UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

ALTERAÇÕES BIOQUÍMICAS E CELULARES CAUSADAS PELA HIPÓXIA-ISQUEMIA NEONATAL: CONTRIBUIÇÃO DO DIMORFISMO SEXUAL

SIMONE NARDIN WEIS

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"Um homem precisa viajar. Por sua conta, não por meio de histórias, imagens, livros ou TV. Precisa viajar por si, com seus olhos e pés, para entender o que é seu. Para um dia plantar as suas próprias árvores e dar-lhes valor. Conhecer o frio para conhecer o calor. E o oposto. Sentir a distância e o desabrigo para estar bem sob o próprio teto. Um homem precisa viajar para lugares que não conhece para quebrar essa arrogância que nos faz ver o mundo como o imaginamos, e não simplesmente como é ou pode ser; que nos faz professores e doutores do que não vimos, quando deveríamos ser alunos, e simplesmente ir ver."

Amyr Klink, Mar sem fim.

Dedicatória

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Ao CNPq pela bolsa concedida e à CAPES.

SUMÁRIO

APRESENTAÇÃO

Esta tese está organizada em tópicos, a saber: **Introdução**, **Objetivos**, **Capítulos** (1 a 3 – referentes aos artigos publicados e ao manuscrito a ser submetido), **Discussão**, **Conclusões**, **Perspectivas** e **Bibliografia**.

A **Introdução** apresenta o embasamento teórico que nos levou a formular a proposta de trabalho. Os **Objetivos** – geral e específicos – estão dispostos no corpo da tese e em maiores detalhes inseridos dentro de cada trabalho científico. Os **Capítulos** contêm os artigos publicados e o manuscrito a ser submetido, realizados durante o período do doutorado. Os dois primeiros trabalhos foram desenvolvidos no Laboratório de Isquemia Cerebral e parte no Laboratório de Neuroproteção e Doenças Metabólicas (Departamento de Bioquímica, ICBS, UFRGS) e o terceiro trabalho foi realizado durante o estágio de doutorado *sandwich* no laboratório coordenado pelo Prof. Dr. Frank R. Sharp (MIND Institute, Department of Neurology, UC Davis Health System, Sacramento, CA, USA) durante o período de 6 meses.

O tópico **Discussão** apresenta uma interpretação geral dos resultados obtidos nos diferentes trabalhos. Nas seções **Conclusões** e **Perspectivas** há uma abordagem geral das conclusões da tese e as possibilidades de futuros trabalhos a partir dos resultados obtidos na presente tese.

A **Bibliografia** contém somente as referências dos trabalhos citados nos tópicos **Introdução** e **Discussão**.

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LISTA DE ABREVIATURAS

$\Delta\psi$: Potencial de Membrana

AIF: Fator Indutor de Apoptose

AMPA: α-amino-3-hidroxi-5-metil-4-isoxazol propionato

APAF-1: Protease Associada à Apoptose-1

Atg: Genes Relacionados à Autofagia

ATP: Trifosfato de Adenosina

BAD: Promotor de Morte Associado à Bcl-2

BAF: Boc-aspartil-(Ome)-fluorometil-cetona

BAX: Proteina X Assoxiada à Bcl-2

Bcl-2: Linfoma de célula B

Bcl-W: *Bcl-2-like protein 2 (BcL2L2)*

Bcl-xL: *B-cell lymphoma-extra large*

BID: *BH3 interacting domain death agonist*

Ca^{2+}: Íon Cálcio

CA1: Corno de Ammon

CAD: DNAse Ativada por Caspase

Cu^{2+}: Íon Cúprico

DIABLO: *Direct IAP-Binding Protein with Low pI*

DNA: Ácido Desoxirribonucléico

EHI: Encefalopatia Hipóxico-Isquêmica

EROs: Espécies Reativas de Oxigênio

$FADH_2$: Flavina Adenina Dinucleotídeo

Fas (CD95): *Cluster of differentiation 95* ou *Apoptosis inducing protein 1*

Fe^{2+}: Íon Ferroso

H_2O_2: Peróxido de Hidrogênio

HI: Hipóxia-Isquemia

K^+: Íon Potássio

LAMP-2A: Proteína 2A de Membrana Associada aos Lisossomos

LC3 / MAP1LC3: Proteína de Cadeia Leve 3 Associada à Microtúbulos (*Microtubule-associated protein light chain-3*)

Mn-SOD: Superóxido Dismutase Dependente de Manganês

mTOR: Alvo da Rapamicina em Mamíferos

Na^{2+}: Íon Sódio

NADH: Nicotinamida Adenina Dinucleotídeo

NCX: Canais de Troca de Na^+/Ca^{2+}

NMDA: N-metil-D-aspartato

NO: Óxido Nítrico

NOS: Óxido Nítrico Sintase

$O_2^{\bullet-}$: Radical Ânion Superóxido

$^{\bullet}OH$: Radical Hidroxila

PARP-1: Poli(ADP-ribose) polimerase

PE: Fosfatidiletanolamina

RNA: Ácido Ribonucléico

TNF: Fator de Necrose Tumoral

TRM2: *tRNA methyltransferase 2*

TRPM7: *Transient receptor potential melastatin 7*

ULK-1: *Serine/threonine-protein kinase*

RESUMO

A hipóxia-isquemia (HI) encefálica é uma das causas mais frequentes de lesões graves com comprometimento crônico das capacidades neurológicas e também de óbito neonatal do mundo. A HI cerebral resulta em alterações hemodinâmicas, bioquímicas e neurofisiológicas como uma consequência direta da falta de oxigênio e glicose. Esses processos podem levar a um dano cerebral por meio da ativação de mecanismos citotóxicos e apoptóticos, que causam prejuízo e morte à célula. Recentemente, alguns estudos mostraram que os danos gerados pela HI neonatal apresentam dimorfismo sexual. Na presente tese, foram avaliados os efeitos da HI neonatal sobre parâmetros de estresse oxidativo e de dano celular após a lesão encefálica em machos e fêmeas a fim de se detectar a contribuição do dimorfismo sexual para a lesão. Foi observado que a HI aumentou a produção de radicais livres causando peroxidação lipídica, e também aumentou a atividade da enzima antioxidante superóxido dismutase (1h e 2h após a HI). Além disso, a HI inibiu a atividade da enzima Na^+, K^+-ATPase imediatamente após a lesão. Estes dados demonstram que a HI foi capaz de induzir o estresse oxidativo e levar à perda de homeostase celular através da alteração no controle da bomba de Na^+ e K^+ no encéfalo dos neonatos após a lesão. Tendo em vista que a mitocôndria é a principal fonte de espécies reativas de oxigênio (EROs) na célula nós investigamos os efeitos da HI sobre a função mitocondrial. Machos e fêmeas expostos à HI apresentaram diminuição na atividade do complexo II da cadeia respiratória em hipocampo, além de diminuição da massa e do potencial de membrana ($\Delta\psi$) mitocondrial tanto no córtex quanto no hipocampo, 2h após o insulto. Por outro lado, em 18h, a atividade dos complexos (I-III, II e IV) da cadeia respiratória mostrou uma inibição severa que foi acompanhada de diminuição de massa e $\Delta\psi$ mitocondrial em ambos os sexos, exceto pelo fato dos machos não apresentarem diminuição na massa mitocondrial. Esses dados mostram que a formação de espécies reativas bem como a peroxidação lipídica ocorre provavelmente devido à inibição da atividade dos complexos da cadeia respiratória. Tão importante quanto a disfunção mitocondrial induzida pela HI, os resultados apontam a presença de dimorfismo sexual neste parâmetro avaliado, uma vez que as fêmeas, além de apresentarem uma atividade dos complexos da cadeia respiratória *per se* maior quando comparadas aos machos, elas mostraram-se mais vulneráveis ao dano da HI. Com a finalidade de identificar a possível contribuição da autofagia para as diferentes alterações mitocondriais encontradas em machos e fêmeas, a atividade autofágica foi mensurada nos neonatos 18h após a lesão. Nós constatamos que as alterações encontradas – estresse oxidativo e disfunção mitocondrial – foram capazes de induzir a atividade autofágica. Entretanto esta se manifestou de forma distinta em córtex e hipocampo e também de maneira diferente em machos e fêmeas. No córtex, as fêmeas submetidas à HI tiveram aumento no número de autofagossomos (ativação da autofagia), porém diminuição dos autolisossomos, demonstrando uma possível inibição de algum passo final do processo. Já no hipocampo, os machos submetidos à HI tiveram indução da autofagia e as fêmeas apresentaram um aumento *per se* da atividade autofágica tanto nos animais controle quanto nos HI. É possível que o aumento das EROs pela cadeia respiratória e a perda de $\Delta\psi$ mitocondrial tenham induzido a autofagia após a lesão causada pela HI no encéfalo dos neonatos. Essas diferenças sexo-específicas são importantes não somente para entendermos o mecanismo de dano

causado pelo insulto, mas também para direcionarmos os estudos sobre as estratégias terapêuticas de acordo com o sexo do indivíduo afetado.

ABSTRACT

Brain hypoxia-ischemia (HI) is one of the most common causes of severe chronic impairment of neurological abilities and also neonatal death in the world. Brain HI results in hemodynamic, biochemical and neurophysiological changes as a direct consequence of oxygen and glucose absence. These processes can lead to brain damage through activation of cytotoxic and apoptotic mechanisms, which cause injury and death to the cell. Recently, some studies have shown that the damage caused by neonatal HI presents sexual dimorphism. In this thesis, it was evaluated the effects of neonatal HI on oxidative stress parameters and cell damage after brain lesion in males and females to verify sexual dimorphism contribution to the lesion. It was observed that HI increased free radicals production leading to lipid peroxidation and also increased superoxide dismutase activity (1h and 2h after HI). Besides, HI inhibited Na^+, K^+-ATPase activity immediately after injury. These data demonstrated that HI was able to induce oxidative stress and lead to cell homeostase loss through modifications on Na^+ and K^+ pump control in neonatal brain after injury. Considering that mitochondria are the main source of reactive oxygen species (ROS) in cell we investigated the effects of HI on mitochondrial function. Males and females exposed to HI showed a decrease in complex II activity of hippocampal respiratory chain in addition to diminished mass and mitochondrial membrane potential ($\Delta\psi$) in both cortex and hippocampus, 2h after insult. On the other hand, at 18h activity of respiratory chain complexes (I-III, II e IV) showed a severe inhibition that was accompanied by a decrease of mitochondrial mass and $\Delta\psi$ in both sexes, except that males do not show decrease in mitochondrial mass. These data demonstrated that reactive species formation and lipid peroxidation probably occur due to inhibition of respiratory chain complexes activities. Just as important as mitochondrial dysfunction induced by HI, results indicate the presence of sexual dimorphism on this parameter, since females, besides having a higher *per se* activity of respiratory chain complexes compared to males, they were more vulnerable to HI damage. In order to identify the possible contribution of autophagy to the distinct mitochondrial alterations found in males and females, autophagic activity was measured in neonates 18h after injury. We verify that changes found – namely oxidative stress and mitochondrial dysfunction - were able to induce autophagic activity. However, it manifested differently in cortex and hippocampus and also in males and females. In the cortex, females subjected to HI had an increase in autophagosomes (activation of autophagy), but decreased autolysosomes, showing a possible inhibition of a final step of the process. On hippocampus, males subjected to HI had autophagy induction and females showed a *per se* increase in autophagic activity in both control and HI animals. It is possible that ROS increased in respiratory chain and loss of mitochondrial $\Delta\psi$ had induced autophagy after lesion caused by HI in the neonatal brain. These sex-specific differences are important not only to understand the mechanism of damage caused by HI insult, but also to direct studies on the therapeutic strategies according to the sex of the affected subject.

1. INTRODUÇÃO

1. HIPÓXIA-ISQUEMIA ENCEFÁLICA NEONATAL

Nas últimas duas décadas, os estudos sobre a hipóxia-isquemia (HI) neonatal vem despertando um interesse científico e social cada vez maior, visto que a mesma continua sendo uma das causas mais frequentes de óbito neonatal do mundo. Nos sobreviventes a HI é uma importante causa de lesões graves com comprometimento crônico das capacidades neurológicas, como a paralisia cerebral, a epilepsia, o retardo mental e as dificuldades de aprendizado (VANNUCCI, 1990; MARIN-PADILLA, 2000; VANNUCCI & HAGBERG, 2004). Estimativas recentes indicam uma incidência de 2 a 9 casos de asfixia perinatal a cada 1000 nascidos a termo (WU *et al.*, 2004). A encefalopatia hipóxico-isquêmica (EHI) é decorrente de condições patológicas maternas, obstétricas e/ou do próprio concepto devido à falta de oxigênio completa ou parcial em um ou mais tecidos corporais (hipoxemia) e à isquemia, que se refere à diminuição da perfusão de sangue no cérebro. A EHI neonatal caracteriza-se pelo conjunto destes dois fatores, hipóxia e isquemia, que associadas às alterações metabólicas decorrentes do insulto induzem modificações bioquímicas, biofísicas e fisiológicas que se traduzem por manifestações clínicas secundárias ao comprometimento fisiológico ou estrutural (ROTTA, 2002).

1.1.1 Etiologia e Aspectos Clínicos

A causa mais frequente da EHI é a asfixia perinatal, que por sua vez pode ser ocasionada por vários fatores, tais como: interrupção do fluxo sanguíneo (ex.: compressão do cordão umbilical), insuficiente troca de gases pela placenta (ex.: descolamento da placenta), perfusão placentária inadequada do lado materno (ex.: hipotensão materna), comprometimento fetal (ex.: retardo no crescimento intra-uterino) e falha ao inflar o pulmão logo após o nascimento (BERGER & GARNIER, 1999; PROCIANOY & SILVEIRA, 2001). Clinicamente, a EHI é definida como uma síndrome de distúrbios das funções neurológicas presentes na primeira semana de vida e manifesta-se como dificuldade para iniciar e manter a respiração, depressão do tônus muscular e reflexos, alteração do nível de consciência e frequentes convulsões (NELSON & LEVITON, 1991).

1.1.2 Aspectos Neuropatológicos da EHI

Em situações fisiológicas, o tecido encefálico possui uma regulação do fluxo sanguíneo independente das variações de perfusão, graças a mecanismos de auto-regulação da circulação cerebral (CASTILLO, 2000). Em uma situação de isquemia, esses mecanismos tornam-se falhos na zona afetada e a pressão de perfusão passa a ser dependente da tensão arterial (CASTILLO, 2000).

O tecido encefálico é altamente dependente do fluxo sanguíneo contínuo para o suprimento de glicose e oxigênio uma vez que seu metabolismo energético possui uma alta demanda energética, seus estoques de energia são limitados e ainda possuem uma

grande dependência do metabolismo aeróbico da glicose. Por todas estas características é que o encéfalo torna-se completamente vulnerável frente a uma lesão isquêmica (MISHRA & DELIVORIA-PAPADOPOULOS, 1999; LIPTON, 1999).

As alterações hemodinâmicas, bioquímicas e neurofisiológicas resultantes da ausência direta de oxigênio e glicose podem levar a uma lesão cerebral por meio da ativação de mecanismos citotóxicos e apoptóticos, que causam dano e morte celular (PORTERA-CAILLIAU *et al.*, 1997; HOSSAIN, 2005; IKONOMIDOU & KAINDL, 2011, THORNTON et al., 2012). Este processo é agravado no encéfalo dos neonatos já que este é mais suscetível do que o encéfalo dos adultos, uma vez que o período perinatal apresenta grande sinaptogênese e alta diferenciação de oligodendrócitos (PAN & PEREZ-POLO, 1993; JACKSON *et al.*, 1994; SAMPATH *et al.*, 1994; BACK *et al.*, 2002; 2007a; 2007b; HERTZ, 2008).

Os achados neuropatológicos da EHI variam conforme a maturidade fetal, a natureza e a extensão da lesão e a forma de intervenção, podendo resultar em até seis lesões hipóxico-isquêmicas diferentes, a saber: morte seletiva neuronal, lesão cerebral para-sagital, leucomalácia periventricular, *status marmoratus*, hemorragia intra ou periventricular e dano isquêmico cerebral focal e multifocal (GILLES *et al.*, 1983; VOLPE, 1995).

