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Efeitos da administração de progesterona e da hipotermia terapêutica sobre a lesão neural, a reatividade astrocitária e o comportamento em ratos Wistar submetidos à hipóxia-isquemia neonatal

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Fisiologia do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de doutor em Fisiologia.

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Co-orientador(a), Prof. Dr. Luciano Stümer de Fraga

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Apresentação

Esta tese é constituída por:

PARTE I:

1. Introdução: contém o embasamento teórico necessário para a compreensão da proposta de trabalho e objetivos.

2. Hipótese: expõe os principais resultados esperados.

3. Objetivos: definem os propósitos centrais do trabalho, desenvolvidos ao longo dos capítulos 1 e 2.

PARTE II:

Capítulo 1: Artigo publicado no periódico Cellular and Molecular Neurobiology – "Long-Lasting Actions of Progesterone Protect the Neonatal Brain Following Hypoxia-Ischemia".

Capítulo 2: Artigo submetido ao periódico Brain Research – Therapeutic hypothermia for the treatment of neonatal hypoxia-ischemia: sex-dependent modulation of astrocyte reactivity

PARTE III:

Discussão Geral: contém a interpretação dos resultados obtidos nos Capítulos 1 e 2, englobando-os em um contexto geral.

Conclusões: apresenta as conclusões gerais da tese.

Referências: lista as referências citadas nas seções Introdução e Discussão.

Lista de figuras

Lista de abreviaturas

HI: Hipóxia-isquemia

ATP: Adenosina trifosfato

TNF: Fator de necrose tumoral

IL: Interleucina

PI3K: fosfoinositídeo-3-cinase

HT: Hipotermia terapêutica

EEG: Eletroencefalografia

PROG: Progesterona

Bad: Proteína de morte associada ao Bcl-2

SNC: Sistema Nervoso Central

OPCs: Células progenitoras de oligodendrócitos

BHE: Barreira hematoencefálica

GFAP: Proteína glial fibrilar ácida

AIF: Fator indutor de apoptose

NF-κB: Fator nuclear κ-B

GABA: Ácido gama-aminobutírico

MAPK: Proteína cinase ativada por mitógenos

CA1: *Corno de Ammon-1*

TLRs: Receptores Toll-*like*

Sumário

Resumo

A hipóxia-isquemia (HI) neonatal moderada a grave afeta 1–3 a cada 1.000 nascidos a termo e continua sendo uma causa significativa de deficiência do neurodesenvolvimento de longo prazo. A morbidade neurológica nos sobreviventes envolve dificuldades de aprendizado, epilepsia e paralisia cerebral. O uso de hipotermia é a única terapia utilizada na clínica, no entanto, o tratamento deve ser iniciado dentro de uma janela terapêutica de 6h após o evento hipóxico-isquêmico. Os efeitos neuroprotetores da hipotermia terapêutica experimental iniciada em momentos distintos após a HI, bem como a provável influência do dimorfismo sexual sobre tais efeitos ainda não foram elucidados. A eficácia de potenciais agentes neuroprotetores tem sido testada em modelos animais. Há razão para se pensar que a progesterona tem um forte potencial para o tratamento da HI neonatal, já que a sua utilização tem se mostrado benéfica em pesquisas relacionadas à lesão cerebral traumática, lesão cerebral isquêmica e outros modelos de lesão do sistema nervoso central em adultos. Essa Tese tem como objetivos avaliar os efeitos da administração de progesterona e da hipotermia terapêutica sobre a lesão neural, a reatividade astrocitária e o neurodesenvolvimento em ratos Wistar submetidos à hipóxia-isquemia neonatal. No primeiro capítulo da Tese avaliamos o efeito da progesterona em animais machos neonatais submetidos ao modelo de HI neonatal. Os animais foram divididos em cinco grupos experimentais: SHAM, HI, HI+PROG-PRÉ (PRÉ), HI+PROG-PÓS (PÓS), HI+PROG-PRÉ/PÓS (PP). Os termos PRÉ e PÓS referem-se à administração de progesterona (na dose de 5 mg/kg) antes ou após o procedimento de HI neonatal. Dependendo do grupo experimental, os animais foram tratados com progesterona imediatamente antes da isquemia e/ou 6 e 24 horas após o início da hipóxia. Foram analisados volume de lesão do hemisfério e do hipocampo ipsilateral à lesão, além de células degenerativas nas áreas CA1 e hilo do hipocampo, como também a expressão das proteínas Akt, caspase-3 e GFAP. Neste capítulo foi possível observar neuroproteção da administração da progesterona quando administrada 6 e 24h após a lesão, reduzindo todos as propriedades analisadas em comparação ao grupo HI sem tratamento. No segundo capítulo avaliamos os efeitos da hipotermia terapêutica experimental em animais submetidos ao modelo de HI neonatal de ambos os sexos em diferentes janelas terapêuticas. Animais machos e fêmeas foram tratados por hipotermia (32ºC) por 5h, o começo do tratamento foi distinto para cada grupo: 2h, 4h, e 6h após a lesão. Após 7 dias da lesão os animais foram a testes comportamentais e foram avaliados o volume de lesão no hemisfério, hipocampo e córtex cerebral, além de células degenerativas e astrogliose no hipocampo. Neste capítulo foi observado que os efeitos da hipotermia terapêutica podem ser dependentes tanto do momento de início do tratamento como do sexo dos animais. A HT iniciada 2h após a HI causou redução da lesão neural e dos tempos de latência nos testes comportamentais em ratos de ambos os sexos. Também foi possível ver um efeito na astrogliose dependente do sexo, uma vez que apenas fêmeas que iniciaram o tratamento 2h após o início da lesão apresentaram redução dos parâmetros morfológicos de astrogliose. No entanto, quando o tratamento tinha início ao final da janela terapêutica (6h), as fêmeas apresentavam aumento de células degenerativas e do tempo de latência em comparação ao grupo submetido ao modelo, mas sem tratamento. Os resultados na presente tese sugerem que a progesterona é um potencial candidato para o tratamento da HI neonatal, por ter produzido melhoras mesmo quando administrada após 6h da lesão; já os efeitos da hipotermia são dependentes do tempo de início do tratamento e do sexo dos animais.

Palavras-chave: hipóxia-isquemia neonatal (HI); progesterona; hipotermia terapêutica; janela terapêutica, astrogliose.

Abstract

Moderate to severe neonatal hypoxia-ischemia (HI) affects 1–3 per 1,000 full-term births and remains a significant cause of long-term neurodevelopmental deficiency. Neurological morbidity in survivors involves learning disabilities, epilepsy and cerebral palsy. The use of hypothermia is the only therapy used in the clinic, however, treatment must be started within a therapeutic window of 6 hours after the hypoxic-ischemic event. The neuroprotective effects of experimental therapeutic hypothermia initiated at different times after IH, as well as the likely influence of sexual dimorphism on such effects have not yet been elucidated. The effectiveness of potential neuroprotective agents has been tested in animal models. There is reason to think that progesterone has a strong potential for the treatment of neonatal HI, since its use has been shown to be beneficial in research related to traumatic brain injury, ischemic brain injury and other models of central nervous system injury in adults . This Thesis aims to evaluate the effects of progesterone administration and therapeutic hypothermia on neural injury, astrocytic reactivity and neurodevelopment in Wistar rats submitted to neonatal hypoxia-ischemia. In the first chapter of the Thesis we evaluated the effect of progesterone in neonatal male animals submitted to the neonatal HI model. The animals were divided into five experimental groups: SHAM, HI, HI + PROG-PRE (PRE), HI + PROG-POST (POST), HI + PROG-PRE / POST (PP). The terms PRE and POST refer to the administration of progesterone (at a dose of 5 mg / kg) before or after the neonatal HI procedure. Depending on the experimental group, the animals were treated with progesterone immediately before ischemia and / or 6 and 24 hours after the onset of hypoxia. Lesion volume of the hemisphere and hippocampus ipsilateral to the lesion were analyzed, as well as degenerative cells in the CA1 and hilum areas of the hippocampus, as well as the expression of the Akt, caspase-3 and GFAP proteins. In this chapter, it was possible to observe neuroprotection of progesterone administration when administered 6 and 24h after the injury, reducing all the properties analyzed in comparison to the HI group without treatment. In the second chapter, we evaluated the effects of experimental therapeutic hypothermia in animals submitted to the neonatal HI model of both sexes in different therapeutic windows. Male and female animals were treated by hypothermia (32ºC) for 5h, the beginning of treatment was different for each group: 2h, 4h, and 6h after the injury. After 7 days of the injury, the animals underwent behavioral tests and the volume of injury in the hemisphere, hippocampus and cerebral cortex was evaluated, in addition to degenerative cells and astrogliosis in the hippocampus. In this chapter it was observed that the effects of therapeutic hypothermia can be dependent both on the time of initiation of treatment and on the sex of the animals. HT initiated 2h after HI caused a reduction in neural injury and latency times in behavioral tests in rats of both sexes. It was also possible to see an effect on sex-dependent astrogliosis, since only females who started treatment 2h after the beginning of the lesion showed a reduction in the morphological parameters of astrogliosis. However, when treatment started at the end of the therapeutic window (6h), females had increased degenerative cells and latency time compared to the group submitted to the model, but without treatment. The results in the present thesis suggest that progesterone is a potential candidate for the treatment of neonatal HI, as it produced improvements even when administered 6 hours after the injury; the effects of hypothermia, on the other hand, are dependent on the time the treatment started and the sex of the animals.

Keywords: neonatal hypoxia-ischemia (HI); progesterone; therapeutic hypothermia; therapeutic window, astrogliosis.

1. Introdução

A encefalopatia hipóxico-isquêmica neonatal, ou apenas hipóxia-isquemia (HI), é uma das principais causas de mortalidade e morbidade em crianças (Knox et al., 2013). A HI ocorre em cerca de 0,2% dos recém-nascidos, sendo que 60% destes são prematuros. Quando sobrevivem, 25% apresentam deficiências neuropsicológicas permanentes, como dificuldade de aprendizado, epilepsia e paralisia cerebral (McQuillen and Ferriero, 2006). As causas da HI, em sua maioria, ocorrem durante a gestação, porém podem ocorrer também no momento e após o nascimento (Douglas-Escobar and Weiss, 2013).

Segundo estudos clínicos, 20% dos casos de HI são causados por insulto anteparto; em 35% por distúrbios maternos, como diabetes, retardo de crescimento intrauterino e infeções; já 10% dos casos são causados por eventos pós-natais; e em 35% dos casos a HI surge em decorrência de problemas durante o trabalho de parto (Silveira and Procianoy, 2015).

A compreensão da fisiopatologia da HI neonatal é essencial para a concepção de intervenções eficazes para os recém-nascidos que sofrem eventos hipóxico-isquêmicos encefálicos (Vannucci and Hagberg, 2004), já que há poucas opções terapêuticas para o tratamento da HI (Ferriero, 2004). A única terapia bem estabelecida atualmente para a HI em neonatos nascidos a termo é a hipotermia terapêutica (HT). No entanto, a HT é apenas parcialmente protetora, reduzindo a taxa combinada de morte e de deficiências graves aos 18 meses de vida em torno de 11% (Cho et al., 2020). Além disso, ainda se estima um resultado adverso em 45% dos casos nos quais a hipotermia é utilizada. Devido a isso, a eficácia de outras abordagens neuroprotetoras têm sido testada em modelos animais (Rees et al., 2011).

1.1 Modelo experimental de hipóxia-isquemia neonatal

Modelos experimentais tentam reproduzir as lesões do sistema nervoso central (SNC) encontradas em recém-nascidos humanos acometidos por HI (Johnston et al., 2001; Rice et al., 1981). Para a padronização de um melhor modelo experimental, estudos também têm observado certas semelhanças na maturação encefálica de ratos de 7 dias de idade com bebês recém-nascidos a termo (Rice et al., 1981). Utilizando o modelo animal de HI de Rice e Vannucci (Figura 1) é possível observar danos encefálicos semelhantes aos encontrados em recém-nascidos a termo acometidos por HI neonatal (Vannucci, 1990).

O modelo consiste na associação da oclusão unilateral de uma das artérias carótidas comuns com a exposição a uma atmosfera hipóxica (geralmente 8% de oxigênio) para produzir dano cerebral unilateral. Os danos podem variar desde morte neuronal, por necrose e/ou apoptose, infarto tecidual generalizado ou até uma combinação de ambos (Johnston et al., 2001; Rice et al., 1981). A lesão ocasionada por este modelo é normalmente restrita ao hemisfério ipsilateral à ligadura carotídea e é observada principalmente no córtex cerebral, na substância branca periventricular, no estriado, no tálamo e no hipocampo. Esses danos são raramente observados no hemisfério contralateral (Vannucci and Hagberg, 2004).

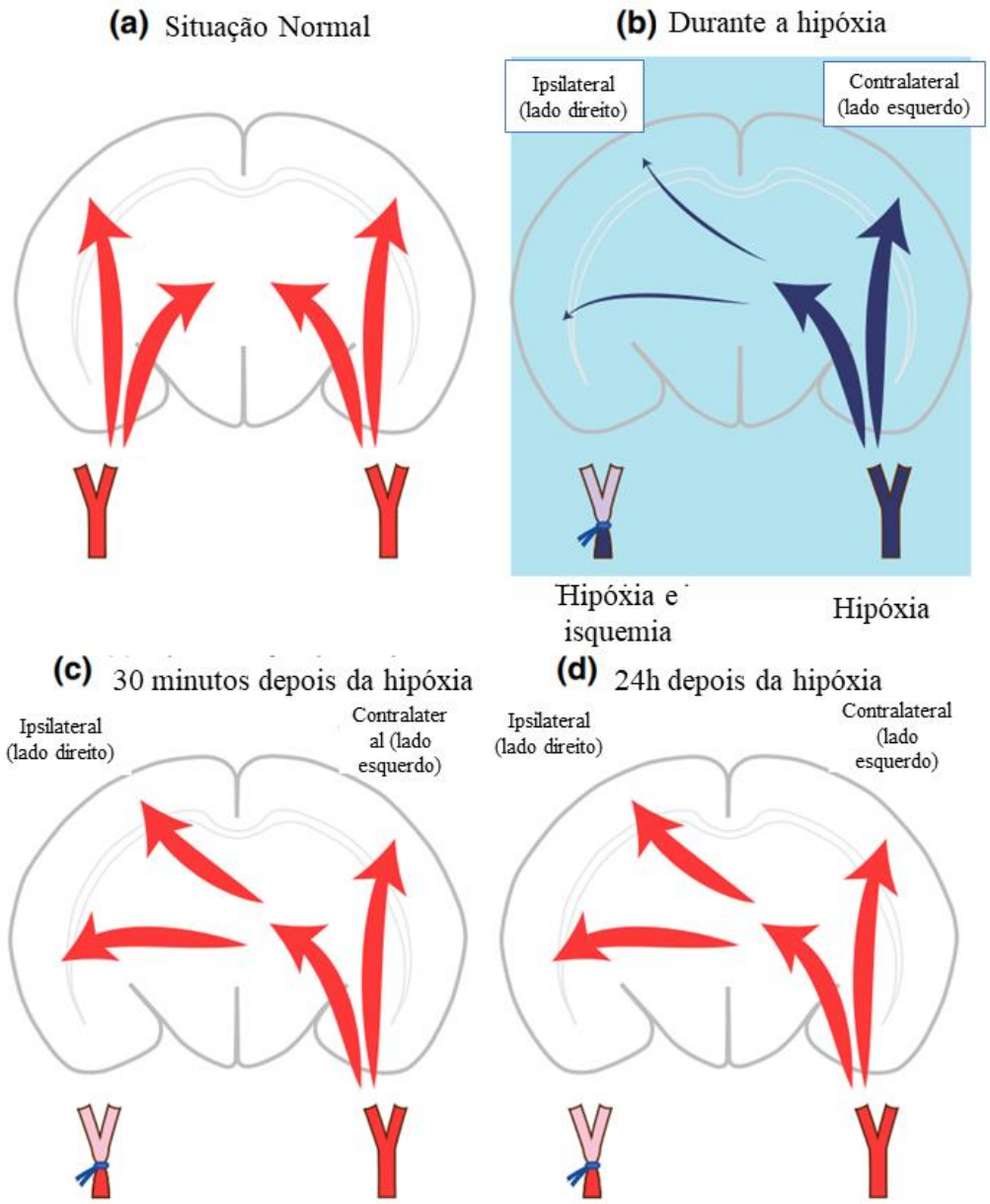

Figura 1. A figura "a" apresenta a situação normal, com fluxo sanguíneo através de ambas artérias carótidas comuns. Em "b", a artéria carótida comum direita está ocluída permanentemente. No entanto, a oclusão não causa dano cerebral por si só. Entretanto, quando o animal é submetido à hipóxia, a combinação entre oclusão da carótida e hipóxia leva a uma redistribuição do fluxo sanguíneo cerebral. O fluxo sanguíneo no hemisfério cerebral contralateral à oclusão não sofre mudanças significativas, enquanto o córtex parietal e frontal, bem como a substância branca subcortical ipsilateral à oclusão, recebem apenas 15-20% do fluxo sanguíneo. Assim, o hemisfério ipsilateral à isquemia está sujeito à hipóxia e isquemia, enquanto o hemisfério contralateral à oclusão está sujeito apenas à hipóxia. Quando a hipóxia termina, o fluxo sanguíneo é restabelecido, e 30 minutos após a hipóxia (c) o hemisfério ipsilateral à isquemia tem fluxo sanguíneo igual em comparação com o hemisfério contralateral à oclusão, ou seja, é perfundido novamente e a isquemia é encerrada. Este fluxo sanguíneo é mantido pelo menos até 24 horas após a hipóxia (d). Adaptado de Brekke e colaboradores (Brekke et al., 2017).

1.2 Fisiopatologia da HI

Estudos experimentais *in vivo* e *in vitro*, e observações clínicas permitiram desenvolver conhecimento sobre a fisiopatologia do evento HI; o entendimento é de que não se trata de um único "evento", mas de um processo evolutivo que leva à morte celular retardada (Figura 2) (Davidson et al., 2015). Durante o período imediato de HI (a fase "primária" da lesão), a deterioração do estado energético está associada à redução da síntese de ATP, prejudicando o equilíbrio iônico através da membrana celular, invertendo as concentrações iônicas e gerando edema cerebral (Korc et al., 1995; Prandini et al., 2005). Em função desta diminuição da concentração de ATP, há uma dificuldade em manter a atividade das bombas iônicas, como a bomba Na^+/K^+-ATPase, levando a uma despolarização da célula que causa uma massiva liberação de neurotransmissores, especialmente do aminoácido excitatório glutamato (du Plessis and Johnston, 1997; E F Sanches et al., 2013). Durante o evento hipóxico, os neurônios pós-sinápticos que respondem a glutamato são ativados pela maior entrada de cálcio devido à despolarização (Delivoria-Papadopoulos and Mishra, 1998), e ocorre acúmulo de neurotransmissores na fenda sináptica devido à falha da recaptação pelos astrócitos e à liberação excessiva mediada pela despolarização (Davidson et al., 2015).

Embora possa haver morte neuronal durante um período suficientemente prolongado de isquemia ou asfixia, muitos neurônios podem se recuperar do insulto, ao menos parcialmente, em um período conhecido como "fase latente", que normalmente se estende ao longo das primeiras 6 horas após a lesão (Cho et al., 2020). Estudos mostraram que neonatos com evidências de asfixia moderada a grave apresentam recuperação transitória do metabolismo oxidativo cerebral após o nascimento, seguida por deterioração secundária com insuficiência energética cerebral entre 6 a 15 horas após o nascimento (Azzopardi et al., 1989).

A gravidade da fase secundária está intimamente relacionada com o neurodesenvolvimento avaliado no primeiro e no 4º anos de vida, e neonatos com encefalopatia que não apresentaram recuperação inicial do metabolismo oxidativo cerebral tiveram piora em seus resultados (Azzopardi et al., 1989). Um padrão idêntico de recuperação inicial do metabolismo oxidativo cerebral seguido por falha energética ("secundária") também é visto após a HI em modelos animais de diversas espécies como porcos, ratos e ovelhas, e está intimamente relacionado à gravidade da lesão neuronal (Bennet et al., 2006; Blumberg et al., 1997; Lorek et al., 1994). O momento da falha energética após a HI está estreitamente associado ao aparecimento de dano cerebral, o que pode ser interpretado como um aumento da morte celular em andamento (Davidson et al., 2015).

Após a morte celular durante a fase secundária, há uma fase terciária de reparo e reorganização. Durante este período, o desenvolvimento de novas células e a "reconfiguração" dos circuitos neuronais sobreviventes são estimulados. Ao mesmo tempo, há evidências de que em alguns ambientes a apoptose fisiológica pode ser regulada positivamente, prejudicando a maturação e a sobrevivência de novas células em longo prazo (Cho et al., 2020; Marín-padilla, 1997). Os mecanismos precisos que contribuem para o desenvolvimento prolongado da lesão não são totalmente conhecidos, mas envolvem inflamação crônica e alterações epigenéticas (Davidson et al., 2015). A reação inflamatória tem início com a infiltração de leucócitos periféricos, indução microglial local e astrogliose (Xiong et al., 2009). Astrócitos reativos liberam citocinas inflamatórias, como o fator de necrose tumoral-α (TNF-α) e a IL-6 por meio de um mecanismo que pode resultar em ação modulatória sustentada em neurônios vizinhos (Davidson et al., 2015; Xiong et al., 2009).

A regulação da proliferação celular e de vias apoptóticas associadas à morte celular é uma abordagem importante para o entendimento do evento hipóxico-isquêmico. PI3K/Akt/mTOR são cinases ativadas por muitos estímulos celulares e regulam funções celulares fundamentais, incluindo transcrição, tradução, proliferação, crescimento e sobrevivência (Asati et al., 2016). A ativação da PI3K por citocinas promove à fosforilação da Akt nos resíduos T308 e S473 (Calautti et al., 2005). A Akt ativada também impede a liberação de cálcio da mitocôndria, um processo importante para a apoptose (Asati et al., 2016).

A ativação da Akt estimula substratos envolvidos na sobrevivência celular e inibe substratos pró-apoptóticos, como Bad e caspase-9, inibindo as vias de apoptose e contribuindo para a sobrevivência celular (Cardone et al., 1998; Kim et al., 2007; Nair and Olanow, 2008).

A maioria dos estudos sobre o envolvimento da proteína Akt na lesão isquêmica observou algum grau de alteração na fosforilação dessa enzima, nos mais variados períodos após a lesão. Os processos de fosforilação/desfosforilação são mecanismos regulatórios que levam à ativação/inativação da Akt (Kitagawa et al., 2002a). Entretanto, outra forma de inativação da enzima é sua clivagem pela caspase-3, que anula os efeitos anti-apoptóticos da Akt (François and Grimes, 1999). A ativação da Akt promove sobrevivência através de diferentes ações celulares. Uma ação que pode mediar os efeitos neuroprotetores da Akt durante eventos hipóxicos é o aumento da captação de glicose pelas células afetadas (Li et al., 2015).

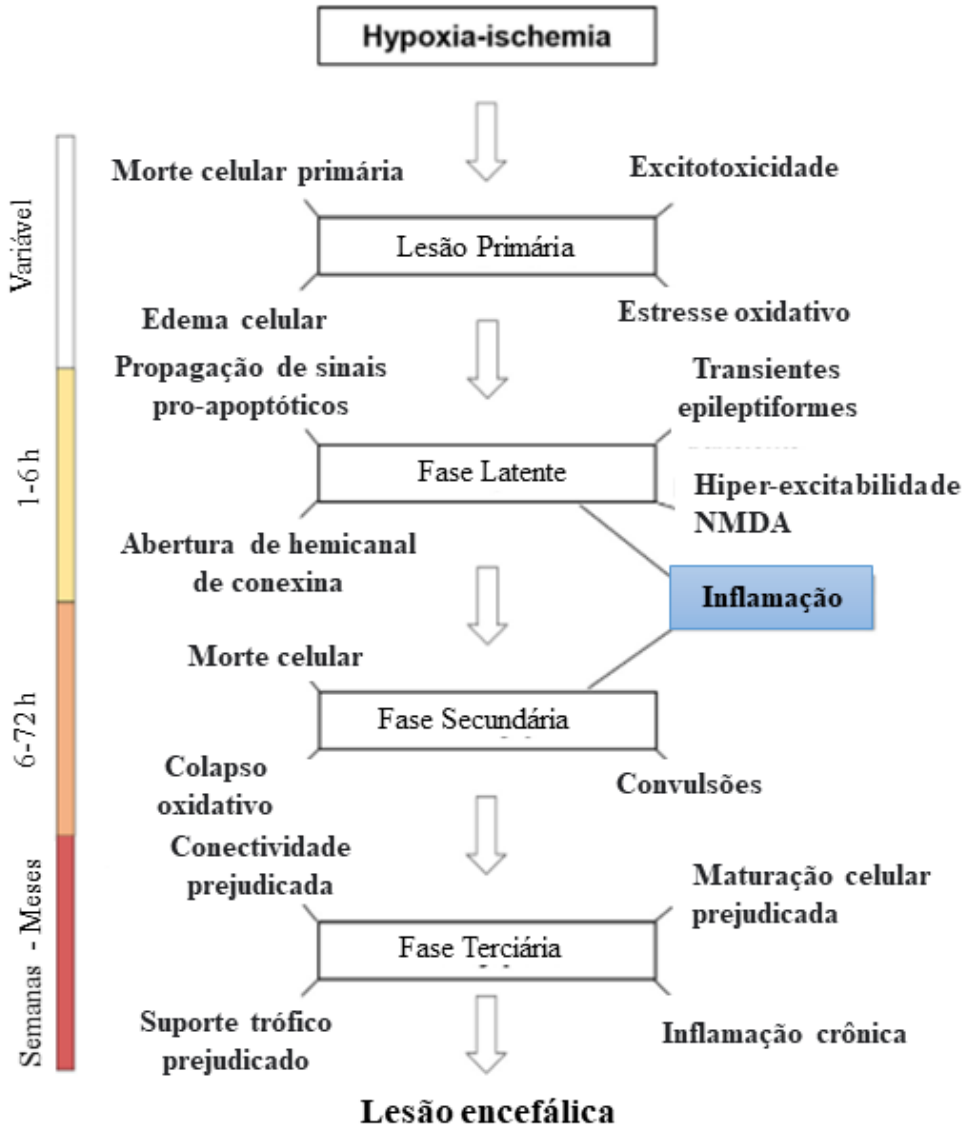

Figura 2. Mecanismos de evolução da lesão neural na fase primária, fase latente, fase secundária e fase terciária que contribuem para a geração de danos cerebrais e incapacidade a longo prazo. Adaptado de Cho e colaboradores (Cho et al., 2020).

1.3 Astrogliose no modelo de HI neonatal

Os astrócitos são o tipo celular mais abundante do SNC, com capacidade de modular o ambiente metabólico e iônico dos neurônios, além de ter funções importantes no tecido neurovascular. Os astrócitos também possuem papel importante no fornecimento aos neurônios de uma fonte renovável de neurotransmissores (Gordon et al., 2008; Mishra et al., 2016). Os astrócitos interagem com as células neurais e não

neurais, incluindo neurônios e suas sinapses, oligodendrócitos, células progenitoras de oligodendrócitos (OPCs), microglia, várias células perivasculares, fibroblastos meníngeos e células imunes circulantes (Sofroniew, 2020). Quando um dano ou doença afeta o SNC, são desencadeadas respostas multicelulares coordenadas que envolvem células da glia, neurônios e células não neurais (M. V. Sofroniew, 2015).

Embora os principais efetores da inflamação do SNC sejam leucócitos circulantes derivados da medula óssea, há um reconhecimento crescente de que as células intrínsecas do SNC têm papéis essenciais no recrutamento, instrução e retenção destes leucócitos em locais do SNC que sofreram insultos. Entre as células intrínsecas do SNC, as células microgliais estão estabelecidas como sensores precoces de dano (Prinz and Priller, 2014). Além disso, os astrócitos podem exercer funções pró-inflamatórias potentes ou funções anti-inflamatórias cruciais para a neuroproteção, dependendo do tipo específico de sinalização (M. V. Sofroniew, 2015).

Astrócitos em tecido saudável expressam muitos receptores para padrões moleculares associados a patógenos e padrões moleculares associados a danos ou perigo, que são conhecidos por desencadear respostas imunes inatas (Gong et al., 2020), em particular receptores Toll-like (TLRs), incluindo o TLR4 (Gong et al., 2020).

Entretanto, além de suas funções no SNC saudável (sem lesão), os astrócitos exibem uma resposta evolutivamente antiga à lesão e à doença do SNC, comumente referida como reatividade astrocitária, que por muito tempo foi considerada homogênea e funcionalmente passiva (Plog and Nedergaard, 2018; Sofroniew and Vinters, 2010). Atualmente, a literatura mostra que os astrócitos reativos podem responder a diversos sinais moleculares que derivam de muitos tipos de células, incluindo neurônios, outras células da glia, células estromais locais, e proteínas séricas, bem como células imunes

transportadas pelo sangue, além de moléculas associadas a distúrbios metabólicos de outros tecidos (Sofroniew, 2020).

Durante o processo de astrogliose, os astrócitos envolvem o tecido danificado que contém infiltração de leucócitos, proliferação de células estromais e fibrose após trauma, isquemia, infecção, inflamação autoimune, acúmulo de toxinas, vazamento da barreira hematoencefálica (BHE) ou doença neurodegenerativa (Sofroniew, 2020).

Além disso, durante a astrogliose há possibilidade de liberação de diversas moléculas que afetam as células próximas de todos os tipos de muitas maneiras distintas. Há evidências clínicas e experimentais acumuladas de que disfunções de astrócitos e astrogliose, seja por perda de funções benéficas ou ganho inadequado de efeitos prejudiciais, têm o potencial de contribuir para ou serem as principais causas de distúrbios do SNC, levando à noção de astrocitopatias (M. V Sofroniew, 2015).

