3 days after ICH (figure 1).
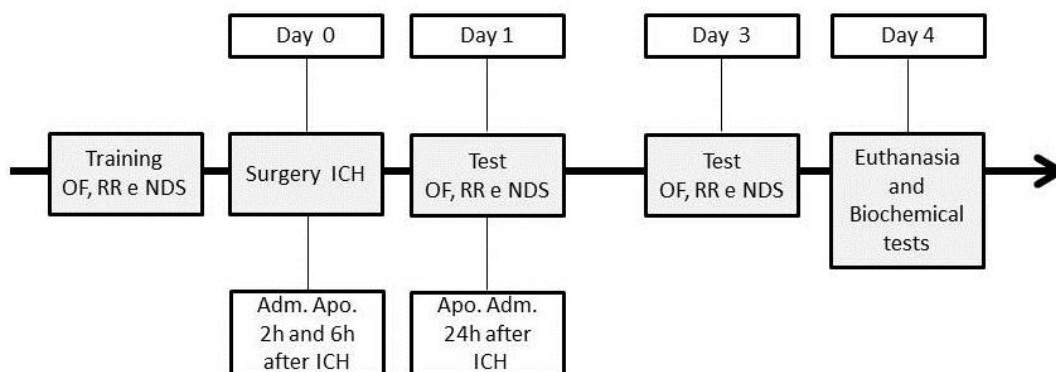

Figure 1.Experimental Design. All rats were submitted to the training on behavior tests (Open Field – OF, rotarod - RR and Neurological Deficit Scale - NDS) previously (24 hours before) to surgery. On day 0, all rats underwent surgery with or without collagenase infusion into the right striatum, and initiated the saline or apocynin administration (i.p.). Twenty-four hours after surgery OF, RR and NDS tests were performed. On the third day after surgery (day3) behavioral tests were performed again and on day 4 the rats were sacrificed and the ipsilateral striatum (considering the surgery side) was removed for further biochemical analysis.

Twenty-four hours after completion of behavioral testing the animals were euthanized for biochemical analysis (figure 1). Levels of reactive oxygen species (ROS), thiobarbituric acidreactive species (TBARS) and total antioxidant capacity (FRAP) were performed.

Protocols

Surgery to induce ICH/Hemorrhagic stroke model

All the surgical procedures were performed with rats anesthetized with ketamine and xylazine, at doses of 0.5mg/kg and 0.25mg/kg i.p., respectively. To induce ICH in rats, after confirmation of anesthesia, they were fixed in the stereotactic table for cleansing the scalp with 70% ethanol and povidone iodine (PVPI). The intrastriatal administration of collagenase was performed according to the coordinates of Paxinos and Watson (1986) to right striatum (0.5 mm anterior to Bregma; 3.5 mm right, and 6.5 mm deep from the surface of the skull. Subsequently, collagenase (1μl; 0.2 U), an enzyme that degrades the basal lamina of blood

vessels, causing bleeding, was administered with an infusion pump over a period of 10 minutes (Nakamura et al., 2004). The needle was removed after an additional 10 minutes to avoid reflux. A small metal screw is inserted into the opening of the skull to avoid intracranial pressure drop, the incision was sutured and lidocaine was applied on site (Caliaperumal et al., 2012). Body temperature was maintained at 37°C during anesthesia using a heating pad.

Behavioral testing

To check possible changes and/or deficits in motor function the following behavioral tests were performed:

Open field test (OF)

To assess the locomotor activity and exploratory behavior of the animals, we analyzed the behavior of locomotion/exploration in the scheme known as "open field" (Bonini et al., 2006). The apparatus consists of a box (60 cm diameter x 45 cm high) divided into 12 quadrants of equal surface area. The animal was gently placed in the open field arena (i.e., the box), so that could explore it freely for 5 min. The number of crossed lines (crossings) and the number of elevations on its hind legs (rearings) were assessed (Bonini et al., 2006).

Rotarod test (RR)

Aiming to evaluate the influence of ICH in the rats motor function was used a RR. The apparatus consists of a rotating cylinder (5 cm diameter x 8 cm wide x 20 cm high), with an automatic recorder of falls. On the training day (prior to ICH) the animals were placed on the static cylinder during 2 minutes for habituation. After the habituation time, the drum rotation was set at 16 rpm for 6 minutes. After that time the animals returned to home box. In the training session the number of falls and the latency for each fall were recorded. In the tests sessions the animals were placed again in the apparatus for 6 minutes with speed set at 20 rpm. The number of falls and the latency of each fall were counted (Stroobants et al., 2013).

Neurological Deficit Scale (NDS)

The NDS is a battery of friendly-user tests that can be applied immediately after the stroke (Schallert, 2006). This battery of tests permits the evaluation of 5 functions (spontaneous spins; retraction of the contralateral hind limb; bilateral seizure of front legs; capacity to walking in the beam and bending of the contralateral forelimb). The animals

receive a score from 0 to 4, according to their neuromotor performance (0 corresponds to no neuromotor deficits and 4 represents a severe neuromotor deficit).

Biochemical analyses

For the biochemical analyzes, rats were sacrificed 24 hours after the end of behavioral testing. The brain was removed, and the right striatum was quickly dissected and homogenized (1:10) in 50 mM TrisHCl, pH 7.4. Tissue samples were centrifuged at 2400g for 20min and the supernatant (S1) were used for subsequent testing

Reactive oxygen species (ROS) levels

The ROS levels were determined by flurimetric method spectrum using 2',7'-dichlorofluorescein diacetate (DCFH-DA). The samples were incubated in the dark with 5 µL of DCFH-DA (1 mM). Oxidation monitoring was made of DCFH-DA to dichlorofluorescein (DCF) fluorescence by reactive oxygen species. The fluorescence emission intensity was performed at 520 nm (with excitation at 480 nm) for 60 minutes after the addition of DCFH-DA in spectrofluorimeter (Shimadzu RF-5301PC Model).

Detection of TBARS level

Lipid peroxidation was assessed by the substances reactive to thiobarbituric acid (TBARS) test (Ohkawa et al., 1979). S1 aliquot was incubated with a solution of 0.8% thiobarbituric acid, acetic acid buffer (pH 3.2) and sodium dodecyl sulfate solution (8%) at 95°C for 2 h. The color reaction was measured at 532 nm. Results were expressed as nmol of malondialdehyde (MDA) per mg protein.