1.1.3 MECANISMOS CITOTÓXICOS DA EHI

A privação de glicose e oxigênio decorrentes da interrupção do suprimento sanguíneo ao encéfalo durante o processo isquêmico reduz a disponibilidade de energia na forma de ATP para as células nervosas (DIRNAGL *et al.*, 1999). A queda nos níveis

de ATP leva a um distúrbio na bomba de sódio e potássio, causando acúmulo de Na^+ intracelular, difusão de K^+ para fora da célula e aumento do influxo de Ca^{2+}. O ganho de soluto é acompanhado de ganho isosmótico de água e consequente edema celular (JAESCHKE, 2003). Com isso, os neurônios tornam-se incapazes de manter o gradiente iônico necessário para a homeostase e funcionamento celular, resultando em despolarização neuronal, excessiva liberação e falha na captação de neurotransmissores excitatórios, mais especificamente o glutamato, no espaço extracelular. O excesso de glutamato na fenda sináptica hiperestimula os seus receptores ionotrópicos NMDA (N-metil-D-aspartato), AMPA (α-amino-3-hidroxi-5-metil-4-isoxazol propionato) e cainato, resultando no influxo de Na^+ e Ca^{2+} para dentro dos neurônios pós-sinápticos e causando neurotoxicidade. Além dos receptores ionotrópicos, o influxo de Ca^{2+} pelos canais de troca Na^+/Ca^{2+} (NCX), TRM2 e TRPM7, por exemplo, e a liberação intracelular excessiva dos estoques desse pelo retículo endoplasmático também contribuem de maneira expressiva para o acúmulo de Ca^{2+} intracelular (MACGREGOR *et al.*, 2003; PIGNATARO *et al.*, 2004; SZYDLOWSKA & TYMIANSKI, 2010) resultando em superativação de um grande número de processos dependentes de Ca^{2+} e dano neuronal. Estes eventos levam a um aumento adicional da liberação de glutamato e redução na sua captação (FERREIRA *et al.*, 1996).

Ao influxo excessivo de cálcio para o interior da célula, segue-se um aumento da formação de espécies reativas de oxigênio e de nitrogênio (LEWÉN *et al.*, 2000; JOHNSTON *et al.*, 2001; GILGUN-SHERKI *et al.*, 2002) que podem iniciar o processo de peroxidação lipídica e outras reações oxidativas em cadeia levando à morte celular após a HI no encéfalo imaturo (NAKAJIMA *et al.*, 2000) (**Figura 1**). Alguns modelos experimentais de HI que utilizam roedores apresentam fortes evidências que suportam os efeitos deletérios do estresse oxidativo e dos radicais livres, da ruptura da barreira

hemato-encefálica, da liberação e sinalização de citocinas e quimiocinas e da atividade das metaloproteinases de matriz após a HI (CHEN *et al.*, 2009; DEGOS *et al.*, 2010).

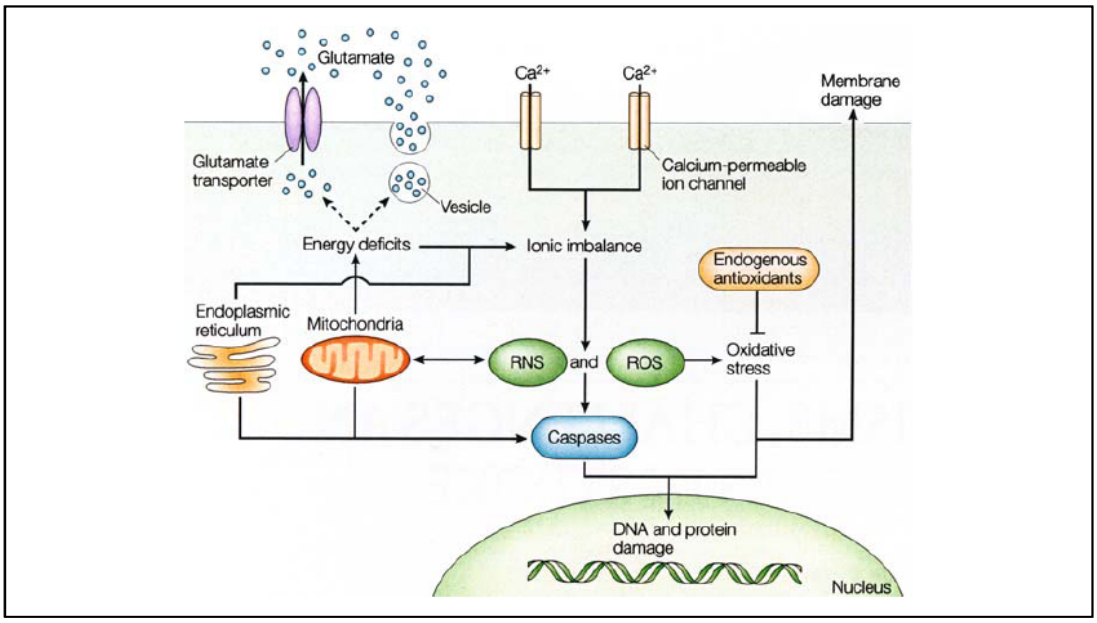

Figura 1. Mecanismos neuropatológicos básicos do dano celular causado pela isquemia cerebral. Extraído de SINGHAL *et al.*, 2011.

1.1.4 O MODELO EXPERIMENTAL DA HI NEONATAL

O modelo experimental de HI neonatal descrito por Levine (1960) e posteriormente modificado por Rice *et al.* (1981) tem sido amplamente utilizado com a finalidade de reproduzir a lesão cerebral hipóxico-isquêmica observada em humanos. É vantajoso por apresentar alta reprodutibilidade (WALTON *et al.*, 1999), baixo custo, baixa mortalidade e por produzir dano em 90% dos ratos, permitindo a recuperação do fluxo sanguíneo, apesar da oclusão da carótida. O dano da HI experimental produz lesões no hemisfério ipsilateral nas regiões do córtex cerebral, substância branca

periventricular e subcortical, estriado (núcleos da base) e hipocampo (ARTENI *et al*., 2003; HOSSAIN, 2005; VANUCCI & VANUCCI, 2005; PEREIRA *et al*., 2007). O modelo utilizado nesta tese, ilustrado na **figura 2**, consiste na obstrução da carótida comum direita (isquemia) seguida da exposição a baixas tensões de oxigênio (8%) por um período de 90 minutos (hipóxia). Nesse modelo experimental, os animais são capazes de resistir a um período de até 3 a 4h de exposição à hipóxia (RICE *et al.*, 1981) e a lesão leva à atrofia tardia das estruturas atingidas.

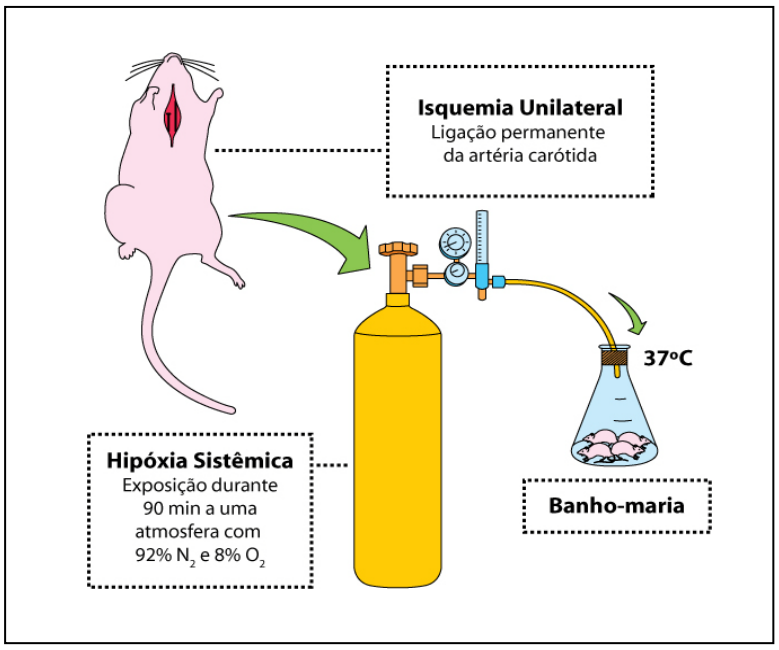

Figura 2. Desenho esquemático do procedimento experimental utilizado para reproduzir a HI neonatal em animais.

1.2 MITOCÔNDRIA

As mitocôndrias são organelas intracelulares com forma e dimensões distintas dependendo do tecido e estado metabólico em que se encontram, com diâmetro que varia de 0,2 a 1μm. Duas membranas envolvem a mitocôndria: uma externa, lisa que reveste o espaço intermembranas e outra interna, com múltiplas invaginações denominadas cristas mitocondriais. Aderido às cristas encontra-se uma grande quantidade de proteínas componentes da cadeia transportadora de elétrons e a F_0F_1ATPase, responsáveis por realizar a fosforilação oxidativa, além de inúmeras proteínas transportadoras (DAUM, 1985). No seu interior – ou matriz mitocondrial – localizam-se as enzimas do ciclo de Krebs, da β-oxidação dos ácidos graxos, do metabolismo do piruvato, RNAs, ribossomos, DNA mitocondrial e outros elementos. No espaço intermembranas estão localizadas proteínas de grande importância fisiológica, com funções importantes para o metabolismo energético como o citocromo *c*, a creatina cinase e a adenilato cinase (DAUM, 1985; FREY & MANNELLA, 2000; REDDY, 2008).

Estas organelas desempenham papel fundamental na sobrevivência celular, pois são responsáveis pela maior parte da energia gerada e utilizada pelas células eucarióticas (NICHOLLS & FERGUSON, 2002). A maior parte do ATP formado na célula provém da cadeia transportadora de elétrons, ou cadeia respiratória, acoplada à fosforilação oxidativa mitocondrial. A cadeia respiratória é um complexo enzimático responsável pela gradativa transferência de elétrons oriundos do metabolismo intermediário para a redução do oxigênio e síntese de ATP. Durante este processo, os elétrons oriundos das coenzimas NADH e $FADH_2$, reduzidas durante o ciclo de Krebs, são transferidos para os complexos I (NADH-ubiquinona oxidorredutase) e II (Succinato-ubiquinona oxidorredutase) e destes para os complexos III (Ubiquinol-Citocromo *c* redutase) e IV (Citocromo *c* oxidase) de uma maneira gradativa, até o

aceptor final de elétrons, o oxigênio molecular, com concomitante formação de água. A passagem de elétrons através dos complexos I, III e IV é acompanhada do bombeamento de prótons da matriz mitocondrial para o espaço intermembranas. Este gradiente eletroquímico, responsável pela formação do potencial de membrana mitocondrial ($\Delta\psi$), dirige o fluxo de prótons de volta à matriz mitocondrial através da F_0F_1ATPase, que utiliza esta energia para a síntese de ATP (HÜTTEMANN *et al.*, 2008) (**Figura 3**). Em condições fisiológicas, aproximadamente 1 a 4% dos elétrons é desviado do fluxo pelos complexos da cadeia respiratória causando a redução incompleta do oxigênio molecular, formando espécies reativas tóxicas como o radical ânion superóxido ($O_2^{\bullet-}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($^{\bullet}OH$), o que torna a mitocôndria uma importante fonte de espécies reativas de oxigênio (EROs) (KOOPMAN *et al.*, 2010).

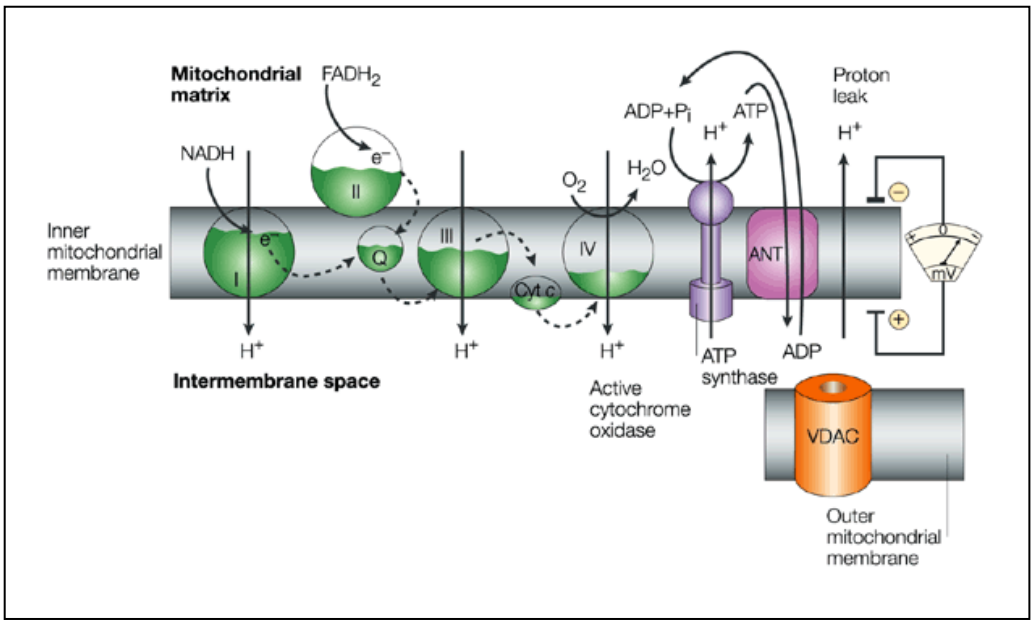

Figura 3. Representação esquemática da cadeia respiratória mitocondrial. Extraído de MONCADA & ERUSALIMSKY, 2002.

O ânion superóxido intramitocondrial, incapaz de se difundir para o citosol, é detoxificado pela enzima antioxidante superóxido dismutase dependente de manganês (Mn-SOD) presente no interior da mitocôndria, gerando peróxido de hidrogênio. A reação do superóxido, $O_2^{\bullet-}$, com o peróxido de hidrogênio forma o radical hidroxila, através da reação de Haber-Weiss. A formação de radical hidroxila a partir do peróxido de hidrogênio também pode ser catalisada pela presença de íons de metais de transição (Fe^{2+} e Cu^{2+}) pela reação de Fenton (GUTTERIDGE & HALLIWELL, 2000; MCCORD, 1987). O aumento de Ca^{2+} intracelular também ativa a enzima óxido nítrico sintase (NOS), localizada na membrana mitocondrial interna, produzindo óxido nítrico ($NO^{\bullet}$). O óxido nítrico pode reagir com o ânion superóxido formando uma espécie altamente reativa denominada peroxinitrito ($ONOO^-$), desencadeando um processo de peroxidação lipídica, oxidação de moléculas sulfidrílicas (RADI *et al.*, 1991) e danos ao RNA e DNA (SALGO *et al.*, 1995) (**Figura 4**). Além disso, o óxido nítrico é um potente inibidor da cadeia respiratória mitocondrial por inativar os complexos I-III (VIEIRA *et al.*, 2001; VIRÁG *et al.*, 2003) e também por inibir diretamente o complexo IV ligando-se ao sítio de ligação específica do O_2 (GIULIVI *et al.*, 1998; BROWN & BORUTAITE, 1999).

Durante a isquemia, a oxidação de proteínas de membrana causa o aumento na permeabilidade da membrana mitocondrial interna devido à abertura do poro de transição de permeabilidade mitocondrial, causando *swelling* mitocondrial (intumescimento osmótico) e ruptura da membrana mitocondrial externa. Assim, proteínas pró-apoptóticas, como o fator indutor de apoptose (AIF) e citocromo *c*, são liberadas no citosol desencadeando a morte celular (MORO *et al.*, 2005; BLOMGREN & HAGBERG, 2006; GALLUZZI *et al.*, 2009; REDDY & REDDY, 2011). Após a

lesão isquêmica inicial, quando a oxigenação e a perfusão cerebral já se encontram restauradas, os níveis de glicose apresentam-se perto dos valores basais em condições normais e o pH intracelular retorna a linha de base, uma falha energética secundária (período de reperfusão) ocorre em um período de 6 a 48h após o insulto inicial (FOLBERGROVA *et al.*, 1992, 1995). Portanto, uma segunda queda dos níveis de fosfato de alta energia intracelular ocorre com concomitante diminuição dos níveis e utilização da glicose pelo encéfalo, ativação das caspases e fragmentação do DNA (BLUMBERG *et al*., 1997; WYATT *et al.*, 1989; GILLAND *et al*., 1998a; 1998b; PUKA-SUNDVALL *et al*., 2000).

No encéfalo imaturo, os altos níveis de lipídios poli-insaturados, o incompleto desenvolvimento do sistema antioxidante e os altos níveis de ferro livre disponíveis para reagirem com as EROs o tornam altamente suscetível ao dano oxidativo (JACKSON & PEREZ-POLO, 1996). Logo, a formação de espécies reativas na mitocôndria bem como a inibição das enzimas antioxidantes, características do processo de estresse oxidativo, parecem ser os principais mediadores do dano neurológico causado pela HI (SIMS & ANDERSON, 2002; ADAM-VIZI, 2005).