Estudos com roedores já demonstraram que os astrócitos localizados na substância cinzenta do hipocampo e do córtex cerebral apresentam morfologia complexa (Nedergaard et al., 2003). Por exemplo, astrócitos nestas regiões podem envolver milhares de sinapses individuais (Fields et al., 2015; Halassa et al., 2009, 2007) e uma infinidade de vasos sanguíneos do parênquima (Iadecola and Nedergaard, 2007), sendo células-chave para a modulação da atividade sináptica e a regulação do fluxo sanguíneo local, permitindo ajustar o fluxo sanguíneo cerebral aos níveis de atividade neuronal (Sheikhbahaei et al., 2018). Embora o significado funcional desses arranjos astrocitários complexos não tenha sido definitivamente estabelecido, sugere-se que a sua morfologia esteja intimamente relacionada ao seu papel na função encefálica (Nedergaard et al., 2003; Sheikhbahaei et al., 2018).

A proteína glial fibrilar ácida (GFAP) é um componente vital do citoesqueleto dos astrócitos durante o desenvolvimento do encéfalo (Middeldorp and Hol, 2011). A GFAP é essencial para o funcionamento dos astrócitos, principalmente na formação da barreira hematoencefálica e na regulação das atividades do SNC em desenvolvimento (Tripathi et al., 2017), além de desempenhar papel benéfico, estimulando as interações neurônio-glia e regulando a transdução sináptica (Albee and Michael, 2003; Middeldorp and Hol, 2011).

Por outro lado, a mudança nos níveis de GFAP altera o funcionamento dos astrócitos com consequências prejudiciais aos neurônios circundantes (Tripathi et al., 2017). Dessa forma, a astrogliose torna-se prejudicial, impedindo a plasticidade neural e, portanto, o crescimento do encéfalo (Pekny and Pekna, 2015). O aumento da expressão de GFAP e a hipertrofia glial estão associados a uma gama de patologias do SNC, incluindo trauma, isquemia e neurodegeneração (Middeldorp and Hol, 2011). O aumento da expressão de GFAP também induz astrogliose, agravando distúrbios neurológicos inflamatórios e a lesão cerebral (Williams et al., 2001). Portanto, a expressão de GFAP e a morfologia astrocitária são variáveis importantes para entender o funcionamento do astrócito.

Estudos sugerem que o aumento da expressão GFAP em modelos de lesão aumentam a expressão da caspase-3 (Aras et al., 2012). No entanto, um estudo mostrou que a via da Akt induzida por FABP7 é essencial para o desenvolvimento glial e facilita a gliogênese em linhagens gliais anormais (Hegedus et al., 2007). Estudo anterior de nosso grupo mostrou que Akt-p (Akt fosforilada) pode regular negativamente a expressão de GFAP. Portanto, o aumento da expressão da Akt-p poderia levar à redução da expressão da caspase-3 de forma direta, além de reduzir a expressão da GFAP, podendo levar a uma redução da caspase-3 de forma indireta (Fabres et al., 2020).

1.4 Dimorfismo sexual

As diferenças do sexo em resposta à hipóxia já foram demonstradas em recém-nascidos prematuros humanos e em roedores nos períodos pré-natal e pós-natal (Burnsed et al., 2015). Estudos experimentais com animais de ambos os sexos descreveram diferenças no metabolismo basal e na expressão e atividade das enzimas mitocondriais (Brekke et al., 2017; Dukhande et al., 2009), com as fêmeas apresentando maior atividade de transporte de elétrons mitocondrial do que os machos (Weis et al., 2012). Além disso, estudos recentes com humanos e animais adultos indicam que o sexo pode ser um modulador da morte de células isquêmicas cerebrais: há evidências de que tanto a vulnerabilidade para o dano cerebral HI quanto os mecanismos de morte celular após a HI diferem entre os sexos (Netto et al., 2017).

Foi recentemente demonstrado em ratos submetidos à HI neonatal que uma translocação mais pronunciada do fator indutor de apoptose (AIF) ocorreu em machos, enquanto a caspase-3 foi mais ativada em fêmeas (Zhu et al., 2006). A diferença sexual em modelos experimentais de lesão encefálica pode ter influência em mecanismos apoptóticos dependentes de caspase: liberação de citocromo C da mitocôndria e subsequente ativação da caspase-3, culminando em aumento da apoptose (Netto et al., 2017). Como consequência, o dimorfismo sexual pode ser um fator determinante no desenvolvimento de novos tratamentos para a HI neonatal.

1.5 Hipotermia terapêutica

Há mais de uma década surgiram evidências experimentais e, posteriormente, estudos clínicos, sugerindo que a HT pode reduzir a lesão encefálica, e assim melhorar o desfecho neurológico, em recém-nascidos, após a HI (Silveira and Procianoy, 2015).

Nas décadas de 1950 e 1960, Miller e Westin estudaram a base fisiológica do papel neuroprotetor da hipotermia no tratamento da "asfixia neonatal", primeiro em animais neonatos e depois em recém-nascidos humanos. Eles e outros demonstraram melhora na sobrevida, sem paralisia cerebral ou retardo mental, nos recém-nascidos apneicos que foram resfriados rapidamente para 23°C a 32°C após o parto, quando as técnicas convencionais de ressuscitação falharam. Apesar de seus resultados, a hipotermia não se tornou uma terapia aceita no cuidado neonatal, em parte porque nunca foi avaliada por ensaios clínicos randomizados (Blackmon et al., 2006).

Ainda na década de 1960, a parada circulatória hipotérmica (temperatura central de 18–20°C) foi introduzida para facilitar o reparo de cardiopatias congênitas complexas (Kirklin et al., 1961). Essa técnica permitiu o reparo precoce de alterações e, portanto, menos morbidade cardíaca na primeira infância. Logo a técnica foi amplamente adotada, embora a duração da isquemia aceita para que o tratamento fosse iniciado sem ter chances de efeito que intensificasse a lesão no SNC não foi estabelecida. A heterogeneidade das lesões e a falta de uniformidade dos procedimentos operatórios e da duração da parada circulatória dificultaram conclusões sobre a segurança do tratamento com hipotermia (Blackmon et al., 2006).

A patogênese da lesão neural de um insulto hipóxico-isquêmico foi melhor definida e mecanismos tecnicamente aplicáveis para resfriar neonatos foram desenvolvidos. Reduções na temperatura do encéfalo entre 2°C e 5°C fornecem neuroproteção em modelos animais recém-nascidos e adultos de isquemia cerebral (Cho et al., 2020; Gunn et al., 1997; Sabir et al., 2012; Shankaran et al., 2005). A temperatura alvo de 33,5°C foi selecionada com base em estudos em animais que mostraram atenuação da lesão cerebral nesta temperatura sem os efeitos adversos (por exemplo, lesão miocárdica) que ocorrem em temperaturas mais baixas (Weinrauch; et al., 1992). O

resfriamento do encéfalo tem um efeito redutor da fatores que contribuem para a lesão cerebral, incluindo aminoácidos excitatórios, o estado de energia cerebral, fluxo sanguíneo cerebral e metabolismo, produção de óxido nítrico e apoptose (Shankaran et al., 2005).

A proteção parcial, e não total, com os atuais protocolos de hipotermia encontrados em estudos clínicos está em grande parte relacionada às dificuldades clínicas envolvidas para que se possa iniciar a hipotermia dentro da janela de oportunidade ideal ou janela terapêutica. Os protocolos clínicos atuais recomendam que, para recém-nascidos com HI moderada a grave, a hipotermia deve ser iniciada nas primeiras 6 horas de vida com uma temperatura entre 33-34°C, e continuada por 72h (Cho et al., 2020).

Já estudos pré-clínicos utilizando fetos de ovelha mostraram que, quando a hipotermia é iniciada na fase latente, em 90 minutos ou 3h após o final da isquemia, a morte de neurônios e de oligodendrócitos é reduzida e a atividade cerebral retorna aos níveis basais encontrados em animais controle, que não foram submetidos à HI (Bennet et al., 2006; Rutherford et al., 2010). No entanto, quando o tratamento é iniciado ao final da fase latente, em torno de 5,5h após a isquemia, é observada apenas uma melhora parcial na sobrevida neuronal e na recuperação da atividade no eletroencefalograma (EEG), sem melhora na sobrevida dos oligodendrócitos (Fleiss and Gressens, 2012; Vannucci et al., 2004). Se a HT for adiada para 8,5h após o fim da isquemia, o tratamento não mais se associa a qualquer melhora na sobrevivência celular ou recuperação da atividade do EEG (Fleiss and Gressens, 2012). Logo, é importante ressaltar que há evidências consistentes de que a hipotermia iniciada o mais cedo possível na fase latente melhora seus efeitos neuroprotetores em animais submetidos à HI neonatal (Sabir et al., 2012).

Ademais, existem poucos estudos publicados sobre o efeito da HT iniciada tardiamente após HI de gravidades diferentes, e ainda menos estudos que questionam se a HT pode aumentar a lesão se iniciada tardiamente (Sabir et al., 2012; Taylor et al., 2002). Ainda, em estudos experimentais com fetos de ovelha, a extensão da duração da hipotermia para 120h foi associada a sobrevida neuronal prejudicada em algumas regiões, em comparação com 72h de HT. Os resultados dos estudos que utilizam HT como tratamento para animais submetidos ao modelo de HI neonatal podem apresentar resultados distintos por haver diferenças de espécie, idade, intensidade de lesão e diferença de sexo entre os estudos realizados (Burnsed et al., 2015; Sabir et al., 2012; Smith et al., 2015, 2016).

Os mecanismos de proteção que fundamentam a HT são multifatoriais (Wassink et al., 2018). A neuroproteção produzida pela hipotermia após HI grave está intimamente associada à supressão de processos apoptóticos e necróticos, provavelmente por meio da redução da permeabilidade mitocondrial e ativação reduzida de vias extrínsecas de morte celular (Cho et al., 2020). A hipotermia também pode ter efeitos que reduzem propriedades excitatórias no SNC, conforme mostrado pela restauração da expressão (reduzida por isquemia) do mRNA do GluR2, a subunidade que limita o influxo de íons de cálcio por meio do receptor de ácido alfa-amino-3-hidroxi-5-metil-4-isoxazolpropiônico (AMPA) e, portanto, sugerindo que a hipotermia pode ajudar a prevenir a excitação excessiva (Colbourne et al., 2002).

Um mecanismo chave da proteção da HT é a imunossupressão. A hipotermia após a HI tem característica de inibir a ativação microglial e a indução de citocinas pró-inflamatórias (Wassink et al., 2018, 2014). A hipotermia também pode suprimir a translocação e a ligação dos principais fatores de transcrição da inflamação, incluindo o

fator nuclear κ-B (NF-κB), a interleucina-1β (IL-1β) e TNF-α após isquemia transitória focal (Han et al., 2003).

No entanto, estudos clínicos e ensaios experimentais mostraram que a hipotermia não é neuroprotetora em todos os casos de HI moderada, e também não mostra efeitos benéficos em casos de HI grave (Sabir et al., 2012; Silveira and Procianoy, 2015; Wood et al., 2016). Portanto, é necessário o desenvolvimento de novas estratégias de tratamento baseadas na fisiopatologia do evento hipóxico-isquêmico.

1.6 Progesterona

Os mecanismos moleculares que ativam a cascata de lesão isquêmica no encéfalo adulto são semelhantes aos encontrados pela HI encefálica em animais neonatos (Peterson et al., 2015). Ambos os modelos de lesão encefálica, adulta e neonatal, produzem edema cerebral, infiltração de macrófagos e ativação microglial e aumento de astrogliose, o que pode levar a uma resposta inflamatória exacerbada, excitotoxicidade e morte celular (Sayeed et al., 2009). À medida que a cascata de lesão progride, pode ocorrer desmielinização, geração de radicais livres e indução de apoptose, o que significa mais danos e, consequentemente, morte celular (PETERSON et al., 2015).

A progesterona (PROG) pode amenizar a lesão cerebral ao afetar muitos mecanismos diferentes para prevenir danos imediatos e de longo prazo. O conceito de que os esteroides gonadais têm funções apenas de controle reprodutivo, de que sua síntese é realizada apenas pelas glândulas adrenais, e de que regulam apenas respostas adaptativas e a homeostase de fluidos corporais tem mudado com o passar dos anos (Schumacher et al., 2004). Diversos estudos mostram ações diversas tanto da PROG, quanto de outros esteroides como estradiol e androgênios no SNC. A influência desses

esteroides pode afetar o funcionamento de neurônios e das células gliais (Sayeed et al., 2007; Schumacher et al., 2004; Stein, 2011).

Os esteroides não apresentam funções apenas endócrinas, mas também exercem ação como neurotransmissores (Baulieu, 1998). Portanto, foi criado o termo "esteroides neuroativos" (Paul and Purdy, 1992) referindo-se a todos os esteroides capazes de regular as funções neurais, incluindo hormônios esteroides, neuroesteroides e esteroides sintéticos (Melcangia et al., 2008).

Como mencionado, a PROG é um neuroesteroide e pode ser sintetizada a partir do colesterol ou a partir de precursores esteroides importados de estruturas periféricas que são metabolizados *"in situ"* (Dubrovsky, 2006), no SNC e periférico, nas células gliais e em neurônios (Chen et al., 2008; Stein, 2009). A PROG é capaz de modular a atividade neural e de regular diversas outras funções do organismo, como desenvolvimento, diferenciação, metabolismo e reprodução de fêmeas de várias espécies (Dubrovsky, 2006).

Sendo um esteroide, a PROG pode atravessar a membrana plasmática e atuar em receptores intracelulares, produzindo efeitos genômicos de longo-prazo (minutos a horas) como fatores de transcrição, regulando a expressão de genes de redes neurais para o início ou para a manutenção de respostas fisiológicas (Schumacher et al., 2004). Por outro lado, a PROG também pode produzir efeitos não-genômicos, que podem ser observados de milissegundos até poucos segundos, ou seja, são muito mais rápidos do que os efeitos clássicos sobre a expressão gênica (Arbo et al., 2014). Muitos desses efeitos são mediados por metabólitos da PROG, como por exemplo, a alopregnanolona. Esses efeitos incluem a modulação da excitabilidade neuronal através de ações na superfície celular e efeitos moduladores sobre a função e composição de alguns receptores, como por exemplo, os

receptores serotoninérgicos e o receptor $GABA_A$ (Arbo et al., 2014). Este efeito da ativação do receptor $GABA_A$ também é dependente da idade do animal, como explicado por Tsuji e colaboradores (Tsuji et al., 2012). Especula-se que a lesão produzida pela hipóxia-isquemia poderia atrasar o desenvolvimento do receptor $GABA_A$ ou afetar outros mecanismos, além da ação do GABA, que podem ocorrer em animais imaturos (Tsuji et al., 2012).

Alguns autores já demonstraram que a PROG também pode diminuir o edema cerebral, diminuir a peroxidação lipídica, diminuir a apoptose e anormalidades neuronais, promover a estabilidade da BHE, além de melhorar a cognição após danos cerebrais (AGGARWAL et al., 2008; SARKAKI et al., 2013). A PROG também pode diminuir o edema, a inflamação, a excitoxicidade do glutamato, os radicais livres e a apoptose em lesões de encéfalos adultos (Jiang et al., 2017; Wang et al., 2013).

O possível mecanismo neuroprotetor da PROG ainda não está completamente esclarecido. Estudos sobre o efeito neuroprotetor da PROG têm focado principalmente nos danos encefálicos em ratos adultos, enquanto aqueles em ratos recém-nascidos são raramente relatados (Wang et al., 2013). As concentrações séricas da PROG e da alopregnanolona, principal metabólito da PROG, em uma gestante, tendem a aumentar durante a gravidez, mostrando uma elevação em torno de 10 a 100 vezes no momento do parto (LUISI et al., 2000). Já foi demonstrado que a concentração de alopregnanolona encontrada no cordão umbilical é praticamente a mesma daquela encontrada no sangue materno (Hill et al., 2000). Além disso, esses esteroides têm a característica de cruzar facilmente a BHE (Li et al., 2013). O encéfalo do rato recém-nascido também está exposto a altas concentrações de PROG e alopregnanolona (Grobin et al., 2003). Estes dois neuroesteroides são fornecidos pela circulação materna, além de serem produzidos pelo encéfalo do feto. Por outro lado, as concentrações de PROG e de alopregnanolona

diminuem muito no encéfalo do neonato após o nascimento, devido à perda do fornecimento de sangue materno (Wang et al., 2010).

Sabe-se que a PROG ativa algumas vias de sinalização envolvidas com estímulos pró-sobrevivência em diferentes áreas encefálicas, promovendo a fosforilação da Akt e a fosforilação da Erk (componente da via das proteínas cinases ativadas por mitógenos - MAPK) (GUERRA-ARAIZA et al., 2009; KAUR et al., 2007). Essas cinases participam das ações da PROG envolvendo o controle da diferenciação e da função neuronal e do comportamento reprodutivo, além das ações de neuroproteção (KAUR et al., 2007). A PROG pode, ainda, reduzir a expressão e a atividade da enzima caspase-3, que é considerada a caspase efetora central e final, sendo responsável pela maior parte da apoptose biológica. O efeito preventivo ao dano cerebral e o mecanismo de ação da PROG têm atraído cada vez mais a atenção dos pesquisadores (WANG et al., 2013).

As amplas propriedades neuroprotetoras da PROG já foram demonstradas em várias espécies animais e em uma variedade de modelos de lesão neurológica (Skolnick et al., 2014). Há razões para se pensar que o neuroesteroide PROG tem um forte potencial para o tratamento da HI neonatal já que o mesmo apresenta efeitos benéficos em pesquisas relacionadas com lesão cerebral traumática, lesão cerebral isquêmica e outros modelos de lesão do sistema nervoso central (SNC) de adultos (KAORE et al., 2012; LUOMA; STERN; MERMELSTEIN, 2012; STEIN, 2011).

A PROG também tem característica modulatória na astrogliose após lesões do SNC (Djebaili et al., 2004; Fabres et al., 2020; Labombarda et al., 2011). A astrogliose é um fenômeno comum em casos de lesão do SNC, dano excitotóxico, envelhecimento, neurodegeneração, neuroinflamação, isquemia e doenças metabólicas (Benarroch, 2005; Sofroniew, 2009). Astrócitos podem ter efeitos prejudiciais após lesões do SNC, levando

à exacerbação da cascata inflamatória, liberação de níveis neurotóxicos de espécies reativas de oxigênio e glutamato e ao comprometimento da função da BHE, aumentando a formação de edema citotóxico (Sofroniew, 2009). Em muitas dessas condições, os hormônios esteróides desempenham um papel anti-gliótico, diminuindo a astrogliose. O efeito anti-gliótico já foi demonstrado para muitos esteróides, incluindo estradiol, moduladores seletivos do receptor de estrogênio, PROG, andrógenos e glicocorticóides (Djebaili et al., 2004; Garcia-Segura et al., 1999). Uma vez que os astrócitos expressam receptores intracelulares e de membrana para a PROG (Labombarda et al., 2011; Schumacher et al., 2004; Waters et al., 2008), especula-se que os efeitos dos esteróides sobre os astrócitos podem ser diretos. Exemplos de efeitos da PROG administrada *in vivo* sobre da função dos astrócitos incluem a inibição da óxido nítrico sintase, da GFAP e de citocinas pró-inflamatórias como TNF-α e IL-18 (Coughlan et al., 2005; Labombarda et al., 2011; Meyer et al., 2010). Também é conhecido que uma inativação seletiva do fator NF-kB astroglial, um regulador chave da inflamação e da cascata de lesão secundária, leva a uma melhora da recuperação funcional em modelos de esclerose múltipla (Brambilla et al., 2009). A este respeito, a inibição de NF-kB pela PROG sugere que a PROG possa agir como um regulador da astrogliose e dos danos secundários provocados pela HI.

1.6 Hipótese

A HI neonatal envolve a ativação de diversas vias moleculares, o que dificulta a busca por um tratamento único adequado. Entretanto, um dos processos centrais associados à lesão cerebral pós-HI é a astrogliose, a qual intensifica o processo neuroinflamatório. A HT, tratamento usado na clínica neonatológica, apresenta efeitos multifatoriais como redução do metabolismo e da neuroinflamação; porém esse método não é efetivo em todos os casos e deve ser iniciado em até 6 horas após o início da lesão. Tendo em vista essa limitação de janela terapêutica, novos tratamentos têm sido buscados.

A PROG apresenta características multifatoriais agindo em diversas vias do SNC (inclusive como anti-inflamatório), podendo ser uma boa candidata a agente neuroprotetor mesmo se administrada tardiamente após a HI.

Com base no que foi exposto, nossa hipótese é de que a PROG irá reduzir a lesão encefálica em animais neonatos submetidos ao modelo de HI, mesmo sendo utilizada após a janela terapêutica da HT. Também prevemos que os benefícios do tratamento hipotérmico serão dependentes do período de início da HT, com tratamentos iniciados mais cedo levando a um maior grau de neuroproteção. Ambos os tratamentos (PROG e HT) reduzirão a reatividade astrocitária causada pela HI, contribuindo para a diminuição da lesão cerebral e melhora dos parâmetros comportamentais. Por outro lado, os efeitos do resfriamento podem ser dependentes do dimorfismo sexual, com uma melhor resposta neuroprotetora sendo observada nas fêmeas (cuja morte celular é dependente de caspase-3), devido a uma reversão ou inibição das vias apoptóticas ativadas pela HI.

2. Objetivos

2.1 Geral

Avaliar os efeitos da administração de PROG e da HT sobre a lesão neural, a reatividade astrocitária e o comportamento em ratos Wistar submetidos à hipóxia-isquemia neonatal.

2.1. Específicos

- Avaliar o grau de severidade da lesão encefálica, a expressão de proteínas anti- e pró-apoptóticas e a astrogliose no hipocampo ipsilateral à lesão em ratos machos submetidos à HI neonatal e tratados com PROG;

- Investigar, em ratos machos e fêmeas submetidos à HI, os efeitos da TH iniciada em diferentes intervalos pós-HI (janela terapêutica), sobre parâmetros do neurodesenvolvimento (peso corporal e reflexos de geotaxia negativa e endireitamento), parâmetros morfológicos (volume de lesão cerebral e porcentagem de células hipocampais em degeneração) e parâmetros astrocitários (imunorreatividade à GFAP e morfologia astrocitária);

- Avaliar os efeitos do dimorfismo sexual sobre os desfechos produzidos pela TH iniciada em diferentes intervalos pós-HI (janela terapêutica).

3. Capitulo I

Long-Lasting Actions of Progesterone Protect the Neonatal Brain Following Hypoxia-Ischemia

Rafael Bandeira Fabres, Nathalia Lima Montes, Yahi de Menezes Camboim, Samir Khal de Souza, Fabrício Nicola, Isadora D'Ávila Tassinari, Maria Flavia Marques Ribeiro, Carlos Alexandre Netto, Luciano Stürmer de Fraga

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ORIGINAL RESEARCH

Long-Lasting Actions of Progesterone Protect the Neonatal Brain Following Hypoxia-Ischemia

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Abstract
Neonatal hypoxia–ischemia (HI) is the leading cause of mortality and morbidity in newborns, occurring in approximately 2% of live births. Neuroprotective actions of progesterone (PROG) have already been described in animal models of brain lesions. However, PROG actions on neonates are still controversial. Here, we treated male Wistar rats exposed to HI with PROG. Five experimental groups were defined ($n=6$/group) according to the scheme of PROG administration (10 mg/kg): SHAM (animals submitted to a fictitious surgery, without ischemia induction, and maintained under normoxia), HI (animals undergoing HI), BEFORE (animals undergoing HI and receiving PROG immediately before HI), AFTER (animals undergoing HI and receiving PROG at 6 and 24 h after HI) and BEFORE/AFTER (animals undergoing HI and receiving PROG immediately before and 6 and 24 h after HI). At P14 (7 days following HI), the volumes of lesion of the cerebral hemisphere and the hippocampus ipsilateral to the cerebral ischemia were evaluated, along with p-Akt, cleaved caspase-3 and GFAP expression in the hippocampus. PROG reduces the loss of brain tissue caused by HI. Moreover, when administered after HI, PROG was able to increase p-Akt expression and reduce both cleaved caspase-3 and GFAP expression in the hippocampus. In summary, it was possible to observe a neuroprotective action of PROG on the brain of neonatal animals exposed to experimental HI. This is the first study suggesting PROG-dependent Akt activation is able to regulate negatively cleaved caspase-3 and GFAP expression protecting neonatal hypoxic-ischemic brain tissue from apoptosis and reactive gliosis.

Keywords Neonatal hypoxia–ischemia · Akt · GFAP · Caspase-3 · Astrogliosis · Neuroprotection

Introduction

Neonatal hypoxia–ischemia (HI) is a condition associated with a variety of harmful events, such as perinatal asphyxia, intraventricular hemorrhage, stroke among others. Therefore, HI is a major cause of mortality and morbidity for neonates (Shankaran et al. 2005). In underdeveloped countries the rate of HI incidents exceeds that of developed countries up to ten times (Silveira and Procianoy 2015). Therapeutic hypothermia is the only clinical treatment for newborns suffering from HI. However, clinical studies and experimental trials have shown hypothermia is not neuroprotective in all cases of moderate HI, and also does not show beneficial effects in cases of severe HI (Sabir et al. 2012; Silveira and Procianoy 2015; Wood et al. 2016). Hence, it is necessary to develop new strategies of treatment based on the pathophysiology of the hypoxic-ischemic event.

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Here, we proposed to use the administration of the steroid progesterone (PROG) as a treatment for neonatal HI. A neuroprotective role of PROG has already been shown in several studies of lesions in the adult central nervous system (for review see Deutsch et al. 2013), including stroke (for review see Guennoun et al. 2019). On the other hand, just a few studies using PROG have been performed in animal models of neonatal HI; still, results are controversial (Tsuji et al. 2012; Wang et al. 2013; Li et al. 2014b, 2015; Peterson et al. 2015; Fabres et al. 2018).

PROG regulates cell proliferation, apoptosis, excitotoxicity and anti-inflammatory features during brain development (Whitaker-azmitia et al. 2014; Atif et al. 2016). In addition, PROG also modulates cell survival pathways by increasing p-Akt expression and inhibiting caspase-3 expression (Djebaili et al. 2004; Li et al. 2015; Stanojlović et al. 2019).

Caspase-3 is the leading factor in the activation of the apoptosis pathway (Djebaili et al. 2004; Li et al. 2015). After an initial phase of necrosis caused by HI, apoptosis is the main process involved in the increase of neural cells death (Davidson et al. 2015). Afterwards, neuroinflammation plays a role in the extension of the brain lesion with astrocytic hypertrophy being the main component of the neuroinflammatory process (Li et al. 2014a; Odorcyk et al. 2017). Astrocytes have an important role in the metabolism, glutamate uptake and maintenance of the blood–brain barrier following a HI event. However, astrocytes hyperactivation produces harmful effects (Teo et al. 2015; Concepcion and Zhang 2018; Burda and Sofroniew 2014). Hyperactivation of astrocytes is associated with secretion of proinflammatory cytokines, leading to a secondary neuronal damage (Rocha-Ferreira and Hristova 2016; Burda and Sofroniew 2014).

In a previous study, we did not find any effect of PROG on the expression of procaspase-3 and p-Akt when animals were evaluated just 48 h following HI. Here, we used the same schedule of PROG administration (before HI and/or 6 h and 24 h after HI) but evaluated the animals a week later. One-week post-injury period was chosen because it encompasses brain damage due to all mechanisms of injury (Teo et al. 2015). Notwithstanding we did not observe any neuroprotective effect of PROG 24 h following HI (Fabres et al. 2018), PROG neuroprotective effects had already been demonstrated when neonatal animals submitted to HI reach adulthood (Peterson et al. 2015).

Our hypothesis is that astrogliosis is increased after injury, which may be related to the activation of apoptosis pathways. In addition, administration of PROG a few hours following HI will produce long-lasting effects reducing brain damage when it is evaluated a week following HI insult. Thus, the aim of the present study was to evaluate the effect of PROG on the volume of brain lesion, astrogliosis and cell survival and apoptosis pathways a week later neonatal cerebral HI.

Material and Methods

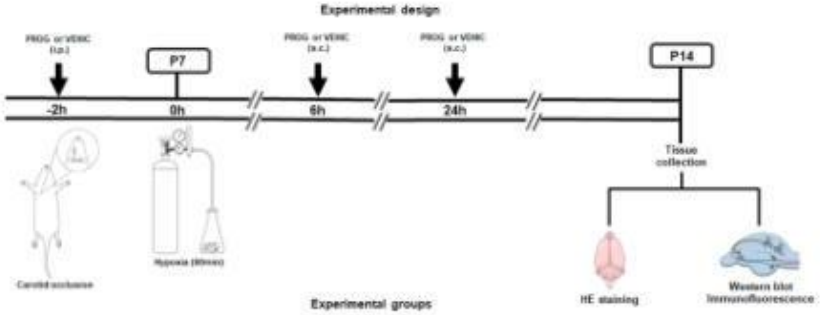
Animals

For this study we used 60 seven-day-old (P7) male Wistar rats ($n = 6$/group), since at this age the level of brain maturation is comparable to that of full-term human neonates (Dobbing and Sands 1979; Netto et al. 2017). Puppies were kept together with their dams which received food and water ad libitum. Light–dark cycle (12 h light/12 h dark) and temperature (22 °C ± 2 °C) were controlled. This study was approved by the Institutional Animal Care and Use Committee of Federal University of Rio Grande do Sul (UFRGS, #26669) and Hospital de Clínicas de Porto Alegre (HCPA, #14-0578).

HI procedure was performed according to the model of Rice et al. (1981) (see Fig. 1 for details). Animals were anesthetized with isoflurane (5% for induction and 3% for maintenance), and then submitted to surgical occlusion of the right common carotid artery. Animals were exposed to isoflurane for no more than 10 min during surgery. After recovering under a heat lamp, pups were left with their mothers during 1–2 h and then placed in a hypoxic chamber (8% O_2/92% N_2, 5L/min) for 60 min at 37°C (animal body temperature). After hypoxic exposure, the puppies were kept in a box under heating for approximately 30 min and then returned to their mothers.