Ferric Reducing/Antioxidant Power (FRAP) Assay

Briefly, 50 µl S1 was added 1.5 ml of freshly prepared (300 mM acetate buffer (37ºC) FRAP reagent (pH 3.6), 10 mM HCl TPTZ 40 mM and 20 mM $FeCl_3 \cdot 6H_2$ in the ratio of 10: 1: 1) in a test tube and incubated for 10 min at 37ºC. The absorbance of the blue color complex was read against a reagent blank (1.5 ml of distilled water FRAP reagent + 50°C) in 593 nm. The FRAP values were expressed as nmol of ferric ions reduced to ferrous form/mg tissue (Ghahremanitamadon et al., 2014).

Statistical analysis

The data distribution was verified using the Shapiro-Wilk test. Two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc was used subsequently to compare the differences between groups in behavioral and biochemical tests. In all cases, statistical significance was consider when $P \leq 0.05$.

Results

Behavioral results

The results of the open field test showed that the model of ICH causes locomotor and exploratory change in the first (crossing: $P < 0.001$ and rearing: $P < 0.05$) and third day (crossing: $P < 0.05$ and rearing: $P < 0.05$) after ICH. However, the results of the open field test did not show significant differences between the animals of ICH and ICH treated with apocynin group, both on day 1 ($P > 0.05$ for crossings and $P > 0.05$ for rearings) as in day 3 ($P > 0.05$ for crossings and $P > 0.05$ for rearings) (Figure 2A and 2B).

Similarly, the ICH decreased the balance of the rats evaluated in RR in the first ($P < 0.001$ for number of falls and $P < 0.001$ for latency to first fall) and third day ($P < 0.01$ for number of falls and $P < 0.01$ for latency to first fall). However, we did not observe significant differences on the RR performance across the ICH and ICH+apocynin group on the first day ($P > 0.05$ for number of falls and $P > 0.05$ for latency to the first fall), and on the third day ($P > 0.05$ for number of falls and $P > 0.05$ for latency to the first fall) (Figure 2C and 2D).

Regarding the analysis NDS, an increase in neurological damage in the first ($P < 0.001$) and third day ($P < 0.01$) after ICH was observed. There were no significant differences between the ICH and ICH treated with apocynin group on days 1 or 3 ($P > 0.05$) (Figure 2E).
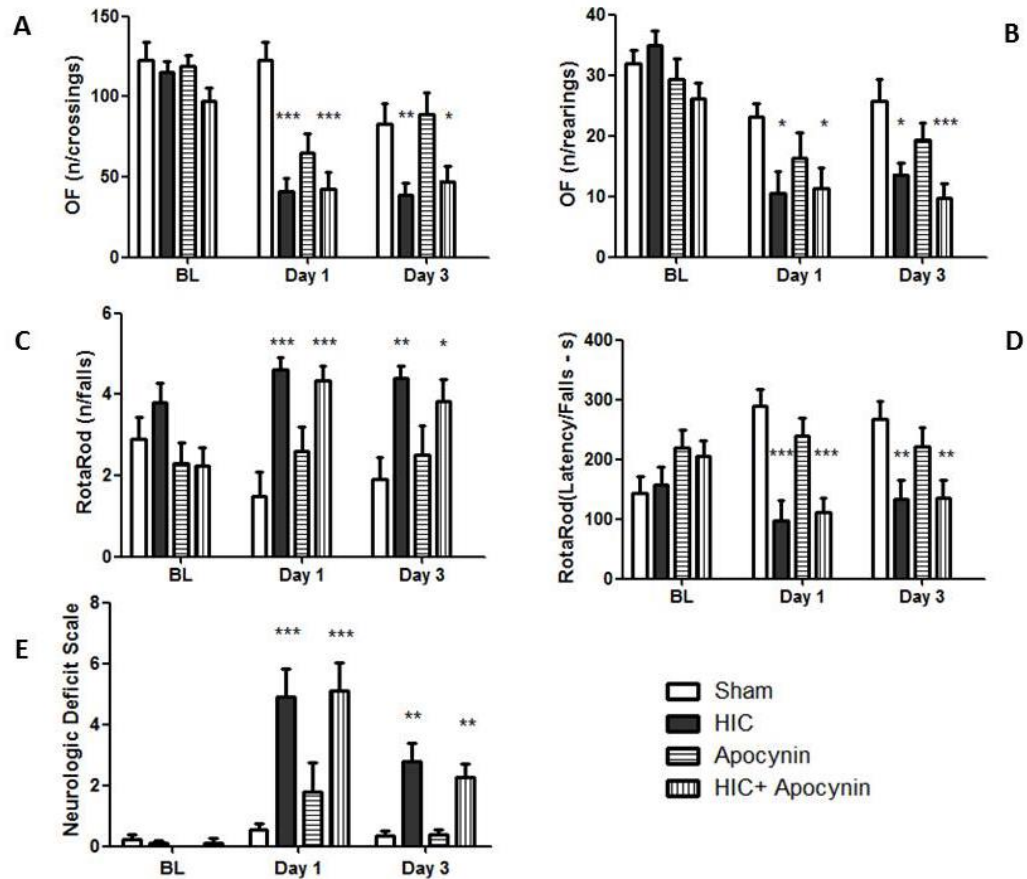
Figure 2. ICH leads to motor disorders and apocynin administration did not reverse these alterations. A. Number of crossings on OF task. B. Number of reagings on OF test. C. Latency (s) to first fall on RR task. D. Number of falls on RR task. E. Score of neurological deficit by NDS test. Data are presented as mean $\pm$ SD. n = 10 rats/group. * P < 0.05 compared to Sham (Two-way ANOVA followed by Bonferroni posthoc test).