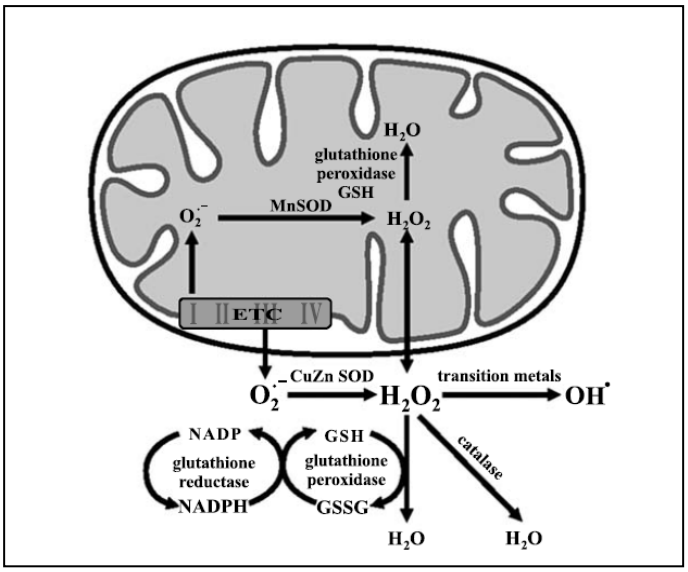

Figura 4. Formação e detoxificação de EROs pela mitocôndria.
Extraído de ADAM-VIZI, 2005.

1.3 MORTE CELULAR

A morte celular isquêmica depende da magnitude do dano isquêmico, ou seja, do grau de severidade e duração do insulto. Na morte celular por apoptose ocorre um dano moderado, onde a permeabilidade da membrana mitocondrial permite a liberação de fatores pró-apoptóticos para o citosol, mas a mitocôndria ainda mantém os níveis de ATP normais, tornando a lesão reversível. Entretanto, em um dano severo, onde a concentração de ATP encontra-se abaixo dos níveis necessários para o processo de apoptose se instaurar, o dano é irreversível e a morte celular acontece por necrose (LIPTON, 1999).

1.3.1 NECROSE

A necrose é um processo patológico e desordenado de morte celular desencadeado geralmente por estímulos extremamente agressivos, tais como a exposição a altas concentrações de agentes tóxicos, lesão por hipóxia/isquemia ou agentes biológicos que causem dano direto ou desencadeiem resposta imunológica danosa. Morfologicamente, as células em necrose apresentam perda da integridade das membranas com extravasamento do conteúdo citoplasmático causando inflamação no tecido adjacente, eosinofilia, presença de vacúolos após a digestão de organelas citoplasmáticas, picnose (condensação da cromatina), cariorrexe (fragmentação nuclear) e cariólise (destruição, por dissolução, do núcleo) (SYNTICHAKI & TAVERNARAKIS, 2010). As alterações morfológicas correspondem à depleção brusca de ATP, perda do controle do balanço iônico, extravasamento do material intracelular devido a um aumento no volume da célula, dano intenso às organelas, ruptura de lisossomos e lise celular (LOCKSHIN & ZAKERI, 2004; YAKOVLEV & FADEN, 2004). Soma-se a esses fatores uma intensa resposta inflamatória no local, oriunda do recrutamento de mastócitos e da liberação de citocinas (LEIST & JÄÄTTELÄ, 2001; FESTJENS *et al.*, 2006).

1.3.2 APOPTOSE

Basicamente, as células em apoptose apresentam características morfológicas específicas que incluem: diminuição do tamanho celular, picnose e cariorrexe. Os conteúdos nuclear e citoplasmático se fragmentam originando os corpos apoptóticos que são fagocitados pelas células saudáveis adjacentes e por macrófagos (GALLUZZI *et al.*,

2009). Ao contrário da necrose, a apoptose é deflagrada por uma cascata de eventos moleculares que, por sua vez, são iniciados por diversos mecanismos culminando na ativação de cisteíno-proteases denominadas caspases (STRASSER *et al.*, 2000). Estímulos externos podem induzir apoptose por meio da ativação de receptores específicos presentes na superfície celular (via extrínseca ou via do receptor de morte celular), chamados de receptores de morte, como os pertencentes à família dos receptores do Fator de Necrose Tumoral (TNF) e da Proteína Fas (CD95). A via intrínseca, ou mitocondrial, é ativada por estresse intracelular ou extracelular, como privação de fatores de crescimento, danos no DNA, hipóxia, perturbações no ciclo celular ou nas vias metabólicas, dentre outros (BERKKANOGLU *et al.*, 2004) (**Figura 5**). Em resposta a estes fatores, a mitocôndria sofre modificações no $\Delta\psi$, na permeabilidade de membrana e no aumento de densidade da matriz. Como desfecho, a mitocôndria libera o citocromo *c* no citoplasma que interage com o Fator de Ativação de Protease Associada à Apoptose-1 (APAF-1) e com a pró-caspase-9 formando um complexo denominado apoptossomo, resultando na clivagem e ativação da caspase-9 que por sua vez cliva e ativa as caspases efetoras, como a caspase-3 (GALLUZZI *et al.*, 2009). Tanto na via extrínseca como na intrínseca, as caspases iniciadoras (caspases 8 e 9) são ativadas e podem catalisar a maturação proteolítica das caspases efetoras (caspase-3 e -7, por exemplo) que clivam outros substratos protéicos da célula resultando no processo apoptótico. A permeabilidade anormal da membrana mitocondrial externa é determinante para o início da morte celular apoptótica, ativando tanto mecanismos dependentes quanto independentes das caspases. Diversas proteínas intracelulares regulam diretamente o processo de ativação das caspases por controlarem a permeabilidade da membrana mitocondrial, como as proteínas da família Bcl-2 (Linfoma de célula B) (DESAGHER & MARTINOU, 2000). Os membros dessa família

podem ser divididos em moléculas pró-apoptóticas (por exemplo: BAX, BAD e BID) e antiapoptóticas (por exemplo: Bcl-2, Bcl-xL e Bcl-w). O equilíbrio relativo entre as diferentes proteínas, refletindo a formação de homodímeros e heterodímeros, define a via de atuação sobre o mecanismo de morte celular programada (ZÖRNIG *et al.*, 2001). Na presença de um sinal apoptótico, a Proteína X Associada à Bcl-2 (BAX), por exemplo, é translocada do citoplasma para as proximidades das mitocôndrias, onde sofre ativação e modificação conformacional, aderindo à membrana mitocondrial externa. Estas proteínas agrupam-se formando oligômeros que acabam por penetrar a membrana mitocondrial externa. Essa integração possibilita a rápida liberação do citocromo *c* (ANTONSSON & MARTINOU, 2000). Por outro lado, a Bcl-2 favorece a sobrevida celular por impedir o escape do citocromo *c*, possivelmente pela formação de heterodímeros com moléculas próapoptóticas como a proteína BAX (AMARANTE-MENDES & GREEN, 1999).

Juntamente com o citocromo *c*, outras proteínas são liberadas da mitocôndria para o citoplasma, dentre elas DIABLO, AIF e endonuclease G. A proteína próapoptótica DIABLO parece estar envolvida na inibição dos IAPs (Inibidores da Apoptose), promovendo a ativação das caspases. A endonuclease G está relacionada com a replicação do DNA mitocondrial, entretanto quando liberada da mitocôndria exerce a função de clivagem internucleossomal do DNA. Já o AIF, migra da mitocôndria para o núcleo ativando a Poli(ADP-ribose) Polimerase-1 (PARP-1) causando morte celular por apoptose independente da ativação das caspases (LI *et al.*, 2001; MODJTAHEDI *et al.*, 2006; YAKOVLEV & FADEN, 2004).

A maioria dos estudos na literatura analisa as duas vias de morte celular – apoptose e necrose – que ocorrem após um insulto hipóxico-isquêmico; entretanto,

poucos autores têm se dedicado à autofagia, um processo que também pode levar à morte celular quando superativado.

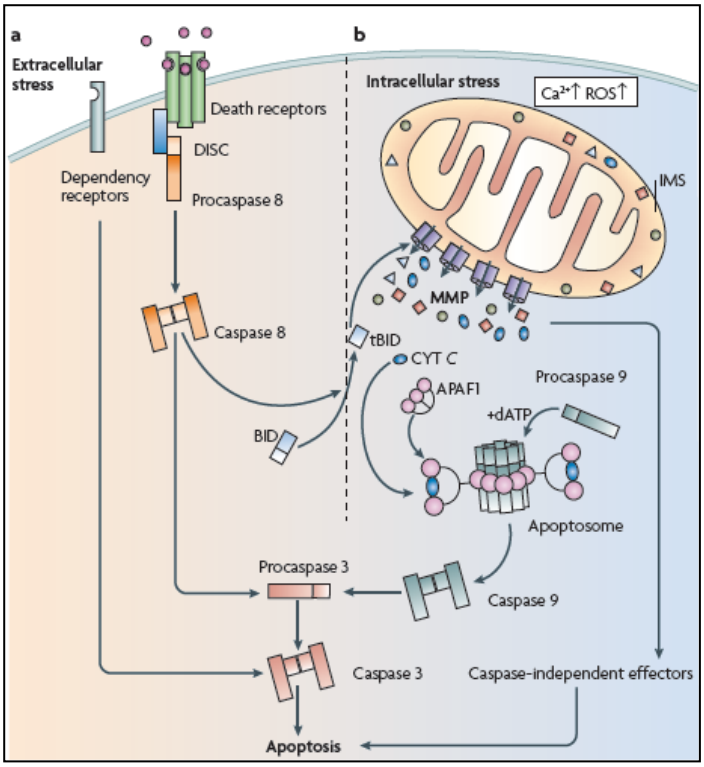

Figura 5. Vias extrínsecas e intrínsecas da morte celular por apoptose. Extraído de GALLUZZI *et al.*, 2009.

1.3.3 AUTOFAGIA

A autofagia é um processo evolucionário conservado nos lisossomos, que tem por objetivo regular a renovação dos constituintes celulares, pela degradação de proteínas e organelas, através da via autofagossomo/lisossomo. A autofagia é importante para manter a homeostase e serve como um mecanismo de adaptação quando há uma baixa disponibilidade de nutrientes como ocorre imediatamente após o

nascimento e nos processos de diferenciação e desenvolvimento normal (KUMA *et al.*, 2004; UCHIYAMA *et al.*, 2008). Entretanto ela também é uma estratégia que as células utilizam para sobreviver frente a uma situação de estresse. Apesar de a autofagia ser considerada essencial para a manutenção da homeostase celular, a ativação excessiva dessa via pode destruir partes do citosol e das organelas levando ao colapso total de todas as funções celulares. Por estes efeitos antagônicos da autofagia é que seu papel nos processos celulares de sobrevivência e morte ainda não está completamente esclarecido (CODOGNO & MEIJER, 2005; ERLICH *et al.*, 2006).

Três tipos diferentes de autofagia estão descritos: a autofagia mediada por chaperonas, a microautofagia e a macroautofagia (MIZUSHIMA & KOMATSU, 2011) (**Figura 6**). Na autofagia mediada por chaperonas, as proteínas citoplasmáticas que sofreram dobramento incorreto permanecem ligadas às chaperonas, sendo então direcionadas aos lisossomos onde são reconhecidas pela proteína LAMP-2A (Proteína de Membrana Associada ao Lisossomo Tipo 2), internalizadas e degradadas. Na microautofagia, pequenas porções de componentes citoplasmáticos são englobadas por invaginação, protusão e/ou septação da membrana lisossomal, internalizadas e degradadas. A macroautofagia é a mais estudada e conhecida de todas as vias e responde por mais de 90% da autofagia celular. Pela macroautofagia, o conteúdo citoplasmático a ser degradado (proteínas e organelas) é isolado por uma dupla membrana chamada autofagossomo que irá se fusionar com um lisossomo, formando o autolissosomo (MIZUSHIMA & KOMATSU, 2011).

Para a execução deste processo, uma série de proteínas codificadas pelos genes relacionados à autofagia (Atg) é necessária (CARLONI *et al.*, 2008; UCHIYAMA *et al.*, 2008; GOLDMAN *et al.*, 2010; MIZUSHIMA *et al.*, 2010). Na etapa inicial (nucleação), uma estrutura de membrana lipídica dupla chamada fagóforo ou membrana

de isolamento alonga-se iniciando o processo de envolvimento dos componentes celulares citoplasmáticos. O recrutamento e a formação de complexos protéicos pelo fagóforo inicia-se pela ativação do complexo ULK1, composto por proteínas como a ULK1, Atg13 e Atg10, que se desloca para um sítio específico no retículo endoplasmático regulando o complexo de proteínas fosfatidilinositol 3-cinase classe III, que incluem a Beclina 1, Atg 14, entre outras. A Beclina 1, em especial, encontra-se ligada à proteína Bcl-2. Em condições de indução da autofagia, este complexo é desfeito e a Beclina 1 torna-se livre para formar o autofagossomo.

Dois sistemas de conjugação são requeridos para o alongamento e fusão da dupla membrana para formar o autofagossomo: o complexo Atg12-Atg5-Atg16 que se une formando um tetrâmero e o complexo Atg8. Este último é clivado pelo Atg4, ativado pelo Atg7 e entregue ao Atg3 que o une a uma fosfatidiletanolamina (PE), formando o Atg8-PE. O sistema de conjugação do Atg8 em leveduras é homólogo ao LC3 (ou MAP1LC3 – *Microtubule-associated protein light chain*-3) em mamíferos (MEIJER & CODOGNO, 2009). O LC3 atua como uma proteína de membrana, contribuindo para a nucleação do autofagossomo (KLIONSKY & EMR, 2000). A proteína LC3-PE tem sido amplamente utilizada como marcador específico de autofagossomos, uma vez que sua forma ligada a PE está presente somente no interior destes (KLIONSKY, 2008; 2009). É importante ressaltar que existem três isoformas da proteína LC3 (LC3A, LC3B e LC3C) que sofrem modificações pós-traducionais durante o processo autofágico. A clivagem do LC3 após a tradução origina a forma citosólica do LC3, denominada LC3-I. Como discutido anteriormente, quando o processo de autofagia é ativado, o LC3-I sofre clivagem e une-se à PE, sendo então convertido em LC3-II (ou LC3-PE). Após a formação do autofagossomo, ocorre a fusão deste com o lisossomo, dando origem ao autolisossomo, que posteriormente irá liberar

os produtos de degradação para reutilização em novos processos celulares (MEIJER & CODOGNO, 2009).

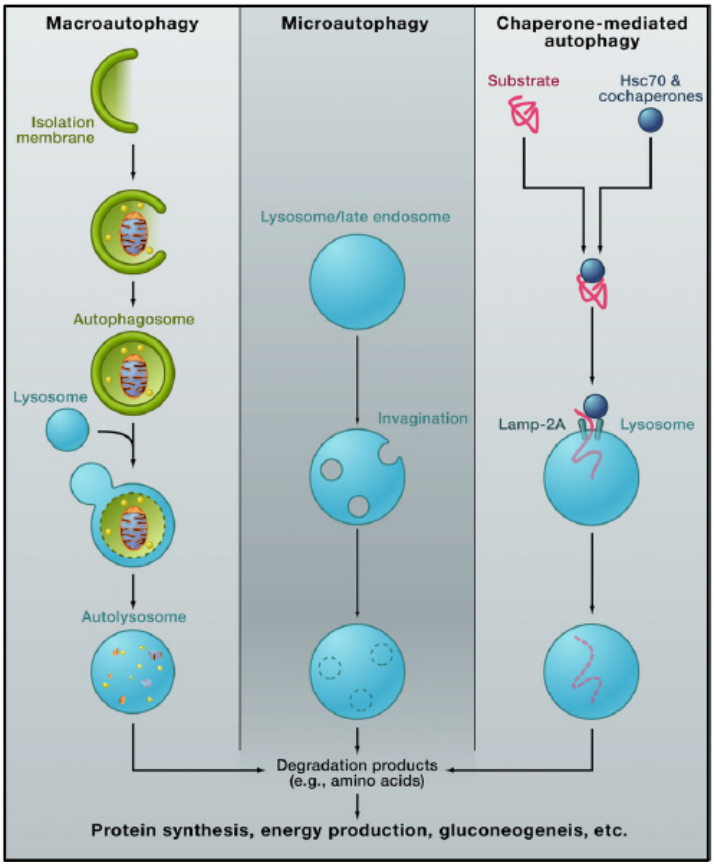

Figura 6. As diferentes vias do processo de autofagia. Adaptado de MIZUSHIMA & KOMATSU, 2011.

Devido à escassez de estudos sobre o papel da autofagia na morte celular causada pela HI, ainda não se sabe qual é a sua relação com os outros tipos de morte celular (apoptose e necrose) após o insulto. De acordo com Balduini *et al.* (2009), a enzima mTOR (Alvo da Rapamicina em Mamíferos) inibe a autofagia em condições normais, ou seja, na presença de nutrientes. Uma vez que durante a HI há depleção dos

nutrientes disponíveis, a autofagia pode ser ativada através da redução na atividade da mTOR. Por outro lado, a autofagia pode atrasar a morte celular por preservar a homeostase celular através da produção de energia pelo catabolismo e por eliminar proteínas e mitocôndrias danificadas (**Figura 7**). Ou seja, estes eventos permitem que as células sobrevivam por mais tempo após um "estresse metabólico". Entretanto, se a autofagia for bloqueada, as células isquêmicas, ou seja, pobres em nutrientes, interrompem a programação do processo de apoptose fazendo com que as células entrem em processo de morte celular por necrose. Por outro lado, se a autofagia estiver superativada, ocorrerá uma massiva ativação lisossomal e por consequência a morte celular.