Experimental Groups And Progesterone Administration

PROG (Sigma) was dissolved in 22.5% cyclodextrin (2-hydroxypropyl-β-cyclodextrin) and administered at a dose of 10 mg/kg of body weight (at a concentration of 5 mg/ml). Animals were assigned to five experimental groups depending on the schedule of PROG and/or vehicle (cyclodextrin) administration, according to our previous paper (Fabres et al. 2018) (see Fig. 1 for details): SHAM, HI, HI + PROG-BEFORE (BEF), HI + PROG-AFTER (AFT), HI + PROG-BEFORE/AFTER (BA). The first administration of PROG was performed immediately before ischemia (to produce in P7 rats high levels of PROG similar to the high prenatal physiological ones) (Tsuji et al. 2012). This first administration was performed intraperitoneally (with animals anesthetized) for quick absorption. The next two administrations were performed 6 h and 24 h after the onset of hypoxia and were done subcutaneously for slower absorption. SHAM group was used as a control group and injected only with vehicle. SHAM animals were submitted to a fictitious surgery (without



Experimental group	1st injection Intraperitoneal injection	2nd injection Subcutaneous injection	3rd injection Subcutaneous injection
SHAM	VEHIC	VEHIC	VEHIC
HI	VEHIC	VEHIC	VEHIC
BEF	PROG	VEHIC	VEHIC
AFT	VEHIC	PROG	PROG
BA	PROG	PROG	PROG

Fig. 1 Experimental design (*upper panel*) and experimental groups (*lower panel*). The procedure of hypoxia–ischemia (HI) was performed according to the model of Rice et al. (1981). Animals were submitted to HI on the seventh postnatal day (P7) and euthanized at P14 for tissue collection and processing. The HI procedure consisted of two steps: animals were first anesthetized and submitted to a surgery to occlusion of the right common carotid artery (time − 2 h). Then, after a period of recovery, animals were placed inside a hypoxic chamber (8% O_2) for 60 min at 37ºC (body temperature). The beginning of hypoxia exposure was taken as time zero (0 h). Every animal was injected three times with progesterone (PROG) dissolved in cyclodextrin and/or vehicle (cyclodextrin), depending on the specific experimental group. First injection was performed with animals anesthetized immediately before the surgery to carotid occlusion (time − 2 h). Second and third injections were administered 6 h e 24 h after the beginning of hypoxia, respectively. Animals were assigned to five experimental groups (*lower panel*) depending on the schedule of PROG and/or vehicle administration, according to our previous paper (Fabres et al. 2018): SHAM, HI, HI + PROG-BEFORE (BEF), HI + PROG-AFTER (AFT), HI + PROG-BEFORE/AFTER (BA). SHAM group was used as a control group and injected only with vehicle. SHAM animals were submitted to a fictitious surgery (without carotid occlusion) and kept under normoxia throughout the experiment; HI animals were submitted to the hypoxic-ischemic procedure (carotid occlusion followed for exposure to a hypoxic atmosphere) and were also injected with vehicle; BEF animals were submitted to HI and received PROG before ischemia); AFT animals were submitted to HI and received PROG after hypoxia; BA animals were submitted to HI and received PROG before ischemia and after hypoxia. CO = carotid occlusion, PROG = progesterone, VEHIC = vehicle, i.p. = intraperitoneal, s.c. = subcutaneous

carotid occlusion) and kept under normoxia throughout the experiment; HI animals were submitted to the hypoxic-ischemic procedure (carotid occlusion followed for exposure to a hypoxic atmosphere) and were also injected with vehicle; BEF animals were submitted to HI and received PROG before ischemia; AFT animals were submitted to HI receiving PROG after hypoxia; BA animals were submitted to HI and received PROG before ischemia and after hypoxia.

Brain Volume Measurement

In order to estimate the volume of lesion of the cerebral hemisphere and hippocampus ipsilateral to carotid occlusion animals were deeply anesthetized with isoflurane at P14 and transcardiacally perfused with cooled saline (0.9% NaCl) followed by 4% paraformaldehyde (PFA). Brains were dissected out, post-fixed in 4% PFA overnight and

dehydrated using an alcoholic series. Next, brains were embedded in paraffin and then sectioned coronally (7 µm) using a microtome (Microm HM 340E, ThermoScientific). Sections were collected on gelatin-coated slides and stained with hematoxylin and eosin (HE).

Sections were collected as from the corpus callosum was visualized until the disappearance of the hippocampus, corresponding from bregma + 2.52 to − 6.84 mm in adult rats, according to Paxinos and Watson rat brain atlas (Paxinos and Watson 2007). Twenty-five sections were collected per animal (one every thirty sections, i.e., one every 210 µm of interval) in order to evaluate the whole cerebral hemisphere volume. For analysis of hippocampal volume, fifteen sections per animal were analyzed (− 2.04 to − 6.12 mm), also with an interval of 210 µm in between. The images were captured and digitalized and the area of each hemisphere and hippocampus was measured using ImageJ software (NIH, Bethesda, USA). The Cavalieri method was used to calculate the volume of the structures (Arteni et al. 2010). Briefly, the areas of the analyzed structures were summed up and multiplied by the distance between each section. The results were expressed as a percentage of volume of lesion relative to the volume of the contralateral hemisphere, according to Sun et al. (Sun et al. 2015).

Cell Counting

Using the HE stained slides we were able to capture images of the hippocampal areas of CA1 and hilus of the dentate gyrus, in order to quantify the number of degenerated and total pyramidal cells (Fig. 2). The images were captured with a microscopy (Zeiss) at magnification of ×400. The average of 70 pyramidal neurons per captured image was counted and the mean value of five different sections was calculated (one field for each section totalizing five images per animal). The cells which showed shrinkage and deformity of the cell body or karyorrhectic and pyknotic nuclei were defined as degenerated cells (Garman 2011; Mano et al. 2014). The results were expressed as the percentage of degenerative cells, i.e., the mean of the degenerative cells divided by the mean of the total cells × 100 (%).

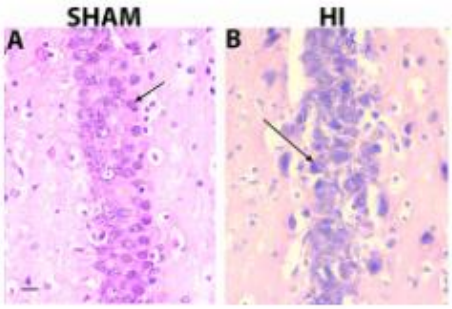


Fig. 2 Representative images of HE stained sections from hippocampal neurons ipsilateral to cerebral ischemia of 14-day-old rats for visualization of normal cells and degenerative cells. **a** representative brain section of a SHAM animal not submitted to HI. Arrow is indicating a normal cell. **b** Representative brain section of an animal from the HI group. Arrow indicates a degenerative cell. Bar = 100 µm

GFAP Immunofluorescence

Sectioned slices were washed with phosphate-buffer saline (PBS) and cell membranes were permeabilized in 0.25% PBS-Triton X-100. Sections were then blocked with 1% albumin for 30 min and incubated with the anti-glial fibrillary acidic protein antibody (anti-GFAP, #G9269, rabbit IgG, 1:200, Sigma-Aldrich) to identify astrocytes. This procedure was carried out in 1% albumin in PBS-Triton X-100 at 4 °C for 24 h. Following PBS washes, sections were incubated with the Alexa 555 anti-rabbit secondary antibody (1:500, A32732, Molecular Probes, Invitrogen). Slices were mounted with mounting medium containing DAPI (Merck, F6057) and coverslipped. In order to quantitatively compare GFAP immunofluorescence among groups, we choose two hippocampal areas (CA1 and hilus) for fluorescence intensity analysis. An area of optical interest was determined (3800 μm^2) and positioned on each of five images. A total of ten images (five for each area, i.e., CA1 and hilus) was evaluated per animal. Images were analyzed with the ImageJ software (NIH, Bethesda, USA), and the value of the integrated density per unit of area was obtained (Nicola et al. 2016).

Western Blotting

After euthanasia, the hippocampus was quickly dissected out and frozen in liquid nitrogen and stored at − 80 °C for subsequent homogenization. Right hippocampus (i.e., hippocampus ipsilateral to carotid occlusion) was processed apart, and 40 µg of proteins were separated by SDS-PAGE (polyacrylamide gel electrophoresis with sodium dodecyl sulfate) and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies anti-phospho-Akt (1:250, #sc-293125, Santa Cruz), anti-GFAP (1:1000, #G9269, Sigma), and anti-cleaved caspase-3 (1:1000, #9661, Cell Signaling). The results were normalized by the expression of glyceraldehyde 3-phosphate dehydrogenase (GFAP/GAPDH), total Akt (phospho-Akt/total Akt) and procaspase-3 (cleaved caspase-3/procaspase-3). The concentration

used for the anti-GAPDH antibody (#MAB-374, Millipore) was 1:2000, for the total anti-Akt antibody (#sc-5298, Santa Cruz) was 1:250 and for the anti-procaspase-3 (#9662, Cell Signalling) was 1:1000. The following secondary antibodies coupled to peroxidase were used: anti-mouse IgG (1:10,000, #AP124P, Millipore) for GAPDH and GFAP and anti-rabbit IgG (1:10,000, #AP132P, Millipore) for phospho-Akt, total Akt, caspase-3 and cleaved caspase-3. Membranes were developed by chemiluminescence and were analyzed densitometrically using the ImageJ software (NIH, Bethesda, USA).

Data Analysis

All data were analyzed using a one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. The data were expressed as mean ± SEM. The significance level was set as $p < 0.05$.

Results

Volume of Cerebral Hemisphere and Hippocampus

Figure 3 (upper panel) shows representative images of histological slices stained with HE from animals of all experimental groups. Images were used to calculate the volume of lesion of the cerebral hemisphere and hippocampus ipsilateral to carotid occlusion of each group. An increase in the volume of lesion of both hemisphere (Fig. 3a) and hippocampus (Fig. 3b) ipsilateral to carotid occlusion was seen in animals of the HI group ($p < 0.05$). On the other hand, the volume of lesion in the brain of animals from groups receiving PROG was similar to the SHAM group ($p > 0.05$).

Cell Counting

The percentage of degenerative neuronal cells in relation to the total neuronal cells was analyzed in the CA1 and hilus of the hippocampus ipsilateral to carotid occlusion (Fig. 4). A significant increase in the percentage of degenerative cells was seen in the hippocampus of animals submitted to HI as compared to SHAM animals (Fig. 4a, b, $p < 0.05$). Administration of PROG caused a significant decrease in the percentage of degenerative cells compared to the animals of the HI group ($p < 0.05$) in both areas, CA1 and hilus. However, PROG was not able to return the number of degenerative cells to values similar to those of the SHAM group ($p < 0.05$).

GFAP Immunoreactivity and Immunocontent

GFAP immunofluorescence was evaluated in the CA1 and hilus of the hippocampus ipsilateral to the carotid occlusion 7 days after the hypoxic-ischemic event (Fig. 5). Animals from groups HI and BEF showed a significant increase in GFAP immunofluorescence in both areas (CA1

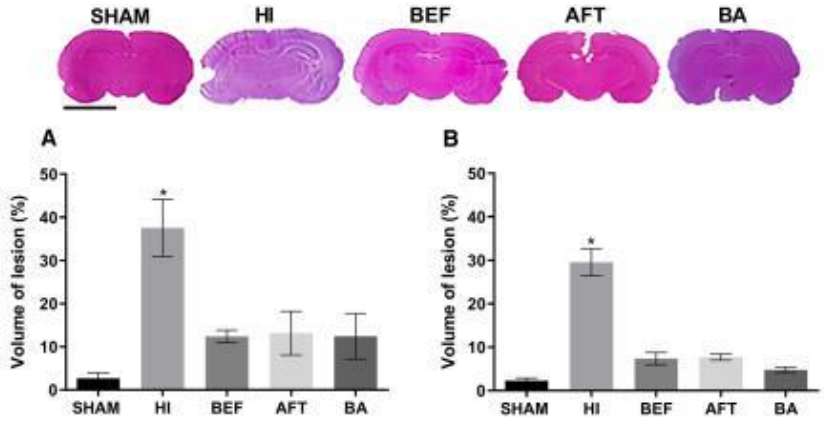


Fig. 3 Volume of lesion (%) in the hemisphere (a) and hippocampus (b) ipsilateral to carotid occlusion. *Upper panel* representative images of HE stained sections of the brain from animals of each experimental group. Data were analyzed by one-way ANOVA followed by Bonferroni and expressed as mean ± SEM. *significant differences compared to the SHAM group ($p < 0.05$). Bar = 0.5 cm

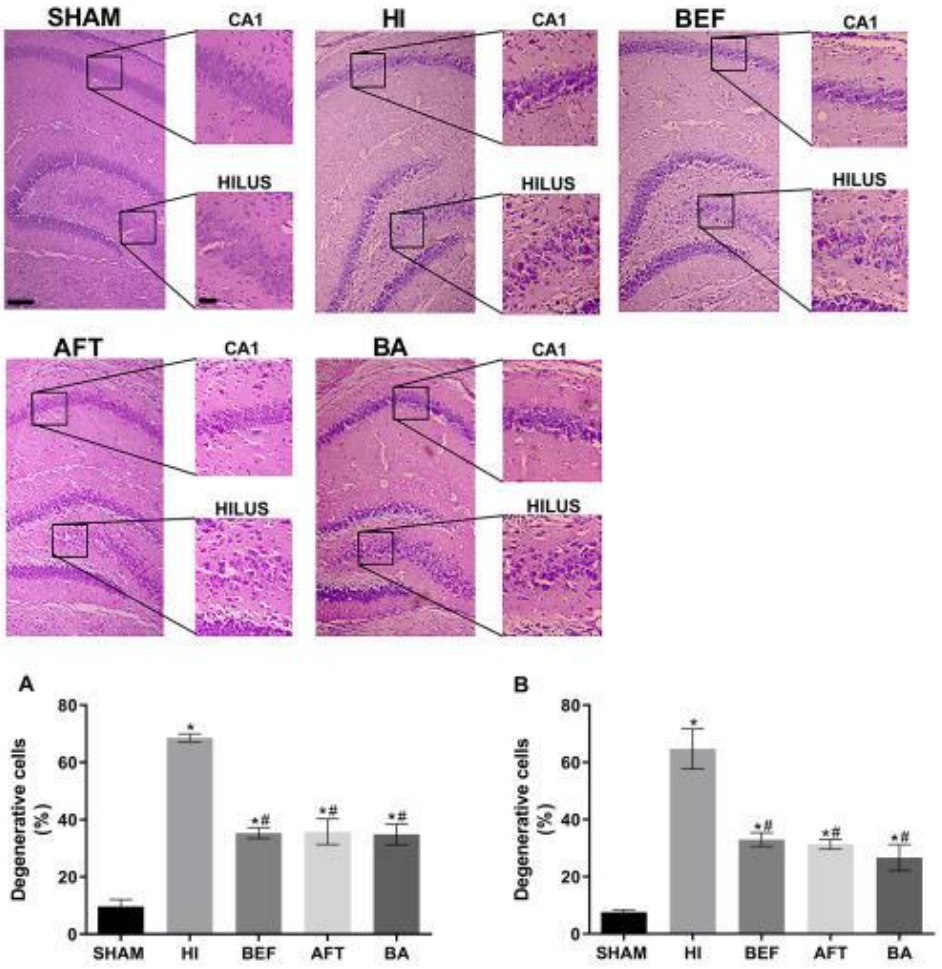


Fig. 4 Percentage of degenerative neurons in the areas of CA1 (a) and hilus (b) of the hippocampus ipsilateral to carotid occlusion. *Upper panel* representative images of HE stained sections of the brain from animals of each experimental group. Data were analyzed by one-way ANOVA followed by Bonferroni and expressed as mean ± SEM. *significant differences compared to the SHAM group ($p < 0.05$); #significant differences compared to the HI group ($p < 0.05$). Bars: main photomicrographs = 200 μm; insets = 100 μm

and hilus) relative to SHAM animals (Fig. 5a, b, $p < 0.05$). Groups AFT and BA did not present significant differences in relation to the SHAM group ($p > 0.05$). These results were corroborated when GFAP immunocontent from the hippocampus ipsilateral to ischemia was analyzed by Western blotting (Fig. 6).

Expression of Survival and Apoptosis Proteins

The ratios of p-Akt/total Akt and cleaved caspase-3/procaspase-3 are represented in Fig. 7. The p-Akt/total

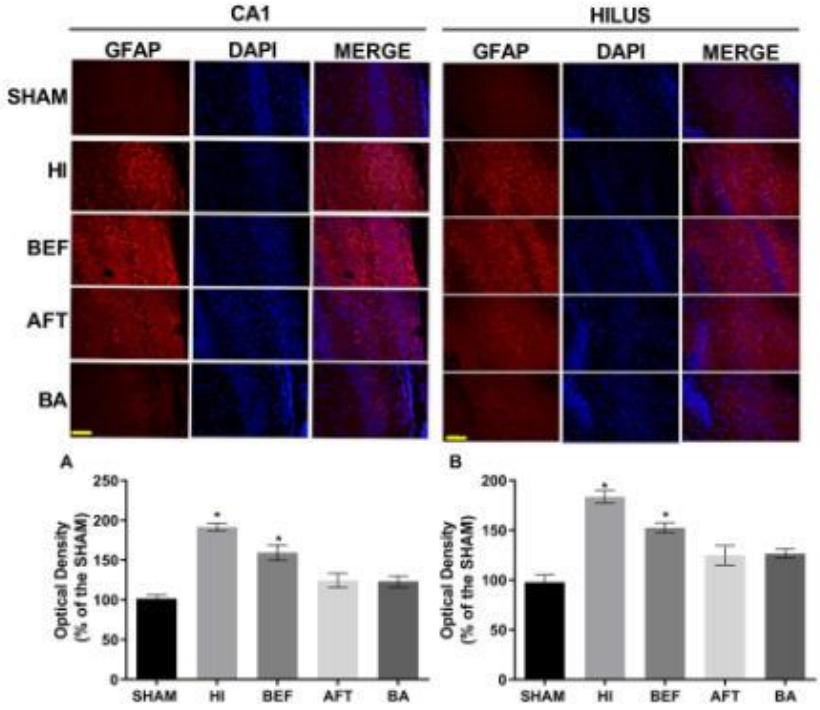


Fig. 5 GFAP immunofluorescence in the areas of CA1 (**a**) and hilus (**b**) of the hippocampus ipsilateral to carotid occlusion. *Upper panel* representative images of GFAP immunofluorescence staining of the brain from animals of each experimental group. Data were analyzed by one-way ANOVA followed by Bonferroni and expressed as mean ± SEM. *significant differences compared to the SHAM group ($p < 0.05$). Bar = 100 µm

Akt ratio in the hippocampus of HI animals did not show significant differences in relation to the SHAM group (Fig. 7a, $p > 0.05$). Despite that, when PROG was administered in neonates submitted to HI, an increase in p-Akt immunocontent was seen (Fig. 7a, $p < 0.05$). The opposite effect was observed when analyzing the cleaved caspase-3 content (Fig. 7b). The HI group showed higher values of cleaved caspase-3 in relation to all the other groups ($p < 0.05$).

Discussion

Most studies using PROG administration in animal models of brain injury in both adults and newborns show that this steroid displays neuroprotective effects (Ishrat et al. 2010, 2012; Li et al. 2014b; Dong et al. 2018; Guennoun et al. 2019). However, there are also studies showing an absence of protective effects (Fabres et al. 2018) or even harmful effects (Murphy et al. 2002; Tsuji et al. 2010) of PROG in neonates submitted to HI. Therefore, there is a need for further studies in order to elucidate the mechanism of action and the physiological effects of PROG.

Here, we have calculated the hemispheric and hippocampal volumes of lesion of neonates exposed to HI whether or not receiving PROG administration. It was possible to observe a reduction of the volume of brain lesion in animals treated with PROG as compared to animals that received only vehicle, regardless of the time-point of hormone administration (before and/or after HI). As compared to HI groups, animals that received PROG showed a significant reduction in the volume of lesion, suggesting a beneficial effect of PROG. It is worth to mention brain volume of lesion was evaluated a week following HI. In a previous study using

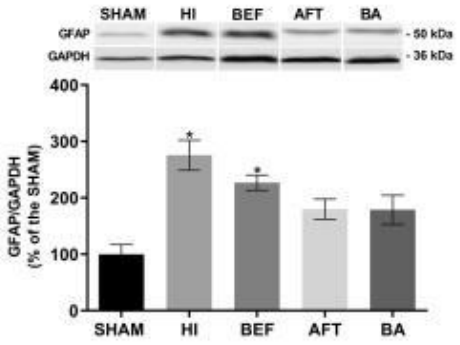


Fig. 6 GFAP immunocontent in the hippocampus ipsilateral to carotid occlusion. *Upper panel* representative Western blotting bands of GFAP immunocontent in the ipsilateral hippocampus from animals of each experimental group. GAPDH was used as a loading control (GFAP/GAPDH). Data were analyzed by one-way ANOVA followed by Bonferroni and expressed as mean ± SEM. *significant differences compared to the SHAM group ($p < 0.05$)

the same experimental design presented here we evaluated animals just 48 h following HI (Fabres et al. 2018). In that case, we were not able to see any effect of PROG administration in reducing the brain volume of lesion. We are confident these differences are due to the distinctions in the time-point of analysis. It is well known HI lesion continue extending for days or even months following the HI event (Mano et al. 2014) and PROG could act reducing this later neuroinflammatory process.

For example, in a study by Peterson et al. (Peterson et al. 2015) animals submitted to HI in P7 and receiving PROG right after injury showed a reduction in the hemisphere-injured area ipsilateral to ischemia when evaluated at 52 days of life (P52). A more recent study also showed a decrease in the area of lesion 8 weeks after injury in mice receiving PROG treatment for one week following HI (Dong et al. 2018).

However, in the literature, only our previous (Fabres et al. 2018) and current papers have evaluated the effects of PROG using histological and biochemical analysis in a short period after the injury, respectively 48 h and 7 days following neonatal HI. The evaluation of the animals following a longer period after the insult may be more adequate to observe the effects of PROG on the brain volume. Nevertheless, in order to understand the biochemical pathways responsible for the extension of injury it is necessary to study short periods after the HI event, since cell signaling pathways activated there could be responsible for structural changes observed in more advanced ages.

Thus, the maintenance of brain volume observed in PROG-treated animals could be related to the activation of signaling pathways involved in cell survival (Ishrat et al. 2012). When we evaluated the percentage of degenerative pyramidal neurons in the areas of CA1 and hilus of the ipsilateral hippocampus, it was possible to note HI animals showed a 7–8.5-fold increase in the number of degenerative cells as compared to SHAM animals. Otherwise, animals submitted to HI and which received PROG (groups BEF, AFT and BA) showed a reduction about 50% in the number of degenerative cells. This result suggests a

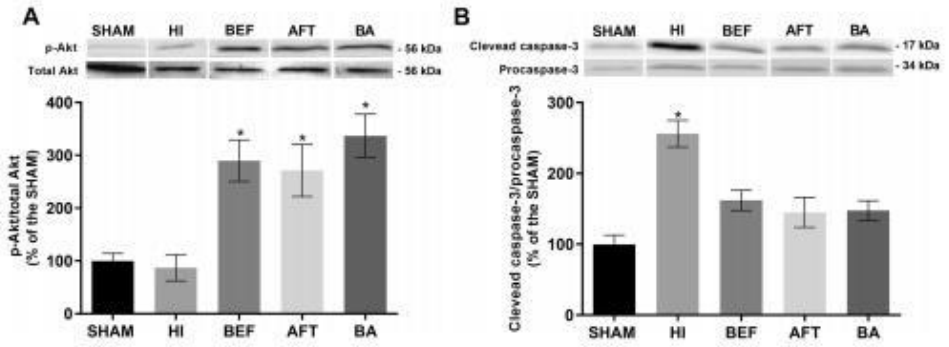


Fig. 7 p-Akt (a) and cleaved caspase-3 (b) immunocontent in the hippocampus ipsilateral to carotid occlusion. *Upper panel* representative Western blotting bands of p-Akt and cleaved caspase-3 immunocontent in the ipsilateral hippocampus from animals of each experimental group. Total Akt and procaspase-3 were used as loading controls (p-Akt/total Akt and cleaved caspase-3/procaspase-3). Data were analyzed by one-way ANOVA followed by Bonferroni and expressed as mean ± SEM. *significant differences compared to the SHAM group ($p < 0.05$)

neuroprotective action of PROG on cell survival and corroborates with our brain volume data.

Since PROG reduces the volume of brain lesion along with reducing degeneration of neurons, we decided also check the effects of PROG administration on astrocytes. Some studies have shown that increases in GFAP immunoreactivity are related to increases in neuronal death indicative of brain injury (Widestrand et al. 2007; Li et al. 2008). In the present study, an increase in GFAP immunoreactivity was observed in CA1 and hilus of hippocampus ipsilateral to injury in HI and BEF groups. When PROG was administered after injury (groups AFT and BA), GFAP immunoreactivity was similar to the SHAM group. These same results were obtained to the ipsilateral hippocampus when the immunocontent of GFAP was evaluated by Western blotting.

The effects of changes in GFAP expression on neuronal injury are controversial. Some studies show that increases in GFAP expression are neuroprotective (Li et al. 2008). Otherwise, it has also been suggested GFAP expression may inhibit normal restorative processes in later stages following adult central nervous system injury (Wilhelmsson 2004; Widestrand et al. 2007). However, a study using GFAP-knockout mice showed no effect on the size of the lesion, besides improved neurogenesis following HI (Järlestedt et al. 2010). It shows the importance of reducing GFAP-overexpression following neonatal HI. The administration of PROG following HI seems to have a beneficial effect on the reduction of GFAP expression.

In the present study, the expression of p-Akt and cleaved caspase-3 in PROG-treated animals was also evaluated at P14. The PROG-treated groups showed an increase in the expression of p-Akt relative to HI and SHAM groups. Whereas, a reduction in the expression of cleaved caspase-3 could be observed in the groups receiving administration of PROG. Altogether, these findings suggest the effects of PROG can last for many days following injury, inhibiting the process of apoptosis along this first week post brain injury (Ishrat et al. 2012).

Apoptosis is one of the major processes of cell death activated after HI injury in neonates (Davidson et al. 2015). Moreover, caspase-3 is one of the main proteins involved in the effector phase of apoptosis (Srinivasan et al. 1998). Inhibition of caspase-3 may be a consequence of the effects of Akt phosphorylation (Asati et al. 2016). Thus, PROG-dependent increase of Akt expression may be involved in the beneficial effects of PROG in animal models of brain injury (Ishrat et al. 2012; Li et al. 2014b, 2015; Stanojlović et al. 2019). In our previous paper (Fabres et al. 2018), PROG (when administrated immediately before ischemia) was unable to increase p-Akt expression 48 h after HI. Li and coworkers (Li et al. 2015) administered PROG 30 min before ischemia and observed an increased expression of p-Akt 24 h after HI. These results show PROG can act at different periods after its administration. Thus, in order to improve PROG-beneficial effects is necessary to elucidate the best therapeutic window for PROG administration.

Along with Akt activation, inhibiting caspase pathway is the best way to block apoptosis. We showed cleaved caspase-3 expression in hippocampus was raised even 7 days following HI. This finding is similar to that of Teo and colleagues (Teo et al. 2015) which showed an increase in caspase-3 expression in the somatossensory cortex neurons 7 days after HI.

Nevertheless, PROG administration was able to revert this increase in cleaved caspase-3 caused by HI, as shown here. In a previous study (Fabres et al. 2018), we showed the expression of procaspase-3 in the hippocampus ipsi and contralateral to ischemia was similar between HI and SHAM animals when evaluated 48 h after HI. Thus, it is likely that an increase in caspase-3 is occurring later, between 48 h and 7 days following HI. In other words, it is likely the existence of distinct periods of increase in caspase-3 expression between the onset of injury, related to the HI event itself, and the later neuroinflammatory process which occurs along days after HI. It is important to pursue this temporal pattern of caspase activation in order to determine more accurately the timing of an intervention as PROG administration.

Our results suggest a relationship between astrogliosis and increased caspase-3 expression, consequently a rise in apoptosis following HI. Tripathi et al. (Tripathi et al. 2017) also suggest p-Akt can regulate negatively GFAP expression through FABP7-PPARγ and MKP3. In the present study, animals which showed increased p-Akt expression (because they were treated with PROG) also showed a decrease in GFAP expression. Thus, we have shown for the first time an increase in p-Akt expression associated with a decrease in GFAP expression in neonates submitted to HI.

Here, we have shown that the administration of PROG in a dose of 10 mg/kg is effective in protect the neonatal brain from male Wistar rats exposed to HI irrespective of the moment of administration (immediately prior ischemia or after hypoxia). All groups treated with PROG showed increased expression of p-Akt and a decrease in cleaved caspase-3 expression. PROG exerts a long-lasting effect since this neuroprotection was observed 7 days after the HI event. On the other hand, a decrease in GFAP was observed only in animals that received PROG after HI (AFT and BA groups), suggesting the timing of administration is relevant for PROG regulation of astrogliosis (Fig. 8). It is a limitation of the present study we have evaluated just one time-point (P14) following PROG administration. However, it does not exclude the fact that animals receiving PROG showed a positive outcome one week after the induction of brain injury when compared to animals in the HI group.