Biochemical results

Striatum biochemical data are shown in Figure 3. The rats submitted to the ICH induction had increase in the pro-oxidant markers levels in relation to sham animals, considering the levels of reactive oxygen species (DCFH) (fig 3A, P < 0.05) and reactive species to thiobarbituric acid (TBARS) (fig 3B, P < 0.05). However the animals that received treatment with apocynin did not show reduction in these pro-oxidant markers in the striatal region (P > 0.05, figs 3A and 3B).
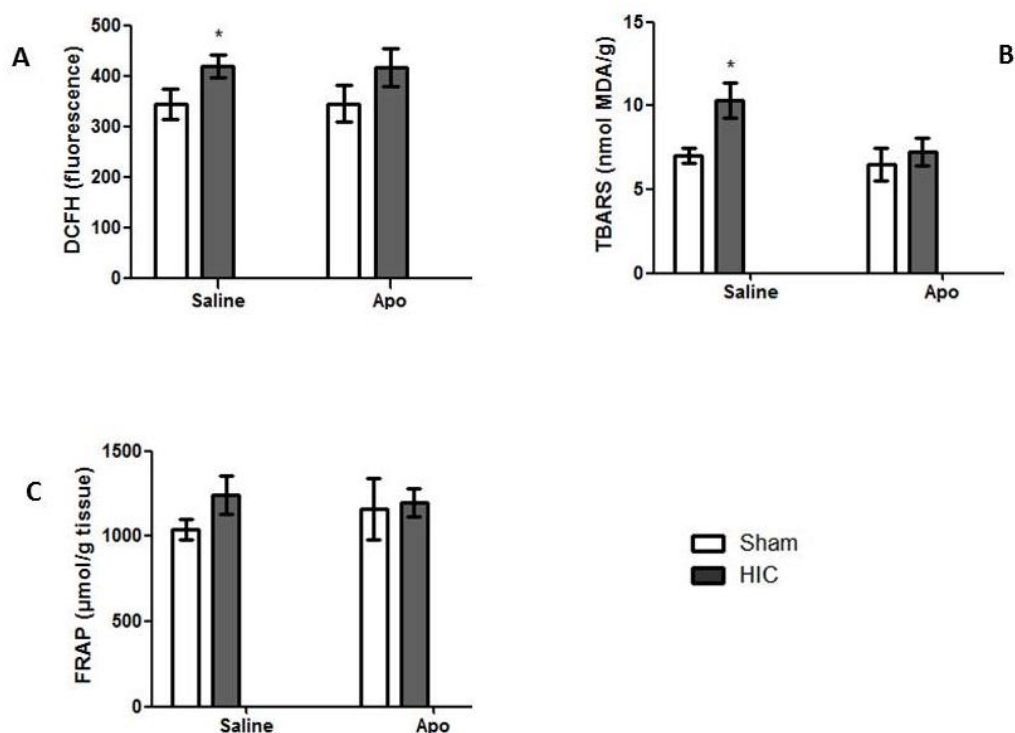
Figure 3: ICH promotes an increase of reactive oxygen species and oxidative damage (lipoperoxidation) in the striatum. Apocynin does not prevent these alterations. A. Reactive oxygen species (ROS) levels in the striatum. B. Lipoperoxidation in the striatum assessed by TBARS. C. Total antioxidant capacity in the striatum by FRAP. Data are reported as means ± SD. *P ≤ 0.05 (Two-way ANOVA followed by Bonferroni post hoc test).

In the analysis of the total antioxidant capacity (FRAP) the rats of ICH group showed no significant difference in the antioxidant defenses compared to the sham group (P > 0.05, fig 3C). As expected in this case, when the results of ICH group were compared to ICH apocynin treated group, no significant difference between groups (P > 0.05, Fig 3C).

Discussion

Here we sought to evaluate the possible neuroprotective role of apocynin on the striatum oxidative balance and neuromotor deficits resulting from an ICH in rats. Our results demonstrate that ICH leads to significant changes in oxidative balance and neuromotor deficit, but contrary to our first hypothesis, apocynin was not able to avoid these deficits.

The results demonstrating the neuromotor deficit are similar to the ones of several recent studies, which demonstrated that the intracerebral hemorrhage generates decreased locomotor and exploratory capacity, balance and neurological damage (Choi et al., 2015; Titova et al., 2007; Quin et al., 2015). Our results also allow us to affirm that the apocynin is not able to reverse the neurological deficits induced by ICH. A previously study using a model of ischemia-reperfusion demonstrate that apocynin administration prior to injury (24h

and 30 min before intravenous dose of 50 mg/kg) reversed considerably neurological deficits caused by this brain injury (Zhang et al., 2015). In contrast, a study using the same model of ischemic injury and a curve of crescent apocynin doses (2.5, 3.75 and 5 mg/kg) administered intravenously 30 minutes prior to ischemic injury showed that only the lowest dose had a protective effect against the ischemic neurological (Tang et al., 2008). These results, although referred to ischemic stroke, highlight the importance of time and dose, considering that the differences in these parameters led to different results.

The hemorrhagic stroke induced neuronal degeneration is directly connected to oxidative stress conditions, as well as to a large area of edema and volume of lesion (Titova et al., 2007). The striatum is one of the brain structures most affected by the redox imbalance (Lin et al., 2015), which may be related to its large amount of GABAergic and glutamatergic receptors (Park et al., 2013). In this work, the ICH group presented increased levels of ROS and lipid peroxidation (TBARS), what represents oxidative stress and damage, so, the motor deficits found in this group can be closely related to this oxidative imbalance (Masoud et al., 2015).

Brain oxidative imbalance plays an important role in various conditions, as during aging (Flores et al., 2014), exposure to chronic stress (Che et al., 2015) and neurodegenerative diseases such as Alzheimer's disease (Han et al., 2015) as well as stroke (Cechetti et al., 2012). Also, there are crescent evidences that oxidative stress contributes to brain damage induced by hemorrhagic stroke (Hackett et al., 2015; Wu et al., 2002). Corroborating with these findings, we observed in this study that after a hemorrhagic event the striatum present a significant increase in ROS and lipid peroxidation.

Considering that apocynin had success in preventing brain damage in different injury models related to oxidative stress (Tang et al., 2007; Wang et al., 2006b) we investigated its effects on ICH. In our case the apocynin administration in 0.5 mg/kg (2, 6 and 24h after ICH – i.p.) was not able to mitigate or reverse the redox imbalance in the striatum. Apart from the different models of injury, an important aspect to consider is that previously works conducted to evaluate the effects of apocynin in brain damage used different doses and administration time. In a recent study evaluating the neuroprotective effect of apocynin on a model of traumatic brain injury in mice, for example, it was reported that i.p. apocynin administration in a dose of 5mg/kg 3, 6 and 24 hours after injury shows positive results, avoiding lipid peroxidation (Ferreira et al., 2013). Another study reported apocynin inefficiency in the same parameter (lipid peroxidation) when administered at doses 3 and 30 mg/kg i.p. two hours after the hemorrhage-induced brain injury in rats (Titova et al., 2007). Beside this study don't

showed significant results in other parameters, such edema and neurological damage (Titova et al., 2007).

These results lead us to believe that the use of apocynin to promote neuroprotection is rather divergent as regards the dose and routes of administration, factors that may be closely related to its effects. Still, we can associate the efficiency of the apocynin use on other models vs. the inefficiency observed in our model (ICH) to the different neurochemical events related to each model/injury, what highlight the importance of uniqueness in treating each disease. Lastly, most studies using apocynin found effects prior to surgery (Wu et al., 2002; Zhang et al., 2015a; Zhang et al., 2015b), while our model uses the same treatment after the hemorrhagic stroke, which may have influence on our results. We prefer this time considering that normally is not possible predict this type of vascular event in daily life.