Estudos com camundongos *knockout* para o gene da caspase-3 e da CAD (DNase ativada por caspase), submetidos à HI mostraram uma grande ocorrência de autofagia nos neurônios piramidais da região CA1 do hipocampo, sendo que estes neurônios que continham um grande número de autofagossomos/autolisossomos sofreram morte neuronal tardia (UCHIYAMA *et al.*, 2008). Da mesma forma, quando foi analisada a morte celular em camundongos *knockout* para o gene Atg7 (impossibilitados de executar a autofagia), notou-se que esta deleção específica preveniu a morte celular tanto por apoptose quanto por necrose, mostrando que a autofagia parece ser um mecanismo inicial que regula as vias de morte celular (UCHIYAMA *et al.*, 2008).

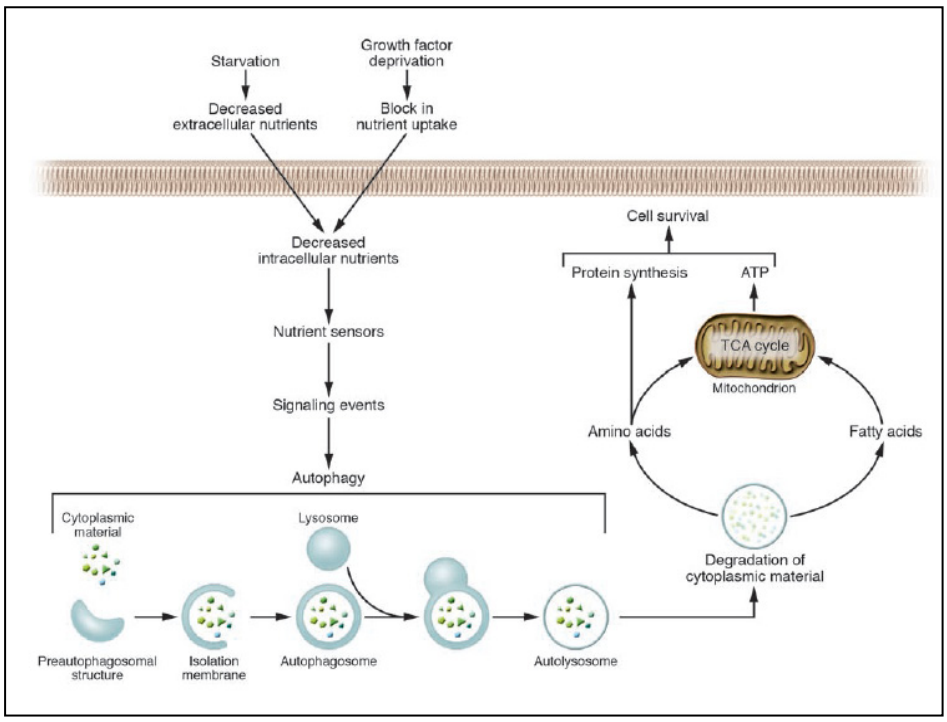

Figura 7. A via de autofagia e a adaptação celular em resposta à privação de nutrientes. Extraído de LEVINE & YUAN, 2005.

1.4 DIMORFISMO SEXUAL

Muitos processos fisiológicos e patológicos do sistema nervoso central apresentam dimorfismo sexual, mais especificamente em relação à predileção por um gênero ou uma resposta sexo-específica para um determinado tratamento. Estudos publicados na literatura mostram que os neurônios de encéfalos de ratos machos neonatos com 9 dias de vida pós-natal, submetidos à HI, apresentaram uma translocação mais pronunciada do AIF da fração mitocondrial para o núcleo após a HI, enquanto que as fêmeas apresentaram elevada ativação de caspase-3 (ZHU *et al.*, 2006). Da mesma forma, foi encontrada uma ativação quatro vezes maior de caspase-3 no encéfalo de fêmeas após a HI quando comparadas com os machos no sétimo dia pós-natal

(RENOLLEAU *et al.*, 2007). Estas diferenças sexo-específicas também são encontradas quando se avaliam os efeitos de estratégias neuroprotetoras. A administração sistêmica de Boc-aspartil-(Ome)-fluorometil-cetona (BAF), um inibidor específico da caspase-3, foi capaz de reduzir o volume do infarto cortical apenas nas fêmeas submetidas à HI (JOLY *et al.*, 2004; RENOLLEAU *et al.*, 2008). Já a deficiência da PARP-1 em camundongos com 7 dias de vida pós-natal protegeu completamente o encéfalo dos machos da lesão hipóxico-isquêmica, sem produzir nenhum efeito nas fêmeas (HAGBERG *et al.*, 2004). As evidências na literatura sugerem que o dimorfismo sexual estende-se também aos humanos (HINDMARSH *et al.,* 2000; AHMED *et al.*, 2008).

Estudos clínicos demonstraram que as crianças do sexo feminino obtiveram uma maior evolução no quadro clínico neurológico após uma lesão traumática encefálica (DONDERS & HOFFMAN, 2002) além de responderem mais favoravelmente ao tratamento utilizado (WEIL *et al.*, 1998). A maior incidência de prematuridade em crianças do sexo masculino também pode representar um grau maior de risco para a lesão cerebral perinatal e, posteriormente, a um comprometimento cognitivo e neurológico mais severo (WOLKE, 1998). Os estudos realizados apontam que as diferenças sexo-específicas estão correlacionadas com a idade e o grau de severidade do insulto ao qual foram submetidas. Esta neuroproteção mais evidente nas fêmeas pode estar em parte relacionada aos efeitos protetores tanto do estrógeno circulante quanto dos receptores do estrógeno no encéfalo (BEYER, 1999; LIU *et al.*, 2009). Isto porque a administração exógena desse hormônio comprovou ser efetiva em melhorar a evolução do quadro clínico neurológico em mulheres após uma isquemia ou trauma cerebral (HURN & MACRAE, 2000).

Baseado no exposto acima, nós decidimos avaliar a influência do dimorfismo sexual no dano celular induzido pela HI em neonatos machos e fêmeas. A **hipótese de**

trabalho deste estudo é que as alterações sexo-específicas demonstradas na literatura podem estar fortemente relacionadas às diferenças no metabolismo energético e nos mecanismos de morte celular após a exposição à lesão da HI neonatal. Para estudar esta hipótese de trabalho, foram avaliados tanto parâmetros de estresse oxidativo quanto a função mitocondrial e o processo de autofagia.

2. OBJETIVOS

2.1 OBJETIVO GERAL

O objetivo da presente tese foi investigar os efeitos celulares da HI no encéfalo de neonatos através da avaliação do estresse oxidativo, do dano mitocondrial e a ativação da via da autofagia e avaliar se estes efeitos apresentam diferenças sexo-específicas.

2.1.1 OBJETIVOS ESPECÍFICOS

A. Investigar os efeitos da HI em hipocampo e córtex cerebral de ratos neonatos através da avaliação de parâmetros de estresse oxidativo, como a formação de espécies reativas, peroxidação aos lipídios e atividade das enzimas antioxidantes glutationa peroxidase, catalase e superóxido dismutase. Ainda, mensurar a atividade da enzima Na^+, K^+-ATPase devido à sua grande sensibilidade ao estresse oxidativo;

B. Avaliar os efeitos da HI sobre a função mitocondrial em hipocampo e córtex cerebral em dois períodos distintos após o dano considerando as possíveis diferenças sexo-específicas, através da mensuração da atividade dos complexos I-III, II e IV da cadeia respiratória mitocondrial e das medidas de massa e potencial de membrana mitocondrial;

C. Verificar a contribuição da via da autofagia no dano encefálico causado pela HI em neonatos levando-se em consideração as possíveis diferenças

sexo-específicas, mensurando-se a formação de vacúolos específicos da via e expressão de proteínas específicas da autofagia.

3. CAPÍTULO 1

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Early biochemical effects after unilateral hypoxia–ischemia in the immature rat brain

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ABSTRACT

Perinatal hypoxia–ischemia (HI) gives rise to inadequate substrate supply to the brain tissue, resulting in damage to neural cells. Previous studies at different time points of development, and with different animal species, suggest that the HI insult causes oxidative damage and changes Na^+, K^+–ATPase activity, which is known to be very susceptible to free radical-related lipid peroxidation. The aim of the present study was to establish the onset of the oxidative damage response in neonatal Wistar rats subjected to brain HI, evaluating parameters of oxidative stress, namely nitric oxide production, lipoperoxidation by thiobarbituric acid reactive substances (TBA-RS) production and malondialdehyde (MDA) levels, reactive species production by DCFH oxidation, antioxidant enzymatic activities of catalase, glutathione peroxidase, superoxide dismutase as well as Na^+, K^+–ATPase activity in hippocampus and cerebral cortex. Rat pups were subjected to right common carotid ligation followed by exposure to a hypoxic atmosphere (8% oxygen and 92% nitrogen) for 90 min. Animals were sacrificed by decapitation 0, 1 and 2 h after HI and both hippocampus and cerebral cortex from the right hemisphere (ipsilateral to the carotid occlusion) were dissected out for further experimentation. Results show an early decrease of Na^+, K^+–ATPase activity (at 0 and 1 h), as well as a late increase in MDA levels (2 h) and superoxide dismutase activity (1 and 2 h after HI) in the hippocampus. There was a late increase in both MDA levels and DCFH oxidation (1 and 2 h) and an increase in superoxide dismutase activity (2 h after HI) in cortex; however Na^+, K^+–ATPase activity remained unchanged. We suggest that neonatal HI induces oxidative damage to both hippocampus and cortex, in addition to a decrease in Na^+, K^+–ATPase activity in hippocampus early after the insult. These events might contribute to the later morphological damage in the brain and indicate that it would be essential to pursue neuroprotective strategies, aimed to counteract oxidative stress, as early as possible after the HI insult.

1. Introduction

Perinatal hypoxia–ischemia (HI) is an important cause of acquired brain damage in infants with subsequent life-long sequelae (Johnston et al., 2001; Volpe, 2001; Dixon et al., 2002; Levene and Evans, 2005; Marlow et al., 2005; Gonzalez and Miller, 2006; Rennie et al., 2007), including cognitive impairment (Marlow et al., 2005; Gonzalez and Miller, 2006; Rennie et al., 2007). Much of our current understanding of the mechanisms of HI brain damage, as well as potential therapeutic interventions, derives from an extensive literature on experimental stroke models in the adult animals. However, direct application of the findings obtained in the adult brain to the newborn animal has been hampered by a paradox, in that the immature brain has generally been considered 'resistant' to the damaging effects of HI, while at the same time exhibiting periods of heightened sensitivity to injury, dependent on the specific developmental stage of the brain (Vannucci and Hagberg, 2004).

Cerebral HI in the newborn has profound molecular consequences that begin with energy failure. This process involves glutamate release, activation of glutamate receptors, coupled to nitric oxide synthase activation, calcium influx and release of

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nitric oxide (NO) (McLean and Ferriero, 2004; Buonocore and Groenendaal, 2007), that may result in mitochondrial dysfunction, generation of reactive species (Lipton, 1999; Taylor et al., 1999) and induction of oxidative stress. In addition, data from the literature demonstrates that mitochondrial electron transport chain is impaired in the postnatal day 26 of rats submitted to HI insult (Clarkson et al., 2007) and in other models of cerebral ischemia (Almeida et al., 1995). Also, HI induces an enhancement of inducible nitric oxide synthase activity and an inhibition of lactate dehydrogenase (Clarkson et al., 2004). It is also important to consider that the newborn brain is especially vulnerable since antioxidant levels are lower in neonates relative to adults (Smith et al., 1993; Nishida et al., 1994).

A significant influx of Na^+ enters neurons both during action potentials depolarizing phase and by flux via glutamate receptors, which are activated during and after HI. Coupled with failure of membrane ion pumps, these processes result in an accumulation of intracellular Na^+, cellular edema (Choi, 1992) and possibly neuron necrosis (Martin et al., 1997, 2000). Na^+, K^+–ATPase is an ubiquitous enzyme responsible for the establishment of cell membrane electrochemical Na^+ and K^+ gradients, including the maintenance of resting membrane potential in neurons. This enzyme contains two major subunits: a larger α protein responsible for the majority of the catalytic activity, and a smaller, glycosylated β protein required for maturation of the enzyme and transport to the cell surface (Lingrel, 1992). Multiple α and β isoforms have been identified in the mammalian brain (McGrail et al., 1991; Peng et al., 1997), likely reflecting both the heterogeneity of cell subtypes and the possible need for variability in enzyme expression based on tissue-specific requirements (Lingrel, 1992). It is well known that Na^+, K^+–ATPase is inhibited by reactive oxygen species and this has been demonstrated in vitro by Thomas and Reed (1990), Huang et al. (1992), Boldyrev et al. (1995) and in vivo by Andreoli et al. (1993) and Mintorovitch et al. (1994). A number of reports indicate that Na^+, K^+–ATPase activity is very sensitive to hypoxia, and post-hypoxia inhibition persists during reoxygenation (Chang et al., 1998; Mishra and Delivoria-Papadopoulos, 1999); therefore, this enzyme activity has been suggested to be a sensitive marker for the grading of HI brain injury (Mishra and Delivoria-Papadopoulos, 1999). It has also been shown that Na^+, K^+–ATPase is inhibited in the adult rat brain subjected to cerebral ischemia (Wyse et al., 2000), however the possible mechanism remains unclear.

It has been shown that HI injury causes oxidative stress by inducing lipoperoxidation and reactive species production, changes the activity of antioxidant enzymes in a variety of animal species and inhibits Na^+, K^+–ATPase activity, known to be sensitive to free radical-related lipid peroxidation. For this reason, the goal of the present study was to establish the onset of the oxidative damage through the measurement of some oxidative stress parameters namely nitric oxide (NO) production, lipoperoxidation by thiobarbituric acid reactive substances (TBA-RS) production and malondialdehyde (MDA) levels, reactive species production by 2′,7′-dichlorofluorescein (DCFH) oxidation, and antioxidant activities of catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD), as well as Na^+, K^+–ATPase activity in hippocampus and cerebral cortex, two brain structures known to be affected in rats subjected to neonatal HI. The understanding of acute pathologic processes preceding the onset of irreversible perinatal injury after hypoxic–ischemic brain damage, that happens 24–48 h post-event, may help to establish effective neuroprotective treatments.

2. Experimental procedures

2.1. Animals

Fifteen pregnant Wistar rats were obtained from the Central Animal House of the Biochemistry Department, Institute of Basic Health Sciences, at the Universidade Federal do Rio Grande do Sul. The day of birth was considered day 0. Litters were culled within 48 h of birth to a maximum of eight pups per litter, summing up 116 rat pups, and were maintained undisturbed until the surgical procedure. Animals were maintained in a 12/12 h light/dark cycle in an air-conditioned constant temperature room (22 ± 1 °C), with free access to food (SUPRA, Porto Alegre, RS, Brazil) and water. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by National Institute of Health (USA) and with the Federation of Brazilian Societies for Experimental Biology. All efforts were done to minimize animal suffering as well as to reduce the number of animals.

2.2. Hypoxia–ischemia

The Levine's (1960) procedure for neonatal HI, as refined by Rice et al. (1981), was used to produce unilateral brain injury to the neonate. This procedure provides a valuable rat model that replicates much of the neuropathology seen in human neonates. By 15–50 h of recovery, the damage, largely restricted to the brain hemisphere ipsilateral to the common carotid artery occlusion, is observed in cerebral cortex, subcortical and periventricular white matter, striatum (basal ganglia) and hippocampus. Tissue injury takes the form of either selective neuronal necrosis (glia and blood vessels spared) or infarction (all elements destroyed). Such neuropathological damage is not often seen in the contralateral hemisphere and never in pups rendered hypoxic without carotid artery ligation (Towfighi et al., 1995; Vannucci and Vannucci, 1997). Rat pups were maintained with their dams until surgical procedure, at postnatal day 7. Then, half of the animals in each litter were subjected to HI, the remaining received sham surgery.

The neonates were anesthetized with halothane, an incision was made to the ventral surface of the neck, parallel and just lateral to the trachea; the right common carotid artery was assessed, isolated from the nerve and vein, and permanently occluded with surgical silk thread. Animals were then allowed to recover for 10 min under a heating lamp and returned to their dams. After 90 min, pups were exposed to the hypoxic atmosphere (8% oxygen and 92% nitrogen) during 90 min in a 1500 mL chamber partially immersed in a 37 °C water bath (Arteni et al., 2003; Rodrigues et al., 2004). Subsequent to the HI procedure, animals were returned to their home cages until sacrifice. Controls were sham-operated, i.e., they were submitted to anesthesia and neck incision, but did not receive artery occlusion or hypoxia; mortality rate was less than 10% of all operated animals. As in the HI procedure, sham-operated animals were allowed to recover for 10 min before returning to their dams. After 90 min, sham pups were separated from the dams, placed under a heating lamp for the same period of hypoxia procedure and returned to their home cages together with the HI pups until sacrifice. Pups were sacrificed by decapitation without anesthesia, immediately (0 h), 1 or 2 h after the end of HI. Each litter was divided so that pups were used for all the different time points.