In summary, the present study was the first one to evaluate the effect of PROG on the astrogliosis in rodents

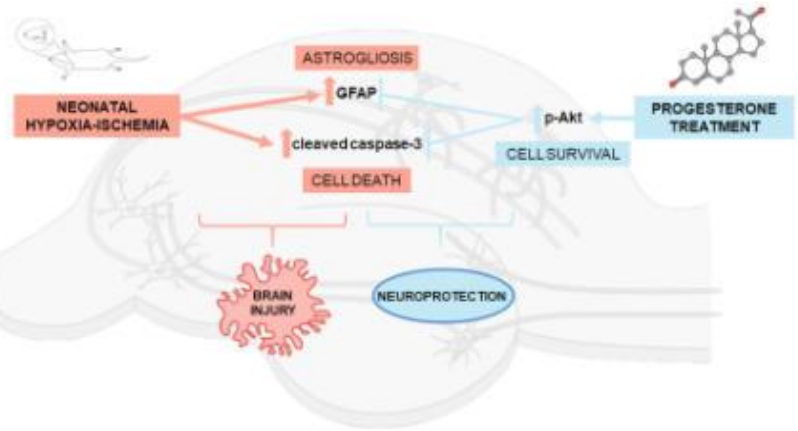


Fig. 8 Schematic diagram of the proposed mechanism for progesterone (PROG) neuroprotection following neonatal hypoxia–ischemia. Neonatal hypoxia–ischemia activates caspase-3 and induces exacerbated astrogliosis which, in turn, causes hippocampal damage (*left*). PROG promotes Akt phosphorylation. p-Akt inhibits activation of caspase-3 as well as expression of GFAP, reducing cell death and astrogliosis which, in turn, leads to hippocampal neuroprotection (*right*)

submitted to neonatal HI. Results suggest PROG promotes Akt signal and decreases caspase-3 and GFAP expression 7 days after HI. However, we have also shown that PROG administration before ischemia was not able to reduce astrogliosis. Thus, studies evaluating different doses of PROG, gender effects and other cell signaling pathways are necessary to elucidate the effects of PROG and allow its use as a clinical therapy for HI treatment.

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Author Contributions All authors contributed in this work. RBF, LSF, CAN and MFMR planned the study design. RBF, NLM, YMC, SKS and FN conducted the experiments. RBF and LSF performed data analysis and wrote the manuscript. RBF, LSF and IDT prepared the figures. RBF, LSF, NLM and IDT revised critically the manuscript. LSF, CAN and MFMR were recipient of funding used to support the study.

Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflicts of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This study was approved by the Institutional Animal Care and Use Committees of Federal University of Rio Grande do Sul (UFRGS, #26669) and Hospital de Clínicas de Porto Alegre (HCPA, #14–0578).

References

Arteni NS, Pereira LO, Rodrigues AL et al (2010) Lateralized and sex-dependent behavioral and morphological effects of unilateral neonatal cerebral hypoxia-ischemia in the rat. Behav Brain Res 210:92–98. https://doi.org/10.1016/j.bbr.2010.02.015

Asati V, Mahapatra DK, Bharti SK (2016) PI3K/Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways inhibitors as anticancer agents: structural and pharmacological perspectives. Eur J Med Chem 109:314–341

Atif F, Yousuf S, Stein DG (2016) Neuropharmacology Progesterone in the treatment of neonatal arterial ischemic stroke and acute seizures: Role of BDNF / TrkB signaling. Neuropharmacology 107:317–328. https://doi.org/10.1016/j.neuropharm.2016.03.052

Burda JE, Sofroniew MV (2014) Reactive gliosis and the multicellular response to CNS damage and disease. Neuron 81(2):229–248

Concepcion KR, Zhang L (2018) Corticosteroids and perinatal hypoxic-ischemic brain injury. Drug Discov Today 23:1718–1732

Davidson JO, Wassink G, Van Den HLG et al (2015) Therapeutic hypothermia for neonatal hypoxic—ischemic encephalopathy—where to from here? Front Neurol. https://doi.org/10.3389/fneur.2015.00198

Deutsch ER, Espinoza TR, Atif F et al (2013) Progesterone's role in neuroprotection, a review of the evidence. Brain Res 1530:82–105. https://doi.org/10.1016/j.brainres.2013.07.014

Djebaili M, Hoffman S, Stein D (2004) Allopregnanolone and progesterone decrease cell death and cognitive deficits after a contusion of the rat pre-frontal cortex. Neuroscience 123:349–359. https://doi.org/10.1016/j.neuroscience.2003.09.023

Dobbing J, Sands J (1979) Comparative aspects of the brain growth spurt. Early Hum Dev 3:79–83. https://doi.org/10.1016/0378-3782(79)90022-7

Dong S, Zhang Q, Kong D et al (2018) Gender difference in the effect of progesterone on neonatal hypoxic/ischemic brain injury

in mouse. Exp Neurol 306:190–198. https://doi.org/10.1016/j.expneurol.2018.05.013

Fabres RB, da Rosa LA, de Souza SK et al (2018) Effects of progesterone on the neonatal brain following hypoxia-ischemia. Metab Brain Dis 33:813–821. https://doi.org/10.1007/s11011-018-0193-7

Garman RH (2011) Histology of the central nervous system. Toxicol Pathol 39:22–35. https://doi.org/10.1177/0192623310389621

Guennoun R, Zhu X, Fréchou M, Gaignard P, Slama A, Liere P, Schumacher M (2019) Steroids in stroke with special reference to progesterone. Cell Mol Neurobiol 39:551–568

Ishrat T, Sayeed I, Atif F et al (2010) Progesterone and allopregnanolone attenuate blood-brain barrier dysfunction following permanent focal ischemia by regulating the expression of matrix metalloproteinases. Exp Neurol 226:183–190

Ishrat T, Sayeed I, Atif F et al (2012) Progesterone is neuroprotective against ischemic brain injury through its effects on the phosphoinositide 3-kinase/protein kinase B signaling pathway. Neuroscience 210:442–450. https://doi.org/10.1016/j.neuroscience.2012.03.008

Järlestedt K, Rousset CI, Faiz M et al (2010) Attenuation of reactive gliosis does not affect infarct volume in neonatal hypoxic-ischemic brain injury in mice. PLoS ONE 5:1–7. https://doi.org/10.1371/journal.pone.0010397

Li L, Lundkvist A, Andersson D et al (2008) Protective role of reactive astrocytes in brain ischemia. J Cereb Blood Flow Metab 28:468–481. https://doi.org/10.1038/sj.jcbfm.9600546

Li S-J, Liu W, Wang J-L et al (2014a) The role of TNF-α, IL-6, IL-10, and GDNF in neuronal apoptosis in neonatal rat with hypoxic-ischemic encephalopathy. Eur Rev Med Pharmacol Sci 18:905–909

Li X, Zhang J, Chai S, Wang X (2014b) Progesterone alleviates hypoxic—ischemic brain injury via the Akt / GSK—3 β signaling pathway. Exp Ther Med 8:1241–1246. https://doi.org/10.3892/etm.2014.1858

Li X, Zhang J, Zhu X et al (2015) Progesterone reduces inflammation and apoptosis in neonatal rats with hypoxic ischemic brain damage through the PI3K/Akt pathway. Int J Clin Exp Med 8:8197–8203

Mano Y, Kotani T, Ito M et al (2014) Maternal molecular hydrogen administration ameliorates rat fetal hippocampal damage caused by in utero ischemia-reperfusion. Free Radic Biol Med 69:324–330. https://doi.org/10.1016/j.freeradbiomed.2014.01.037

Murphy SJ, Littleton-Kearney MT, Hurn PD (2002) Progesterone administration during reperfusion, but not preischemia alone, reduces injury in ovariectomized rats. J Cereb Blood Flow Metab 22:1181–1188. https://doi.org/10.1097/00004647-200210000-00005

Netto CA, Sanches E, Odorcyk FK et al (2017) Sex-dependent consequences of neonatal brain hypoxia-ischemia in the rat. J Neurosci Res 95:409–421

Nicola FC, Rodrigues LP, Crestani T et al (2016) Human dental pulp stem cells transplantation combined with treadmill training in rats after traumatic spinal cord injury. Brazilian J Med Biol Res 49:1–11. https://doi.org/10.1590/1414-431x20165319

Odorcyk FK, Nicola F, Duran-Carabali LE et al (2017) Galantamine administration reduces reactive astrogliosis and upregulates the anti-oxidant enzyme catalase in rats submitted to neonatal hypoxia ischemia. Int J Dev Neurosci 62:15–24. https://doi.org/10.1016/j.ijdevneu.2017.07.006

Paxinos G, Watson C (2007) The rat brain in stereotaxic coordinates. Elsevier, SanDiego

Peterson BL, Won S, Geddes RI et al (2015) Sex-related differences in effects of progesterone following neonatal hypoxic brain injury. Behav Brain Res 286:152–165. https://doi.org/10.1016/j.bbr.2015.03.005

Rice JE, Vannucci RC, Brierley JB (1981) The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann Neurol 9:131–141. https://doi.org/10.1002/ana.410090206

Rocha-Ferreira E, Hristova M (2016) Plasticity in the neonatal brain following hypoxic-ischaemic injury. Neural Plast. https://doi.org/10.1155/2016/4901014

Sabir H, Scull-Brown E, Liu X, Thoresen M (2012) Immediate hypothermia is not neuroprotective after severe hypoxia-ischemia and is deleterious when delayed by 12 hours in neonatal rats. Stroke 43:3364–3370. https://doi.org/10.1161/STROKEAHA.112.674481

Shankaran S, Laptook AR, Ehrenkranz RA et al (2005) Whole-body hypothermia for neonates with hypoxic–ischemic encephalopathy. N Engl J Med 353:1574–1584. https://doi.org/10.1056/NEJMcps050929

Silveira RC, Procianoy RS (2015) Hypothermia therapy for newborns with hypoxic ischemic encephalopathy. J. Pediatr (Rio J) 91:78–83

Srinivasan A, Roth KA, Sayers RO et al (1998) In situ immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system. Cell Death Differ 5:1004–1016. https://doi.org/10.1038/sj.cdd.4400449

Stanojlović M, Guševac Stojanović I, Zarić M et al (2019) Progesterone protects prefrontal cortex in rat model of permanent bilateral common carotid occlusion via progesterone receptors and Akt/Erk/eNOS. Cell Mol Neurobiol. https://doi.org/10.1007/s10571-019-00777-2

Sun H-S, Xu B, Chen W et al (2015) Neuronal K(ATP) channels mediate hypoxic preconditioning and reduce subsequent neonatal hypoxic—ischemic brain injury. Exp Neurol 263:161–171. https://doi.org/10.1016/j.expneurol.2014.10.003

Teo JD, Morris MJ, Jones NM (2015) Hypoxic postconditioning reduces microglial activation, astrocyte and caspase activity, and inflammatory markers after hypoxia-ischemia in the neonatal rat brain. Pediatr Res 77:757–764. https://doi.org/10.1038/pr.2015.47

Tripathi S, Kushwaha R, Mishra J et al (2017) Docosahexaenoic acid up-regulates both PI3K/AKT-dependent FABP7–PPARγ interaction and MKP3 that enhance GFAP in developing rat brain astrocytes. J Neurochem 140:96–113. https://doi.org/10.1111/jnc.13879

Tsuji M, Aoo N, Harada K et al (2010) Sex differences in the benefits of rehabilitative training during adolescence following neonatal hypoxia-ischemia in rats. Exp Neurol 226:285–292. https://doi.org/10.1016/j.expneurol.2010.09.002

Tsuji M, Taguchi A, Ohshima M et al (2012) Progesterone and allopregnanolone exacerbate hypoxic-ischemic brain injury in immature rats. Exp Neurol 233:214–220

Wang X, Zhang J, Yang Y et al (2013) Progesterone attenuates cerebral edema in neonatal rats with hypoxic-ischemic brain damage by inhibiting the expression of matrix metalloproteinase-9 and aquaporin-4. Exp Ther Med 6:263–267. https://doi.org/10.3892/etm.2013.1116

Whitaker-azmitia PM, Lobel M, Moyer A (2014) Low maternal progesterone may contribute to both obstetrical complications and autism. Med Hypotheses 82:313–318. https://doi.org/10.1016/j.mehy.2013.12.018

Widestrand Å, Faijerson J, Wilhelmsson U et al (2007) Increased Neurogenesis and Astrogenesis from Neural Progenitor Cells Grafted in the Hippocampus of $GFAP^{-/-}Vim^{-/-}$ Mice. Stem Cells 25:2619–2627. https://doi.org/10.1634/stemcells.2007-0122

Wilhelmsson U (2004) Absence of Glial Fibrillary Acidic Protein and Vimentin Prevents Hypertrophy of Astrocytic Processes and Improves Post-Traumatic Regeneration. J Neurosci 24:5016–5021. https://doi.org/10.1523/JNEUROSCI.0820-04.2004

Wood T, Osredkar D, Puchades M et al (2016) Treatment temperature and insult severity influence the neuroprotective effects of therapeutic hypothermia. Sci Rep 6:23430. https://doi.org/10.1038/srep23430

4. Capítulo II

Therapeutic hypothermia for the treatment of neonatal hypoxia-ischemia: sex-dependent modulation of astrocyte reactivity

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Therapeutic hypothermia for the treatment of neonatal hypoxia-ischemia: sex-dependent modulation of astrocyte reactivity

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Abstract

Therapeutic hypothermia (TH) is the standard treatment used for neonatal hypoxia-ischemia (HI) with the time window limited to 6h post the injury event. However, the therapeutic window to TH in animal models of HI and the influence of sexual dimorphism has not been elucidated. Therefore, the aim of present study was to investigate the effective time window to start TH in male and female rats submitted to neonatal HI. Wistar rats (P7) were divided in groups: NAIVE and SHAM (control groups), HI (animals submitted to hypoxia-ischemia) and TH (animals submitted to HI and treated with TH; 32ºC for 5h). TH was started at 2h (TH-2h group), 4h (TH-4h group), or 6h (TH-6h group) after HI. Seven days after HI animals were subjected to behavioural tests, analysis of volume of lesion and astrogliosis. Male and female rats from the TH-2h group showed a reduction in the volume of lesion and in the intensity of GFAP immunofluorescence. However, TH-2h females showed a reduction of degenerative cells and in the number of primary processes of astrocytes. Interestingly, females from the TH-6h group showed greater volume of lesion and of degenerative hippocampal cells. These were associated with worse performance in behavioral neurodevelopmental tests. Altogether, results suggest TH-induced neuroprotection is time- and sex-dependent. TH started in later periods, 6h post-event, can even worsen the HI lesion in females. These data indicate the need of developing specific therapeutic protocols for each sex and reinforce the early beginning of hypothermic treatment.

Key-words: neonatal hypoxia-ischemia; therapeutic hypothermia; GFAP; hippocampus, neurodevelopment, astrocytes

1. Introduction

Neonatal hypoxia-ischemia (HI) is one of the main causes of mortality and morbidity in neonates (Nelson and Lynch, 2004; Shankaran et al., 2005). Most survivors present with cerebral palsy, neurosensory deficits and cognitive impairments throughout life (Shankaran et al., 2005). Currently, therapeutic hypothermia (TH) is established as the standard treatment used for neonates with moderate to severe injuries caused by neonatal HI events. TH must be started within a period of 6 hours after the hypoxic event (therapeutic window) in order to achieve the desired neuroprotective effects (Cho et al., 2020; Gunn et al., 1997). In humans, the recommended protocol for TH is the reduction of the temperature of the newborn to 33-34ºC for 72 hours (Davies et al., 2019). However, at the experimental setting efficacy of most hypothermia protocols depends on the species studied (Gunn et al., 1997; Wood et al., 2016). For example, hypothermia of 30ºC lasting 120h appears to present the best neuroprotection in sheep when started 5.5h after the hypoxic-ischemic insult (Gunn et al., 1997). In rats, however, TH protocols that use longer periods of time and depth temperatures do not show additional neuroprotective effects and can even intensify the brain injury (Sabir et al., 2014, 2012; Wood et al., 2016).

TH is used in the clinic irrespective of the sex of patients (Cho et al., 2020; Shankaran et al., 2005). However, there is growing evidence of sex dimorphism in neural functioning, especially in animal models (Netto et al., 2017); it is then conceivable that the neuroprotective effects of hypothermia could be influenced by sexual dimorphism. In fact, some studies in animal models of HI has shown that the neuroprotective effects of TH may depend on the sex of animals (Burnsed et al., 2015; Nie et al., 2016; Smith et al., 2015, 2016; Thoresen et al., 2009) as better results in behavioural tests were seen on females treated with hypothermia compared to males, whereas other studies found no sex-

related differences nor in reducing the volume of injury or in behavioural tests (Fang et al., 2013; Sabir et al., 2014, 2012; Wagner et al., 2002).

Interestingly, studies showing sex-dependent neuroprotective responses of TH report divergent data, some with positive behavioural and morphological effects only in males (Burnsed et al., 2015) and others reporting improvements in behavioural results in females (Smith et al., 2015; Thoresen et al., 2009). Conversely, there are differences in HI-induced brain damage between male and female rats (Netto et al., 2017; Sanches et al., 2015, 2013): in females, cell death after HI is mainly dependent on caspase pathways, whereas in males it appears to be related to pathways independent of caspase activation (Askalan et al., 2015; Joly et al., 2004; Zhu et al., 2006). Another difference observed is on the neuroinflammatory process, one of the main causes of progressive brain lesion even for months after the HI event: males show higher microglia activation and peripheral inflammatory response, as compared to females (Mirza et al., 2015; Netto et al., 2017).

Together with microglia activation and peripheral leukocyte infiltration into the nervous tissue, reactive astrogliosis plays a fundamental role in the extension of injury following HI. Astrocyte hyperactivity is associated with secondary neuronal damage due to the release of proinflammatory cytokines after HI (Ahn et al., 2018). TH is able to reduce astrogliosis (Ahn et al., 2018; Sabir et al., 2016); however, it is also not clear whether this effect can be influenced by sex dimorphism.

Clinical studies demonstrate that the therapeutic window for hypothermia in neonatalogy has been set to a maximum of 6 hours after the hypoxic-ischemic event (Davies et al., 2019; Gunn et al., 1997). However, there is no such definition when it comes to animal models of HI, with experimental hypothermia being applied during hypoxia or until 12 hours post-event (Burnsed et al., 2015; Reinboth et al., 2016; Sabir et

al., 2012; Smith et al., 2015). In order to better understand the mechanisms through which TH produces its beneficial effects, an experimental time-window shall be established.

Therefore, the present study aims to elucidate the effective time window of TH in male and female rats submitted to neonatal hypoxia-ischemia. The variables under scrutiny are developmental (body weight, negative geotaxis and righting reflex), morphological (brain lesion volume and degenerating cells in the hippocampus) and structural (GFAP immunoreactivity and astrocyte morphology). The working hypothesis is that the effects of therapeutic hypothermia can be influenced by sexual dimorphism.

2.Results

2.1 Body weight

There were no differences in the body weight among groups before the animals underwent neonatal HI; the two-way ANOVA did not detected effect of the factors group ($F(5,180) = 0.07140$, $p=0.9964$), and sex ($F(1,180) = 0.03382$, $p=0.8543$). However, after reaching the age of 14 days there was a significant effect of both factors, group ($F(5, 176) = 20{,}56$, $p<0.0001$) and sex ($F(1, 176) = 14{,}65$, $p=0.0002$), but no interaction between them ($F(5, 176) = 0{,}4278$, $p=0.8289$). The animals that were submitted to HI showed a significant reduction in the body weight as compared to the control groups ($p<0.05$), except for the TH-2h group ($p>0.05$), in both males and females (Fig. 2).

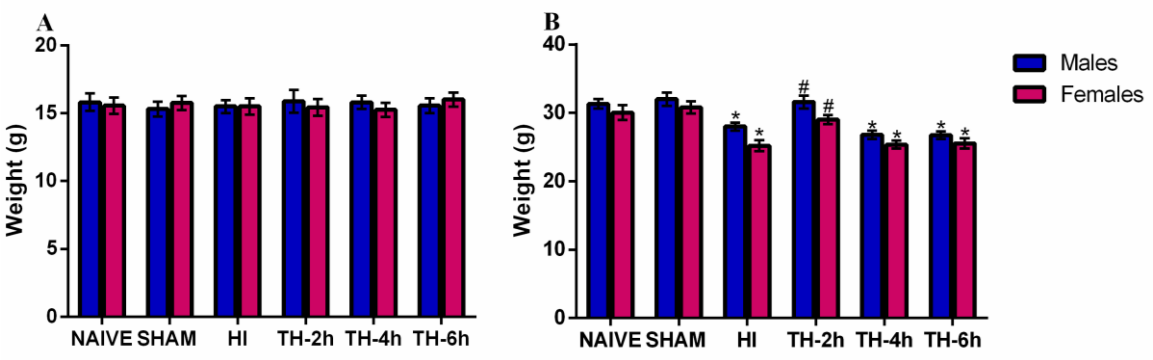

Figure 2. Results of body weight of male and female animals. (A) Body weight of the male's animals at the ages of p7 and p14, respectively. (B) Body weight of the female's animals at the ages of p7 and p14, respectively. Data were analyzed by two-way ANOVA followed by Bonferroni and expressed as mean ±SEM. * significant differences compared to the NAÏVE and SHAM group (p<0.05); # significant differences compared to the HI group (p<0.05).

2.2 Volume of brain injury

Fig. 3A shows the volume of the right hemisphere brain lesion. Two-way ANOVA showed a significant effect of the factor group (F (5,180) = 149.7, p<0.0001), with no effect of sex (F (1,180) =1.626, p=0.2050). The HI group showed a greater volume of injury of the hemisphere as compared to the NAÏVE and SHAM groups (p<0.05). In the males, the TH-2h group was the only one to show a reduction in the hemispheric lesion as compared to the HI group (p <0.05). In females, a significant reduction of the volume of the hemispheric lesion was seen in the TH-2h and TH-4h groups as compared to the HI group (p<0.05). In addition, a larger volume of lesion in the hemisphere of males of the group TH-2h relative to the TH-2h females' group (p<0.05) was also observed.

Two-way ANOVA detected a significant effect of the factor group (F (5,180) = 123.9, p<0.0001) but not of the factor sex (F (1,180) = 0.5032, p=0.4796) for the volume of lesion relative to the hippocampus (Fig. 3B). The animals of the HI group showed a significant lesion as compared to the NAÏVE and SHAM groups (p<0.05). The TH-2h group showed a reduction in the lesion volume relative to the HI group for both sexes (p<0.05).

Analysis of the volume of lesion of the cerebral cortex (Fig. 3C) showed a significant effect of both factors, group (F (5,180) = 110.5, p<0.0001) and sex (F (1,180) = 5.480, p=0.0211), as well as an interaction between the factors (F(5,108)=2.392 p=0.0423). All groups submitted to the HI bear a significant cortical lesion relative to the

NAÏVE and SHAM groups (p<0.05). However, the TH-2h group showed a reduction in the volume of lesion as compared to the HI group (p<0.05), for both sexes. It was also observed that males of the TH-4h group showed smaller lesion in the cerebral cortex as compared to females of the TH-4h group (p<0.05).

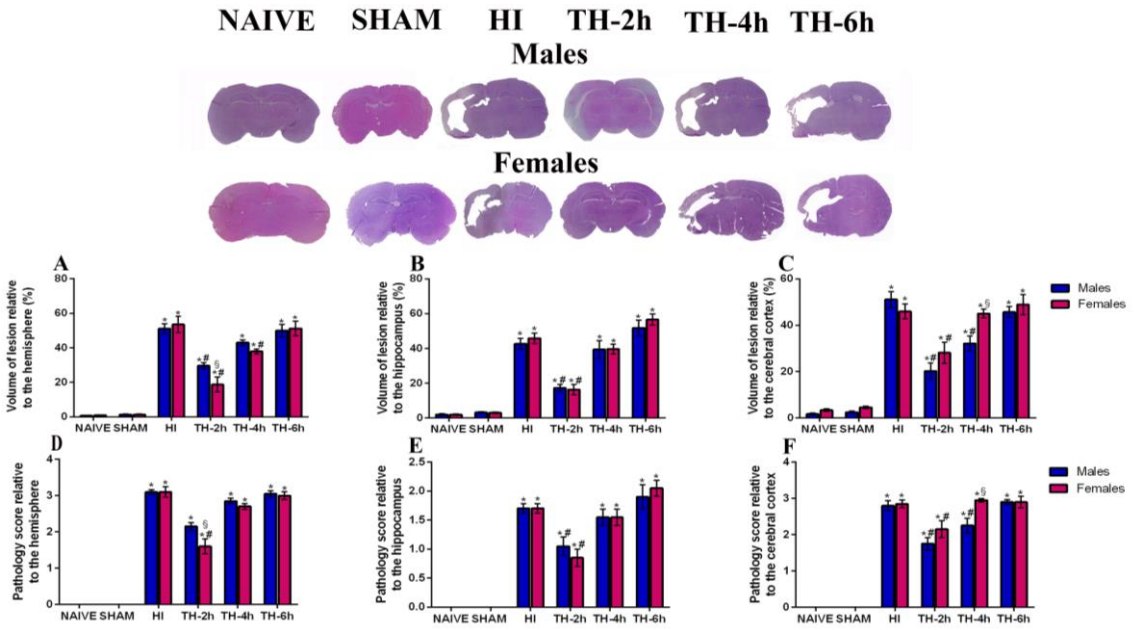

Figure 3. *Upper panel* representative figure of the lesion volume. Percentage of volume of lesion of the hemisphere, hippocampus and cortex cerebral ipsilateral to ischemia of the males and females (A, B, C). Pathology score of the hemisphere, hippocampus and cortex cerebral ipsilateral to ischemia of the male and females animals. (C, D, E). Data were analyzed by two-way ANOVA followed by Bonferroni and expressed as mean ±SEM.* significant differences compared to the NAÏVE and SHAM group (p<0.05); # significant differences compared to the HI group (p<0.05); § significant differences between males and females (p<0.05). Bar = 0.5 cm

2.3 Pathology score

Pathology scores of the hemisphere (Fig 3D) differed between groups. There was a significant effect of the factors group (F (5,180) = 455.7 p<0.0001), and sex (F (1,180) = 5.025, p=0.0270) and an interaction between the factors (F(5,108) = 2.506 p=0.0346). The HI group showed a greater pathology score as compared to the NAÏVE and SHAM

groups ($p<0.05$). Both males and females of the TH-2h group had reduced scores as compared to the HI group ($p<0.05$), however females of the TH-2h group had a smaller scores compared to the male of the TH-2h group ($p<0.05$).

The two-way ANOVA of pathology scores of the hippocampus (Fig. 3E), detected a significant effect of the factor group ($F (5,180) = 111.2$, $p<0.0001$), with no effect of sex ($F (1,180) = 0.01549$, $p=0.5012$). HI rats showed a significant higher pathological score as compared to NAÏVE and SHAM groups ($p<0.05$), and the TH-2h group showed a lower pathological score compared to the HI group, for both sexes ($p<0.05$).

Two-way ANOVA of pathology score of the cerebral cortex (Fig. 3F) showed a significant effect of both factors, group ($F(5,180) = 238.8$, $p<0.0001$) and sex ($F (1,180) = 7.032$, $p=0.0092$), as well as a significant interaction ($F(5,108) = 8.369$, $p = 0.0220$). It was also observed that the HI group had a greater pathology score compared to NAÏVE and SHAM groups, for both sexes ($p<0.05$). In the males, both TH-2h and TH-4h groups showed a reduction in the pathology score relative to the HI group ($p<0.05$). Among females, only the TH-2h group reduced the score compared to the HI group ($p<0.05$). In addition, females of the TH-4h group showed a higher pathology score in the cerebral cortex compared to the males of the group TH-4h ($p<0.05$).

2.4 Cell Counting

The percentage of degenerative cells was analyzed in the CA1 and hilus subfields of the hippocampus ipsilateral to ischemia (Fig. 4A). For CA1, two-way ANOVA detected a significant effect of factors group ($F (5,180) = 350.4$ $p<0.0001$) and sex ($F (1,180) = 14.22$, $p=0.0003$), with an interaction between them ($F(5,108) = 8.369$, $p<0.0001$). The HI group showed a higher percentage of degenerative cells in relation to the NAÏVE and SHAM groups ($p<0.05$). In males, the TH-2h group showed a reduced

percentage of degenerative cells in the CA1 area as compared to the HI group ($p<0.05$). In females this reduction in the percentage of degenerative cells in CA1 was observed in the groups TH-2h and TH-4h, as compared to the HI group ($p<0.05$). Moreover, females belonging to the TH-2h and TH-4h groups showed a higher percentage of degenerative cells in the CA1 area as compared to males ($p<0.05$).

When the hilus was evaluated (Fig. 4B), two-way ANOVA showed a significant effect of the factors group ($F(5,180) = 350.4$ $p<0.0001$) and sex ($F(1,180) = 14.22$, $p=0.0003$), with an interaction between them ($F(5,108) = 6.056$, $p<0.0001$). For both sexes, the HI group followed the same pattern observed in the CA1 area, showing an increase in the percentage of degenerative cells as compared to the NAÏVE and SHAM groups ($p<0.05$). Males of the TH-2h and TH-4h groups showed a decrease in the percentage of degenerative cells in the hilus relative to the HI group ($p<0.05$). Among females, only the animals of the TH-2h group showed a reduction in the percentage of degenerative cells in the hilus as compared to the HI group ($p<0.05$). However, a sex-related difference was also observed: females of the groups HI and TH-2h showed a reduced percentage of degenerative cells compared to the males of the same groups, respectively ($p<0.05$). Besides that, females belonging to the TH-6h group showed an increase in the percentage of degenerative cells in relation to the HI group ($p<0.05$).

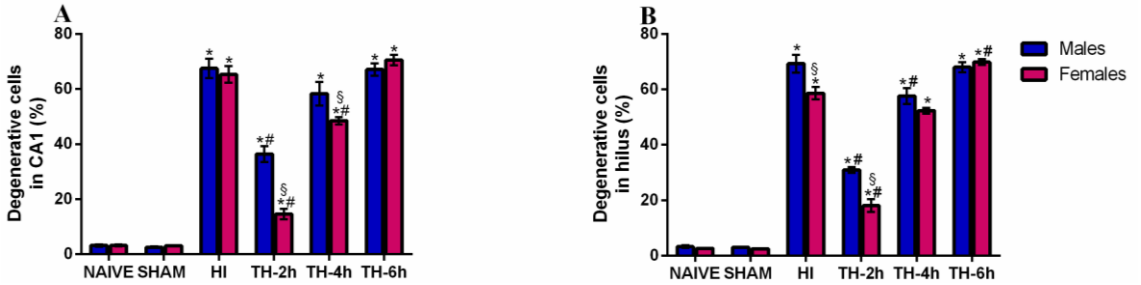

Figure 4. Percentage of degenerative neurons in the areas of CA1 and hilus of the hippocampus ipsilateral to ischemia of male and female animals. (A, B). Data were analyzed by two-way ANOVA followed by Bonferroni and expressed as mean ±SEM. * significant differences compared to the NAÏVE and SHAM group ($p<0.05$); # significant

differences compared to the HI group ($p<0.05$); § significant differences between males and females ($p<0.05$).