The understanding of brain damage events after an ICH and their relation with the motor disorders resulting from injury is key to the search for new treatment strategies after hemorrhagic stroke. Our study contributes to the knowledge in this area showing that apocynin is not an effective alternative in the treatment of neuromotor and neurochemical damage induced by hemorrhagic stroke, at least when administrated in the dose of 0.5kg/kg i.p., 2, 6 and 24h after ICH. Therefore, more researches, testing other doses, routes and times of administration, are necessary to establish if apocynin could be used to prevent ICH neuromotor and oxidative damage.

References

Abdul-Muneer, P.M., Schuetz, H., Wang, F., Skotak, M., Jones, J., Gorantla, S., Zimmerman, M.C., Chandra, N., Haorah, J., 2013. Induction of oxidative and nitrosative damage leads to cerebrovascular inflammation in an animal model of mild traumatic brain injury induced by primary blast. Free Radic Biol Med 60, 282-291.
Aronowski, J., Zhao, X., 2011. Molecular pathophysiology of cerebral hemorrhage: secondary brain injury. Stroke 42, 1781-1786.
Balami, J.S., Buchan, A.M., 2012. Complications of intracerebral haemorrhage. Lancet Neurol 11, 101-118.
Bonini, J.S., Bevilaqua, L.R., Zinn, C.G., Kerr, D.S., Medina, J.H., Izquierdo, I., Cammarota, M., 2006. Angiotensin II disrupts inhibitory avoidance memory retrieval. Horm Behav 50, 308-313.
Caliaperumal, J., Ma, Y.L., Colbourne, F., 2012. Intra-parenchymal ferrous iron infusion causes neuronal atrophy, cell death and progressive tissue loss: Implications for intracerebral hemorrhage. Exp Neurol 237, 363-369.
Cechetti, F., Worm, P.V., Elsner, V.R., Bertoldi, K., Sanches, E., Ben, J., Siqueira, I.R., Netto, C.A., 2012. Forced treadmill exercise prevents oxidative stress and memory deficits following chronic cerebral hypoperfusion in the rat. Neurobiol Learn Mem 97, 90-96.

Che, Y., Zhou, Z., Shu, Y., Zhai, C., Zhu, Y., Gong, S., Cui, Y., Wang, J.F., 2015. Chronic unpredictable stress impairs endogenous antioxidant defense in rat brain. Neurosci Lett 584, 208-213.

Ferreira, A.P., Rodrigues, F.S., Della-Pace, I.D., Mota, B.C., Oliveira, S.M., Velho Gewehr Cde, C., Bobinski, F., de Oliveira, C.V., Brum, J.S., Oliveira, M.S., Furian, A.F., de Barros, C.S., Ferreira, J., Santos, A.R., Fighera, M.R., Royes, L.F., 2013. The effect of NADPH-oxidase inhibitor apocynin on cognitive impairment induced by moderate lateral fluid percussion injury: role of inflammatory and oxidative brain damage. Neurochem Int 63, 583-593.

Flores, M.F., Martins, A., Schimidt, H.L., Santos, F.W., Izquierdo, I., Mello-Carpes, P.B., Carpes, F.P., 2014. Effects of green tea and physical exercise on memory impairments associated with aging. Neurochem Int 78, 53-60.

Ghahremanitamadon, F., Shahidi, S., Zargooshnia, S., Nikkhah, A., Ranjbar, A., Soleimani Asl, S., 2014. Protective effects of Borago officinalis extract on amyloid beta-peptide(25-35)-induced memory impairment in male rats: a behavioral study. Biomed Res Int 2014, 798535.

Hackett, M.J., DeSouza, M., Caine, S., Bewer, B., Nichol, H., Paterson, P.G., Colbourne, F., 2015. A new method to image heme-Fe, total Fe, and aggregated protein levels after intracerebral hemorrhage. ACS Chem Neurosci 6, 761-770.

Kahles, T., Brandes, R.P., 2013. Which NADPH oxidase isoform is relevant for ischemic stroke? The case for nox 2. Antioxid Redox Signal 18, 1400-1417.

Kahles, T., Luedike, P., Endres, M., Galla, H.J., Steinmetz, H., Busse, R., Neumann-Haefelin, T., Brandes, R.P., 2007. NADPH oxidase plays a central role in blood-brain barrier damage in experimental stroke. Stroke 38, 3000-3006.

Liang, Z., Wu, G., Fan, C., Xu, J., Jiang, S., Yan, X., Di, S., Ma, Z., Hu, W., Yang, Y., 2016. The emerging role of signal transducer and activator of transcription 3 in cerebral ischemic and hemorrhagic stroke. Prog Neurobiol 137, 1-16.

Lin, H.J., Chen, S.T., Wu, H.Y., Hsu, H.C., Chen, M.F., Lee, Y.T., Wu, K.Y., Chien, K.L., 2015. Urinary biomarkers of oxidative and nitrosative stress and the risk for incident stroke: a nested case-control study from a community-based cohort. Int J Cardiol 183, 214-220.

Masoud, S.T., Vecchio, L.M., Bergeron, Y., Hossain, M.M., Nguyen, L.T., Bermejo, M.K., Kile, B., Sotnikova, T.D., Siesser, W.B., Gainetdinov, R.R., Wightman, R.M., Caron, M.G., Richardson, J.R., Miller, G.W., Ramsey, A.J., Cyr, M., Salahpour, A., 2015. Increased expression of the dopamine transporter leads to loss of dopamine neurons, oxidative stress and l-DOPA reversible motor deficits. Neurobiol Dis 74, 66-75.

Mayer, S.A., Rincon, F., 2005. Treatment of intracerebral haemorrhage. Lancet Neurol 4, 662-672.

Nakamura, T., Keep, R.F., Hua, Y., Schallert, T., Hoff, J.T., Xi, G., 2004. Deferoxamine-induced attenuation of brain edema and neurological deficits in a rat model of intracerebral hemorrhage. J Neurosurg 100, 672-678.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95, 351-358.

Park, C.Y., Lee, S.H., Kim, B.K., Shin, M.S., Kim, C.J., Kim, H., 2013. Treadmill exercise ameliorates impairment of spatial learning ability through enhancing dopamine expression in hypoxic ischemia brain injury in neonatal rats. J Exerc Rehabil 9, 406-412.

Paxinos, G., & Watson, C. (1986). The rat brain in stereotaxic coordinates (2 ed.). New York: Academic Press.

Schallert, T., 2006. Behavioral tests for preclinical intervention assessment. NeuroRx 3, 497-504.

Stroobants, S., Gantois, I., Pooters, T., D'Hooge, R., 2013. Increased gait variability in mice with small cerebellar cortex lesions and normal rotarod performance. Behav Brain Res 241, 32-37.