2.3. Tissue preparation

The brain was rapidly excised on a Petri dish placed on ice and cortex and hippocampus from the right hemisphere were dissected out. Samples were collected and instantaneously placed on liquid nitrogen and stored at −70 °C until biochemical assays, when they were homogenized in 10 vol (w:v) of 20 mM ice-cold phosphate buffer (pH 7.4) containing 1 mM EDTA for determination of antioxidant enzyme activities. For the determination of other oxidative stress parameters, samples were homogenized in 10 vol (w:v) of 20 mM ice-cold potassium phosphate buffer (pH 7.4), containing 140 mM KCl in a teflon–glass homogenizer. As for the Na^+, K^+–ATPase activity determination, cerebral structures were homogenized in 10 vol (w:v) of 0.32 M sucrose solution containing 5 mM HEPES and 1 mM EDTA, pH 7.4 (Wyse et al., 2000). The homogenate was centrifuged at $1700 \times g$ for 10 min at 4 °C, pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and used for the assays.

2.4. Biochemical assays

2.4.1. Determination of thiobarbituric acid reactive substances

The formation of TBA-RS is used as an indicator of lipoperoxidation. MDA, a product of lipoperoxidation, reacts with two molecules of thiobarbituric acid (TBA) at low pH and high temperature to form a pink-colored complex. TBA-RS formation was determined according to Esterbauer and Cheeseman (1990). Briefly, aliquots of samples were pre-incubated for 1 h on a 37 °C water bath and, afterwards, 10% TCA was added. Samples were centrifuged at $960 \times g$ for 10 min at 4 °C. The supernatant was mixed with 0.67% TBA (1:2) and this mixture was heated on a boiling water bath. Afterwards, n-butanol was added in order to separate the organic phase that was collected and the production of color was assessed spectrophotometrically at 532 nm. The calibration curve was performed using 1,1,3,3-tetramethoxypropane as standard.

2.4.2. Malondialdehyde determination

The measurement of MDA by the TBA-RS assay is an easy and quick assay for the assessment of lipoperoxidation, however, it is non-specific, and is generally poor when applied to biological samples. For this reason, we also decided to measure MDA by HPLC, a procedure likely to be more sensitive than TBA-RS. MDA levels were measured by HPLC according to the method described by Esterbauer and Cheeseman

(1990). Briefly, an aqueous sample of the homogenate, pH 6.5–8.0, was separated by HPLC using an amino-phase column with acetonitrile, 30 mM Tris buffer, pH 7.4 (1:9 v/v). The effluent was monitored at 267 nm, the maximum absorption of the enolate anion form of free MDA.

2.4.3. 2′,7′-Dihydrodichlorofluorescein oxidation

Production of reactive oxygen/nitrogen species was measured following the method of LeBel et al. (1992), which is based on DCFH oxidation. 2′,7′-Dihydrodichlorofluorescein diacetate (DCFH-DA) is cleaved by cellular esterases and the DCFH formed is eventually oxidized by reactive oxygen species or reactive nitrogen species presented in samples. Briefly, DCFH-DA was incubated for 30 min at 37 °C with an aliquot of the supernatant obtained from cortex or hippocampus. Fluorescence was measured using excitation and emission wavelengths of 488 nm and 525 nm, respectively. A calibration curve was performed with standard DCF and the levels of reactive species were calculated as nmol DCF formed per mg protein.

2.4.4. Nitric oxide production

NO production was determined by measuring its metabolites nitrate (NO^{3-}) and nitrite (NO^{2-}), according to Miranda et al. (2001). Eighty-five microliters of vanadium chloride was mixed to 85 μL of the supernatants of cerebral structures for the complete reduction of nitrate to nitrite. Then, 85 μL of Griess reagent (a mixture of N-1-naphthylethylenediamine dihydrochloride and sulfanilamide) was added and incubated for 1 h at 37 °C in a water bath in a dark room. The resulting pink-stained pigment was determined in a spectrophotometer at 540 nm. A calibration curve was performed using sodium nitrite and each curve point was subjected to the same treatment as supernatants. Nitric oxide production values were calculated as nmol per mg protein.

2.4.5. Catalase activity

CAT activity assessment is based upon establishing the rate of hydrogen peroxide (H_2O_2) degradation spectrophotometrically at 240 nm at 25 °C (Aebi, 1984). CAT activity was calculated in terms of micromoles of H_2O_2 consumed per minute per mg protein.

2.4.6. Glutathione peroxidase activity

GPx activity was determined according to Wendel (1981), with modifications. The reaction was carried out at 37 °C in 200 μL of solution containing 20 mM potassium phosphate buffer (pH 7.7), 2 mM EDTA, 0.8 mM sodium azide, 0.5 mM NADPH, 2 mM glutathione and 0.4 U glutathione reductase. The activity of GPx was measured using tert-butylhydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was always subtracted from the overall reaction ratio. GPx activity was expressed as nmol NADPH oxidized per minute per mg protein.

2.4.7. Superoxide dismutase activity

SOD activity was determined using a RANSOD kit (RANDOX) which is based on the procedure described by Delmas-Beauvieux et al. (1995). This method uses xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye, which is assessed spectrophotometrically at 505 nm wavelength at 37 °C. The specific activity is reported as units (one unit of SOD is that which causes a 50% inhibition of the rate of reduction of I.N.T. under the conditions of the assay) per mg protein.

2.4.8. Determination of Na^+, K^+–ATPase activity

The reaction mixture for the Na^+, K^+–ATPase assay contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl and 40 mM Tris–HCl buffer, pH 7.4, in a final volume of 200 μL. The reaction was started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3 mM. Controls were assayed under the same conditions with the addition of 1 mM ouabain. Na^+, K^+–ATPase activity was calculated by the difference between the two assays as described by Wyse et al. (2000). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986); enzyme specific activity was expressed as nmol Pi released per minute per mg protein.

2.4.9. Protein assay

Total protein concentration for the oxidative stress biomarkers and antioxidant enzyme activities was determined using the method described by Lowry et al. (1951) using bovine serum albumin as the standard. For determination of Na^+, K^+–ATPase activity, total protein concentration was measured by Bradford (1976).

2.5. Statistics

Results are expressed as mean ± standard error of the mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA), followed by *post hoc* Duncan's test for multiple comparisons. A value of $p < 0.05$ was considered significant. All statistical analysis was performed using the Statistica® software package running on a compatible personal computer.

3. Results

3.1. Oxidative stress biomarkers

HI changed neither nitrite production nor TBA-RS levels in hippocampus or cortex, as showed by one-way ANOVA ($p > 0.05$) (Table 1). Although DCFH oxidation levels in cerebral cortex were significantly increased 1 and 2 h after the event [$F(3,20) = 7.32$, $p < 0.01$] (Table 1), the same alteration was not observed in the hippocampus. MDA levels were increased 2 h after the insult in the hippocampus [$F(3,16) = 5.18$, $p < 0.05$] and 1 and 2 h after HI in the cortex [$F(3,16) = 10.17$, $p < 0.01$] (Fig. 1).

3.2. Antioxidant enzymes activities

CAT and GPx activities were not affected by HI, considering time points and brain structures studied ($p > 0.05$) (Table 2). However, SOD activity was increased in hippocampus 1 h and 2 h after the event [$F(3,30) = 3.72$, $p < 0.05$], and 2 h in cortex [$F(3,28) = 4.24$, $p < 0.05$] (Table 2).

3.3. Na^+, K^+–ATPase activity

In the hippocampus, Na^+, K^+–ATPase activity was reduced 0 and 1 h after HI, when compared to the sham group [$F(3,22) = 6.39$, $p < 0.01$] (Fig. 2). In contrast, neonatal HI did not affect Na^+, K^+–ATPase activity in cerebral cortex at any analyzed time points ($p > 0.05$).

3.4. Discussion

Present study was delineated to investigate the onset of the oxidative stress response and Na^+, K^+–ATPase activity alterations in the immature rat brain following unilateral HI in hippocampus and cerebral cortex, two particularly vulnerable brain structures. Results demonstrate that HI induced oxidative stress, changed the activity of antioxidant enzymes and decreased Na^+, K^+–ATPase activity in both cerebral structures as early as 2 h after the ischemic insult.

Ischemic brain injury in the newborn leads to the formation of free radical species such as superoxide anion radical ($O_2^{\bullet -}$), nitric oxide radicals (NO•) and hydroxyl radicals (•OH) among others in both in vivo and in vitro experiments (Ohsawa et al., 2007; Kumar et al., 2008). These free radicals perpetuate further injury by initiating lipoperoxidation of cell membranes and damaging DNA and proteins (Kumar et al., 2008). In the present study, it was shown an increase in MDA levels in the right hippocampus, ipsilateral to the carotid occlusion, 2 h after HI, suggesting lipoperoxidation in this structure. An enhancement in DCFH oxidation, which represents an index of non-specific reactive species production, and in MDA levels also suggest an increase in generalized reactive species production and in consequence, increase in lipoperoxidation in cerebral cortex. These results are in agreement with data in the literature, which suggest increased peroxidation of brain cell membrane lipids and generation of free radicals after HI using other models, such as prenatal hypoxia in newborn piglets and guinea pigs (Andersen et al., 1996; Maulik et al., 1998; Mishra et al., 1989; Numagami et al., 1997; Razdan et al., 1993). We must stress that, in most published studies, experimental models are different from those used in present study, either in the animal species chosen, or the period of development, or in the HI model utilized.

As regard the enzyme defense system, SOD activity showed an increment 1 and 2 h after HI in the hippocampus, and 2 h after HI in cerebral cortex, suggesting a possible compensatory effect on enzyme activity that had occurred to overcome the elevated superoxide production elicited by HI damage, since increased

Table 1
Oxidative stress biomarkers in hippocampus and cortex of neonatal rats submitted to unilateral hypoxia–ischemia.

	Sham	HI 0 h	HI 1 h	HI 2 h
Hippocampus				
TBA-RS	0.950 ± 0.09	1.100 ± 0.12	0.927 ± 0.03	0.750 ± 0.07
DCFH oxidation	7.13 ± 0.18	7.21 ± 0.49	6.64 ± 0.40	7.62 ± 0.36
Nitrites	36.91 ± 1.39	39.25 ± 3.03	38.55 ± 1.44	39.35 ± 1.75
Cortex				
TBA-RS	0.271 ± 0.036	0.324 ± 0.046	0.317 ± 0.052	0.314 ± 0.058
DCFH oxidation	8.01 ± 0.51	8.18 ± 0.33	10.32 ± 0.29**	9.97 ± 0.56**
Nitrites	17.80 ± 1.70	21.88 ± 4.17	19.52 ± 4.08	19.95 ± 3.68

Data are expressed as mean ± SEM for 5–8 animals in each group. TBA-RS are expressed as nmol MDA per mg protein, DCFH oxidation as pmol DCF formed per mg protein and nitrites as nmol NO_2 per mg protein.
** Significant difference from sham-operated animals ($p < 0.01$).

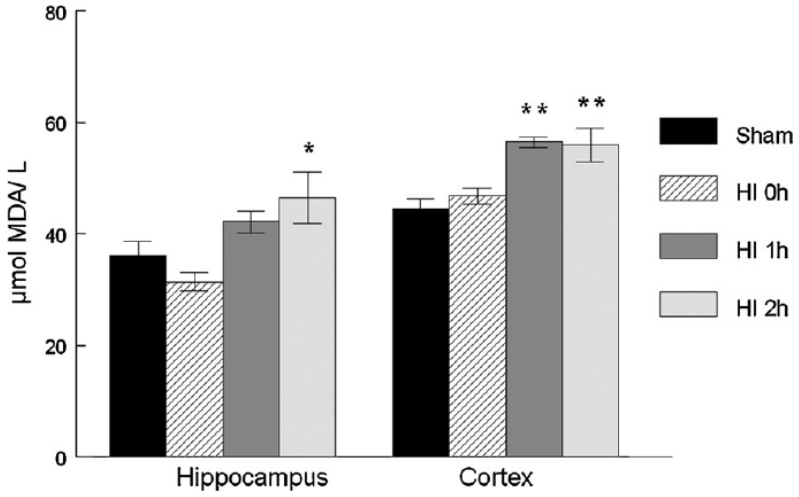

Fig. 1. MDA levels in hippocampus and cortex of neonatal rats submitted to unilateral hypoxia–ischemia. Data are expressed as mean ± SEM for 5 animals in each group. Asterisks indicate significant difference from sham-operated animals (*$p < 0.05$; **$p < 0.01$).

superoxide production may stimulate SOD activity due to the substrate allosteric activation (Misra and Fridovich, 1972). In contrast, there were no changes in CAT or GPx activities in the studied structures. The reactive oxygen species scavenging activity of SOD is effective only when it is followed by CAT and GPx activities, because the dismutase action of SOD generates H_2O_2 from superoxide anion, which requires to be further scavenged by CAT and GPx (Halliwell, 2001). The H_2O_2 can interact with superoxide anion leading to the formation of the highly reactive hydroxyl radical (Haber and Weiss, 1934), and this can also happen when H_2O_2 is in the presence of iron (Halliwell, 2001; Haber and Weiss, 1934). Previous studies have verified that SOD1 overexpression results in marked neuroprotection in adult rats after ischemia–reperfusion (Chan et al., 1998), whereas in the neonatal animal, it exacerbates the injury (Ditelberg et al., 1996). The variable effect of SOD1 in the brain during different stages of development may be explained by the fact that SOD1 transgenic adult mice show an adaptative rise in catalase activity (Przedborski et al., 1992), whereas neonatal SOD1 transgenic brains do not show an adaptative increase in either GPx or catalase (Fullerton et al., 1998), similar to the results reported here. Therefore, the increment in the production of reactive species may lead to the increased SOD activity, and the imbalance in the activity of these enzymes is suggested to further induce oxidative stress in these cerebral structures. The present data support that reactive species are part of the injurious cascades in the immature brain as the levels of free radicals are increased after HI, possibly compromising the antioxidant defense system (Bågenholm et al., 1998).

Table 2
Activities of catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) in hippocampus and cortex of neonatal rats submitted to unilateral hypoxia–ischemia.

	Sham	HI 0 h	HI 1 h	HI 2 h
Hippocampus				
CAT activity	3.29 ± 0.25	3.34 ± 0.31	3.94 ± 0.42	3.22 ± 0.23
GPx activity	37.45 ± 2.39	46.57 ± 1.68	44.28 ± 2.59	42.09 ± 2.89
SOD activity	10.14 ± 0.93	12.22 ± 0.78	13.61 ± 1.53*	14.87 ± 0.59*
Cortex				
CAT activity	6.22 ± 0.37	4.72 ± 0.42	5.37 ± 0.51	4.52 ± 0.43
GPx activity	34.71 ± 2.56	42.29 ± 2.74	41.80 ± 3.53	40.33 ± 3.16
SOD activity	14.00 ± 0.80	12.16 ± 1.51	13.25 ± 1.08	19.36 ± 2.37*

Data are expressed as mean ± SEM for 7–9 animals in each group. CAT activity is expressed as µmol H_2O_2 oxidized per minute per mg protein, GPx activity as nmol NADPH oxidized per minute per mg protein and SOD activity as U/mg protein.
* Significant difference from sham-operated animals ($p < 0.05$).

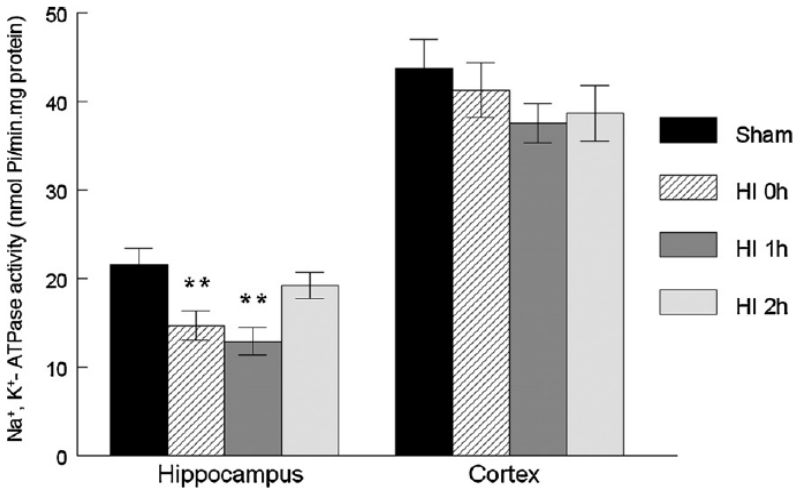

Fig. 2. Na^+, K^+–ATPase activity in hippocampus and cortex of neonatal rats submitted to unilateral hypoxia–ischemia. Data are expressed as mean ± SEM for 5–7 animals in each group. Asterisks indicate significant difference from sham-operated animals (**$p < 0.01$).