2.5 GFAP immunoreactivity and astrocytes morphology

GFAP immunoreactivity and astrocyte morphology were studied in the CA1 subfield of the hippocampus ipsilateral to the carotid occlusion (Fig. 5). Two-way ANOVA showed a significant effect of the factors group ($F(4,40) = 19.87$ $p<0.0001$) and sex ($F(1,40) = 6.625$ $p=0.00139$) on GFAP fluorescence intensity (Fig. 5A). Animals of the HI group showed an increase in the GFAP fluorescence intensity compared to the control group, for both sexes. Males from the TH-2h and TH-4h groups showed a reduction in GFAP fluorescence relative to the HI group ($p<0.05$). However, only females from the TH-2h group showed this same pattern of reduction ($p<0.05$).

In the analysis of the length of the astrocyte processes (Fig. 5B), two-way ANOVA detected a significant effect of both factors, group ($F(4,37) = 55.28$ $p<0.3598$) and sex ($F(1,37) = 0.8596$ $p=0.3598$). Animals from both sexes showed an increase in the length of the processes in the HI group as compared to controls; however, only the females from the TH-2h group had a reduced length of the processes as compared to the HI group.

In addition, two-way ANOVA showed a significant effect of the factor group ($F(4,39) = 31.42$, $p<0.0001$) but not of the factor sex ($F(1,39) = 1.865$, $p=0.1799$) for the number of primary process from the astrocytes (Fig. 5C). The HI group of both sexes showed the same pattern observed in previous astrocyte analyses: there was an increase in the number of astrocyte primary processes as compared to the control group ($p<0.05$). TH treatment reduced the number of processes compared to the HI group only in the TH-2h group of females ($p<0.05$).

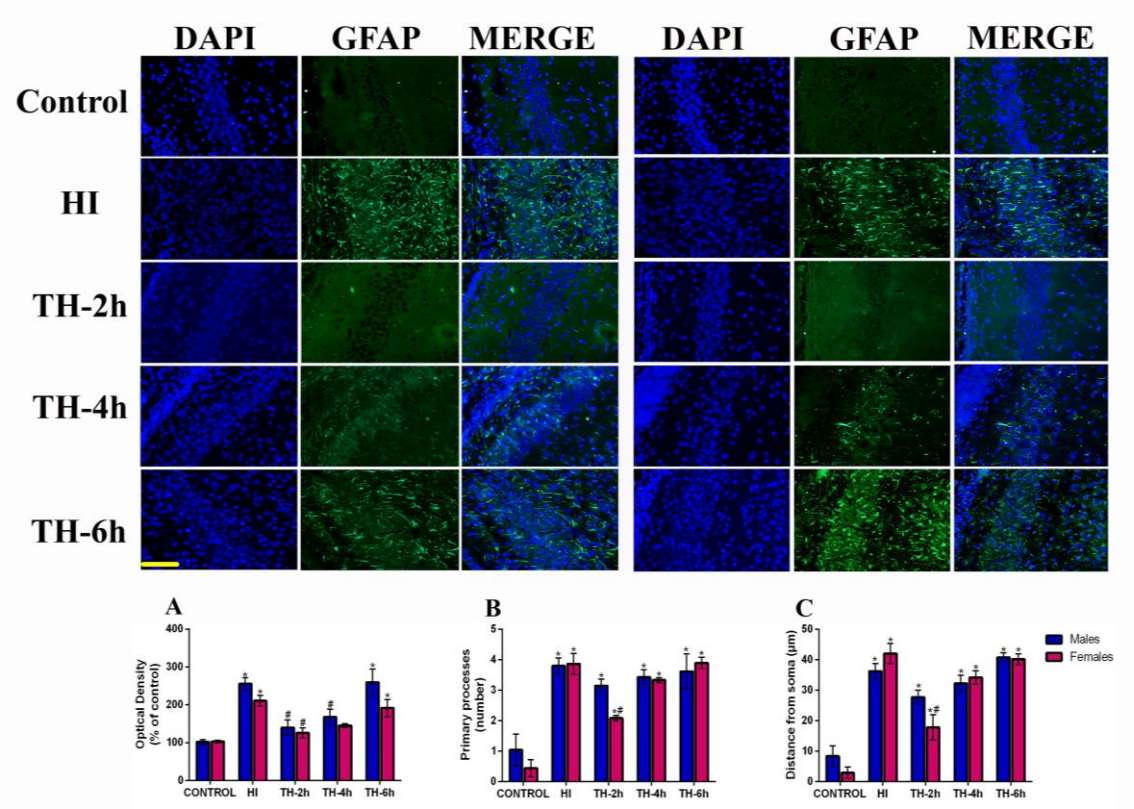

Figure 5. *Upper panel* representative images of GFAP immunofluorescence staining from each experimental group in the area CA1. GFAP immunofluorescence, length of process of soma and the number of primary processes of the area of CA1 of the hippocampus ipsilateral to carotid occlusion in males and females (A, B and C). Data were analyzed by two-way ANOVA followed by Bonferroni and expressed as mean ±SEM. * significant differences compared to the control group (p<0.05); # significant differences compared to the HI group (p<0.05). Bar = 100 µm

2.6 Behavioral Assessment

The results of negative geotaxis and righting reflex tests are shown in Figure 6. Two-way ANOVA of negative geotaxis data showed a significant effect of the factor group (F (5,180) = 85.66 p<0.0001) but not of the factor sex (F (1,180) = 1.361, p=0.2449); however, there was a significant interaction between the factors (F (5,108) = 5.231, p=0.0002). The HI group from both sexes showed an increase in the latency to complete the test as compared to the NAIVE groups. In males from TH-2h and TH-4h groups there was a decrease in the latency to complete the test relative to the HI group.

In females, however, only the TH-2h group had reduced latency to complete the test relative to the HI group. It was also observed an increase in the latency in the female TH-6h group relative to the HI group (Fig. 6B).

The righting reflex analysis showed a significant effect of the factor group ($F(5,180) = 25.55$ $p<0.0001$), but not of the factor sex ($F(1,180) = 0.2714$ $p=0.6030$); an interaction between the factors was also found ($F(5,180) = 2.637$, $p=0.0250$). Similar to the results of negative geotaxis, the HI group from both sexes showed an increase in the latency to complete the test relative to the NAÏVE and SHAM groups. However, only the males from the TH-2h group reduced the latency as compared to the HI group. Moreover, females from the TH-6h group showed an increase in the latency to complete the test relative to the HI group (Fig. 6B), as already observed in the negative geotaxis test.

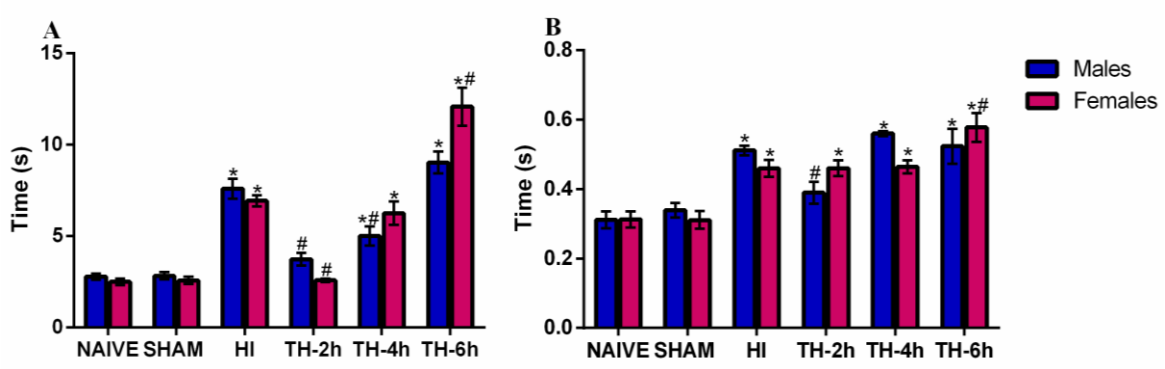

Figure 6. Results of righting reflex and negative geotaxis to animals (p14) of male and female animals. (A, B) Negative geotaxis and righting reflex of the males and females, respectively. Data were analyzed by two-way ANOVA followed by Bonferroni and expressed as mean ±SEM. * significant differences compared to the NAÏVE and SHAM group ($p<0.05$); # significant differences compared to the HI group ($p<0.05$).

2.7 Data Correlation

2.7.1 Correlation between the volume of lesion and the pathology score

Some studies use the pathological score in order to estimate the level of brain lesion following neonatal HI (Sabir et al., 2014; Thoresen et al., 1996). In order to assess the validity of our pathological score we tested if it was correlated with the volume of lesion. There was a positive correlation between the pathological score and the volume of lesion for all structures evaluated (hemisphere, hippocampus, and cerebral cortex) in both sexes ($p<0.05$), (Table 1).

2.7.2 Correlation between volume of lesion, pathology score and behavioral performance

When the latency to complete the behavioral tests was plotted against the results of the volume of lesion or the pathology score a positive correlation was observed for all brains structures evaluated (hemisphere, hippocampus and cerebral cortex) in both sexes ($p<0.05$).

2.7.3 Correlation among the volume of lesion, pathology score and percentage of degenerative cells and assessment of astrogliosis in hippocampus

The data of lesion volume, pathological score and percentage of degenerative cells in the hippocampus showed a positive correlation with the level of astrogliosis (mainly related to the length of the processes and the number of primary processes) for both sexes ($p<0.05$).

Table 1. Correlation between histological and behavioural data separated by males and females. † = very weak association, ‡ = weak association, § = moderate association, * = strong association, and ** = very strong association.

Males	**Volume of Lesion**			**Behavioral Assessment**		**Immunofluorescence (GFAP)**		
	Hemisphere	Hippocapus	Cerebral Cortex	Negative Geotaxis	Righting Relex	Fluorescence Intensity	Primary process	Distance from SOMA
Volume of Lesion	**r^2**	**r^2**	**r^2**	**r^2**	**r^2**	**r^2**	**r^2**	**r^2**
Hemisphere	-	-	-	0.4831§	0.3017‡	-	-	-
Hippocapus	-	-	-	0.3835‡	0.3243‡	0.3430‡	0.2905‡	0.5307§
Cerebral Cortex	-	-	-	0.5017§	0.3110‡	-	-	-
Degenerative Cells								
CA1 of Hippocampus	-	-	-	-	-	0.6516*	0.8092**	0.7782*
Pathology Score								
Hemisphere	0.9538**	-	-	0.4368§	0.3334‡	-	-	-
Hippocapus	-	0.9084**	-	0.4505§	0.2969‡	0.3247‡	0.3250‡	0.5946§
Cortex Cerebral	-	-	0.9066**	0.4361§	0.3155‡	-	-	-
Females	**Volume of Lesion**			**Behavioral Assessment**		**Immunofluorescence (GFAP)**		
	Hemisphere	Hippocapus	Cortex Cerebral	Negative Geotaxis	Righting Relex	Fluorescence Intensity	Primary process	Distance from SOMA
Volume of Lesion	**r^2**	**r^2**	**r^2**	**r^2**	**r^2**	**r^2**	**r^2**	**r^2**
Hemisphere	-	-	-	0.4321§	0.1882†	-	-	-
Hippocapus	-	-	-	0.5500§	0.4158‡	0.4065§	0.7136*	0.6611*
Cortex Cerebral	-	-	-	0.3514‡	0.2056‡	-	-	-
Degenerative Cells								
CA1 of Hippocampus	-	-	-	-	-	0.6516*	0.8092**	0.7782*
Pathology Score								
Hemisphere	0.9259**	-	-	0.4269§	0.2175‡	-	-	-
Hippocapus	-	0.9237**	-	0.4505§	0.2106‡	0.5821§	0.7026*	0.6726*
Cortex Cerebral	-	-	0.9264**	0.3103‡	0.2548‡	-	-	-

3. Discussion

The best results of therapeutic hypothermia are achieved when treatment starts immediately or at the shortest interval after the hypoxic-ischemic event (Cho et al., 2020; Sabir et al., 2012). However, although this can be achieved in animal models, in clinical practice it is not always possible to start HT shortly after HI, since testing for confirmation of brain damage is necessary before hypothermia starts (Cho et al., 2020).

Here, results showed the effective time window to start experimental TH is sex-dependent. TH initiated earlier, i.e., 2 hours after the injury reduced the extent of lesion in the hemisphere, hippocampus, and cerebral cortex for both sexes. However, starting hypothermia as late as 6 hours after hypoxia worsened outcomes for morphological (percentage of degenerative cells in the hippocampus) and behavioral parameters.

Body weight is a developmental parameter which can also provide important information about the protective effects of hypothermia. It is well known that HI can lead to motor deficits which, in turn, lead to feeding difficulties and affect weight gain of the animals (Fabres et al., 2018; Sanches et al., 2013). Animals from the HI group showed a reduction in the body weight relative to the SHAM and NAIVE groups when evaluated 7 days after HI (P14). However, the TH-2h group did not show this reduction, indicating a beneficial effect of hypothermia when started 2h after HI. Such an important developmental parameter was not seen when hypothermia was started later (TH-4h and TH-6h groups).

Sabir and colleagues (2012 and 2014) did not observe any difference in weight gain between HI animals and animals of both sexes treated with hypothermia (32ºC, 5h) started immediately or 3, 4, 6 or 12h after the injury (Sabir et al., 2014, 2012). However, these studies did not use any group of animals not submitted to neonatal HI, which makes it difficult the comparison with present work.

This prevention of weight loss seen in the TH-2h group may be associated with a lower volume of brain damage and, consequently, less motor damage. Here, animals belonging to the TH-2h group also showed a reduction in the lesion volume in every brain structure evaluated as compared to the HI group (Fig. 3). However, when TH was started later (TH-4h and TH-6h groups), this neuroprotective effect of TH was blunted, suggesting TH started later may not be able to protect the brain.

There are experimental and clinical evidence showing that the closer the onset of hypothermia is to the moment of HI, the greater will be the reduction of brain injury (Sabir et al., 2014, 2012; Thoresen et al., 2013). Sabir *et al*. (2012) observed that hypothermia started immediately or 3h after HI caused a reduction in brain injury, an effect not seen when hypothermia was started later (at 6h and 12h following HI) (Sabir et al., 2012). Park and colleagues (2015) also did not observe neuroprotective effects when hypothermia was started 6 hours after the injury (Park et al., 2015), corroborating with our findings.

To strengthen the analysis of brain injury, we decided to calculate the pathological score (Thoresen et al., 1996) of the brain structures from which the injury volume was assessed. The correlation between the data of the pathological score and the lesion volume showed a very strong association proving both forms of analysis can be used.

Although the analysis of brain injury volume and pathological score demonstrated a beneficial effect of hypothermia initiated early, no sex-related differences were found for these parameters. However, it is known that there is difference in the type of cell death depending on the sex; in females, cell death is mainly dependent on caspase activation, whereas in males cell death is caspase-independent. Therefore, an evaluation at the cellular level could help to find any difference between the sexes. The hippocampus was chosen for this analysis, since it is the most affected brain structure in the animal model of neonatal HI (Dhikav and Anand, 2012).

When the percentage of degenerative cells in the pyramidal layer of the CA1 and hilus was evaluated, a sex-related difference was revealed. Animals from the HI group showed an 8-fold increase in the percentage of degenerative cells for both sexes, which is in accordance to our previous study using only males (Fabres et al., 2020). TH reduced the percentage of cells in degeneration when started up to 4h after the injury, although the most striking neuroprotection was observed in the TH-2h group. In addition, in both hilus and CA1, the percentage of degenerative cells in females from the TH-2h group was around 15%, while in the males of HT-2h this percentage was higher, around 30% in the hilus and 35% in CA1. These results corroborate with data in the literature showing that HT is able to reduce neuronal death in the CA1 area of the hippocampus on the third and seventh days after injury (Wood et al., 2016; Xiong et al., 2009).

The hilus of the dentate gyrus was chosen for being a region considered less vulnerable to injury than the CA1 area. It was showed that hypothermia started earlier was able to reduce cell degeneration in the hilus; on the other hand, when started 6h after HI, the number of degenerating cells in females was even greater than that observed in the HI group.

Reductions in the volume of brain injury and in the percentage of degenerative cells are important factors indicative of the efficacy of TH; however, it is well known that brain injury caused by HI can extend for weeks to months (Davidson et al., 2015). Neuroinflammation is one of the main causes of extension of the injury for longer periods. It is well known that male animals submitted to neonatal HI show increased microglial activation and upregulation of the inflammatory response (Netto et al., 2017). However, astrocytes are also players in this process and astrogliosis is also related to neuroinflammation (Davidson et al., 2015).

In our study, TH started earlier (TH-2h) decreased GFAP fluorescence intensity in the CA1 area, suggesting a reduction of astrogliosis for both, males and females. However, for a more detailed assessment of astrocyte activation, we used Sholl circles to quantify astrocyte complexity (arbor lenght and number of processes) (Mari et al., 2019; Mestriner et al., 2011). Sholl analyses showed females from HT-2h group had a reduction in the number of primary processes as well as in the length of the processes as compared to the HI group, which was not seen for males.

The morphology of astrocytes may reflect the functional significance of neuroglial interactions (Sheikhbahaei et al., 2018). The glial scar formation triggered by neural lesions such as those produced after hypoxic-ischemic events, when uncontrolled, may affect neural communication and cell function (Sheikhbahaei et al., 2018; Sofroniew, 2018). Therefore, although GFAP fluorescence intensity was used to infer increased astrocyte reactivity, females from the group TH-2h showed a smaller number of primary processes and shorter length, indicating a more organized glial scar formation; this is conceivably beneficial for tissue repair.

Astrocyte reactivity parameters (evaluated by GFAP immunofluorescence intensity and Sholl circles) also showed a strong association with the percentage of degenerative cells in CA1 (Table 1). That would be expected since astrogliosis is a phenomenon in which astrocytes branch off and extend their processes to form a glial scar at the locations where neurons died. A study by Reddy and colleagues (2020) also observed a correlation between the volume of the hippocampus and the astrogliosis marker GFAP+, corroborating the hypothesis of glial scar formation (Reddy et al., 2020).

In addition to the morphological parameters, an analysis of neurodevelopmental parameters is also important and can provide broader data on the neuroprotective effects

of TH. Previous studies from our group (Tassinari et al., 2020) and others (Lubics et al., 2005) have shown HI causes an increase in the latency to complete the negative geotaxis test, as seen here.

However, together with the neuroprotective effects of HT on morphological and structural variables, we also observed an improvement in the behavioral response in the test of negative geotaxis in the animals from TH-2h group of both sexes. Jatana and colleagues (2006) showed hypothermia was not able to reduce latency in the negative geotaxis test, 7 days after injury; however, hypothermia protocol lasted 3 hours less than the one used here, which could explain the differences in results. By starting hypothermia 3 hours after injury and using a prolonged period of hypothermia (48h)(Jatana et al., 2006), Ahn and colleagues (2018) observed a reduction in the latency when animals reached 42 days of life (Ahn et al., 2018) but not in P14, P21, P28, and P35. It has already been observed in animal models that hypothermia periods longer than 5h have no additional neuroprotective effects (Sabir et al., 2012). Moreover, prolonged periods of maternal separation produces negative effects on behavior (Lehmann et al., 1999), which could explain why longer periods of hypothermia do not lead to an improvement of behavioral outcomes.

When the righting reflex was evaluated, only males from TH-2h group showed a reduction in the latency to complete the test as compared to the HI group. Yuan *et al* (2015) also observed a tendency of hypothermia in reducing the latency to complete the righting reflex test, as compared to the HI group; however, sex-dependent effects were not assessed (Yuan et al., 2015). In the present study, HT was not able to improve the female response in the righting reflex test, regardless of the period in which it was started (2h, 4h or 6h after HI).

The behavioral improvement observed in males and females in the negative geotaxis test, as well as in males in the righting reflex test, may be related to the reduction in the volume of lesion and in the percentage of degenerative cells observed. To test this hypothesis, a correlation between behavioral results and morphological and structural parameters was run. We found only a weak to moderate association depending on the parameters compared (Table 1). This can be explained because the behavioral tests used here are characterized by reflex movements and they are more related to brain areas not evaluated in the study (Heyser, 2003; Schneider and Przewłocki, 2005).

Our study has some limitations. First, we have evaluated only one time point (P14, 7 days after the HI event) and it is well known the lesion can extend for days to months. Therefore, further studies using animals from both sexes treated with TH and followed for longer periods are needed, which would allow the assessment of cognitive function using specific behavioral tests during adulthood. Second, we did not evaluate the molecular mechanisms underlying the neuroprotective effects of TH. Understanding the molecular basis of TH effects can allow for combined treatments for the achievement of better neuroprotective outcomes.

4. Concluding remarks

Summarizing, it was shown that TH started earlier after the HI injury leads to a reduction in brain damage and astrogliosis, as well as to a decrease in the latencies to complete behavioral tests, for both sexes. However, when TH is initiated later (i.e., 6h after HI), females showed an increase in the percentage of degenerative cells in the hippocampus ipsilateral to the lesion. Females from TH-6h group also showed an increase in the latency in the behavioral tests, as compared to the HI group. Furthermore, this paper is the first one to report that the effect of TH on astrogliosis is dependent on the sex. Therefore, we emphasize the importance of starting TH as soon as possible for both sexes.

If there is a need for the treatment to be started later, even greater care should be given to females, as a late start of the treatment can intensify the injury in female animals.

5. Experimental Procedure

5.1 Animals

In this study we used 192 seven-day-old Wistar rats (P7) (male and female) and 24 dams obtained from the Animal Facility of the Department of Biochemistry of the Federal University of Rio Grande do Sul, Porto Alegre, RS. The puppies were kept in 270 x 260 x 310 mm plastic boxes together with their respective mothers (08 puppies per box together with their respective mother). All animals were kept in a 12h/12h light/dark cycle with controlled temperature (± 22ºC) and the mothers received food and water *ad libitum*. This study was approved by the Institutional Animal Care and Use Committee of the Federal University of Rio Grande do Sul (#31442)

5.2 Neonatal hypoxia-ischemia

The neonatal HI procedure was based on the Rice-Vannuci model and in previous studies (Fabres et al., 2020, 2018; Rice et al., 1981). In P7, under anaesthesia with isoflurane (5% for induction and 3% for maintenance), neonates underwent surgery for occlusion of the right common carotid artery. A longitudinal incision was made two millimetres to the right of the trachea. The right common carotid artery was isolated and permanently occluded using a surgical thread (silk 4.0). After surgery, the pups were left with their mothers for 1h-2h for recovery and then placed in a hypoxia chamber (n = 6 animals per chamber) and exposed to an atmosphere of a certified mixture of 8% O_2 and 92% N_2 for 90 minutes at 37°C. After the hypoxic exposure, pups were removed from the chamber and kept in a warm box, for approximately 15 minutes, and then returned to their mothers.

5.3 Therapeutic Hypothermia

Animals were placed in an acrylic chamber inside a temperature-controlled water bath to receive hypothermia (18-20ºC). The animals reached a body temperature of 32ºC (temperature indicated for therapeutic hypothermia in newborn animals (Wood et al., 2016) within 20 minutes. Animals failing to reach this temperature within the initial 20 minutes were discarded from the experiment. Animals were maintained in the hypothermic chamber for 5 uninterrupted hours. After hypothermia, the animals were rewarmed for 30 minutes until a body temperature of 33-34ºC and then returned to their mothers (Wood et al., 2016).

For this study three different therapeutic time windows were used, i.e., three different intervals of time to start hypothermia after HI. Hypothermia was started two hours (TH-2h group), four hours (TH-4h) or six hours (TH-6h) after the hypoxic-ischemic event. During the period between the end of the HI and the beginning of hypothermia, the animals were kept in their boxes together with their mothers. For each time window (2, 4 or 6h to start hypothermia) there were three respective control groups: a NAIVE group (animals not manipulated), a SHAM group and a HI group (animals submitted to HI but kept in normothermia). These groups allowed us to check for differences caused by the time animals stayed away from their mothers, for example. However, as no differences were verified among these control groups, we grouped them into a single SHAM and a single HI group. All experimental groups were additionally separated in males and females. The timeline of experiments is depicted in Figure 1.

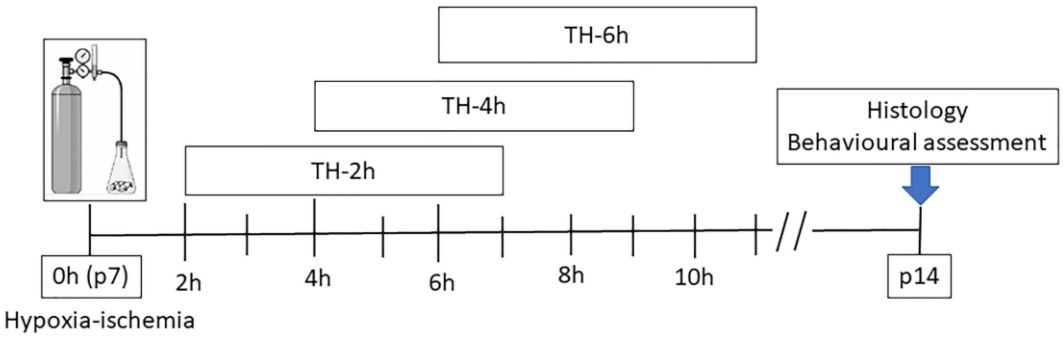

Figure 1. Representative figure of the experimental design. The animals treated for hypothermia were divided into groups by sex and by the time of beginning of treatment, 2, 4 or 6 hours after the end of hypoxia.

5.4 Body temperature and body weight

The measurement of the temperature of each animal during the experiments was made by using an infrared thermometer (Incoterm, TCl 1000). In a pilot evaluation, the accuracy of the infrared thermometer was compared to that of a rectal thermometer; a difference lower than 0.5ºC was observed between both (data not shown). Thus, we decided to continue the experiments using only the infrared thermometer because it is a non-invasive one (Smith et al., 2015).

Animal's body temperature was measured every 5 minutes during the first 20 minutes of hypothermia. All animals in the TH groups reached the temperature of 32±0.5ºC within these first 20 minutes. After that, body temperature of animals from groups SHAM, HI and TH was measured every 30 minutes. Temperature of the animals of the NAIVE group (kept in their own boxes) was not measured in order to reduce manipulation effects, as well as to reduce mother's stress. Every animal was weighed in two moments: before the surgery for carotid occlusion (P7) and just before euthanasia (P14).

5.5 Histological analysis

In order to evaluate the volume of lesion of the cerebral hemisphere, hippocampus and cerebral cortex, animals (P14) were deeply anesthetized with isoflurane and transcardially perfused with saline solution (0.9% NaCl) followed by 4% paraformaldehyde (PFA). Brains were removed and submerged in a solution of 4% PFA overnight, then dehydrated in an alcoholic series (80%, 90%, 96% and 100%) and cleared in xylene. Afterwards, the brains were embedded in paraffin, sectioned (7 μm) using a microtome (Microm HM 340E ThermoScientific) and stained with hematoxylin and eosin (HE).

The following antero-posterior coordinates were used for brain analysis: +2.52 mm to -6.84 mm relative to bregma for analysis of the cerebral hemisphere; +1.70 mm to -4.16 mm for the cerebral cortex and -2.04 mm to -6.12 mm for the hippocampus. All coordinates were according to the Paxinos and Watson rat brain atlas (Paxinos and Watson, 2007) and to a previous study (Fabres et al., 2020). To reduce possible errors due to brain oedema the equation by Sun *et al.* (2015) was used, according previous studies (Fabres et al., 2020; Sun et al., 2015).

The same images used to evaluate the volume of brain lesion were used to calculate a score indicative of lesion severity (n = 10), according to (Thoresen et al., 1996). The severity of the injury was divided into 9 levels varying from 0 (without injury) to 4 (maximum injury), with 0.5 intervals. The classification was performed based on the percentage of injury according to the scale: 0 (without lesion), 1 (injury equal to or less than 10%), 2 (indicative of 20-30% of lesion), 3 (injury between 40-60%) and 4 (more than 75% of injury). For the calculations of the pathological score of the hippocampus, animals with lesions below or equal to 20% were classified with a score of 1; a score of 2 indicates 50% of injury, a pathological score of 3 indicates 75% of lesion; and a score

of 4 indicates a value of 100% of injury. The evaluation criteria were based on the paper by Thoresen *et al.* (Thoresen et al., 1996) with modifications.

5.6 Cell counting

For estimating the number of cells in degeneration (cells showing shrinkage and deformity of the cell body or karyorrhectic and pyknotic nuclei) we used slides stained with haematoxylin and eosin as described in our previous study (Fabres et al., 2020). The subfields CA1 and hilus of the dentate gyrus of the hippocampus ipsilateral to the lesion were evaluated. Five different sections containing the CA1 and hilus were evaluated per animal. The images were obtained using a microscope (Zeiss) at magnification of × 400. The mean of the number of degenerative cells divided by the mean of the total number of cells × 100 was used to estimate the percentage of degenerative cells in each subfield.

5.7 Immunofluorescence and morphological analysis

For immunofluorescence analysis the sections were deparaffinized using xylene (10 minutes) and rehydrated using a decreasing alcoholic series. After that, the protocol followed the one used in a previous study (Fabres et al., 2020). For identification of astrocytes, the primary anti-glial fibrillary acidic protein antibody (anti-GFAP, #G9269, rabbit IgG, 1:200, Sigma-Aldrich) was used while the secondary antibody of choice was Alexa 488 anti-rabbit (1:500, Molecular Probes, Invitrogen).The slides were assembled with mounting medium containing DAPI (Merck, F6057) and coverslipped.