Tang, L.L., Ye, K., Yang, X.F., Zheng, J.S., 2007. Apocynin attenuates cerebral infarction after transient focal ischaemia in rats. J Int Med Res 35, 517-522.

Titova, E., Ostrowski, R.P., Sowers, L.C., Zhang, J.H., Tang, J., 2007. Effects of apocynin and ethanol on intracerebral haemorrhage-induced brain injury in rats. Clin Exp Pharmacol Physiol 34, 845-850.

Walder, C.E., Green, S.P., Darbonne, W.C., Mathias, J., Rae, J., Dinauer, M.C., Curnutte, J.T., Thomas, G.R., 1997. Ischemic stroke injury is reduced in mice lacking a functional NADPH oxidase. Stroke 28, 2252-2258.

Wang, J., Zhuang, H., Dore, S., 2006a. Heme oxygenase 2 is neuroprotective against intracerebral hemorrhage. Neurobiol Dis 22, 473-476.

Wang, Q., Tompkins, K.D., Simonyi, A., Korthuis, R.J., Sun, A.Y., Sun, G.Y., 2006b. Apocynin protects against global cerebral ischemia-reperfusion-induced oxidative stress and injury in the gerbil hippocampus. Brain Res 1090, 182-189.

Wang, T., Jacobson-Kram, D., Pilaro, A.M., Lapadula, D., Jacobs, A., Brown, P., Lipscomb, J., McGuinn, W.D., 2010. ICH guidelines: inception, revision, and implications for drug development. Toxicol Sci 118, 356-367.

Wu, J., Hua, Y., Keep, R.F., Schallert, T., Hoff, J.T., Xi, G., 2002. Oxidative brain injury from extravasated erythrocytes after intracerebral hemorrhage. Brain Res 953, 45-52.

Xi, G., Keep, R.F., 2012. Intracerebral hemorrhage: mechanisms and therapies. Transl Stroke Res 3, 1-3.

Zhang, H.F., Li, T.B., Liu, B., Lou, Z., Zhang, J.J., Peng, J.J., Zhang, X.J., Ma, Q.L., Peng, J., Luo, X.J., 2015a. Inhibition of myosin light chain kinase reduces NADPH oxidase-mediated oxidative injury in rat brain following cerebral ischemia/reperfusion. Naunyn Schmiedebergs Arch Pharmacol 388, 953-963.

Zhang, K., Liu, Y., Liu, X., Chen, J., Cai, Q., Wang, J., Huang, H., 2015b. Apocynin improving cardiac remodeling in chronic renal failure disease is associated with up-regulation of epoxyeicosatrienoic acids. Oncotarget 6, 24699-24708.

PARTE III

3. DISCUSSÃO

Os resultados desta dissertação, apresentados no formato de dois artigos científicos, corroboram com a premissa de que, embora compartilhem de mecanismos fisiopatológicos comuns, o AVE isquêmico e o AVE hemorrágico apresentam peculiaridades, de forma que algumas propostas de neuroproteção podem ser efetivas nos dois modelos, enquanto outras podem ser efetivas em um tipo de AVE e em outro não.

O primeiro estudo apresentado teve como objetivo investigar o papel neuroprotetor do exercício físico diante de um quadro de isquemia-reperfusão cerebral em ratos. Para isso, submetemos os animais a 8 semanas de exercício aeróbico em esteira rolante previamente à cirurgia de oclusão das artérias carótidas comuns, que mimetiza um quadro de isquemia global, seguido de reperfusão sanguínea. A função motora dos animais, assim como o equilíbrio oxidativo estriatal foram avaliados e revelaram o potencial neuroprotetor do exercício. Já se sabe que o exercício físico apresenta inúmeros benefícios para o sistema nervoso central, incluindo melhora na sua plasticidade (Lambert e Evans, 2005) e diminuição da formação de espécies reativas de oxigênio (Schimidt et al., 2014). Nós demonstramos a capacidade do exercício físico em proteger contra os déficits neuromotores causados pelo AVE isquêmico, embora a proteção contra o estresse oxidativo tenha sido parcial. Os déficits motores observadas neste estudo provavelmente são resultantes do evento isquêmico induzido nos ratos (Cechetti et al., 2011), estando intimamente associados à condição de estresse oxidativo (Allen e Bayraktutan, 2009).

O corpo estriado é uma das regiões do cérebro mais afetadas pelo estresse oxidativo após a isquemia-reperfusão cerebral e as suas concentrações relativamente elevadas de receptores GABA e neurônios glutamatérgicos podem estar relacionados com esta neurotoxicidade (Park et al., 2013). As deficiências motoras observadas no primeiro estudo parecem estar relacionadas com o aumento dos níveis de EROs e peroxidação lipídica no corpo estriado, causando desbalanço redox nesta região do cérebro (Masoud et al., 2015). Verificamos que o exercício foi eficaz para prevenir os déficits motores e também em diminuir os níveis de EROs, porém, não a peroxidação lipídica. Aparte de seus efeitos positivos sobre o equilíbrio oxidativo, sabe-se que o exercício físico aeróbico promove uma série de respostas benéficas à saúde cerebral, que vão desde liberação de determinados neurotransmissores e hormônios, até o aumento da plasticidade neuronal, de forma que é

provável que os efeitos do exercício neste modelo não sejam restritos unicamente aos seus efeitos no balanço oxidativo.

No segundo estudo apresentado avaliamos o possível papel neuroprotetor da apocinina sobre o dano motor e o balanço oxidativo no estriado em um modelo de AVE hemorrágico (hemorragia intracerebral) em ratos. Neste estudo demonstramos que o modelo de AVE hemorrágico empregado leva a um dano motor considerável, bem como a alterações significativas no equilíbrio e na função neurológica dos ratos, além de desbalanço redox. Esses resultados são semelhantes a diversos estudos recentes, que demostram que a hemorragia intracerebral gera diminuição da capacidade locomotora e exploratória, do equilíbrio, e, gera déficit neurológico em roedores (Choi et al., 2015; Titova et al., 2007; Quin et al., 2015,).

Nossos resultados indicaram que a administração de apocinina (0,5 mg/kg, 2, 6 e 24h após a HIC) foi ineficaz na reversão dos prejuízos neuromotores e estresse oxidativo estriatal resultantes da hemorragia intracerebral. A degeneração neuronal induzida pelo AVE hemorrágico está diretamente ligada à condição de estresse oxidativo causado pela hemorragia intracerebral (Liang et al., 2015). Em particular, a NADPH oxidase parece desempenhar um papel importante na lesão cerebral induzida pelo HIC, já que em modelos nocautes para o seu gene (gp91phox) há proteção cerebral (Han et al., 2015). Considerando estes aspectos, bem como os resultados prévios obtidos com uso de apocinina em outros modelos de injúria cerebral que envolvem dano oxidativo, nossa hipótese de trabalho era de que a apocinina teria efeitos benéficos no tratamento do AVE hemorrágico.