The distinct activation concerning antioxidant enzymes here reported might be explained by possible distinct mechanisms of regulation involved in the early response of these enzymes to oxidative stress. Mavelli et al. (1982) demonstrated that the specific activity of the enzymes involved in cell defense against partially reduced forms of oxygen (namely CAT, GPx and SOD) in the developing brain, does not uniformly parallel the development of aerobic metabolism during postnatal maturation of the rat brain. Moreover, results obtained in that study show that the temporal changes of these enzymes cannot be correlated with each other, indicating that they do not parallel the increasing activity of aerobic brain metabolism during development.

There was a decrease in Na^+, K^+–ATPase activity immediately and 1 h after HI in the ipsilateral hippocampus. This decrease shall not be related to the known loss of high-energy phosphate levels that occurs during HI (Golden et al., 2001), since enzyme assay is run in optimal biochemical conditions, which includes an excess of ATP concentration. Besides, the impaired activity does not appear to be related to decreased expression of isoforms of the enzyme since the effect was observed immediatelly after HI, and previous studies (Jamme et al., 1999; Golden et al., 2001) evidence Na^+, K^+–ATPase inhibition without changes in the expression of subunit isoforms in models of focal cerebral ischemia and HI. This change in Na^+, K^+–ATPase activity may be due to the oxidative damage already reported to happen in the hippocampus; in fact, Na^+, K^+–ATPase inhibition by free radical oxygen species has been described in several tissues, possibly by free radical attack to the lipid bilayer followed by membrane disorganization and suppression of enzyme activity (Kako et al., 1988; Razdan et al., 1993; Jamme et al., 1995; Matté et al., 2006). It has also been demonstrated that both hypoxia and lipid peroxidation changes Na^+, K^+–ATPase function by modifying specific active sites in a selective manner rather than through a non-specific destructive process (Razdan et al., 1993; Graham et al., 1993; Mishra et al., 1989). Interestingly, Na^+, K^+–ATPase activity remained unchanged in the ipsilateral cortex, in spite of the increased reactive species production.

Although the timing of events subsequent to HI that lead to cell death is not clearly established, it is known that significant infarction of the brain is present during the 24–48 h period of post-HI with significant loss in volume of the ipsilateral striatal, cortical and hippocampal regions 1 week after the episode (Hossain et al., 2004). Defining the sequence of molecular events will be important to design effective neuroprotection strategies; in fact, currently available interventions, such as hypothermia and N-acetylcysteine administration, are efficient in human and animal studies only if early instituted (Scher, 2001). Based on present results, we may suggest that it would be essential to evaluate neuroprotective strategies aimed to counteract oxidative stress as early as possible after the HI insult.

Summarizing, this study demonstrates the onset of the oxidative stress response and Na^+, K^+–ATPase activity alterations elicited by HI in both hippocampus and cortex of neonatal rats. These effects suggest that the oxidative insult, in association with the inhibition of ATPase activity, may contribute to the later brain morphological damage found in this experimental model.

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4. CAPÍTULO 2

Artigo: Neonatal hypoxia-ischemia induces sex-related changes in rat brain mitochondria – Publicado no periódico *Mitochondrion*

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Neonatal hypoxia–ischemia induces sex-related changes in rat brain mitochondria

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ABSTRACT

The effects of neonatal hypoxia–ischemia (HI) on energy metabolism in male and female rats were investigated, testing the hypothesis that HI-induced brain mitochondrial dysfunction could present in a dimorphic pattern. Impairment in electron transport chain complex activities at 2 and 18 h after HI was observed in cortex and hippocampus in rats of both sexes, with females presenting an overall activity higher than that of males. Females also showed loss of mitochondrial mass and membrane potential 18 h after HI, while males were only slightly affected. These findings suggest a dimorphism in mitochondrial dysfunction and provide information that may lead to new neuroprotection strategies.

1. Introduction

Perinatal asphyxia is an important contributor to brain injury in preterm neonates that suffer from interruption to brain blood and oxygen supplies (Carty et al., 2008). Mild insults arising from hypoxia–ischemia (HI) include attention-deficit hyperactivity syndrome and minimal brain disorders causing long-term neurodevelopment impairments (Boksa and El-Khodor, 2003; Volpe, 2001), while severe cases may cause spasticity, epilepsy and mental retardation (Hill, 1991; Volpe, 2001).

Brain ischemia is associated with high susceptibility of mitochondrial damage. Mitochondria are motile organelles present in all aerobic organisms that convert the energy released by metabolic processes in the mitochondrial matrix into ATP. They participate in a number of intracellular processes, such as calcium homeostasis and the control of life and death through apoptosis (Koopman et al., 2010). Mitochondria are also a major source of partially reduced derivatives of molecular oxygen (O_2) that include the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) generated through the four protein complexes of electron transport chain (Koopman et al., 2010).The consequences of mitochondrial damage are severe. Mitochondrial respiratory chain damage may lead to opening of the mitochondrial permeability transition pore causing mitochondria depolarization and swelling, leading to both necrotic and apoptotic cell death (Moro et al., 2005; Rodriguez-Enriquez et al., 2009).

Several studies have demonstrated that many central nervous system (CNS) functions and pathological conditions are sexually dimorphic, i.e., a predilection for one gender and specific gender-dependent response to treatment. CNS diseases of childhood and of the elderly demonstrate gender predominance and/or sexually dimorphic responses to therapies (Dewing et al., 2003; Donders and Hoffman, 2002; Morrison et al., 2004; Renolleau et al., 2008), and the exposure to circulating sex steroids seems to contribute to this phenomenon (Renolleau et al., 2008). Recent data revealed that neurons from immature male rat brains submitted to HI presented a pronounced translocation of apoptosis-inducing factor (AIF) from the mitochondrial fraction to the nucleus. On the other hand, female brain neurons displayed higher cleavage and activation of caspase-3, as compared to that of male brains (Renolleau et al., 2007; Zhu et al., 2006). Altogether, these results show that males are more sensitive to mitochondrial dysfunction, with increased mitochondrial permeability on the outer and inner membranes leading to a high quantity of released proteins (AIF, cytochrome c, EndoG and Smac/Diablo) as compared to females (Renolleau et al., 2008). Confirmation of these experiment in human studies has shown that girls have better neurological outcome after traumatic brain injury (Donders and Hoffman, 2002; Morrison et al., 2004) and respond more favorably to treatment (Weil et al., 1998).

Abbreviations: HI, hypoxia–ischemia; MTG, mitotracker green; MTR, mitotracker red; $\Delta\psi$, mitochondrial membrane potential.

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Considering that mitochondrial dysfunction may play a key role in HI-induced neuronal death and that the distinct signaling pathways to cell death are sex-related in neonatal animals, this study investigates the effects of HI on mitochondrial activity as assessed by activities of electron transport chain complexes in the cerebral cortex and hippocampus of male and female neonates. Mitochondrial mass and consequent membrane potential were also evaluated, according to the hypothesis that HI-induced mitochondrial dysfunction presents a sexual dimorphic pattern.

2. Materials and methods

2.1. Animals

Pregnant Wistar rats were obtained from the Central Animal House of the Biochemistry Department at the Institute of Basic Health Sciences from the Universidade Federal do Rio Grande do Sul. The day of birth was considered day zero. Twelve litters were culled within 48 h of birth to a maximum of eight pups per litter (totaling 96 rat pups), and were maintained undisturbed until the surgical procedure. Animals were maintained on a 12 h light/dark cycle in a constant temperature room (22 ± 1 °C), with free access to food (SUPRA, Porto Alegre, RS, Brazil) and water. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA) and with the Federation of Brazilian Societies for Experimental Biology. All efforts were done to minimize animal suffering as well as to reduce the number of used animals.

2.2. Hypoxia–ischemia

The Levine (1960) procedure for neonatal HI, as modified by Rice et al. (1981), was used to produce unilateral brain injury to the neonate. This procedure provides a valuable rat model that replicates much of the neuropathology seen in human neonates. The damage is largely restricted to the brain hemisphere ipsilateral to the common carotid artery occlusion and affects mainly the cerebral cortex, subcortical and periventricular white matter, striatum and the hippocampus. Such neuropathological damage is not often seen in the contralateral hemisphere and is never present in pups rendered hypoxic without previous carotid artery ligation (Towfighi et al., 1995; Vannucci and Vannucci, 1997). Rat pups were maintained with their dams until surgical procedure, at postnatal day 7. Then, half of the rats in each litter were subjected to HI, the remaining animals received sham surgery. Neonates were anesthetized with halothane, an incision was made to the neck ventral surface, parallel and just lateral to the trachea; the right common carotid artery was assessed, isolated from the nerve and vein and permanently occluded with surgical silk thread. Animals were then allowed to recover for 10 min under a heating lamp and returned to their dams. After 120 min, pups were exposed to hypoxic atmosphere (8% oxygen and 92% nitrogen) for 90 min in a 1500 mL chamber partially immersed in a 37 °C water bath (Arteni et al., 2003; Rodrigues et al., 2004). Subsequent to the HI procedure, animals were returned to their home cages until decapitation (2 or 18 h after the procedure). Controls were sham-operated, i.e., they were submitted to anesthesia and the neck incision, but received neither artery occlusion nor hypoxia. Mortality rate was less than 10% of all operated animals.

2.3. Tissue preparation

The brain was rapidly removed from the skull, freed from meninges and the ipsi- and contralateral cerebral cortices and hippocampi were isolated. As for determination of respiratory chain complex activities, brain structures were homogenized with a teflon–glass homogenizer (1:20, w/v) in SETH buffer (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base), pH 7.4. The homogenates were centrifuged at 1000×*g* for 10 min and the supernatants were immediately kept at −70 °C until enzyme activity determination (Pettenuzzo et al., 2006). Mitotracker was used for mitochondrial function analysis of cerebral cortex and hippocampal cell suspensions obtained by mechanical dissociation with PBS containing collagenase to yield digestion to a density of about 200.000 cells/mL. The dissociated contents were then filtered into sterile 50 mL Falcon tubes (BD Biosciences) through 40 μm nylon cell strainer (Cell Filter Strainer — BD Biosciences) and kept on ice until mitochondrial staining.

2.4. Respiratory chain enzyme activity determination

Mitochondrial energy metabolism was evaluated using enzymatic analysis of electron transport chain (ETC) activities. The activities of the ETC complexes I–III, II and IV were determined in homogenates according to standard methods previously described (Fischer et al., 1985; Rustin et al., 1994; Schapira et al., 1990). The activity of complexes I–III (complex I + CoQ + III) was assessed by measuring the increase in absorbance due to cytochrome c reduction at 550 nm according to the method described by Schapira et al. (1990). The reaction mixture contained 8 to 15 μg protein and 20 mM potassium phosphate buffer (2 mM KCN, 10 mM EDTA and 50 mM cytochrome c), pH 8.0. The reaction was initiated by adding 25 mM NADH and was monitored at 25 °C for 3 min before addition of 10 mM rotenone; the activity was measured for an additional 3 min. Complex I–III activity was the rotenone sensitive NADH: cytochrome c reductase activity. The activity of complex II (succinate: DCIP oxiredutase) was determined according to Fischer et al. (1985) by following the decrease in absorbance due to the reduction of 2,6-DCIP at 600 nm. The reaction was carried out at 30 °C in 40 mM potassium phosphate buffer (16.0 mM sodium succinate and 8 mM DCIP), pH 7.4, and was pre-incubated with 30 to 60 μg protein for 20 min. After that, 4 mM sodium azide, 7 mM rotenone and 40 mM DCIP were added to the medium and monitored for 5 min. Cytochrome c oxidase (complex IV, COX) activity was determined according to Rustin et al. (1994), by following the decrease in absorbance due to oxidation of previously reduced cytochrome c at 550 nm. The reaction mixture was initiated by adding 0.7 mg reduced cytochrome c in a medium containing 10 mM potassium phosphate buffer (0.6 mM n-dodecyl-b-D-maltoside), pH 7.0, and 1.5 to 3 μg protein. The activity of complex IV was measured at 25 °C for 10 min. The activities of respiratory chain complexes were calculated and expressed as nmol/min/mg protein.

2.5. Mitochondrial mass and membrane potential measurements

MitoTracker Red (MTR or Chloromethyl-X-rosamine) and MitoTracker Green (MTG) dyes were employed to assess mitochondrial function. MTR is a lipophilic cationic fluorescent dye that is concentrated inside mitochondria because of the negative mitochondrial membrane potential (Pendergrass et al., 2004). The loss of membrane potential results in release of MTR from mitochondria and a subsequent decrease in fluorescence (Khanal et al., 2011). MTG is a green-fluorescing fluorophore which is taken up electrophoretically into mitochondria and has been used as a measure of mitochondrial mass independent of mitochondrial membrane potential. Chloromethyl groups on MTG form covalent adducts with sulfhydryls of mitochondrial matrix proteins such that MTG is retained even after mitochondrial depolarization (Rodriguez-Enriquez et al., 2009). MTR and MTG were dissolved in dimethylsulfoxide (DMSO) to a 1 mM stock concentration. Dissociated cells, previously filtered, were stained with 100 nm MTR and 100 nm MTG for 45 min. at 37 °C in a water bath in a dark room according to method described by Keij et al., 2000 and Pendergrass et al., 2004 with some modifications. Immediately after staining cell suspensions were removed from the water bath and analyzed by flow cytometry.

2.6. Flow cytometry analysis

Samples stained with MTR and MTG dyes were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Mito-Tracker dyes were excited at 488 nm using an air-cooled argon laser. Negative controls (samples without stain) were included for setting up the machine voltages. Controls stained with a single dye were used to set compensation. The emission of fluorochromes was recorded through specific band-pass fluorescence filters: red (FL-3; 670 nm long pass) and green (FL-1; 530 nm/30). Fluorescence emissions were collected using logarithmic amplification. In brief, data from 10.000 events (intact cells) were acquired and mean relative fluorescence intensity was determined after exclusion of debris events from the data set. All flow cytometric acquisitions and analyses were performed using CELLQuest Pro data acquisition (BD Biosciences) and FlowJo analysis software.

Flow cytometry data were analyzed and plotted by density as a single-parameter histogram which shows the relative fluorescence on the x-axis and the number of events (cell count) on the y-axis. Analyzed samples produced a single distinct peak that can be interpreted as the positive dataset. The histogram was divided in two halves (named cells with low and high mass or potential), based on the controls peak for MTR and MTG, and this evaluation was applied to all data in each parameter for both analyzed parameters. Analyses resulted in two cell populations with different mitochondrial $\Delta\psi$ and mass. The first population presents low $\Delta\psi$ and mass and the second, high $\Delta\psi$ and mass. The lower accumulation of MTR or MTG, and thus lower fluorescence values would be indicative of decreased mitochondrial mass or $\Delta\psi$ (Kalbacova et al., 2003; Leira et al., 2001).

2.7. Protein determination

Protein concentrations were determined in homogenates by the method of Lowry et al. (1951) using bovine serum albumin standards.

2.8. Statistical analysis

All assays were performed in duplicate and the mean was used for statistical analysis. Data are presented as mean ± S.E.M. and were analyzed by three-way analysis of variance (ANOVA) splitting the file by time. The statistical factors analyzed were injury (sham x HI), hemisphere (right x left) and sex (male x female). Values of $p<0.05$ were considered as significant. All tests were performed on a microcomputer using the Statistical Package for the Social Sciences software (SPSS).

3. Results

The effects of HI on mitochondrial energy metabolism, function and mass were investigated in cerebral cortex and the hippocampus of neonates, with special attention on sex-related differences. All results were analyzed and presented in two groups, 2 and 18 h after the HI procedure.

3.1. Two hours after HI

3.1.1. Mitochondrial respiratory chain enzyme activity

In the cerebral cortex, ANOVA of complex I + III activity revealed a *sex* effect ($F(1,36)=16.679$, $p<0.001$), with female rats displaying higher activity than males in all groups (Fig. 1A). A similar *sex* effect was seen in complex II activity ($F(1,36)=10.589$, $p=0.002$), that also showed an *injury x hemisphere* interaction ($F(1,36)=7.725$, $p=0.009$) (Fig. 1B). Analysis of complex IV activity revealed a *sex* main effect ($F(1,36)=107.159$, $p<0.001$) and an *injury x sex* interaction, with HI female rats presenting greater activity than males ($F(1,36)=4.877$, $p=0.034$) (Fig. 1C).

No significant differences were observed in complex I + III activity in the hippocampus (Fig. 2A). Analysis showed that females presented higher activity than males in complex II activity ($F(1,40)=9.089$, $p=0.004$). An inhibition of hippocampus complex II activity was seen in both male and female HI neonates, as demonstrated an *injury x hemisphere* interaction ($F(1,40)=32.231$, $p<0.001$) (Fig. 2B). Three-way ANOVA revealed that complex IV activity was reduced by HI injury ($F(1,36)=8.618$, $p=0.006$) (Fig. 2C).