For the analysis of fluorescence intensity (as an indicative of astrogliosis), 5 sections per animal were used (n = 5 animals per group); the NAIVE and SHAM groups were used as controls. All images were captured with high magnification (400 x) using a Nikon Eclipse E-600 microscope (Japan). An area of optical interest of 3800 μm^2 was

defined to delimit all areas of interest and the value of the integrated density per unit of area was obtained using the ImageJ software (NIH, Bethesda, USA).

The assessment of astrocyte morphology in the CA1 area of the hippocampus (number of primary processes and the length of the processes relative to the soma), was run in 3 astrocytes per section (from 5 sections per animal, totalling 15 astrocytes per animal). The Sholl's concentric circles method was used for this analysis: concentric circles with 2 μm of intervals were drawn around each cell analyzed using the Image-Pro Plus software 6.0 (Mestriner et al., 2011).

5.8 Behavioural Assessment

The test of negative geotaxis was performed according to Ahn and colleagues (2018), with minor modifications. At P14, animals were placed individually on a platform with 35º of inclination, with heads turned towards the bottom of the platform. The latency of the animals to rotate 180º and position themselves with their heads facing the top of the platform was registered (Ahn et al., 2018). Each puppy was tested three times and the average was calculated.

To test the righting reflex at P14, animals were removed from their cages and put on a flat surface on their back. The latency necessary to straighten up with all paws on the surface was recorded.

5.9 Data analysis

All statistical analyses were performed using GraphPad Prism 6.0. Normality test was performed, and parametric data were analyzed by a two-way analysis of variance (ANOVA) (factors: sex and groups) followed by Bonferroni post-hoc test. Data were expressed as mean±SEM and the significance level were set as $p<0.05$.

Declaration of interest

The authors declare that they have no conflict of interest.

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References

Ahn, S.Y., Chang, Y.S., Sung, D.K., Sung, S.I., Park, W.S., 2018. Hypothermia broadens the therapeutic time window of mesenchymal stem cell transplantation for severe neonatal hypoxic ischemic encephalopathy. Sci. Rep. 8, 1–10. https://doi.org/10.1038/s41598-018-25902-x

Askalan, R., Gabarin, N., Armstrong, E.A., Fang Liu, Y., Couchman, D., Yager, J.Y., 2015. Mechanisms of neurodegeneration after severe hypoxic-ischemic injury in the neonatal rat brain. Brain Res. 1629, 94–103. https://doi.org/10.1016/j.brainres.2015.10.020

Burnsed, J.C., Chavez-Valdez, R., Hossain, M.S., Kesavan, K., Martin, L.J., Zhang, J., Northington, F.J., 2015. Hypoxia-ischemia and therapeutic hypothermia in the neonatal mouse brain - A longitudinal study. PLoS One 10, 1–20. https://doi.org/10.1371/journal.pone.0118889

Cho, K.H.T., Davidson, J.O., Dean, J.M., Bennet, L., Gunn, A.J., 2020. Cooling and immunomodulation for treating hypoxic-ischemic brain injury. Pediatr. Int. 62, 770–778. https://doi.org/10.1111/ped.14215

Davidson, J.O., Wassink, G., van den Heuij, L.G., Bennet, L., Gunn, A.J., 2015. Therapeutic Hypothermia for Neonatal Hypoxic–Ischemic Encephalopathy – Where to from Here? Front. Neurol. 6. https://doi.org/10.3389/fneur.2015.00198

Davies, A., Wassink, G., Bennet, L., Gunn, A., Davidson, J., 2019. Can we further optimize therapeutic hypothermia for hypoxic-ischemic encephalopathy? Neural Regen. Res. 14, 1678–1683. https://doi.org/10.4103/1673-5374.257512

Dhikav, V., Anand, K., 2012. Hippocampus in health and disease: An overview. Ann. Indian Acad. Neurol. 15, 239. https://doi.org/10.4103/0972-2327.104323

Fabres, R.B., da Rosa, L.A., de Souza, S.K., Cecconello, A.L., Azambuja, A.S., Sanches, E.F., Ribeiro, M.F.M., de Fraga, L.S., 2018. Effects of progesterone on the neonatal brain following hypoxia-ischemia. Metab. Brain Dis. 1–9. https://doi.org/10.1007/s11011-018-0193-7

Fabres, R.B., Montes, N.L., Camboim, Y. de M., de Souza, S.K., Nicola, F., Tassinari, I.D., Ribeiro, M.F.M., Netto, C.A., de Fraga, L.S., 2020. Long-Lasting Actions of Progesterone Protect the Neonatal Brain Following Hypoxia-Ischemia. Cell. Mol. Neurobiol. https://doi.org/10.1007/s10571-020-00827-0

Fang, A.Y., Gonzalez, F.F., Sheldon, R.A., Ferriero, D.M., 2013. Effects of combination therapy using hypothermia and erythropoietin in a rat model of neonatal hypoxia-ischemia. Pediatr. Res. 73, 12–17. https://doi.org/10.1038/pr.2012.138

Gunn, A.J., Gunn, T.R., De Haan, H.H., Williams, C.E., Gluckman, P.D., 1997. Dramatic neuronal rescue with prolonged selective head cooling after ischemia in fetal lambs. J. Clin. Invest. 99, 248–256. https://doi.org/10.1172/JCI119153

Heyser, C.J., 2003. Assessment of Developmental Milestones in Rodents. Curr. Protoc. Neurosci. 25, 1–15. https://doi.org/10.1002/0471142301.ns0818s25

Jatana, M., Singh, I., Singh, A.K., Jenkins, D., 2006. Combination of systemic hypothermia and N-acetylcysteine attenuates hypoxic-ischemic brain injury in neonatal rats. Pediatr. Res. 59, 684–689. https://doi.org/10.1203/01.pdr.0000215045.91122.44

Joly, L.M., Mucignat, V., Mariani, J., Plotkine, M., Charriaut-Marlangue, C., 2004. Caspase Inhibition after Neonatal Ischemia in the Rat Brain. J. Cereb. Blood Flow Metab. 24, 124–131. https://doi.org/10.1097/01.WCB.0000100061.36077.5F

Lehmann, J., Pryce, C.., Bettschen, D., Feldon, J., 1999. The Maternal Separation Paradigm and Adult Emotionality and Cognition in Male and Female Wistar Rats. Pharmacol. Biochem. Behav. 64, 705–715. https://doi.org/10.1016/S0091-3057(99)00150-1

Lubics, A., Reglodi, D., Tamás, A., Kiss, P., Szalai, M., Szalontay, L., Lengvári, I., 2005. Neurological reflexes and early motor behavior in rats subjected to neonatal hypoxic-ischemic injury. Behav. Brain Res. 157, 157–165. https://doi.org/10.1016/j.bbr.2004.06.019

Mari, C., Odorcyk, F.K., Sanches, E.F., Wartchow, K.M., Martini, A.P., Nicola, F., 2019. International Journal of Developmental Neuroscience Arundic acid administration protects astrocytes , recovers histological damage and memory de fi cits induced by neonatal hypoxia ischemia in rats. Int. J. Dev. Neurosci. 76, 41–51. https://doi.org/10.1016/j.ijdevneu.2019.06.003

Mestriner, R.G., Pagnussat, A.S., Boisserand, L.S.B., Valentim, L., Netto, C.A., 2011. Skilled reaching training promotes astroglial changes and facilitated sensorimotor recovery after collagenase-induced intracerebral hemorrhage. Exp. Neurol. 227, 53–61. https://doi.org/10.1016/j.expneurol.2010.09.009

Mirza, M.A., Ritzel, R., Xu, Y., McCullough, L.D., Liu, F., 2015. Sexually dimorphic outcomes and inflammatory responses in hypoxic-ischemic encephalopathy. J. Neuroinflammation 12, 1–10. https://doi.org/10.1186/s12974-015-0251-6

Nelson, K.B., Lynch, J.K., 2004. Stroke in newborn infants. Lancet Neurol. 3, 150–158.

https://doi.org/10.1016/S1474-4422(04)00679-9

Netto, C.A., Sanches, E., Odorcyk, F.K., Duran-Carabali, L.E., Weis, S.N., 2017. Sex-dependent consequences of neonatal brain hypoxia-ischemia in the rat. J. Neurosci. Res. https://doi.org/10.1002/jnr.23828

Nie, X., Lowe, D.W., Rollins, L.G., Bentzley, J., Fraser, J.L., Martin, R., Singh, I., Jenkins, D., 2016. Sex-specific effects of N-acetylcysteine in neonatal rats treated with hypothermia after severe hypoxia-ischemia. Neurosci. Res. 108, 24–33. https://doi.org/10.1016/j.neures.2016.01.008

Park, W.S., Sung, S.I., Ahn, S.Y., Yoo, H.S., Sung, D.K., Im, G.H., Choi, S.J., Chang, Y.S., 2015. Hypothermia augments neuroprotective activity of mesenchymal stem cells for neonatal hypoxic-ischemic encephalopathy. PLoS One 10, 1–13. https://doi.org/10.1371/journal.pone.0120893

Paxinos, G., Watson, C., 2007. The Rat Brain in Stereotaxic Coordinates, Elsevier S. ed, Elsevier Science. SanDiego, CA.

Reddy, S.D., Wu, X., Kuruba, R., Sridhar, V., Reddy, D.S., 2020. Magnetic resonance imaging analysis of long-term neuropathology after exposure to the nerve agent soman: correlation with histopathology and neurological dysfunction. Ann. N. Y. Acad. Sci. 1–20. https://doi.org/10.1111/nyas.14431

Reinboth, B.S., Köster, C., Abberger, H., Prager, S., Bendix, I., Felderhoff-Müser, U., Herz, J., 2016. Endogenous hypothermic response to hypoxia reduces brain injury: Implications for modeling hypoxic-ischemic encephalopathy and therapeutic hypothermia in neonatal mice. Exp. Neurol. 283, 264–275. https://doi.org/10.1016/j.expneurol.2016.06.024

Rice, J.E., Vannucci, R.C., Brierley, J.B., 1981. The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann. Neurol. 9, 131–141. https://doi.org/10.1002/ana.410090206

Sabir, H., Osredkar, D., Maes, E., Wood, T., Thoresen, M., 2016. Xenon combined with therapeutic hypothermia is not neuroprotective after severe hypoxia-ischemia in neonatal rats. PLoS One 11, 1–10. https://doi.org/10.1371/journal.pone.0156759

Sabir, H., Scull-Brown, E., Liu, X., Thoresen, M., 2012. Immediate hypothermia is not neuroprotective after severe hypoxia-ischemia and is deleterious when delayed by 12 hours in neonatal rats. Stroke 43, 3364–3370. https://doi.org/10.1161/STROKEAHA.112.674481

Sabir, H., Walløe, L., Dingley, J., Smit, E., Liu, X., Thoresen, M., 2014. Combined treatment of Xenon and hypothermia in newborn rats - Additive or synergistic effect? PLoS One 9, 1–7. https://doi.org/10.1371/journal.pone.0109845

Sanches, E.F., Arteni, N., Nicola, F., Aristimunha, D., Netto, C.A., 2015. Sexual dimorphism and brain lateralization impact behavioral and histological outcomes following hypoxia-ischemia in P3 and P7 rats. Neuroscience 290, 581–593. https://doi.org/10.1016/j.neuroscience.2014.12.074

Sanches, E.F., Arteni, N.S., Nicola, F., Boisserand, L., Willborn, S., Netto, C. a., 2013. Early hypoxia-ischemia causes hemisphere and sex-dependent cognitive impairment and histological damage. Neuroscience. https://doi.org/10.1016/j.neuroscience.2013.01.066

Schneider, T., Przewłocki, R., 2005. Behavioral alterations in rats prenatally to valproic acid: Animal model of autism. Neuropsychopharmacology 30, 80–89. https://doi.org/10.1038/sj.npp.1300518

Shankaran, S., Laptook, A.R., Ehrenkranz, R.A., Tyson, J.E., McDonald, S.A., Donovan, E.F., Fanaroff, A.A., Poole, W.K., Wright, L.L., Higgins, R.D., Finer, N.N., Carlo, W.A., Duara, S., Oh, W., Cotten, C.M., Stevenson, D.K., Stoll, B.J., Lemons, J.A., Guillet, R., Jobe, A.H., 2005. Whole-Body Hypothermia for Neonates with Hypoxic–Ischemic Encephalopathy. N. Engl. J. Med. 353, 1574–1584. https://doi.org/10.1056/NEJMcps050929

Sheikhbahaei, S., Morris, B., Collina, J., Anjum, S., Znati, S., Gamarra, J., Zhang, R., Gourine, A. V, Smith, J.C., 2018. Morphometric analysis of astrocytes in brainstem respiratory regions. J Comp Neurol 526, 2032–2047. https://doi.org/10.1002/cne.24472

Smith, A., Garbus, H., Rosenkrantz, T., Fitch, R., 2015. Sex Differences in Behavioral Outcomes Following Temperature Modulation During Induced Neonatal Hypoxic Ischemic Injury in Rats. Brain Sci. 5, 220–240. https://doi.org/10.3390/brainsci5020220

Smith, A.L., Rosenkrantz, T.S., Fitch, R.H., 2016. Effects of sex and mild intrainsult hypothermia on neuropathology and neural reorganization following neonatal hypoxic ischemic brain injury in rats. Neural Plast. 2016. https://doi.org/10.1155/2016/2585230

Sofroniew, M. V, 2018. Astrogliosis.

Sun, H.-S., Xu, B., Chen, W., Xiao, A., Turlova, E., Alibraham, A., Barszczyk, A., Bae, C.Y.J., Quan, Y., Liu, B., Pei, L., Sun, C.L.F., Deurloo, M., Feng, Z.-P., 2015. Neuronal KATP channels mediate hypoxic preconditioning and reduce subsequent neonatal hypoxic–ischemic brain injury. Exp. Neurol. 263, 161–171. https://doi.org/10.1016/j.expneurol.2014.10.003

Tassinari, I.D.Á., Andrade, M.K.G., da Rosa, L.A., Hoff, M.L.M., Nunes, R.R., Vogt, E.L., Fabres, R.B., Sanches, E.F., Netto, C.A., Paz, A.H., de Fraga, L.S., 2020. Lactate Administration Reduces Brain Injury and Ameliorates Behavioral Outcomes Following Neonatal Hypoxia–Ischemia. Neuroscience. https://doi.org/10.1016/j.neuroscience.2020.09.006

Thoresen, M., Bågenholm, R., Løberg, E.M., Apricena, F., Kjellmer, I., 1996. Posthypoxic cooling of neonatal rats provides protection against brain injury. Arch. Dis. Child. 74, 0–5. https://doi.org/10.1136/fn.74.1.f3

Thoresen, M., Hobbs, C.E., Wood, T., Chakkarapani, E., Dingley, J., 2009. Cooling combined with immediate or delayed xenon inhalation provides equivalent long-term neuroprotection after neonatal hypoxia-ischemia. J. Cereb. Blood Flow Metab. 29, 707–714. https://doi.org/10.1038/jcbfm.2008.163

Thoresen, M., Tooley, J., Liu, X., Jary, S., Fleming, P., Luyt, K., Jain, A., Cairns, P., Harding, D., Sabir, H., 2013. Time Is Brain: Starting Therapeutic Hypothermia within Three Hours after Birth Improves Motor Outcome in Asphyxiated Newborns. Neonatology 104, 228–233. https://doi.org/10.1159/000353948

Wagner, B.P., Nedelcu, J., Martin, E., 2002. Delayed postischemic hypothermia improves long-term behavioral outcome after cerebral hypoxia-ischemia in neonatal rats. Pediatr. Res. 51, 354–360. https://doi.org/10.1203/00006450-200203000-00015

Wood, T., Osredkar, D., Puchades, M., Maes, E., Falck, M., Flatebø, T., Walløe, L., Sabir, H., Thoresen, M., 2016. Treatment temperature and insult severity influence the neuroprotective effects of therapeutic hypothermia. Sci. Rep. 6, 23430. https://doi.org/10.1038/srep23430

Xiong, M., Yang, Y., Chen, G., Zhou, W., 2009. Post-ischemic hypothermia for 24 h in P7 rats rescues hippocampal neuron : Association with decreased astrocyte activation and inflammatory cytokine expression. Brain Res. Bull. 79, 351–357. https://doi.org/10.1016/j.brainresbull.2009.03.011

Yuan, X., Ghosh, N., McFadden, B., Tone, B., Bellinger, D.L., Obenaus, A., Ashwal, S., 2015. Hypothermia modulates cytokine responses after neonatal rat hypoxic-ischemic injury and reduces brain damage. ASN Neuro 6. https://doi.org/10.1177/1759091414558418

Zhu, C., Xu, F., Wang, X., Shibata, M., Uchiyama, Y., Blomgren, K., Hagberg, H., 2006. Different apoptotic mechanisms are activated in male and female brains after neonatal hypoxia-ischaemia. J. Neurochem. 96, 1016–1027. https://doi.org/10.1111/j.1471-4159.2005.03639.x

5 Discussão

A HI neonatal causa falha energética aguda, devido à falta de oxigênio e de glicose no encéfalo, seguida por normalização transitória durante a reperfusão e por deficiência de energia secundária posterior, no período de 6–24 h. Embora a hipotermia, a intervenção terapêutica padrão, seja eficaz em diversos casos, ela pode ter efeitos adversos. As diretrizes clínicas recomendam que a HT seja iniciada nas primeiras 6 horas após o nascimento, com base nos achados de um modelo de HI fetal de ovelha (equivalente aos bebês nascidos a termo), mostrando neuroproteção significativa quando a HT foi iniciada antes de 5,5 horas após a lesão cerebral (Gunn et al., 1997). No entanto, ainda hoje é difícil identificar de forma confiável bebês que sofreram HI dentre os muitos que precisam de ressuscitação e têm acidose metabólica ao nascimento. Nos ensaios clínicos atuais, o resfriamento não foi iniciado antes de 4 a 5,5 h após o evento hipóxico-isquêmico (Cho et al., 2020).

Portanto, há uma necessidade crucial de terapias adicionais que possam ser utilizadas numa janela terapêutica maior do que aquela da hipotermia para atenuar os danos ao encéfalo em desenvolvimento e diminuir a morbidade nos sobreviventes (Tang et al., 2017). Contudo, estudos clínicos e experimentais demonstraram que a HI neonatal não é um evento único, mas sim um "*processo evolutivo*" que leva à morte celular a longo prazo, ou seja, horas a dias após o evento inicial (Wassink et al., 2014). Logo, uma nova intervenção terapêutica deveria ter efeitos multifatoriais, isto é, acionar vários mecanismos moleculares, além de reduzir a lesão encefálica e produzir melhora cognitiva e motora. Intervenções que estabilizam o metabolismo cerebral após a HI também podem ser uma alternativa (Cho et al., 2020; Davidson et al., 2015; Wassink et al., 2014).

Os hormônios sexuais são conhecidos por influenciar a diferenciação neuronal durante o desenvolvimento fetal. Em adultos, a PROG afeta a função neural modulando

a transcrição gênica e a atividade celular por meio de receptores de membrana e intracelulares abundantes no SNC (Arbo et al., 2014; Schumacher et al., 2004).

A PROG exerce uma ampla gama de ações, dependendo do tecido-alvo. Em um modelo animal de lesão cerebral penetrante, a PROG diminuiu a astrogliose nas proximidades da lesão (Garcia-Segura et al., 1999). Evidências crescentes indicam que a PROG também oferece proteção contra outras formas de lesão neural. Outros autores relataram que a PROG exerce efeitos benéficos sobre a lesão da medula espinhal, incluindo melhora da remielinização e da função motora (Baulieu, 1998), além de favorecer a cognição após eventos distintos de dano cerebral, como lesão cerebral traumática (Sarkaki et al., 2013b) e isquemia cerebral (Aggarwal et al., 2008b) em animais adultos.

5.1 A PROG tem efeitos de longa duração em animais neonatais submetidos ao modelo de HI

No primeiro capítulo testamos os possíveis efeitos neuroprotetores da PROG administrada em 3 momentos distintos: imediatamente antes da isquemia (grupo PRÉ), 6h e 24h após o final da hipóxia (grupo PÓS) e nos 3 momentos (grupo PP), ou seja, imediatamente antes da isquemia, e 6 e 24h após a lesão. Os resultados mostram que a PROG, independente do momento em que foi administrada, causa neuproteção, a qual foi observada 7 dias após a HI (P14). A PROG reduziu a lesão no hemisfério e no hipocampo ipsilateral à oclusão da carótida, diminuiu a porcentagem de células degenerativas nas áreas do CA1 e hilo do hipocampo, aumentou a expressão de proteína relacionada a efeitos anti-apoptóticos e reduziu a ativação das vias de morte celular e a astrogliose.

Estudos corroboram que a PROG pode ser benéfica em diversos modelos de lesões do sistema nervoso, ou seja, atua como um agente neuroprotetor (Cervantes et al., 2002; Chen et al., 1999; Peterson et al., 2015). Um tratamento com PROG mostrou aumentar a proliferação e diferenciação de progenitores de oligodendrócitos em células produtoras de mielina maduras, e aumenta o fator de transcrição (Olig1) necessário para reparar a desmielinização induzida por lesão traumática do SNC em animais adultos (De Nicola et al., 2009). Peterson e colaboradores (Peterson et al., 2015) trabalharam com ratos P7 da linhagem Sprague-Dawley, utilizando machos e fêmeas submetidos aos eventos hipóxicos-isquêmicos. Em um dos grupos de animais, a PROG (8mg/kg) foi administrada em um total de 9 injeções, nos tempos 0 (logo após o fim do evento hipóxico), 2 e 24 horas e 2, 3, 4, 5, 6 e 7 dias após a HI. Os animais foram eutanasiados com 52 dias (P52), para permitir uma análise da resposta da PROG a longo prazo. Foi verificado que a PROG reduziu a lesão nos animais machos submetidos ao procedimento de HI. Por outro lado, os resultados obtidos com fêmeas não mostraram qualquer efeito neuroprotetor capaz de reduzir a lesão. Portanto, além da idade do animal ser um fator de extrema importância para a escolha do modelo e tratamento, como já discutido, a diferença sexual também influencia a resposta, neste caso mostrando um efeito neuroprotetor apenas em animais machos (Peterson et al., 2015).

No entanto, trabalho publicado por nosso grupo verificou que a utilização da PROG em animais de 7 dias submetidos ao modelo de HI, não causou efeitos neuroprotetores e nem adversos quando avaliado o volume de lesão, peso corporal e vias de sobrevivências e de apoptose 48h após a lesão (Fabres et al., 2018). Por outro lado, (Tsuji et al., 2012) relataram que a PROG aumentou o dano encefálico em ratos Wistar de 14 dias de idade submetidos ao modelo de HI, em comparação com animais de 7 dias de idade. Já animais com 21 dias de idade tratados com PROG e submetidos à HI

apresentaram lesão semelhante ao grupo sem a administração de PROG (Tsuji et al., 2012).

Sugerimos que a diferença nos resultados encontrados por Tsuji e colaboradores (2012) possa ser explicada pela diferença de idade dos animais ao serem submetidos ao modelo de HI. Estudos já demonstram que a PROG tem efeitos modulatórios sobre a função do receptor $GABA_A$ e que o receptor GABA pode possuir ações excitatórias em idades neonatais, diferente da característica inibitória encontrada em animais adultos (Arbo et al., 2014; Ben-Ari, 2002; Ben-Ari et al., 2007). Isso se dá por uma diferença de proporção entre os transportadores de cloreto presentes na membrana plasmática (NKCC1 e KCC2) das células neuronais, fazendo com que haja um efluxo de cloreto nos neurônios em resposta ao GABA que, portanto, assume um papel excitatório (Ben-Ari et al., 2012).

Em roedores, já foi demonstrado que a expressão de KCC2 é mínima ao nascimento, baixa durante a primeira semana de vida e comparável ao adulto apenas em P14–15. Já a expressão de NKCC1 em neurônios corticais é mais alta durante a primeira semana pós-natal, diminuindo em P14 para os níveis mais baixos, similares aos encontrados em adultos (Dzhala and Staley, 2015; Dzhala et al., 2005). A excitabilidade excessiva é um dos fatores que levam à morte celular no evento hipóxico-isquêmico neonatal, portanto a idade do animal, para escolha da administração da PROG, deve ser avaliada em futuros experimentos.

Nesse mesmo trabalho (Tsuji et al., 2012), também foi verificado que a administração tanto de alopregnanolona, metabólito da PROG relacionado com efeitos rápidos, quanto de PROG a exógena produziam os mesmos efeitos, levando à hipótese de que a PROG poderia acentuar a lesão (nesse caso) através de seu principal metabólito, a alopregnanolona. A utilização de uma curva utilizando doses diferentes de

alopregnanolona (1, 3 e 10 mg/kg) constatou uma diminuição significativa do volume do hemisfério ipsilateral à lesão HI apenas nos grupos que receberam as doses de 3 mg/kg e 10 mg/kg de alopregnanolona. A dose de 1 mg/kg de alopregnanolona não acentuou a lesão produzida pela HI (Tsuji et al., 2012). Em nosso estudo utilizamos a maior dose testada (10mg/kg) para todos os tempos de administração (grupos PRÉ, PÓS e PP).

Assim, os resultados contraditórios, tanto benéficos como deletérios da PROG, podem ser explicados por diferenças na dose, janela de administração e idade dos animais.

Em nosso estudo anterior (Fabres et al., 2018), utilizando a mesma dose de PROG e os mesmos tempos de administração, não observamos efeitos protetores sobre os parâmetros avaliados em animais com 7 dias (P7) submetidos à HI neonatal avaliados 48h após a lesão (P9). Isso levantou dúvidas em relação à PROG ter efeitos de longa duração ou realmente não ter efeitos neuroprotetores neste modelo. Portanto, no presente estudo, avaliamos os animais 7 dias após a lesão, e foi possível observar uma redução da lesão em todos os grupos, como também redução da expressão de caspase-3 (associadas às vias de morte celular) e aumento da atividade da via da Akt (via de sobrevivência celular). Com isso, mesmo não observando melhora em nossas variáveis no estudo de 2018 (Fabres et al., 2018), os resultados desta tese sugerem que os efeitos estavam ocorrendo e que foi possível observar o seu resultado em P14, quando foi observada redução da lesão encefálica (Fabres et al., 2020).

Estudos utilizando a PROG em outros modelos experimentais de lesão encefálica demonstraram que os efeitos benéficos da PROG têm relação com o aumento da expressão da Akt-p (Ishrat et al., 2012; Li et al., 2015; Stanojlović et al., 2019). A Akt-p tem características anti-apoptóticas por inibir vias que podem levar à clivagem e consequente ativação da caspase-3 (Franke et al., 2003).

A Akt-p promove a fosforilação e inativação da maioria de seus substratos (Franke et al., 2003; Hanada et al., 2004), como o fator de transcrição "*forkhead*" (FKHR), a proteína de morte associada ao Bcl-2 (Bad) e a glicogênio sintase cinase 3 (GSK3), promovendo a sobrevivência celular. Bad e FKHR, são fatores citosólicos e são normalmente fosforilados pela Akt (Zhao et al., 2005). A desfosforilação da Bad causa sua translocação para as mitocôndrias, desencadeando a liberação do citocromo C. O citocromo C então ativa a caspase-3, levando à apoptose (Jiang and Wang, 2004).

O FKHR desfosforilado se transloca para dentro do núcleo e atua como um fator de transcrição, promovendo a superexpressão do mediador de morte celular que interage com Bcl-2 (Bim) e ligante Fas (Brunet et al., 2001; Hanada et al., 2004). Bim causa a liberação do citocromo C, enquanto o ligante Fas leva à ativação da caspase-8 e da caspase-3 (Graham and Chen, 2001). A Akt também regula negativamente a atividade da GSK3β, fosforilando-a no resíduo Ser9 (Bhat et al., 2000). A desfosforilação da GSK3β leva à sua ativação, que fosforila a β-catenina, um fator de transcrição que desempenha papéis importantes na sobrevivência celular (Nusse, 2003).

Dessa forma, a administração da PROG leva ao aumento da expressão da Akt-p e consequente inibição dos substratos FKHR, Bad e GSK3β, resultando em redução da expressão e ativação da caspase-3 (Figura 3). No entanto, esse efeito parece ser de longa duração, como visto em nosso estudo, onde apenas foi possível observar aumento da Akt-p e, portanto, redução da caspase-3 clivada após 7 dias a lesão.