Em um estudo utilizando a administração de apocinina previamente à lesão (24h e 30 min antes por via intravenosa na dose de 50mg/kg), Zhang e cols. (2015) demonstraram que a apocinina reverte consideravelmente os déficits neurológicos causados pela isquemia-reperfusão cerebral. Em contrapartida, um estudo com o mesmo modelo de lesão isquêmica utilizando a administração de apocinina em curva de doses (2.5, 3.75 e 5mg/kg por via intravenosa 30 minutos antes da lesão isquêmica) mostrou que apenas a menor dose foi eficaz, comparada com as outras doses (Tang et al., 2008). Tais resultados demonstram que a dose e o tempo de administração parecem ser aspectos cruciais para determinação dos efeitos da apocinina.

Neste trabalho optamos por estudar os efeitos de diferentes estratégias neuroprotetoras em diferentes modelos de AVE. Entendemos que os efeitos do exercício físico vão além de sua ação modulatória sobre o estresse oxidativo; possivelmente o exercício físico apresente efeitos positivos de prevenção dos danos motores relacionados ao AVE Hemorrágico, assim

como no AVE Isquêmico; por isso, ainda estamos desenvolvendo pesquisas neste sentido. No entanto, considerando as características do AVE Hemorrágico optamos por buscar uma estratégia que pudesse ser empregada logo após o evento hemorrágico, com o intuito de modular o dano, visto que este tipo de evento vascular, embora mais raro, é, muitas vezes imprevisível e as suas sequelas são normalmente muito mais graves e incapacitantes, daí a escolha da apocinina, que, embora tenha sido empregada com sucesso previamente em modelos de isquemia e TCE, não apresentou os mesmos resultados na presente pesquisa.

4. CONCLUSÕES

Diante dos resultados encontrados neste trabalho, podemos afirmar que o exercício físico é uma estratégia eficaz de neuroproteção em casos de AVE isquêmico, prevenindo os danos motores e atenuando o estresse oxidativo no estriado. Por outro lado, a apocinina não se mostrou uma estratégia eficaz para proteção dos danos causados pelo AVE hemorrágico, ao menos na dose e tempos de administração aqui empregados.

Sendo assim, podemos dizer que o estudo fisiopatológico e morfológico de cada tipo de AVE e dos efeitos de diferentes estratégias neuroprotetoras em cada modelo é de extrema importância, pois, embora compartilhem alguns mecanismos patológicos, os dois modelos diferem em outros aspectos.

5. PERSPECTIVAS

Em busca da melhor compreensão dos mecanismos fisiopatológicos das doenças acima mencionadas e também os possíveis alvos terapêuticos de cada uma, nosso grupo pretende dar seguimento às pesquisas com AVE isquêmico e hemorrágico.

Em relação ao exercício físico estamos buscando novos protocolos e diferentes tipos de exercícios, como por exemplo, a atividade física anaeróbica, como estratégia de neuroproteção e já concluímos os primeiros experimentos comportamentais avaliando a neuroproteção das funções motoras induzida pelo exercício no modelo de AVE Hemorrágico. Ainda, pretendemos realizar avaliações histológicas como complemento ao estudo da apocinina como estratégia de neuroproteção.

6. REFERÊNCIAS

ALLEN, C. L.; BAYRAKTUTAN, U. Oxidative stress and its role in the pathogenesis of ischaemic stroke. Int J Stroke, v. 4, n. 6, p. 461-70, Dec 2009. ISSN 1747-4949 (Electronic)
1747-4930 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19930058 >.

Santamaría-del Ángel, D. et al. Comparative effects of catechin, epicatechin and N-Ω-nitroarginine on quinolinic acid-induced oxidative stress in rat striatum slices. Biomedicine & Pharmacotherapy n. 78 p. 210–215. 2016.

ARONOWSKI, J.; ZHAO, X. Molecular pathophysiology of cerebral hemorrhage: secondary brain injury. Stroke, v. 42, n. 6, p. 1781-6, Jun 2011. ISSN 1524-4628 (Electronic)
0039-2499 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21527759 >.

ARRICK, D. M. et al. Vigorous exercise training improves reactivity of cerebral arterioles and reduces brain injury following transient focal ischemia. Microcirculation, v. 21, n. 6, p. 516-23, Aug 2014. ISSN 1549-8719 (Electronic)
1073-9688 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24617555 >.

AUSTIN, M. W. et al. Aerobic exercise effects on neuroprotection and brain repair following stroke: a systematic review and perspective. Neurosci Res, v. 87, p. 8-15, Oct 2014. ISSN 1872-8111 (Electronic)
0168-0102 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24997243 >.

BAEZ-MENDOZA, R.; SCHULTZ, W. The role of the striatum in social behavior. Front Neurosci, v. 7, p. 233, 2013. ISSN 1662-4548 (Print)
1662-453X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24339801 >.

BRUNSWICK, A. S. et al. Serum biomarkers of spontaneous intracerebral hemorrhage induced secondary brain injury. J Neurol Sci, v. 321, n. 1-2, p. 1-10, Oct 15 2012. ISSN 1878-5883 (Electronic)
0022-510X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22857988 >.

CECHETTI, F. et al. Forced treadmill exercise prevents oxidative stress and memory deficits following chronic cerebral hypoperfusion in the rat. Neurobiol Learn Mem, v. 97, n. 1, p. 90-6, Jan 2012. ISSN 1095-9564 (Electronic)
1074-7427 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22001013 >.

CHEN-ROETLING, J.; CAI, Y.; REGAN, R. F. Neuroprotective effect of heme oxygenase-2 knockout in the blood injection model of intracerebral hemorrhage. BMC Res Notes, v. 7, p. 561, 2014. ISSN 1756-0500 (Electronic) 1756-0500 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25149897 >.

CHEN, S. et al. Asiaticoside attenuates memory impairment induced by transient cerebral ischemia-reperfusion in mice through anti-inflammatory mechanism. Pharmacol Biochem Behav, v. 122, p. 7-15, Jul 2014. ISSN 1873-5177 (Electronic) 0091-3057 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24631487 >.

CHOI, D. H. et al. Role of neuronal NADPH oxidase 1 in the peri-infarct regions after stroke. PLoS One, v. 10, n. 1, p. e0116814, 2015. ISSN 1932-6203 (Electronic) 1932-6203 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25617620 >.