3.1.2. Mitochondrial mass and membrane potential measurements

Analyses of cerebral cortex cells labeled with MTG and MTR are shown in Figs. 3 and 4. The number of events considered negative to MTG and MTR fluorescence (events counted but not considered marked with mitotracker) was small in all experimental groups (around 5%); however, in HI groups the number of this negative fluorescent events was greater when comparing to other groups, especially in MTR. Cells from HI damaged neonates comprised a subset of cells that shifted from a population with high mitochondrial mass and $\Delta\psi$ to a population of cells with low mass (Fig. 3A and B) and less polarized mitochondrial membranes (Fig. 4A and B), in both males and females. An *injury x hemisphere* interaction could be observed in all parameters: high mass ($F(1,38)=5.963$, $p=0.019$), low mass ($F(1,39)=9.228$, $p=0.004$) (Fig. 3A and B), high $\Delta\psi$ ($F(1,37)=7.107$, $p=0.011$) and low $\Delta\psi$ ($F(1,40)=13.758$, $p=0.001$) (Fig. 4A and B).

A similar pattern of damage was seen in the hippocampus of neonates submitted to HI. With respect to mitochondrial mass, a greater number of cells with low fluorescence intensity ($F(1,34)=6.013$, $p=0.019$) (Fig. 5B) and a decrease of high fluorescence intensity ($F(1,34)=7.296$, $p=0.011$) (Fig. 5A) were also seen in HI subjects, as an *injury x hemisphere* interaction demonstrates. Fluorescence became less intense on HI, independent of sex, with *injury* ($F(1,39)=11.060$, $p=0.002$) and *hemisphere* ($F(1,39)=12.378$, $p=0.001$) main effects at low $\Delta\psi$, with no statistical differences at high $\Delta\psi$ fluorescence (Fig. 6A and B).

3.2. Eighteen hours after HI

3.2.1. Mitochondrial respiratory chain enzyme activity

In the cerebral cortex, complex I + III activity was significantly higher in females than males, ($F(1,34)=4.999$, $p=0.032$). ANOVA also found an *injury x hemisphere* interaction ($F(1,34)=22.342$, $p<0.001$), with both male and female HI neonates presenting lower activities in the ipsilateral hemisphere (Fig. 1D). A significant reduction of complex II activity was seen in the ipsilateral hemisphere from HI animals ($F(1,35)=13.173$, $p=0.001$ – *injury x hemisphere* interaction), with no sex-related differences (Fig. 1E). Analysis of complex IV activity demonstrated a *sex* main effect ($F(1,33)=61.788$, $p<0.001$) and an *injury x sex x hemisphere* interaction ($F(1,33)=6.510$, $p=0.016$) (Fig. 1F).

The same pattern was seen in the hippocampus; overall complex I + III activity was greater in females as compared to males ($F(1,33)=4.985$, $p=0.032$) and decreased enzyme activity was observed in both sexes from HI ipsilateral group ($F(1,33)=21.676$, $p<0.001$ – *injury x hemisphere* interaction) (Fig. 2D). A main effect of *sex* was seen in complex II activity ($F(1,33)=25.078$, $p<0.001$), with females showing greater activity than males. Complex II activity was reduced in both sexes HI ipsilateral group ($F(1,33)=35.235$, $p<0.001$ – *injury x hemisphere* interaction) comparing to the other groups, and this reduction seems to be sex-related ($F(1,33)=25.503$, $p<0.001$ – *injury x sex* interaction) (Fig. 2E). Similarly to the results in the cortex, ANOVA revealed an *injury x sex x hemisphere* interaction ($F(1,34)=5.198$, $p=0.029$) in complex IV activity (Fig. 2F), since decreased enzyme activity in the HI right hippocampus was observed in females.

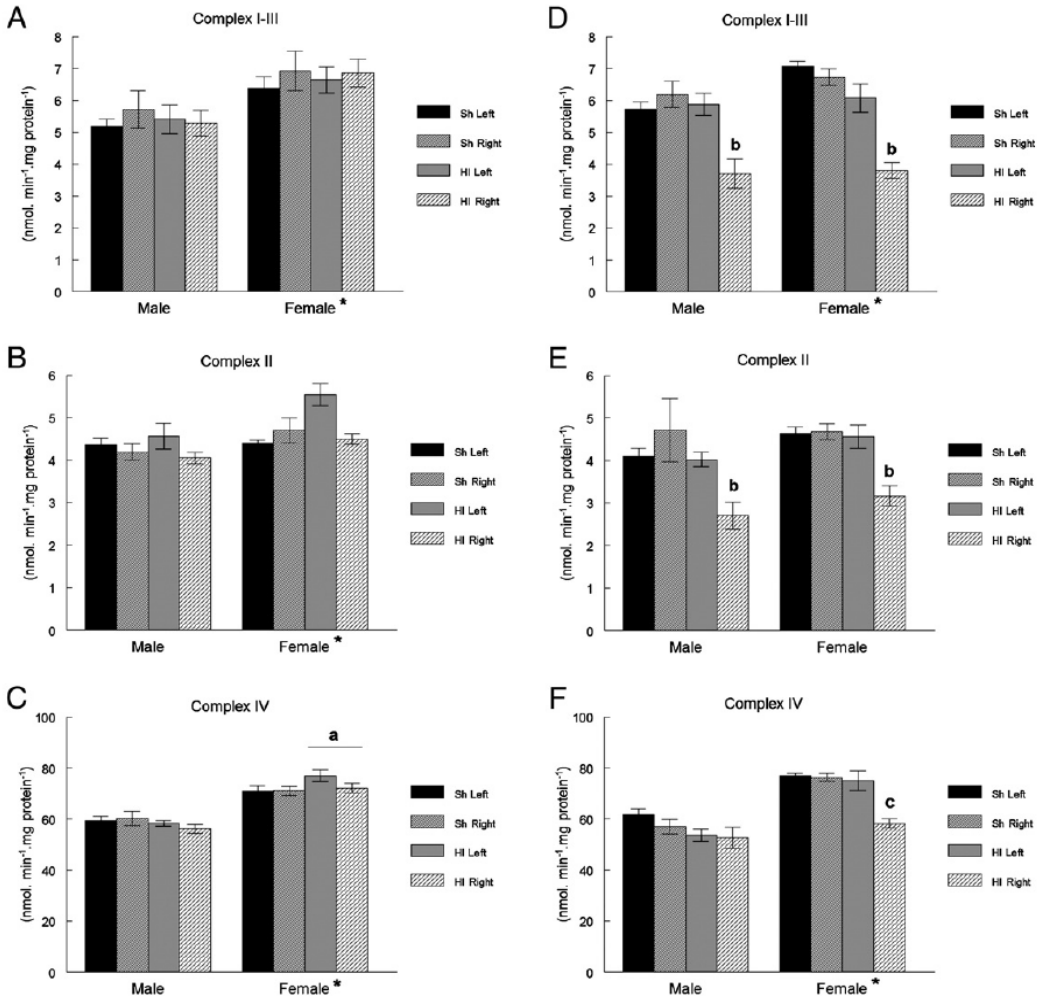

Fig. 1. Mitochondrial respiratory chain activity in cerebral cortex. The activities of mitochondrial electron transport chain complexes were determined in cerebral cortex of male and female neonates 2 h (A, B and C) and 18 h (D, E and F) after HI injury. Data are expressed as nmol/min/mg protein (mean ± SEM) of five to six animals in each group. Significant differences and interaction between sham/HI *versus* left/right hemisphere *versus* male/female were revealed by three-way ANOVA. *illustrates *sex* main effect, [a]*injury x sex* interaction, [b]*injury x hemisphere* interaction and [c]*injury x hemisphere x sex* interaction ($p<0.05$).

3.2.2. Mitochondrial mass and membrane potential measurements

Figs. 3 and 4 summarize results of cells from cerebral cortex labeled with MTG and MTR. With respect to mitochondrial mass, a *sex* main effect ($F(1,38)=34.783$, $p<0.001$), *hemisphere* main effect ($F(1,38)=12.437$, $p=0.001$) and *injury x sex* interaction ($F(1,38)=8.275$, $p=0.007$) were seen on the high fluorescence population, in which a decreased number of cells with high mitochondrial mass was observed only in females (Fig. 3C). In the low mass cell population, a *sex* main effect ($F(1,38)=31.776$, $p<0.001$) and an *injury x hemisphere* interaction ($F(1,38)=8.419$, $p=0.006$) were detected in HI neonates (Fig. 3D). A *sex* main effect was observed at high ($F(1,38)=12.157$, $p=0.001$) and low $\Delta\psi$ ($F(1,39)=8.093$, $p=0.007$) as well as an *injury x hemisphere* interaction at high ($F(1,38)=9.616$, $p=0.004$) (Fig. 4C) and low $\Delta\psi$ ($F(1,39)=12.529$, $p=0.001$) (Fig. 4D). An *injury x sex* interaction ($F(1,38)=9.233$, $p=0.004$) was also seen at high $\Delta\psi$.

In the hippocampus, the number of cells labeled with high MTG fluorescence was smaller in females at high ($F(1,37)=185.464$, $p<0.001$) and low mass ($F(1,38)=162{,}777$ $p<0.001$) and even further reduced in HI female neonates than in males with an *injury x sex* interaction ($F(1,37)=11.490$, $p=0.002$) and *injury x hemisphere* interaction ($F(1,37)=6.182$, $p=0.018$) at high mass (Fig. 5C) and *injury x sex* interaction ($F(1,38)=5.593$, $p=0.023$) at low mass (Fig. 5D). As observed in mitochondrial mass, $\Delta\psi$ from females hippocampi seemed to be more affected than males with a *sex* main effect ($F(1,39)=139.064$, $p<0.001$) and an *injury x sex* interaction ($F(1,39)=6.805$, $p=0.013$) at high $\Delta\psi$ fluorescence (Fig. 6C), while at low $\Delta\psi$ (Fig. 6D), a *sex* ($F(1,40)=118.786$, $p<0.001$) main effect and an *injury x hemisphere* interaction ($F(1,40)=5.789$, $p=0.021$) could be observed.

4. Discussion

This study demonstrates a sex-related dimorphism of mitochondrial dysfunction in neonatal rats after a hypoxic–ischemic insult. Mitochondrial electron transport chain complexes are impaired 2 h and 18 h after the HI event, in the cerebral cortex and the hippocampus of both males and females, with females presenting an overall higher activity than that of males. On the other hand, females presented a loss of mitochondrial mass and $\Delta\psi$ 18 h after HI while males seem to be fairly, or even not, affected.

Cellular functions are either directly or indirectly dependent on cell energy supplies; the brain is particularly sensitive since it requires a continuous supply of oxygen and glucose to maintain its normal function and viability. When this supply is lowered to critical levels,

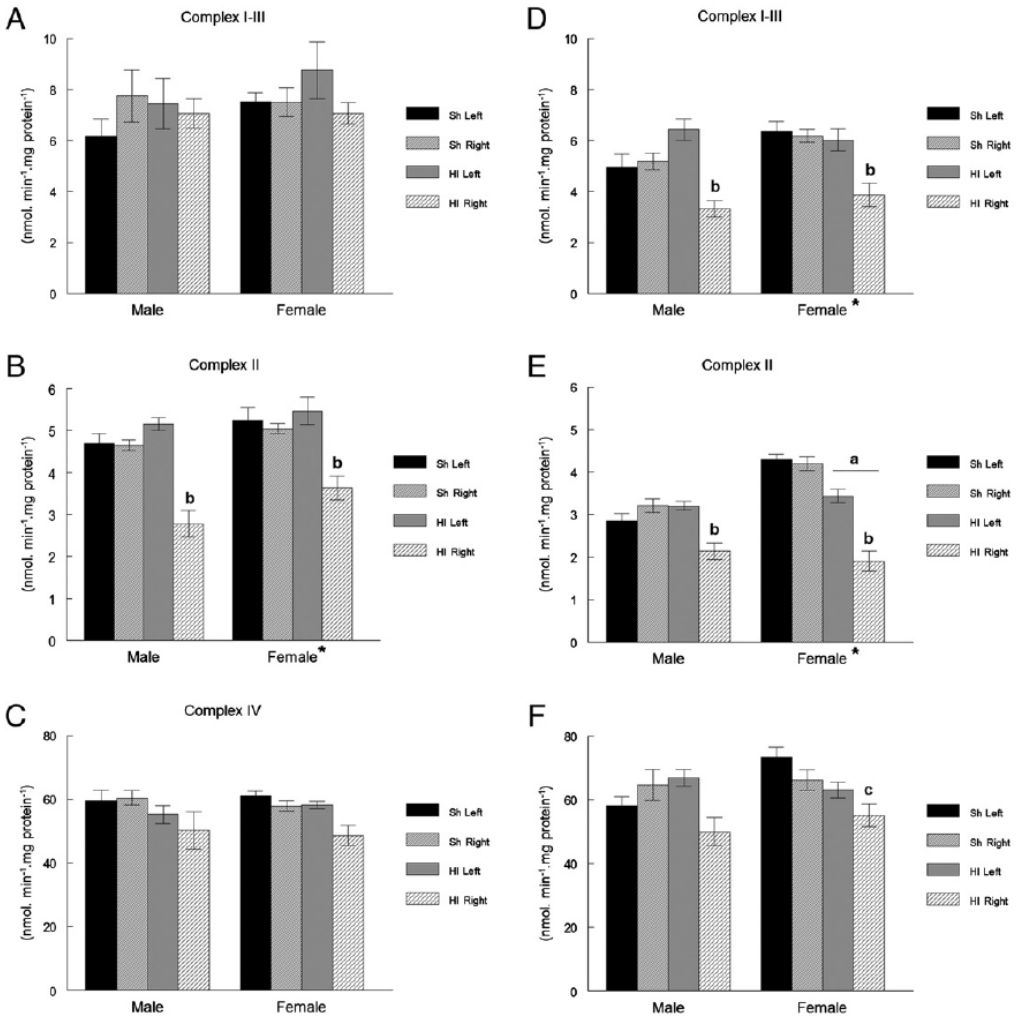

Fig. 2. Mitochondrial respiratory chain activity in the hippocampus. The activities of mitochondrial electron transport chain complexes were determined in the hippocampus of male and female neonates 2 h (A, B and C) and 18 h (D, E and F) after HI injury. Data are expressed as nmol/min/mg protein (mean ± SEM) of five to six animals in each group. Significant differences and interaction between sham/HI *versus* left/right hemisphere *versus* male/female were revealed by three-way ANOVA. *illustrates *sex* main effect, [a]*injury x sex* interaction, [b]*injury x hemisphere* interaction and [c]*injury x hemisphere x sex* interaction ($p<0.05$).

as occurs after severe hypoxia or ischemia, a cascade of events takes place triggered by depletion of the energy fuel ATP (Moro et al., 2005). When mitochondrial function was evaluated 2 h after HI, both males and females showed inhibition of hippocampal electron transport chain complexes II and IV, whereas in cortical tissue only a small variation in activities could be observed in both sexes. On the other hand, there was a pronounced inhibition of complex (I–III, II and IV) activities in all groups 18 h after HI. With the exception of cortical complex II activity, females subjected to HI presented an overall greater activity than males and also proved to be more vulnerable to the injury. Therefore, the electron transference through enzymatic complexes of the respiratory chain was greatly decreased and probably the transference of energy to form ATP was reduced. In fact, several studies demonstrated that mitochondrial respiration is susceptible to disruption or damage in brain ischemia (Allen et al., 1995; Hagberg, 2004; Larsen et al., 2008; Seppet et al., 2009; Sims, 1991).

The final step of cell respiration, in which oxygen is reduced to H_2O, is accompanied by a 1–4% one-electron reduction of oxygen to generate the anion superoxide (Boveris, 1977; Sivitz and Yorek, 2010). The inhibition of respiratory complex activity 2 h and 18 h after HI probably caused slowing down of the flow of electrons in the respiratory chain rendering a more reduced state of the upstream components, favoring the escape of electrons and the formation of reactive oxygen species (ROS) (Adam-Vizi, 2005). This notion is supported by a recent study demonstrating that neonatal HI produces oxidative damage to the cortex and hippocampus, by increasing malondialdehyde levels, reactive species formation, and superoxide dismutase activity (Weis et al., 2011).

In order to see whether the reduced activity of respiratory chain was due to a decrease of mitochondrial mass (quantity) and if this affected the membrane potential, mitochondrial mass and $\Delta\psi$ were evaluated by flow cytometry using mitochondrial dyes. When these parameters were analyzed 2 h after HI, a decrease in the number of cells with high mitochondrial mass and polarized mitochondrial membranes was observed in the cortex and hippocampus of both sexes. However, 18 h after HI, males and females presented a distinct pattern of mitochondrial function injury. In females the inhibition of respiratory chain activity was accompanied by a decrease of mitochondrial mass and depolarized mitochondrial membranes, while in males the mitochondrial mass remained intact despite the depolarization of mitochondrial membranes (diminished activity without diminution of quantity).