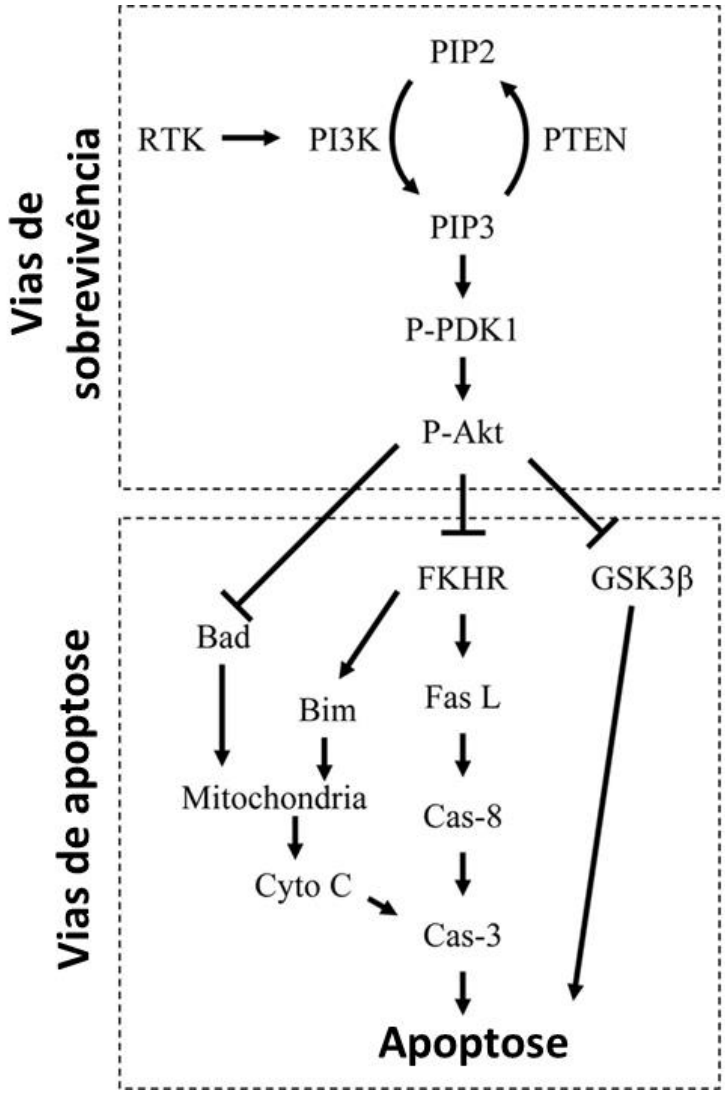

Figura 3 – Um diagrama das vias de sobrevivência e apoptose moduladas pela Akt. Figura modificada de (Zhao et al., 2005)**. PIP:** fosfatidilinositol fosfato; **PIP3:** fosfatidilinositol-3,4,5-trisfosfato, **PIP2:** fosfatidilinositol-4,5-bifosfato **PI3K:** fosfoinositídeo 3-cinase **RTK:** receptor tirosina cinase; **PTEN:** proteína fosfatase e homólogo de tensina; **P-PDK1:** Proteína cinase-1-dependente de fosfoinositídeo **FKHR:** forkhead transcription factor **GK3β:** glicogênio sintase cinase-3β **Cas-8:** caspase-8 **Cas-3:** caspase-3 **Cyto C:** citocromo C

Estudos com modelos de isquemia em animais adultos corroboram com nossos achados, mostrando que a Akt promove a sobrevivência neuronal (Friguls et al., 2002; Noshita et al., 2001). Além disso, a proteção por alguns agentes em modelos de animais transgênicos e *knockout* envolvem a manutenção da atividade Akt após acidente vascular cerebral (Limbourg et al., 2002; Noshita et al., 2003).

Nosso trabalho também sugere que a via da Akt pode modular a expressão da GFAP. Em nosso estudo, a administração da PROG após a lesão se mostrou eficiente em reduzir a expressão da GFAP no hipocampo de animais neonatos. Já foi demonstrado que a Akt-p pode regular negativamente a expressão de GFAP através de FABP7-PPARγ e MKP3 (Tripathi et al., 2017). A ação da GFAP em modelos de lesões encefálicas é contraditória, porém sugere-se que o aumento da expressão da GFAP, consequência da astrogliose, pode ter função neuroprotetora no SNC em encéfalos sem lesão encefálica (Sofroniew, 2020). No entanto, após uma lesão ou doença no SNC pode ocorrer expressão de receptores toll-like (TLRs), além de alterações morfológicas que podem levar a aumento da neuroinflamação e, por consequência, aumento na morte neuronal e extensão da lesão por vários dias (Widestrand et al. 2007; Li et al. 2008).

Neste trabalho foi possível observar que todos os grupos que receberam a administração da PROG reduziram a porcentagem de células degenerativas no hipocampo em comparação ao grupo submetido ao modelo de HI que não recebeu a PROG. É possível que, se analisado em um tempo mais longo, após os 14 dias, fosse possível observar uma redução ainda mais acentuada desta degeneração nos grupos que receberam a PROG após a lesão. Isso porque apenas esses grupos reduziram a expressão de GFAP. Portanto, esse estudo sugere que a PROG, se administrada após a lesão, teria ação redutora da astrogliose por meio do aumento da expressão da Akt-p. Além disso, após a lesão, os astrócitos, como também a micróglia reativa e os oligodendrócitos, podem expressar receptores de membrana do tipo alfa para a progesterona (mPRα), indicando que as células gliais poderiam ser um alvo para a modulação pela PROG nessa condição (Arbo et al., 2014; Meffre et al., 2007).

5.2 A janela terapêutica da hipotermia é a mesma para ambos os sexos?

Os protocolos atuais de HT para HI moderada a grave envolvem iniciar o resfriamento o mais rápido possível, dentro de 6 horas após o nascimento, até 34,5ºC (±0,5ºC) para resfriamento seletivo da cabeça ou 33,5ºC (±0,5ºC) para resfriamento de corpo inteiro, com resfriamento contínuo por 72 horas (Cho et al., 2020). Em estudos experimentais ainda não há um consenso sobre o protocolo de HT ideal. Os protocolos diferenciam-se em relação à temperatura da hipotermia, o tempo de início e também quanto à duração do tratamento (Burnsed et al., 2015; Gunn et al., 1997; Sabir et al., 2012; Smith et al., 2015; Wood et al., 2020).

Em relação a melhor temperatura para utilizar na HT em modelos experimentais, um estudo utilizou ratos Wistar com 7 dias submetidos ao modelo de HI neonatal e que foram resfriados em uma média de 33,5°C, 32°C , 30°C, 26°C ou 18°C por 5 horas após a HI, e foi observado não haver proteção adicional com temperaturas abaixo de 33,5°C (Wood et al., 2016). Além disso, Sabir e colaboradores (2012) observaram que ratos Wistar com idade de P7 submetidos ao modelo de HI e tratados com HT (32ºC) por 5h, apresentavam efeitos neuroprotetores semelhantes aos observados em animais submetidos a um período mais longo de tratamento, de 10h (Sabir et al., 2012). No mesmo estudo publicado por Sabir (2012) foi observado que a HT iniciada 3h após a lesão não foi suficiente para reduzir a lesão encefálica, enquanto que animais que iniciaram o tratamento após 12h apresentaram aumento da lesão em relação ao grupo submetido à HI e não tratado com HT.

Modelos experimentais utilizando ratos já mostraram que existe dimorfismo sexual significativo associado aos mecanismos de lesão e à ativação de vias de morte celular (Burnsed et al., 2015; Netto et al., 2017; E. F. Sanches et al., 2013). No entanto, poucos trabalhos abordam o efeito do dimorfismo sobre a eficácia da HT (Burnsed et al.,

2015; Sabir et al., 2012; Smith et al., 2015, 2016; Wood et al., 2020). Dentre os trabalhos que avaliaram se o efeito da HT poderia ser dependente do sexo do animal não houve um consenso, com os trabalhos apresentando resultados discordantes (Burnsed et al., 2015; Sabir et al., 2012; Smith et al., 2015, 2016; Wood et al., 2020). Portanto, não se sabe se o sexo tem um efeito na resposta ao tratamento com HT na prática clínica ou nos modelos translacionais. Além disso, nenhum trabalho avaliou se o efeito da janela terapêutica da HT poderia ser influenciado pela diferença sexual. Em nosso segundo artigo avaliamos o uso da HT em modelo animal de HI neonatal.

Em camundongos, a neuroproteção mediada pela HT (31ºC por 4h) foi notável em machos, com neuroproteção em todas as regiões analisadas na idade P18; esta neuroproteção persistiu em P30 e confirma a neuroproteção hipotérmica generalizada em vez de específica no encéfalo de camundongos (Burnsed et al., 2015). Neste mesmo estudo de Burnsed e colaboradores (2015) foi observado que a porcentagem de lesão em fêmeas tratadas com HT foi reduzida nas regiões analisadas em P18; no entanto, quando atingiam P30 a lesão era semelhante ao grupo HI sem tratamento. O estudo sugeriu lesão inicial menor e neuroproteção transitória em fêmeas. No mesmo estudo, a hipotermia forneceu melhora da memória em machos, o que não ocorreu em fêmeas.

Nos estudos de Smith e colaboradores (2015 e 2016), em que foi utilizado o modelo de HI neonatal em ratos Wistar P7, a HT foi administrada durante todo o período de hipóxia. Smith avaliou o efeito da HT em ambos os sexos e observou tendência de redução do volume de lesão encefálica (Smith et al., 2016) e melhora na execução dos testes comportamentais em fêmeas tratadas (Smith et al., 2015).

Um estudo mais recente, analisou um grande número de ratos Wistar machos e fêmeas submetidos ao modelo de HI neonatal e tratados com HT (32ºC, iniciada

imediatamente após a hipóxia, por 5h) (Wood et al., 2020). O estudo observou melhora no escore patológico utilizado para avaliar o grau da lesão encefálica apenas em fêmeas.

Esses estudos podem diferir em seus resultados por apresentarem diferenças em suas metodologias, espécies de animais utilizads, e variáveis analisadas. No entanto, nenhum dos trabalhos analisou tempos diferentes de início do tratamento por hipotermia comparando com possíveis diferenças sexuais. Em nosso trabalho foi possível observar que quando a hipotermia é iniciada mais cedo, maior é a neuroproteção em ambos os sexos. No entanto, quanto o início do tratamento é mais demorado, observam-se efeitos neuroprotetores menos significativos ou ausentes, corroborando com os dados encontrados por Sabir e colaboradores (2012).

Quando o tratamento tem início ao final do momento conhecido por janela terapêutica da HT (6 horas após o evento hipóxico-isquêmico), os machos não apresentaram neuroproteção nas estruturas encefálicas avaliadas (hemisfério, hipocampo e córtex cerebral ipsilateral à oclusão da carótida) para volume de lesão, como também no escore patológico. O mesmo foi observado quando foram avaliadas as áreas de CA1 e hilo e a astrogliose no hipocampo. No entanto, as fêmeas que iniciaram a hipotermia após 6h, aumentaram a porcentagem de células em degeneração no hilo do hipocampo ipsilateral à lesão, como também a latência para a realização dos testes comportamentais em comparação ao grupo submetido a HI sem tratamento.

Esses resultados demonstram que a janela terapêutica utilizada como padrão para a HT nos dois sexos deve ser questionada, uma vez que dentro do tempo considerado seguro (por sugerir não apresentar pioras comportamentais e aumento de dano encefálico) ocorre piora em fêmeas, mesmo que em parâmetros diferentes do volume de lesão encefálica. No entanto, os animais foram avaliados apenas 7 dias após a lesão, sendo necessárias análises futuras em idade adulta.

5.3 A hipotermia modula a astrogliose

A lesão encefálica hipóxico-isquêmica pode iniciar uma reação inflamatória estéril, com inflamação periférica e indução microglial local (Hagberg et al., 2015). Estudos em ovelhas fetais prematuras relataram que a HI regula positivamente citocinas circulantes e induz ativação microglial crônica e astrogliose entre 3-21 dias após o insulto (Cho et al., 2019; van den Heuij et al., 2019). Criticamente, a microglia e os astrócitos liberam citocinas pró-inflamatórias durante a evolução da lesão neural, que podem prejudicar a maturação celular e facilitam a morte de oligodendrócitos e neurônios (Cho et al., 2020). Assim, esses dados apoiam fortemente a indução secundária ou crônica da glia e a produção de citocinas como mecanismos-chave na patogênese da lesão cerebral HI.

A hipotermia pós-insulto inibe a ativação microglial e a indução de citocinas pró-inflamatórias. Estudos sugerem que a eficácia da hipotermia após a HI pode ser dependente da via específica TLR (Cho et al., 2020). Esse receptor por muito tempo foi relacionado apenas com as células da microglia, no entanto atualmente se sabe que ele é expresso também pelos astrócitos (Cho et al., 2020; Sofroniew, 2020). Além disso, a utilização da HT em modelos de isquemia cerebral em ratos adultos demonstrou que a HT poderia elevar a expressão da proteína Akt, que através da inibição da Bad, FKHR e GSK3β pode reduzir a astrogliose (Diao et al., 2020; Fukui et al., 2006; Tripathi et al., 2017).

No entanto, um efeito da TH sobre a astrogliose que ocorre de forma dependente do sexo, em modelo de HI neonatal, não havia sido demonstrado até o presente momento. Além disso, já foi demonstrado que a astrogliose aumentada em um SNC saudável tem efeitos não apenas neuroprotetores mas também plásticos. Enquanto que em situações adversas, nas quais o SNC sofre alguma lesão, a astrogliose pode levar a um aumento da

liberação de sinalizadores e citocinas que podem aumentar a inflamação no tecido, podendo estender a morte celular por dias ou até meses (Cho et al., 2020; Sofroniew, 2020). A morfologia dos astrócitos durante o processo de astrogliose após uma lesão no SNC pode ser alterada, aumentando os processos primários e suas ramificações, sinalizando uma desordem e um aumento da inflamação (Sheikhbahaei et al., 2018; Xiong et al., 2009).

Nossos resultados mostram que apenas nas fêmeas que iniciaram o tratamento 2h após a lesão, houve redução no número de processos primários e no comprimento das ramificações dos astrócitos, demonstrando uma possível redução na sinalização da inflamação no hipocampo ipsilateral à oclusão carotídea. Essa redução poderia explicar alguns resultados da literatura que observaram que as fêmeas, quando o tratamento foi iniciado logo após a HI, apresentaram menor lesão encefálica (Wood et al., 2020). Como nosso trabalho avaliou apenas uma idade, não foi possível confirmar a hipótese em animais adultos.

5.4 A PROG pode ser uma alternativa para o tratamento após a fase latente?

A HT é o único tratamento utilizado na clínica atualmente, no entanto, ela tem efeitos em apenas uma pequena parcela de pacientes (em torno de 11%), levando à necessidade de buscas por novos tratamentos (Cho et al., 2020). A eficácia limitada da HT deve-se em parte a uma janela terapêutica curta para o início do tratamento, de apenas 6h após o insulto. Estudos já demonstraram que a média de tempo para o início da TH na clínica é de 4 a 5,5h após o insulto (Cho et al., 2020).

Estudos experimentais que utilizem tratamentos alternativos que possam levar à neuroproteção após esse período de tempo são extremamente necessários. Para que o tratamento seja eficaz ele deve agir em diversas vias que estarão alteradas após o evento

HI. Em razão disso, os neuroesteroides foram escolhidos para serem testados em diversos modelos de lesão encefálica, inclusive após o evento HI. No entanto, os resultados da literatura são contraditórios em relação à PROG e HI neonatal (Fabres et al., 2018; Peterson et al., 2015; Tsuji et al., 2012). Utilizando uma dose de 10 mg/kg e a administração da PROG 6h (tempo máximo da janela terapêutica para uso da HT, que não produziu melhoras nos machos e piorou os desfechos em fêmeas) e 24h após o início da lesão, foi possível observar neuroproteção com redução do volume de lesão no hemisfério e hipocampo, além de redução das células degenerativas nas áreas hipocampais de CA1 e do hilo, além do aumento da expressão da Akt e redução da caspase-3 clivada (Fabres et al., 2020). Além de atuar sobre vias de sinalização semelhantes às vias afetadas pela hipotermia, como a via da Akt, sugere-se que, após a lesão, a PROG seja capaz de se ligar a receptores presentes nos astrócitos e assim reduzir a astrogliose. Portanto, a PROG se mostrou uma candidata para o uso combinado com a hipotermia, podendo ser uma alternativa para casos em que o tempo máximos da janela terapêutica de HT seja ultrapassado.

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6 Conclusão

A presente Tese demonstra que a PROG tem efeitos neuroprotetores em modelo experimental de HI neonatal, apresentando redução da lesão encefálica e na estrutura do hipocampo ipsilateral a oclusão da carótida comum direita. Esse resultado se deve a um aumento da expressão da proteína Akt-p, a qual reduz a expressão da caspase-3 clivada, proteína chave para apoptose. A PROG também se mostrou eficiente em reduzir a astrogliose, mecanismo com efeito neuroinflamatório em lesões encefálicas, sugerindo uma ação através do aumento da Akt-p e uma provável ligação aos receptores mPRα. O efeito neuroprotetor apareceu mesmo quando a PROG foi administrada após 6h da lesão encefálica, portanto, no limite de tempo da janela terapêutica da HT. Resultados presentes na tese também demonstram que a HT tem efeitos neuroprotetores dependentes do dimosfismo sexual, e do tempo de início do tratamento, com efeitos benéficos sendo mais evidentes se iniciada mais próxima possível ao início da lesão por HI. Também observamos que as fêmeas, quando iniciado o tratamento ao final da janela terapêutica, tiveram piora em alguns parâmetros avaliados, aumento de células degenerativas e tempo de latência, em comparação aos animais sem tratamento.

Tanto a PROG quanto a HT reduziram a astrogliose causada pela HI, sendo esse efeito da HT dependente do sexo (com redução mais proeminente em fêmeas). Assim, estudos futuros sobre a HT devem observar com especial cuidado o momento de início e seus efeitos dependente do sexo. Além da PROG ser uma candidata em potencial para utilização juntamente com a HT para tratar HI neonatal.

7 Perspectivas

Avaliar se os efeitos neuroprotetores da HT com janelas terapêuticas diferentes permanecem após a fase adulta.

Avaliar se a associação de HT e PROG causa maior neuroproteção, quando comparado aos efeitos individuais, em animais machos e fêmeas submetidos ao modelo de HI neonatal.

8 Referências

Aggarwal, R., Medhi, B., Pathak, A., Dhawan, V., Chakrabarti, A., 2008a. Neuroprotective effect of progesterone on acute phase changes induced by partial global cerebral ischaemia in mice. J. Pharm. Pharmacol. 60, 731–7. https://doi.org/10.1211/jpp.60.6.0008

Aggarwal, R., Medhi, B., Pathak, A., Dhawan, V., Chakrabarti, A., 2008b. Neuroprotective effect of progesterone on acute phase changes induced by partial global cerebral ischaemia in mice. J. Pharm. Pharmacol. 60, 731–737. https://doi.org/10.1211/jpp.60.6.0008

Albee, M., Michael, B., 2003. GFAP : Functional Implications Gleaned From Studies of Genetically Engineered Mice. Glia 90, 87–90. https://doi.org/10.1002/glia.10219

Aras, R., Barron, A.M., Pike, C.J., 2012. Caspase activation contributes to astrogliosis. Brain Res. 1450, 102–115. https://doi.org/10.1016/j.brainres.2012.02.056

Arbo, B.D., Andrade, S., Osterkamp, G., Ribeiro, M., 2014. Effect of low doses of progesterone in the expression of the GABA (A) receptor a 4 subunit and procaspase-3 in the hypothalamus of female rats. Endocrine 46, 561–567. https://doi.org/10.1007/s12020-013-0126-5

Asati, V., Mahapatra, D.K., Bharti, S.K., 2016. European Journal of Medicinal Chemistry PI3K / Akt / mTOR and Ras / Raf / MEK / ERK signaling pathways inhibitors as anticancer agents : Structural and pharmacological perspectives. Eur. J. Med. Chem. 109, 314–341. https://doi.org/10.1016/j.ejmech.2016.01.012

Azzopardi, D., Wyatt, J.S., Cady, E.B., Delpy, D.T., Baudin, J., Stewart, A.L., Hope, P.L., Hamilton, P.A., Reynolds, E.O.R., 1989. Prognosis of Newborn Infants with Hypoxic-Ischemic Brain Injury Assessed by Phosphorus Magnetic Resonance

Spectroscopy. Pediatr. Res. 25, 445–451. https://doi.org/10.1203/00006450-198905000-00004

Baulieu, E.E., 1998. NEUROSTEROIDS : A NOVEL FUNCTION OF THE BRAIN. Psychoneuroendocrinology 23, 963–987.

Ben-Ari, Y., 2002. Excitatory actions of gaba during development: the nature of the nurture. Nat Rev Neurosci 3, 728–739. https://doi.org/10.1038/nrn920

Ben-Ari, Y., Gaiarsa, J.-L., Tyzio, R., Khazipov, R., 2007. GABA: A Pioneer Transmitter That Excites Immature Neurons and Generates Primitive Oscillations. Physiol. Rev. 87, 1215–1284. https://doi.org/10.1152/physrev.00017.2006

Ben-Ari, Y., Khalilov, I., Kahle, K.T., Cherubini, E., 2012. The GABA excitatory/inhibitory shift in brain maturation and neurological disorders. Neuroscientist 18, 467–486. https://doi.org/10.1177/1073858412438697

Benarroch, E.E., 2005. Neuron-Astrocyte Interactions: Partnership for Normal Function and Disease in the Central Nervous System. Mayo Clin. Proc. 80, 1326–1338. https://doi.org/10.4065/80.10.1326

Bennet, L., Roelfsema, V., Pathipati, P., Quaedackers, J.S., Gunn, A.J., 2006. Relationship between evolving epileptiform activity and delayed loss of mitochondrial activity after asphyxia measured by near-infrared spectroscopy in preterm fetal sheep. J. Physiol. 572, 141–154. https://doi.org/10.1113/jphysiol.2006.105197

Bhat, R. V., Shanley, J., Correll, M.P., Fieles, W.E., Keith, R.A., Scott, C.W., Lee, C.M., 2000. Regulation and localization of tyrosine216 phosphorylation of glycogen synthase kinase-3β in cellular and animal models of neuronal degeneration. Proc. Natl. Acad. Sci. U. S. A. 97, 11074–11079. https://doi.org/10.1073/pnas.190297597

Blackmon, L.R., Stark, A.R., Newborn, the C. on F., Pediatrics, A.A. of, ADepartment, 2006. Hypothermia : A Neuroprotective Therapy for. Pediatrics 117, 942–8. https://doi.org/10.1542/peds.2005-2950

Blumberg, R.M., Cady, E.B., Wigglesworth, J.S., McKenzie, J.E., Edwards, A.D., 1997. Relation between delayed impairment of cerebral energy metabolism and infarction following transient focal hypoxia-ischaemia in the developing brain. Exp. Brain Res. 113, 130–137. https://doi.org/10.1007/BF02454148

Brambilla, R., Persaud, T., Hu, X., Karmally, S., Shestopalov, V.I., Dvoriantchikova, G., Ivanov, D., Nathanson, L., Barnum, S.R., Bethea, J.R., 2009. Transgenic Inhibition of Astroglial NF- □ B Improves Functional Outcome in Experimental Autoimmune Encephalomyelitis by Suppressing Chronic Central Nervous System Inflammation 1. J. Immunol. 182, 2628–2640. https://doi.org/10.4049/jimmunol.0802954

Brekke, E., Berger, H.R., Widerøe, M., Sonnewald, U., Morken, T.S., 2017. Glucose and Intermediary Metabolism and Astrocyte–Neuron Interactions Following Neonatal Hypoxia–Ischemia in Rat. Neurochem. Res. 42, 115–132. https://doi.org/10.1007/s11064-016-2149-9

Brunet, A., Datta, S.R., Greenberg, M.E., 2001. Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr. Opin. Neurobiol. 11, 297–305. https://doi.org/10.1016/S0959-4388(00)00211-7

Burnsed, J.C., Chavez-Valdez, R., Hossain, M.S., Kesavan, K., Martin, L.J., Zhang, J., Northington, F.J., 2015. Hypoxia-ischemia and therapeutic hypothermia in the neonatal mouse brain - A longitudinal study. PLoS One 10, 1–20. https://doi.org/10.1371/journal.pone.0118889

Calautti, E., Li, J., Saoncella, S., Brissette, J.L., Goetinck, P.F., 2005. Phosphoinositide

3-Kinase Signaling to Akt Promotes Keratinocyte Differentiation Versus Death. J. Biol. Chem. 280, 32856–32865. https://doi.org/10.1074/jbc.M506119200

Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S., Reed, J.C., 1998. Regulation of cell death protease caspase-9 by phosphorylation. Science 282, 1318–21.

Cervantes, M., González-Vidal, M.D., Ruelas, R., Escobar, A., Moralí, G., 2002. Neuroprotective effects of progesterone on damage elicited by acute global cerebral ischemia in neurons of the caudate nucleus. Arch. Med. Res. 33, 6–14.

Chen, G., Shi, J.-X., Qi, M., Wang, H.-X., Hang, C.-H., 2008. Effects of progesterone on intestinal inflammatory response, mucosa structure alterations, and apoptosis following traumatic brain injury in male rats. J. Surg. Res. 147, 92–8. https://doi.org/10.1016/j.jss.2007.05.029

Chen, J., Chopp, M., Li, Y., 1999. Neuroprotective effects of progesterone after transient middle cerebral artery occlusion in rat. J. Neurol. Sci. 171, 24–30.

Cho, K.H.T., Davidson, J.O., Dean, J.M., Bennet, L., Gunn, A.J., 2020. Cooling and immunomodulation for treating hypoxic-ischemic brain injury. Pediatr. Int. 62, 770–778. https://doi.org/10.1111/ped.14215

Cho, K.H.T., Wassink, G., Galinsky, R., Xu, B., Mathai, S., Dhillon, S.K., van den Heuij, L.G., Davidson, J.O., Weaver-Mikaere, L., Bennet, L., Gunn, A.J., Fraser, M., 2019. Protective effects of delayed intraventricular TLR7 agonist administration on cerebral white and gray matter following asphyxia in the preterm fetal sheep. Sci. Rep. 9, 1–17. https://doi.org/10.1038/s41598-019-45872-y

Colbourne, F., Grooms, S.Y., Zukin, R.S., Buchan, A.M., Bennett, M.V.L., 2002. Hypothermia rescues hippocampal CA1 neurons and attenuates down-regulation of

the AMPA receptor GluR2 subunit after forebrain ischemia. PNAS 100, 3–7.

Coughlan, T., Gibson, C., Murphy, S., 2005. Modulatory effects of progesterone on inducible nitric oxide synthase expression in vivo and in vitro. J. ofNeurochemistry 93, 932–942. https://doi.org/10.1111/j.1471-4159.2005.03068.x

Davidson, J.O., Wassink, G., van den Heuij, L.G., Bennet, L., Gunn, A.J., 2015. Therapeutic Hypothermia for Neonatal Hypoxic–Ischemic Encephalopathy – Where to from Here? Front. Neurol. 6. https://doi.org/10.3389/fneur.2015.00198

De Nicola, A.F., Labombarda, F., Deniselle, M.C.G., Gonzalez, S.L., Garay, L., Meyer, M., Gargiulo, G., Guennoun, R., Schumacher, M., 2009. Progesterone neuroprotection in traumatic CNS injury and motoneuron degeneration. Front. Neuroendocrinol. 30, 173–187. https://doi.org/10.1016/j.yfrne.2009.03.001

Delivoria-Papadopoulos, M., Mishra, O.P., 1998. Mechanisms of cerebral injury in perinatal asphyxia and strategies for prevention. J. Pediatr. 132, S30-4.

Diao, M.Y., Zhu, Y., Yang, J., Xi, S.S., Wen, X., Gu, Q., Hu, W., 2020. Hypothermia protects neurons against ischemia/reperfusion-induced pyroptosis via m6A-mediated activation of PTEN and the PI3K/Akt/GSK-3β signaling pathway. Brain Res. Bull. 159, 25–31. https://doi.org/10.1016/j.brainresbull.2020.03.011

Djebaili, M., Hoffman, S.., Stein, D.., 2004. Allopregnanolone and progesterone decrease cell death and cognitive deficits after a contusion of the rat pre-frontal cortex. Neuroscience 123, 349–359. https://doi.org/10.1016/j.neuroscience.2003.09.023

Douglas-Escobar, M., Weiss, M.D., 2013. Biomarkers of brain injury in the premature infant. Front. Neurol. 3 JAN, 1–7. https://doi.org/10.3389/fneur.2012.00185

du Plessis, A., Johnston, M., 1997. Hypoxic-ischemic brain injury in the newborn. Cellular mechanisms and potential strategies for neuroprotection. Clin. Perinatol. 24,

627–654.

Dubrovsky, B., 2006. Neurosteroids , neuroactive steroids , and symptoms of affective disorders. harmacology, Biochem. Behav. 84, 644–655. https://doi.org/10.1016/j.pbb.2006.06.016

Dukhande, V. V, Isaac, A.O., Chatterji, T., Lai, J.C.K., 2009. Reduced glutathione regenerating enzymes undergo developmental decline and sexual dimorphism in the rat cerebral cortex. Brain Res. 1286, 19–24. https://doi.org/10.1016/j.brainres.2009.05.029

Dzhala, V., Staley, K.J., 2015. Acute and Chronic Efficacy of Bumetanide in an in vitro Model of Posttraumatic Epileptogenesis. CNS Neurosci. Ther. 21, 173–180. https://doi.org/10.1111/cns.12369

Dzhala, V.I., Talos, D.M., Sdrulla, D.A., Brumback, A.C., Mathews, G.C., Benke, T.A., Delpire, E., Jensen, F.E., Staley, K.J., 2005. NKCC1 transporter facilitates seizures in the developing brain. Nat. Med. 11, 1205–1213. https://doi.org/10.1038/nm1301

Fabres, R.B., da Rosa, L.A., de Souza, S.K., Cecconello, A.L., Azambuja, A.S., Sanches, E.F., Ribeiro, M.F.M., de Fraga, L.S., 2018. Effects of progesterone on the neonatal brain following hypoxia-ischemia. Metab. Brain Dis. 1–9. https://doi.org/10.1007/s11011-018-0193-7

Fabres, R.B., Montes, N.L., Camboim, Y. de M., de Souza, S.K., Nicola, F., Tassinari, I.D., Ribeiro, M.F.M., Netto, C.A., de Fraga, L.S., 2020. Long-Lasting Actions of Progesterone Protect the Neonatal Brain Following Hypoxia-Ischemia. Cell. Mol. Neurobiol. https://doi.org/10.1007/s10571-020-00827-0

Ferriero, D.M., 2004. Neonatal Brain Injury. N. Engl. J. Med. 351, 1985–1995. https://doi.org/10.1056/NEJMra041996

Fields, R.D., Woo, D.H., Basser, P.J., 2015. Perspective Glial Regulation of the Neuronal Connectome through Local and Long-Distant Communication. Neuron 86, 374–386. https://doi.org/10.1016/j.neuron.2015.01.014

Fleiss, B., Gressens, P., 2012. Tertiary mechanisms of brain damage: a new hope for treatment of cerebral palsy? Lancet Neurol. 11, 556–566. https://doi.org/10.1016/S1474-4422(12)70058-3

François, F., Grimes, M.L., 1999. Phosphorylation-dependent Akt cleavage in neural cell in vitro reconstitution of apoptosis. J. Neurochem. 73, 1773–6.