DISHMAN, R. K. et al. Neurobiology of exercise. Obesity (Silver Spring), v. 14, n. 3, p. 345-56, Mar 2006. ISSN 1930-7381 (Print) 1930-7381 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16648603 >.

DONNAN, G.A, Fisher M, Macleod M and Davis SM: Stroke. Lancet 371: 1612-1623, 2008.

DURUKAN, A.; TATLISUMAK, T. Acute ischemic stroke: overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia. Pharmacol Biochem Behav, v. 87, n. 1, p. 179-97, May 2007. ISSN 0091-3057 (Print) 0091-3057 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17521716 >.

ENGELS, F. et al. Effects of apocynin, a drug isolated from the roots of Picrorhiza kurroa, on arachidonic acid metabolism. FEBS Lett, v. 305, n. 3, p. 254-6, Jul 6 1992. ISSN 0014-5793 (Print) 0014-5793 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1299626 >.

EXTRAMIANA, F.; MAISON-BLANCHE, P. Stroke and atrial fibrillation: where to go from here? Stroke, v. 46, n. 3, p. 605-7, Mar 2015. ISSN 1524-4628 (Electronic) 0039-2499 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25634002 >.

FARKOUH, M. E. et al. Strategies for multivessel revascularization in patients with diabetes. N Engl J Med, v. 367, n. 25, p. 2375-84, Dec 20 2012. ISSN 1533-4406 (Electronic) 0028-4793 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23121323 >.

FLORES, M. F. et al. Effects of green tea and physical exercise on memory impairments associated with aging. Neurochem Int, v. 78, p. 53-60, Dec 2014. ISSN 1872-9754 (Electronic)

0197-0186 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25195719 >.

GERFEN, C. R. The neostriatal mosaic: multiple levels of compartmental organization. Trends Neurosci, v. 15, n. 4, p. 133-9, Apr 1992. ISSN 0166-2236 (Print)
0166-2236 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1374971 >.

GO, A. S. et al. Heart disease and stroke statistics--2014 update: a report from the American Heart Association. Circulation, v. 129, n. 3, p. e28-e292, Jan 21 2014. ISSN 1524-4539 (Electronic)
0009-7322 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24352519 >.

HAN, B. H. et al. Contribution of reactive oxygen species to cerebral amyloid angiopathy, vasomotor dysfunction, and microhemorrhage in aged Tg2576 mice. Proc Natl Acad Sci U S A, v. 112, n. 8, p. E881-90, Feb 24 2015. ISSN 1091-6490 (Electronic)
0027-8424 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25675483 >.

HEUMULLER, S. et al. Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant. Hypertension, v. 51, n. 2, p. 211-7, Feb 2008. ISSN 1524-4563 (Electronic)
0194-911X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18086956 >.

JAVED, H. et al. S-allyl cysteine attenuates oxidative stress associated cognitive impairment and neurodegeneration in mouse model of streptozotocin-induced experimental dementia of Alzheimer's type. Brain Res, v. 1389, p. 133-42, May 10 2011. ISSN 1872-6240 (Electronic)
0006-8993 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21376020 >.

KIM, J. H. et al. Post-treatment of an NADPH oxidase inhibitor prevents seizure-induced neuronal death. Brain Res, v. 1499, p. 163-72, Mar 7 2013. ISSN 1872-6240 (Electronic)
0006-8993 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23313582 >.

KIRKMAN, M. A.; ALLAN, S. M.; PARRY-JONES, A. R. Experimental intracerebral hemorrhage: avoiding pitfalls in translational research. J Cereb Blood Flow Metab, v. 31, n. 11, p. 2135-51, Nov 2011. ISSN 1559-7016 (Electronic)
0271-678X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21863040 >.

LAI, T. W.; ZHANG, S.; WANG, Y. T. Excitotoxicity and stroke: identifying novel targets for neuroprotection. Prog Neurobiol, v. 115, p. 157-88, Apr 2014. ISSN 1873-5118 (Electronic)
0301-0082 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24361499 >.

LAMBERT, C. P.; EVANS, W. J. Adaptations to aerobic and resistance exercise in the elderly. Rev Endocr Metab Disord, v. 6, n. 2, p. 137-43, May 2005. ISSN 1389-9155 (Print)
1389-9155 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15843885 >.

LI, P. F. et al. Lactuside B decreases aquaporin-4 and caspase-3 mRNA expression in the hippocampus and striatum following cerebral ischaemia-reperfusion injury in rats. Exp Ther Med, v. 7, n. 3, p. 675-680, Mar 2014. ISSN 1792-0981 (Print)
1792-0981 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24520266 >.

LIANG, D. et al. Cytotoxic edema: mechanisms of pathological cell swelling. Neurosurg Focus, v. 22, n. 5, p. E2, 2007. ISSN 1092-0684 (Electronic)
1092-0684 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17613233 >.

LIANG, Z. et al. The emerging role of signal transducer and activator of transcription 3 in cerebral ischemic and hemorrhagic stroke. Prog Neurobiol, v. 137, p. 1-16, Feb 2016. ISSN 1873-5118 (Electronic)
0301-0082 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/26738445 >.

LIN, T. W.   et al. Running exercise delays neurodegeneration in amygdala and hippocampus of Alzheimer's disease (APP/PS1) transgenic mice. Neurobiol Learn Mem, v. 118, p. 189-97, Feb 2015. ISSN 1095-9564 (Electronic)
1074-7427 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25543023 >.

MASOUD, S. T. et al. Increased expression of the dopamine transporter leads to loss of dopamine neurons, oxidative stress and l-DOPA reversible motor deficits. Neurobiol Dis, v. 74, p. 66-75, Feb 2015. ISSN 1095-953X (Electronic)
0969-9961 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25447236 >.

MINNERUP, J.   et al. Neuroprotection for stroke: current status and future perspectives. Int J Mol Sci, v. 13, n. 9, p. 11753-72, 2012. ISSN 1422-0067 (Electronic)
1422-0067 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23109881 >.

MORETTI, A.; FERRARI, F.; VILLA, R. F. Neuroprotection for ischaemic stroke: current status and challenges. Pharmacol Ther, v. 146, p. 23-34, Feb 2015. ISSN 1879-016X (Electronic)
0163-7258 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25196155 >.

NOTARIUS, C. F. et al. Divergent muscle sympathetic responses to dynamic leg exercise in heart failure and age-matched healthy subjects. J Physiol, v. 593, n. 3, p. 715-22, Feb 1 2015. ISSN 1469-7793 (Electronic)

0022-3751 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25398528 >.

O'DONNELL, M. J. et al. Risk factors for ischaemic and intracerebral haemorrhagic stroke in 22 countries (the INTERSTROKE study): a case-control study. Lancet, v. 376, n. 9735, p. 112-23, Jul 10 2010. ISSN 1474-547X (Electronic)
0140-6736 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20561675 >.