Franke, T.F., Hornik, C.P., Segev, L., Shostak, G.A., Sugimoto, C., 2003. PI3K/Akt and apoptosis: Size matters. Oncogene 22, 8983–8998. https://doi.org/10.1038/sj.onc.1207115

Friguls, B., Petegnief, V., Justicia, C., Pallàs, M., Planas, A.M., 2002. Activation of ERK and Akt signaling in focal cerebral ischemia: Modulation by TGF-α and involvement of NMDA receptor. Neurobiol. Dis. 11, 443–456. https://doi.org/10.1006/nbdi.2002.0553

Fukui, O., Kinugasa, Y., Fukuda, A., Fukuda, H., Tskitishvili, E., Hayashi, S., Song, M., Kanagawa, T., Hosono, T., Shimoya, K., Murata, Y., 2006. Post-ischemic hypothermia reduced IL-18 expression and suppressed microglial activation in the immature brain. Brain Res. 1121, 35–45. https://doi.org/10.1016/j.brainres.2006.08.121

Garcia-Segura, L.M., Naftolin, F., Hutchison, J.B., Azcoitia, I., Chowen, J.A., 1999. Role of astroglia in estrogen regulation of synaptic plasticity and brain repair. J. Neurobiol. 40, 574–584. https://doi.org/10.1002/(SICI)1097-4695(19990915)40:4<574::AID-NEU12>3.0.CO;2-8

Gong, T., Liu, L., Jiang, W., Zhou, R., 2020. DAMP-sensing receptors in sterile inflammation and inflammatory diseases. Nat. Rev. Immunol. 20, 95–112. https://doi.org/10.1038/s41577-019-0215-7

Gordon, G.R.J., Choi, H.B., Rungta, R.L., Ellis-davies, G.C.R., Macvicar, B.A., 2008. Brain metabolism dictates the polarity of astrocyte control over arterioles. Nature 456. https://doi.org/10.1038/nature07525

Graham, S.H., Chen, J., 2001. Programmed cell death in cerebral ischemia. J. Cereb. Blood Flow Metab. 21, 99–109. https://doi.org/10.1097/00004647-200102000-00001

Grobin, A.C., Heenan, E.J., Lieberman, J.A., Morrow, A.L., 2003. Perinatal neurosteroid levels influence GABAergic interneuron localization in adult rat prefrontal cortex. J. Neurosci. 23, 1832–9.

Gunn, A.J., Gunn, T.R., De Haan, H.H., Williams, C.E., Gluckman, P.D., 1997. Dramatic neuronal rescue with prolonged selective head cooling after ischemia in fetal lambs. J. Clin. Invest. 99, 248–256. https://doi.org/10.1172/JCI119153

Hagberg, H., Mallard, C., Ferriero, D.M., Vannucci, S.J., Levison, S.W., Vexler, Z.S., Gressens, P., 2015. The role of inflammation in perinatal brain injury. Nat. Rev. Neurol. 11, 192–208. https://doi.org/10.1038/nrneurol.2015.13

Halassa, M.M., Fellin, T., Haydon, P.G., 2009. Neuropharmacology Tripartite synapses : Roles for astrocytic purines in the control of synaptic physiology and behavior. Neuropharmacology 57, 343–346. https://doi.org/10.1016/j.neuropharm.2009.06.031

Halassa, M.M., Fellin, T., Takano, H., Dong, J., Haydon, P.G., 2007. Synaptic Islands Defined by the Territory of a Single Astrocyte. J. Neurosci. 27, 6473–6477.

https://doi.org/10.1523/JNEUROSCI.1419-07.2007

Han, H.S., Karabiyikoglu, M., Kelly, S., Sobel, R.A., Yenari, M.A., 2003. Mild Hypothermia Inhibits Nuclear Factor- □ B Translocation in Experimental Stroke 6, 589–598. https://doi.org/10.1097/01.WCB.0000059566.39780.8D

Hanada, M., Feng, J., Hemmings, B.A., 2004. Structure, regulation and function of PKB/AKT - A major therapeutic target. Biochim. Biophys. Acta - Proteins Proteomics 1697, 3–16. https://doi.org/10.1016/j.bbapap.2003.11.009

Hegedus, B., Dasgupta, B., Shin, J.E., Emnett, R.J., Hart-mahon, E.K., Elghazi, L., Bernal-mizrachi, E., Gutmann, D.H., 2007. Article Neurofibromatosis-1 Regulates Neuronal and Glial Cell Differentiation from Neuroglial Progenitors In Vivo by Both cAMP- and Ras-Dependent Mechanisms. Cell Stem Cell 1, 443–457. https://doi.org/10.1016/j.stem.2007.07.008

Hill, M., Parízek, A., Bicíková, M., Havlíková, H., Klak, J., Fait, T., Cibula, D., Hampl, R., Cegan, A., Sulcová, J., Stárka, L., 2000. Neuroactive steroids, their precursors, and polar conjugates during parturition and postpartum in maternal and umbilical blood: 1. Identification and simultaneous determination of pregnanolone isomers. J. Steroid Biochem. Mol. Biol. 75, 237–44.

Iadecola, C., Nedergaard, M., 2007. Glial regulation of the cerebral microvasculature. Nat. Neurosci. 10, 1369–1376. https://doi.org/10.1038/nn2003

Ishrat, T., Sayeed, I., Atif, F., Hua, F., Stein, D.G., 2012. Progesterone is neuroprotective against ischemic brain injury through its effects on the phosphoinositide 3-kinase/protein kinase B signaling pathway. Neuroscience 210, 442–450. https://doi.org/10.1016/j.neuroscience.2012.03.008

Jiang, C., Zuo, F., Wang, Y., Lu, H., Yang, Q., Wang, J., 2017. Progesterone Changes

VEGF and BDNF Expression and Promotes Neurogenesis After Ischemic Stroke. Mol. Neurobiol. 54, 571–581. https://doi.org/10.1007/s12035-015-9651-y

Jiang, X., Wang, X., 2004. Cytochrome C-mediated apoptosis. Annu. Rev. Biochem. 73, 87–106. https://doi.org/10.1146/annurev.biochem.73.011303.073706

Johnston, M. V, Trescher, W.H., Ishida, A., Nakajima, W., 2001. Neurobiology of hypoxic-ischemic injury in the developing brain. Pediatr. Res. 49, 735–41.

Kim, N.H., Kim, K., Park, W.S., Son, H.S., Bae, Y., 2007. PKB/Akt inhibits ceramide-induced apoptosis in neuroblastoma cells by blocking apoptosis-inducing factor (AIF) translocation. J. Cell. Biochem. 102, 1160–70.

Kirklin, J.W., Dawson, B., Devloo;, R.A., Theye;, R.A., 1961. Open Intracardiac Operations : Use of Circulatory Arrest During Hypothermia Induced by Blood Cooling. Ann. Surg. 154, 769–776.

Kitagawa, K., Takasawa, K., Kuwabara, K., Sasaki, T., Tanaka, S., Mabuchi, T., Sugiura, S., Omura-Matsuoka, E., Matsumoto, M., Hori, M., 2002a. Differential Akt phosphorylation at Ser473 and Thr308 in cultured neurons after exposure to glutamate in rats. Neurosci. Lett. 333, 187–190.

Kitagawa, K., Takasawa, K., Kuwabara, K., Sasaki, T., Tanaka, S., Mabuchi, T., Sugiura, S., Omura-Matsuoka, E., Matsumoto, M., Hori, M., 2002b. Differential Akt phosphorylation at Ser473 and Thr308 in cultured neurons after exposure to glutamate in rats. Neurosci. Lett. 333, 187–90.

Knox, R., Zhao, C., Miguel-Perez, D., Wang, S., Yuan, J., Ferriero, D., Jiang, X., 2013. Enhanced NMDA receptor tyrosine phosphorylation and increased brain injury following neonatal hypoxia–ischemia in mice with neuronal Fyn overexpression. Neurobiol. Dis. 51, 113–119. https://doi.org/10.1016/j.nbd.2012.10.024

Korc, I., Bidegain, M., Martell, M., 1995. Radicales libres Bioquímica y sistemas antioxidantes Implicancia en la patología neonatal. Rev. Médica del Uruguay 11, 121–135.

Labombarda, F., González, S., Lima, A., Roig, P., Guennoun, R., Schumacher, M., Nicola, A.F. De, 2011. Progesterone attenuates astro- and microgliosis and enhances oligodendrocyte differentiation following spinal cord injury. Exp. Neurol. 231, 135–146. https://doi.org/10.1016/j.expneurol.2011.06.001

Li, X., Han, H., Hou, R., Wei, L., Wang, G., Li, C., Li, D., 2013. Progesterone treatment before experimental hypoxia-ischemia enhances the expression of glucose transporter proteins GLUT1 and GLUT3 in neonatal rats. Neurosci. Bull. 29, 287–94. https://doi.org/10.1007/s12264-013-1298-y

Li, X., Zhang, J., Zhu, X., Wang, P., Wang, X., Li, D., 2015. Progesterone reduces inflammation and apoptosis in neonatal rats with hypoxic ischemic brain damage through the PI3K/Akt pathway. Int. J. Clin. Exp. Med. 8, 8197–203.

Limbourg, F.P., Huang, Z., Plumier, J.-C., Simoncini, T., Fujioka, M., Tuckermann, J., Schütz, G., Moskowitz, M.A., Liao, J.K., 2002. Rapid nontranscriptional activation of endothelial nitric oxide synthase mediates increased cerebral blood flow and stroke protection by corticosteroids. J. Clin. Invest. 110, 1729–1738. https://doi.org/10.1172/JCI15481

Lorek, A., Takei, Y., Cady, E.B., Wyatt, J.S., Penrice, J., Edwards, A.D., Peebles, D., Wylezinska, M., Owen-Reece, H., Kirkbride, V., Cooper, C.E., Aldridge, R.F., Roth, S.C., Brown, G., Delpy, D.T., Reynolds, E.O.R., 1994. Delayed ("Secondary") Cerebral Energy Failure after Acute Hypoxia-Ischemia in the Newborn Piglet: Continuous 48-Hour Studies by Phosphorus Magnetic Resonance Spectroscopy.

Pediatr. Res. 36, 699–706. https://doi.org/10.1203/00006450-199412000-00003

Luisi, S., Petraglia, F., Benedetto, C., Nappi, R.E., Bernardi, F., Fadalti, M., Reis, F.M., Luisi, M., Genazzani, A.R., 2000. Serum allopregnanolone levels in pregnant women: changes during pregnancy, at delivery, and in hypertensive patients. J. Clin. Endocrinol. Metab. 85, 2429–33. https://doi.org/10.1210/jcem.85.7.6675

Marín-padilla, M., 1997. Developmental Neuropathology and Impact of Perinatal Brain Damage. II. J. Neuropathol. Exp. Neurol. 56, 219–235. https://doi.org/10.1097/00005072-199703000-00001

McQuillen, P.S., Ferriero, D.M., 2006. Perinatal Subplate Neuron Injury: Implications for Cortical Development and Plasticity. Brain Pathol. 15, 250–260. https://doi.org/10.1111/j.1750-3639.2005.tb00528.x

Meffre, D., Pianos, A., Liere, P., Eychenne, B., Cambourg, A., Schumacher, M., Stein, D.G., Guennoun, R., 2007. Steroid Profiling in Brain and Plasma of Male and Pseudopregnant Female Rats after Traumatic Brain Injury: Analysis by Gas Chromatography/Mass Spectrometry. Endocrinology 148, 2505–2517. https://doi.org/10.1210/en.2006-1678

Melcangia, R.C.., Garcia-Segura, L.M.., Mensah-Nyagan., A.G., 2008. Review Neuroactive steroids: State of the art and new perspectives. Cell. Mol. Life Sci. 65, 777–797. https://doi.org/10.1007/s00018-007-7403-5

Meyer, M., Claudia, Æ.M., Deniselle, G., Garay, Æ.L.I., Schumacher, M., Nicola, Æ.A.F. De, 2010. Stage Dependent Effects of Progesterone on Motoneurons and Glial Cells of Wobbler Mouse Spinal Cord Degeneration. Cell Mol Neurobiol 30, 123–135. https://doi.org/10.1007/s10571-009-9437-8

Middeldorp, J., Hol, E.M., 2011. Progress in Neurobiology GFAP in health and disease.

Prog. Neurobiol. 93, 421–443. https://doi.org/10.1016/j.pneurobio.2011.01.005

Mishra, A., Reynolds, J.P., Chen, Y., Gourine, A. V, Rusakov, D.A., Attwell, D., 2016. Astrocytes mediate neurovascular signaling to capillary pericytes but not to arterioles. Nat. Neurosci. 19. https://doi.org/10.1038/nn.4428

Nair, V.D., Olanow, C.W., 2008. Differential modulation of Akt/glycogen synthase kinase-3beta pathway regulates apoptotic and cytoprotective signaling responses. J. Biol. Chem. 283, 15469–78.

Nedergaard, M., Ransom, B., Goldman, S.A., 2003. New roles for astrocytes : Redefining the functional architecture of the brain. Trends Neurosci. 26, 523–530. https://doi.org/10.1016/j.tins.2003.08.008

Netto, C.A., Sanches, E., Odorcyk, F.K., Duran-Carabali, L.E., Weis, S.N., 2017. Sex-dependent consequences of neonatal brain hypoxia-ischemia in the rat. J. Neurosci. Res. https://doi.org/10.1002/jnr.23828

Noshita, N., Lewén, A., Sugawara, T., Chan, P.H., 2001. Evidence of phosphorylation of Akt and neuronal survival after transient focal cerebral ischemia in mice. J. Cereb. Blood Flow Metab. 21, 1442–1450. https://doi.org/10.1097/00004647-200112000-00009

Noshita, N., Sugawara, T., Lewén, A., Hayashi, T., Chan, P.H., 2003. Copper-zinc superoxide dismutase affects Akt activation after transient focal cerebral ischemia in mice. Stroke 34, 1513–1518. https://doi.org/10.1161/01.STR.0000072986.46924.F4

Nusse, R., 2003. Wnts and Hedgehogs: Lipid-modified proteins and similarities in signaling mechanisms at the cell surface. Development 130, 5297–5305. https://doi.org/10.1242/dev.00821

Paul, M.S., Purdy, H.R., 1992. Neuroactive steroids. FASEB J. 6, 2311–2322.

Pekny, M., Pekna, M., 2015. Center for Brain Repair and Rehabilitation , Department of Clinical Neuroscience and. BBA - Mol. Basis Dis. 1862, 483–491. https://doi.org/10.1016/j.bbadis.2015.11.014

Peterson, B.L., Won, S., Geddes, R.I., Sayeed, I., Stein, D.G., 2015. Sex-related differences in effects of progesterone following neonatal hypoxic brain injury. Behav. Brain Res. 286, 152–165. https://doi.org/10.1016/j.bbr.2015.03.005

Plog, B.A., Nedergaard, M., 2018. The glymphatic system in CNS health and disease: past, present and future. Ammu Rev Pathol 13, 379–394. https://doi.org/10.1146/annurev-pathol-051217-111018

Prandini, M.N., Neves Filho, A., Lapa, A.J., Stavale, J.N., 2005. Mild hypothermia reduces polymorphonuclear leukocytes infiltration in induced brain inflammation. Arq. Neuropsiquiatr. 63, 779–84. https://doi.org//S0004-282X2005000500012

Prinz, M., Priller, J., 2014. Microglia and brain macrophages in the molecular age: From origin to neuropsychiatric disease. Nat. Rev. Neurosci. 15, 300–312. https://doi.org/10.1038/nrn3722

Rees, S., Harding, R., Walker, D., 2011. International Journal of Developmental Neuroscience The biological basis of injury and neuroprotection in the fetal and neonatal brain. Int. J. Dev. Neurosci. 29, 551–563. https://doi.org/10.1016/j.ijdevneu.2011.04.004

Rice, J.E., Vannucci, R.C., Brierley, J.B., 1981. The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann. Neurol. 9, 131–41.

Rutherford, M., Malamateniou, C., McGuinness, A., Allsop, J., Biarge, M.M., Counsell, S., 2010. Magnetic resonance imaging in hypoxic-ischaemic encephalopathy. Early

Hum. Dev. 86, 351–360. https://doi.org/10.1016/j.earlhumdev.2010.05.014

Sabir, H., Scull-Brown, E., Liu, X., Thoresen, M., 2012. Immediate hypothermia is not neuroprotective after severe hypoxia-ischemia and is deleterious when delayed by 12 hours in neonatal rats. Stroke 43, 3364–3370. https://doi.org/10.1161/STROKEAHA.112.674481

Sanches, E. F., Arteni, N.S., Nicola, F., Boisserand, L., Willborn, S., Netto, C. a., 2013. Early hypoxia-ischemia causes hemisphere and sex-dependent cognitive impairment and histological damage. Neuroscience. https://doi.org/10.1016/j.neuroscience.2013.01.066

Sanches, E F, Arteni, N.S., Scherer, E.B., Kolling, J., Nicola, F., Willborn, S., Wyse, A.T.S., Netto, C.A., 2013. Are the consequences of neonatal hypoxia – ischemia dependent on animals ' sex and brain lateralization ? Brain Res. https://doi.org/10.1016/j.brainres.2013.02.040

Sarkaki, A.R., Khaksari Haddad, M., Soltani, Z., Shahrokhi, N., Mahmoodi, M., 2013a. Time- and dose-dependent neuroprotective effects of sex steroid hormones on inflammatory cytokines after a traumatic brain injury. J. Neurotrauma 30, 47–54. https://doi.org/10.1089/neu.2010.1686

Sarkaki, A.R., Khaksari Haddad, M., Soltani, Z., Shahrokhi, N., Mahmoodi, M., 2013b. Time- and Dose-Dependent Neuroprotective Effects of Sex Steroid Hormones on Inflammatory Cytokines after a Traumatic Brain Injury. J. Neurotrauma 30, 47–54. https://doi.org/10.1089/neu.2010.1686

Sayeed, I., Parvez, S., Wali, B., Siemen, D., Stein, D.G., 2009. Direct inhibition of the mitochondrial permeability transition pore: A possible mechanism for better neuroprotective effects of allopregnanolone over progesterone. Brain Res. 1263,

165–173. https://doi.org/10.1016/j.brainres.2009.01.045

Sayeed, I., Wali, B., Stein, D.G., 2007. Progesterone inhibits ischemic brain injury in a rat model of permanent middle cerebral artery occlusion. Restor. Neurol. Neurosci. 25, 151–9.

Schumacher, M., Guennoun, R., Robert, F., Carelli, C., Gago, N., Ghoumari, A., Gonzalez Deniselle, M.C., Gonzalez, S.L., Ibanez, C., Labombarda, F., Coirini, H., Baulieu, E.-E., De Nicola, A.F., 2004. Local synthesis and dual actions of progesterone in the nervous system: neuroprotection and myelination. Growth Horm. IGF Res. 14, 18–33. https://doi.org/10.1016/j.ghir.2004.03.007

Shankaran, S., Laptook, A.R., Ehrenkranz, R.A., Tyson, J.E., McDonald, S.A., Donovan, E.F., Fanaroff, A.A., Poole, W.K., Wright, L.L., Higgins, R.D., Finer, N.N., Carlo, W.A., Duara, S., Oh, W., Cotten, C.M., Stevenson, D.K., Stoll, B.J., Lemons, J.A., Guillet, R., Jobe, A.H., 2005. Whole-Body Hypothermia for Neonates with Hypoxic–Ischemic Encephalopathy. N. Engl. J. Med. 353, 1574–1584. https://doi.org/10.1056/NEJMcps050929

Sheikhbahaei, S., Morris, B., Collina, J., Anjum, S., Znati, S., Gamarra, J., Zhang, R., Gourine, A. V, Smith, J.C., 2018. Morphometric analysis of astrocytes in brainstem respiratory regions. J Comp Neurol 526, 2032–2047. https://doi.org/10.1002/cne.24472

Silveira, R.C., Procianoy, R.S., 2015. Hypothermia therapy for newborns with hypoxic ischemic encephalopathy. J. Pediatr. (Rio. J). https://doi.org/10.1016/j.jped.2015.07.004

Skolnick, B.E., Maas, A.I., Narayan, R.K., Hoop, R.G. Van Der, Macallister, T., Ward, J.D., Nelson, N.R., Stocchetti, N., 2014. A Clinical Trial of Progesterone for Severe

Traumatic Brain Injury. new engl J. Med. Orig. 371, 2467–2476. https://doi.org/10.1056/NEJMoa1411090

Smith, A., Garbus, H., Rosenkrantz, T., Fitch, R., 2015. Sex Differences in Behavioral Outcomes Following Temperature Modulation During Induced Neonatal Hypoxic Ischemic Injury in Rats. Brain Sci. 5, 220–240. https://doi.org/10.3390/brainsci5020220

Smith, A.L., Rosenkrantz, T.S., Fitch, R.H., 2016. Effects of sex and mild intrainsult hypothermia on neuropathology and neural reorganization following neonatal hypoxic ischemic brain injury in rats. Neural Plast. 2016. https://doi.org/10.1155/2016/2585230

Sofroniew, M. V., 2015. Astrocyte barriers to neurotoxic inflammation. Nat. Rev. Neurosci. 16, 249–263. https://doi.org/10.1038/nrn3898

Sofroniew, M. V, 2020. Astrocyte Reactivity : Subtypes , States , and Functions in CNS Innate Immunity. Trends Immunol. 41, 758–770. https://doi.org/10.1016/j.it.2020.07.004

Sofroniew, M. V, 2015. Astrogliosis perspectives. Cold Spring Harb. Perspect. Biol. 7, 1–16.

Sofroniew, M. V, 2009. Molecular dissection of reactive astrogliosis and glial scar formation. https://doi.org/10.1016/j.tins.2009.08.002

Sofroniew, M. V, Vinters, H. V, 2010. Astrocytes : biology and pathology. Acta Neuropathol 119, 7–35. https://doi.org/10.1007/s00401-009-0619-8

Stanojlović, M., Guševac Stojanović, I., Zarić, M., Martinović, J., Mitrović, N., Grković, I., Drakulić, D., 2019. Progesterone Protects Prefrontal Cortex in Rat Model of Permanent Bilateral Common Carotid Occlusion via Progesterone Receptors and

Akt/Erk/eNOS. Cell. Mol. Neurobiol. https://doi.org/10.1007/s10571-019-00777-2

Stein, D.G., 2011. Progesterone in the treatment of acute traumatic brain injury: a clinical perspective and update. Neuroscience 191, 101–106. https://doi.org/10.1016/j.neuroscience.2011.04.013

Stein, D.G., 2009. PROGESTERONE EXERTS NEUROPROTECTIVE EFFECTS AFTER BRAIN INJURY. Brain Res. Rev. 57, 386–397. https://doi.org/10.1016/j.brainresrev.2007.06.012.PROGESTERONE

Tang, S., Xu, S., Lu, X., Gullapalli, R.P., McKenna, M.C., Waddell, J., 2017. Neuroprotective Effects of Acetyl- L -Carnitine on Neonatal Hypoxia Ischemia-Induced Brain Injury in Rats. Dev. Neurosci. 38, 384–396. https://doi.org/10.1159/000455041

Taylor, D.L., Mehmet, H., Cady, E.B., Edwards, A.D., 2002. Improved neuroprotection with hypothermia delayed by 6 hours following cerebral hypoxia-ischemia in the 14-day-old rat. Pediatr. Res. 51, 13–19. https://doi.org/10.1203/00006450-200201000-00005

Tripathi, S., Kushwaha;, R., Mishra;, J., Gupta;, M.K., Kumar, H., Sanyal, Somali, Singh, D., Sanyal, Sabyasachi, Sahasrabuddhe, A.A., Kamthan, M., Mudiam, M.K.R., Bandyopadhyay, S., 2017. Docosahexaenoic acid up-regulates both PI3K/AKT-dependent FABP7-PPARy interaction and MKP3 that enhance GFAP in developing rat brain astrocytes. J. Neurochem. 140, 96–113. https://doi.org/10.1111/jnc.13879

Tsuji, M., Taguchi, A., Ohshima, M., Kasahara, Y., Ikeda, T., 2012. Progesterone and allopregnanolone exacerbate hypoxic-ischemic brain injury in immature rats. Exp. Neurol. 233, 214–20.

van den Heuij, L.G., Fraser, M., Miller, S.L., Jenkin, G., Wallace, E.M., Davidson, J.O.,

Lear, C.A., Lim, R., Wassink, G., Gunn, A.J., Bennet, L., 2019. Delayed intranasal infusion of human amnion epithelial cells improves white matter maturation after asphyxia in preterm fetal sheep. J. Cereb. Blood Flow Metab. 39, 223–239. https://doi.org/10.1177/0271678X17729954

Vannucci, R.C., 1990. Experimental biology of cerebral hypoxia-ischemia: relation to perinatal brain damage. Pediatr. Res. 27, 317–26. https://doi.org/10.1203/00006450-199004000-00001

Vannucci, R.C., Towfighi, J., Vannucci, S.J., 2004. Secondary Energy Failure after Cerebral Hypoxia–Ischemia in the Immature Rat. J. Cereb. Blood Flow Metab. 24, 1090–1097. https://doi.org/10.1097/01.WCB.0000133250.03953.63

Vannucci, S.J., Hagberg, H., 2004. Hypoxia-ischemia in the immature brain. J. Exp. Biol. 207, 3149–3154. https://doi.org/10.1242/jeb.01064

Wang, J.M., Singh, C., Liu, L., Irwin, R.W., Chen, S., Chung, E.J., Thompson, R.F., Brinton, R.D., 2010. Allopregnanolone reverses neurogenic and cognitive deficits in mouse model of Alzheimer's disease. Proc. Natl. Acad. Sci. U. S. A. 107, 6498–503. https://doi.org/10.1073/pnas.1001422107

Wang, X., Zhang, J., Yang, Y., Dong, W., Wang, F., Wang, L., Li, X., 2013. Progesterone attenuates cerebral edema in neonatal rats with hypoxic-ischemic brain damage by inhibiting the expression of matrix metalloproteinase-9 and aquaporin-4. Exp. Ther. Med. 6, 263–267. https://doi.org/10.3892/etm.2013.1116

Wassink, G., Davidson, J.O., Lear, C.A., Gunn, A.J., Bennet, L., Juul, S.E., Northington, F., 2018. A working model for hypothermic neuroprotection. J. Physiol. 23, 5641–5654. https://doi.org/10.1113/JP274928

Wassink, G., Gunn, E.R., Paul, P., Bennet, L., Gunn, A.J., 2014. The mechanisms and

treatment of asphyxial encephalopathy. Front. Neurosci. 8, 1–11. https://doi.org/10.3389/fnins.2014.00040

Waters, E.M., Torres-reveron, A., Ewen, B.S.M.C., 2008. Ultrastructural Localization of Extranuclear Progestin Receptors in the Rat Hippocampal Formation. J. Comp. Neurol. 511, 34–46. https://doi.org/10.1002/cne.21826

Weinrauch;, V., Safar;, P., Tisherman, S., Kuboyama, K., Radovsky, A., 1992. Beneficial Effect of Mild Hypothermia and Detrimental Effect of Deep Hypothermia After Cardiac Arrest in Dogs. Stroke 23, 1454–1462.

Weis, S.N., Pettenuzzo, L.F., Krolow, R., Valentim, L.M., Mota, C.S., Dalmaz, C., Wyse, A.T.S., Alexandre, C., 2012. Mitochondrion Neonatal hypoxia – ischemia induces sex-related changes in rat brain mitochondria. Mitochondrion 12, 271–279. https://doi.org/10.1016/j.mito.2011.10.002

Williams, C.S., Sheng, H., Brockman, J.A., Armandla, R., Shao, J., Washington, M.K., Elkahloun, A.G., Dubois, R.N., 2001. A Cyclooxygenase-2 Inhibitor (SC-58125) Blocks Growth of Established Human Colon Cancer Xenografts 1. Neoplasia 3, 428–436. https://doi.org/10.1038/sj.neo.7900177

Wood, T., Osredkar, D., Puchades, M., Maes, E., Falck, M., Flatebø, T., Walløe, L., Sabir, H., Thoresen, M., 2016. Treatment temperature and insult severity influence the neuroprotective effects of therapeutic hypothermia. Sci. Rep. 6, 23430. https://doi.org/10.1038/srep23430

Wood, T.R., Gundersen, J.K., Falck, M., Maes, E., Osredkar, D., Løberg, E.M., Sabir, H., Walløe, L., Thoresen, M., 2020. Variability and sex-dependence of hypothermic neuroprotection in a rat model of neonatal hypoxic–ischaemic brain injury: a single laboratory meta-analysis. Sci. Rep. 10, 1–13. https://doi.org/10.1038/s41598-020-

67532-2

Xiong, M., Yang, Y., Chen, G., Zhou, W., 2009. Post-ischemic hypothermia for 24 h in P7 rats rescues hippocampal neuron : Association with decreased astrocyte activation and inflammatory cytokine expression. Brain Res. Bull. 79, 351–357. https://doi.org/10.1016/j.brainresbull.2009.03.011

Zhao, H., Shimohata, T., Wang, J.Q., Sun, G., Schaal, D.W., Sapolsky, R.M., Steinberg, G.K., 2005. Akt contributes to neuroprotection by hypothermia against cerebral ischemia in rats. J. Neurosci. 25, 9794–9806. https://doi.org/10.1523/JNEUROSCI.3163-05.2005

Zhu, C., Xu, F., Wang, X., Shibata, M., Uchiyama, Y., Blomgren, K., Hagberg, H., 2006. Different apoptotic mechanisms are activated in male and female brains after neonatal hypoxia-ischaemia. J. Neurochem. 96, 1016–1027. https://doi.org/10.1111/j.1471-4159.2005.03639.x