OHK, T. G. et al. Neuronal damage using fluoro-jade B histofluorescence and gliosis in the striatum after various durations of transient cerebral ischemia in gerbils. Neurochem Res, v. 37, n. 4, p. 826-34, Apr 2012. ISSN 1573-6903 (Electronic)
0364-3190 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22219128 >.

PARK, C. Y. et al. Treadmill exercise ameliorates impairment of spatial learning ability through enhancing dopamine expression in hypoxic ischemia brain injury in neonatal rats. J Exerc Rehabil, v. 9, n. 4, p. 406-12, 2013. ISSN 2288-176X (Print)
2288-176X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24278893 >.

Pawlowski, J. et al.Memory Evaluation of Patients with Lesion in the Basal Ganglia and Thalamus Following Stroke. Psicologia em Pesquisa v. 7 n .1, p.79-88. 2013

QIN, J. et al. Transplantation of Induced Pluripotent Stem Cells Alleviates Cerebral Inflammation and Neural Damage in Hemorrhagic Stroke. PLoS One, v. 10, n. 6, p. e0129881, 2015. ISSN 1932-6203 (Electronic)
1932-6203 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/26086994 >.

SARKAKI, A. et al. Improvement in Memory and Brain Long-term Potentiation Deficits Due to Permanent Hypoperfusion/Ischemia by Grape Seed Extract in Rats. Iran J Basic Med Sci, v. 16, n. 9, p. 1004-10, Sep 2013. ISSN 2008-3866 (Print)
2008-3866 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24171080 >.

SCHIMIDT, H. L. et al. Memory deficits and oxidative stress in cerebral ischemia-reperfusion: neuroprotective role of physical exercise and green tea supplementation. Neurobiol Learn Mem, v. 114, p. 242-50, Oct 2014. ISSN 1095-9564 (Electronic)
1074-7427 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25062645 >.

SMITH, W. S. Pathophysiology of focal cerebral ischemia: a therapeutic perspective. J Vasc Interv Radiol, v. 15, n. 1 Pt 2, p. S3-12, Jan 2004. ISSN 1051-0443 (Print)
1051-0443 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15101511 >.

SOSA, P. M. et al. Physical exercise prevents motor disorders and striatal oxidative imbalance after cerebral ischemia-reperfusion. Braz J Med Biol Res, v. 48, n. 9, p. 798-804, Sep 2015. ISSN 1414-431X (Electronic)

0100-879X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/26222650 >.

SPATZ, M. Past and recent BBB studies with particular emphasis on changes in ischemic brain edema: dedicated to the memory of Dr. Igor Klatzo. Acta Neurochir Suppl, v. 106, p. 21-7, 2010. ISSN 0065-1419 (Print)
0065-1419 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19812915 >.

SUTHERLAND, B. A. et al. Neuroprotection for ischaemic stroke: translation from the bench to the bedside. Int J Stroke, v. 7, n. 5, p. 407-18, Jul 2012. ISSN 1747-4949 (Electronic)
1747-4930 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22394615 >.

SUTO, Y. et al. Relationship between three-year survival and functional outcome at discharge from acute-care hospitals in each subtype of first-ever ischemic stroke patients. Internal Medicine 2011;50:1377–83.

TANIGUCHI, M. et al. Induction of aquaporin-4 water channel mRNA after focal cerebral ischemia in rat. Brain Res Mol Brain Res, v. 78, n. 1-2, p. 131-7, May 31 2000. ISSN 0169-328X (Print)
0169-328X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10891592 >.

TOUYZ, R. M. Apocynin, NADPH oxidase, and vascular cells: a complex matter. Hypertension, v. 51, n. 2, p. 172-4, Feb 2008. ISSN 1524-4563 (Electronic)
0194-911X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18086948 >.

WAKISAKA, Y. et al. Oxidative stress through activation of NAD(P)H oxidase in hypertensive mice with spontaneous intracranial hemorrhage. J Cereb Blood Flow Metab, v. 28, n. 6, p. 1175-85, Jun 2008. ISSN 0271-678X (Print)
0271-678X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18301433 >.

WILMORE J.H; Costil D. Fisiologia do esporte e do exercício. São Paulo: Manole; 2001

WONG, S. P.; FONG, V. H.; VIEIRA, A. Activities of apocynin in cytotoxicity assays of potential pathological relevance. Biomed Pharmacother, v. 76, p. 6-10, Dec 2015. ISSN 1950-6007 (Electronic)
0753-3322 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/26653543 >.

XI, G.; KEEP, R. F. Intracerebral hemorrhage: mechanisms and therapies. Transl Stroke Res, v. 3, n. Suppl 1, p. 1-3, Jul 2012. ISSN 1868-601X (Electronic)
1868-4483 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24323857 >.

XIONG, L. et al. Intracerebral hemorrhage and cognitive impairment. Biochim Biophys Acta, Dec 12 2015. ISSN 0006-3002 (Print)
0006-3002 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/26692171 >.

ZHOU, Y. et al. Inflammation in intracerebral hemorrhage: from mechanisms to clinical translation. Prog Neurobiol, v. 115, p. 25-44, Apr 2014. ISSN 1873-5118 (Electronic)
0301-0082 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24291544 >.

ZIAI, W. C. Hematology and inflammatory signaling of intracerebral hemorrhage. Stroke, v. 44, n. 6 Suppl 1, p. S74-8, Jun 2013. ISSN 1524-4628 (Electronic)
0039-2499 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23709738 >.

ZUHAID, M. et al. Frequency of modifiable risk factors in stroke patients. J Ayub Med Coll Abbottabad, v. 26, n. 2, p. 235-8, Apr-Jun 2014. ISSN 1025-9589 (Print)
1025-9589 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25603685 >.

7. ANEXOS

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

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CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: 002/2014

Título: ANÁLISE DAS DISFUNÇÕES MOTORAS PROVENIENTES DE DIFERENTES INJÚRIAS IMPOSTAS AO SISTEMA NERVOSO CENTRAL EM RATOS

Data da aprovação: 28/05/2014

Período de vigência do projeto: De: 05/2014   Até: 05/2017

Pesquisador: PÂMELA BILLIG MELLO CARPES

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MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

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CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: **032/2014**

Titulo: **Efeitos da administração de apocinina no dano oxidativo e déficit neuromotor induzido pelo Acidente Vascular Encefálico do subtipo hemorrágico em ratos**

Data da aprovação: **13/11/2014**

Período de vigência do projeto: De: **11/2014**   Até: **11/2017**

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