Electron transfer through the four protein complexes is accompanied by pumping of protons outward from the matrix into the

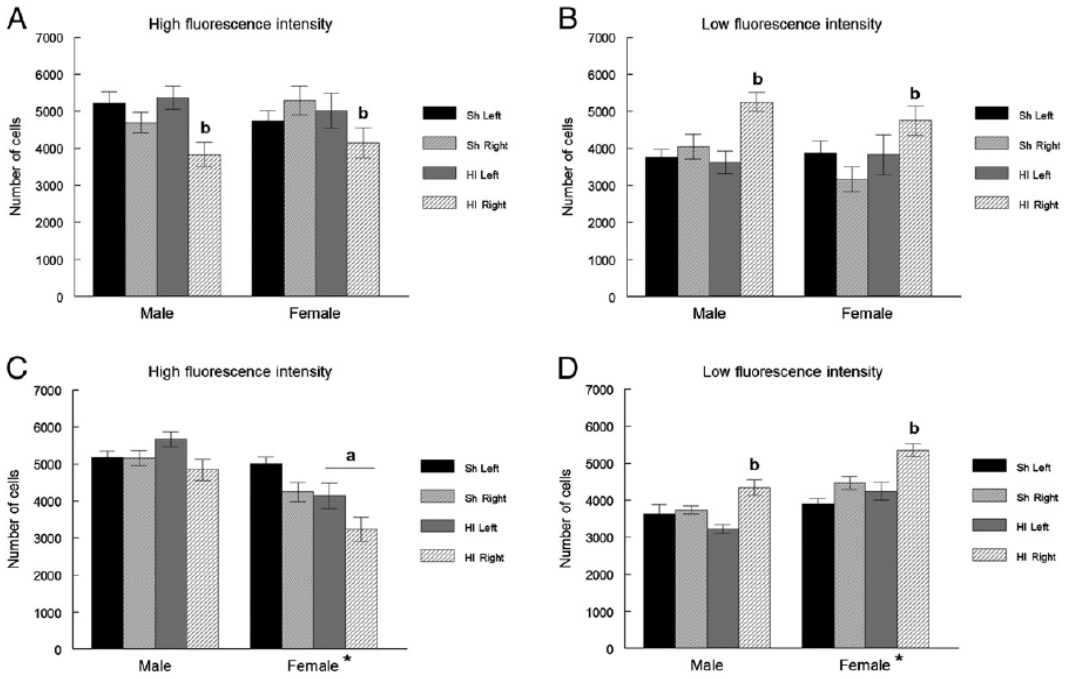

Fig. 3. Mitochondrial mass measurement in cerebral cortex. Flow cytometry analysis of MitoTracker Green fluorescence intensity from cerebral cortex cells of male and female neonates subjected to HI at 2 h (A and B) and 18 h (C and D) after injury. Data are expressed as number of cells (mean ± SEM) of five to six animals in each group and were plotted as a histogram of two populations with high and low mitochondrial mass. Significant differences and interaction between sham/HI *versus* left/right hemisphere *versus* male/female were revealed by three-way ANOVA. *illustrates *sex* main effect, [a]*injury x sex* interaction, and [b]*injury x hemisphere* interaction ($p<0.05$).

intermembrane space generating an electrochemical gradient, referred to as mitochondrial membrane potential ($\Delta\psi$). Membrane potential can be considered a key indicator of mitochondrial function, health, metabolic activity (Distelmaier et al., 2008; Solaini et al., 2007) and perturbances suggest indication of a pathological disorder of the system (Iijima, 2006). The present study reports that at 18 h

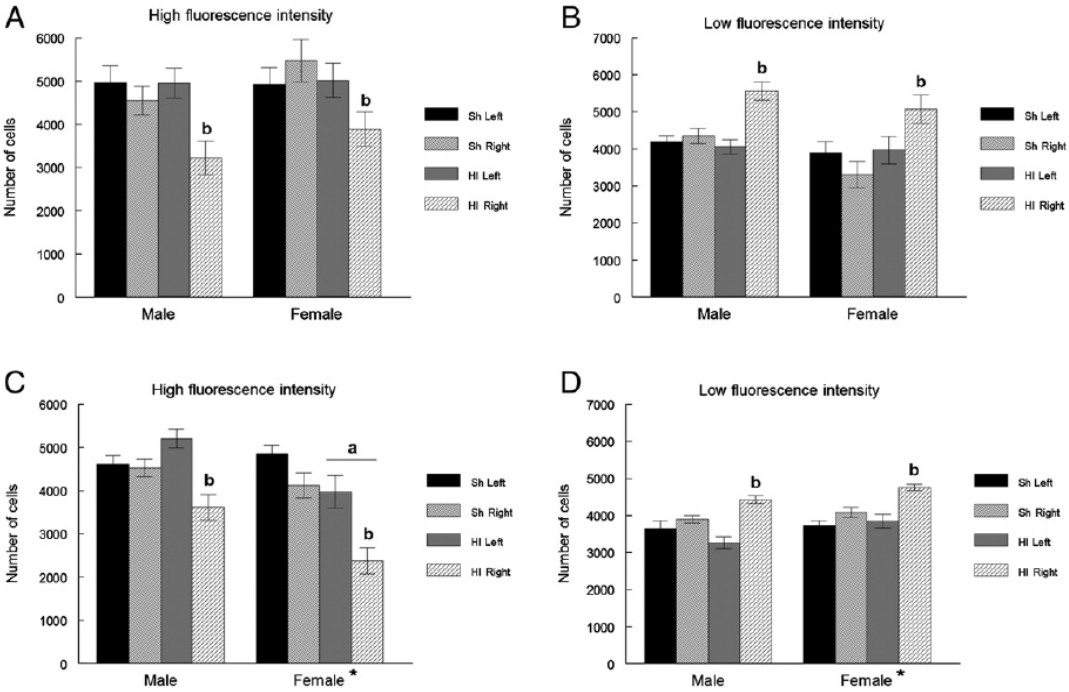

Fig. 4. Mitochondrial membrane potential measurement in cerebral cortex. Flow cytometry analysis of MitoTracker Red fluorescence intensity from cerebral cortex cells of male and female neonates subjected to HI at 2 h (A and B) and 18 h (C and D) after injury. Data are expressed as number of cells (mean ± SEM) of five to six animals in each group and were plotted as a histogram producing two populations with high and low mitochondrial $\Delta\psi$. Significant differences and interaction between sham/HI *versus* left/right hemisphere *versus* male/female were revealed by three-way ANOVA. *illustrates *sex* main effect, [a]*injury x sex* interaction, and [b]*injury x hemisphere* interaction ($p<0.05$).

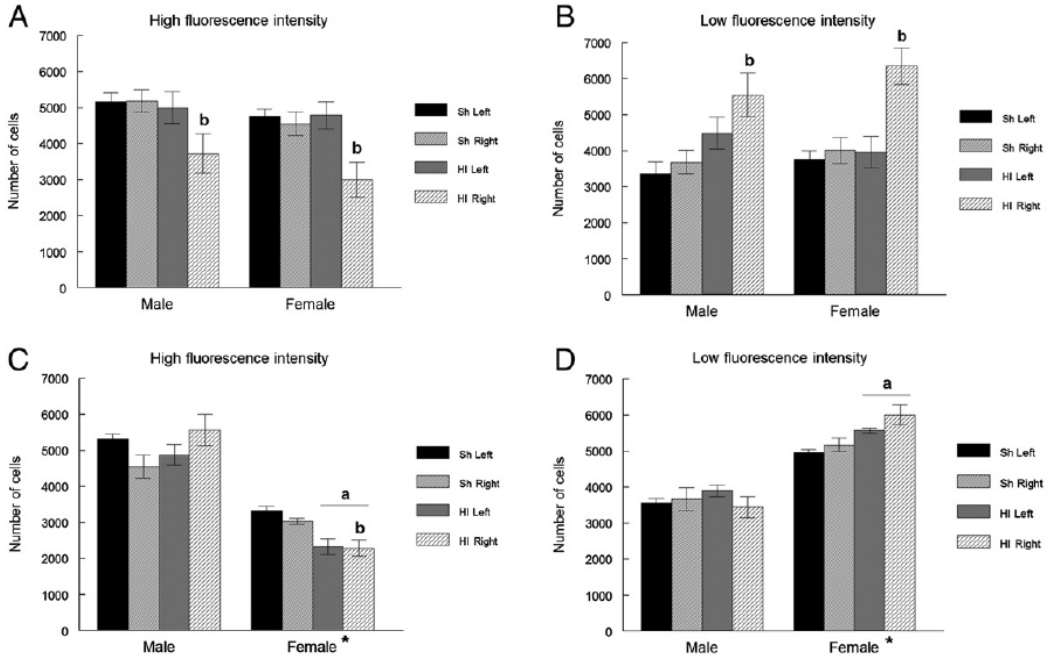

Fig. 5. Mitochondrial mass measurement in hippocampus. Flow cytometry analysis of MitoTracker Green fluorescence intensity from hippocampus cells of male and female neonates subjected to HI at 2 h (A and B) and 18 h (C and D) after injury. Data are expressed as number of cells (mean ± SEM) from five to six animals in each group and were plotted as a histogram of two populations with high and low mitochondrial mass. Significant differences and interaction between sham/HI *versus* left/right hemisphere *versus* male/female were revealed by three-way ANOVA. *illustrates *sex* main effect, [a]*injury x sex* interaction, and [b]*injury x hemisphere* interaction ($p<0.05$).

the mitochondrial depolarization is accompanied by disruption of mitochondrial mass only in females, while males presented with slightly fewer polarized mitochondria. This result differs from those observed 2 h after HI, where both sexes presented a disruption in mass and depolarization. This depolarization in mitochondrial membrane, both at 2 and 18 h, can led to substantial effects on cell energy homeostasis

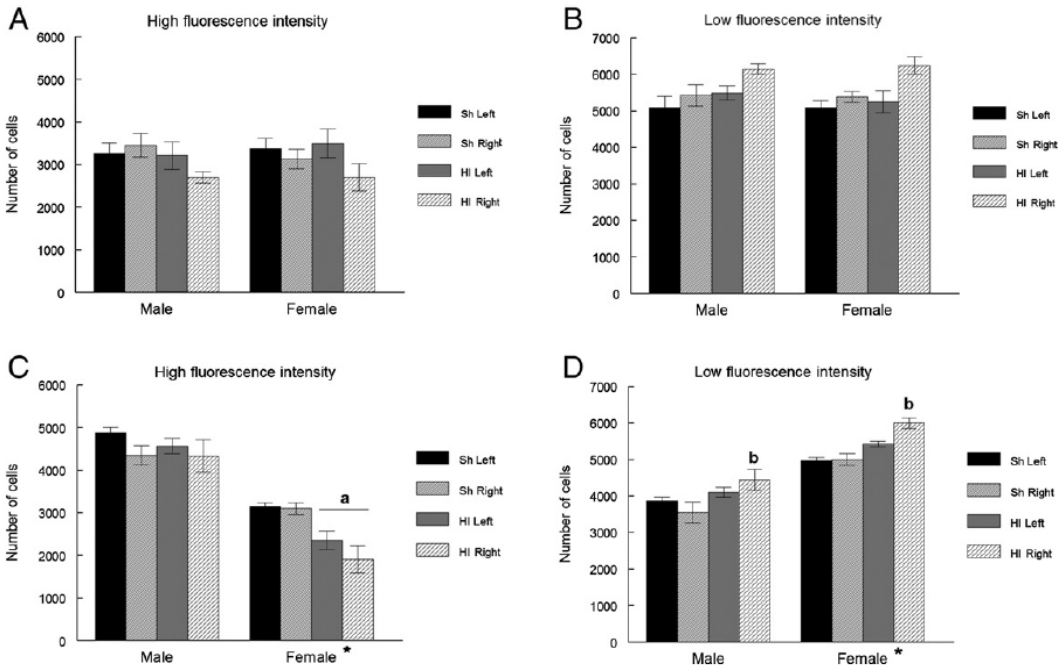

Fig. 6. Mitochondrial membrane potential measurement in hippocampus. Flow cytometry analysis of MitoTracker Red fluorescence intensity from hippocampus cells of male and female neonates subjected to HI at 2 h (A and B) and 18 h (C and D) after injury. Data are expressed as number of cells (mean ± SEM) of five to six animals in each group and were plotted as a histogram resulting in two populations with high and low mitochondrial $\Delta\psi$. Significant differences and interaction between sham/HI *versus* left/right hemisphere *versus* male/female were revealed by three-way ANOVA. *illustrates *sex* main effect, [a]*injury x sex* interaction, and [b]*injury x hemisphere* interaction ($p<0.05$).

since the phosphorylation state of the cytosolic ATP pool has been demonstrated to be very sensitive to minor changes in $\Delta\psi$ (Distelmaier et al., 2008, 2009).

Sexual dimorphism can be determined in mammalian brain for many characteristics such as brain architecture, neurochemical composition and susceptibility to several disorders (Baron-Cohen et al., 2005; Cahill, 2006). Previous experimental studies from our research group demonstrated lateralized and sex-dependent performance deficits in neonates submitted to HI, in which males had increased locomotion and females had impaired working memory performance (Arteni et al., 2010). This difference was also seen when animals were exposed to early-stage cage enrichment (Pereira et al., 2008) in which adolescent females exhibited performance recovery in object recognition and a partial improvement in a working memory spatial task. Previous works have shown that the female brain, even in the neonatal period, may have greater capacity than the male to combat oxidative stress since it has higher antioxidant capacity (Dukhande et al., 2009). It is also known that liver mitochondria from adult female rats have higher oxidative capacity accompanied by a greater differentiation degree than male rats' liver (Valle et al., 2007).

It is already known that there are sex-specific differences in cell death pathways after HI injury even during the pre-pubertal period (Tsuji et al., 2010). Males may be more vulnerable to oxidative stress leading to an important production of mitochondrial reactive oxygen species and a caspase-independent cell death. In contrast, in response to injury in the female, an estrogen microenvironment can upregulate antiapoptotic proteins (Subramanian and Shaha, 2007) preserving, in part, the stability of the mitochondrial membrane and reducing the release of proapoptotic proteins. Cell death in females seems to be mainly dependent on caspase pathways (Renolleau et al., 2008). Sex-specific differences are also seen in our current report. Female neonates showed a basal activity of enzymatic complexes higher than that of males. When exposed to HI injury, male and female brains had distinct patterns of mitochondrial dysfunction at 18 h after damage. In males, decreased activity of respiratory chain and $\Delta\psi$ was observed without mitochondrial mass being affected. This is tentatively explained by: 1) mitochondria could be losing function leading to opening of the mitochondrial permeability transition pore, resulting in mitochondria swelling, release of cytochrome *c*, cell damage and apoptosis (Sivitz and Yorek, 2010); therefore, the swollen mitochondria would have an increase in its contact surface, thus increasing the staining with MTG dye, which would explain the lack of change in mitochondrial mass; and 2) a delayed mitochondria effect could have occurred. Evidence from studies in neonatal HI animal models strongly demonstrates that there is an induction of AIF translocation from the mitochondria to the nucleus in males from 16 h until 24 h after injury (Nijboer et al., 2007; Renolleau et al., 2007, 2008). Females displayed an increase of cleaved caspase-3 at the same observed time points, without sex-specific differences on elevated cytochrome *c* levels (Nijboer et al., 2007; Renolleau et al., 2007, 2008). The AIF translocation and activation of nuclear poly (ADP-ribose) polymerase (PARP) in males could induce further damage to the mitochondria leading to secondary leakage of cytochrome *c* to the cytosol and subsequent caspase-3 activation (Wang et al., 2004; Yu et al., 2002), so delaying the loss of mitochondrial mass. On the other hand, females had depolarized mitochondrial membrane and inhibited activity of complexes with disruption of mitochondrial mass, suggesting a decreased intracellular number of mitochondria. This mitochondrial disappearance from cells could be due to mitophagy, a process of selective elimination of aged and dysfunctional mitochondria to protect cells from the harm of disordered mitochondrial metabolism and release of proapoptotic proteins (Kim et al., 2007). Perhaps the mitochondrial damage signals, e.g. ROS formed inside mitochondria due to enzymatic complex inhibition, loss of $\Delta\psi$ and initiation of apoptosis demonstrated here and in other studies (Renolleau et al., 2008; Weis et al., 2011) would increase the autophagic envelopment of mitochondria thus preventing proapoptotic protein release and further cell death. In fact, neurons induced to undergo apoptosis in the absence of caspase inhibitors present autophagic particles that appear before the onset of visible signs of apoptosis (Xue et al., 1999).

5. Conclusions

In conclusion, the present study shows for the first time that neonatal HI injury produces sex-related differences in measurements of mitochondrial function, namely inhibition of mitochondrial electron transport chain complexes activities and diminished mitochondrial mass and membrane potential in female neonate rats. Taken with other reports, our findings highlight mitochondria as good therapeutic targets for neuroprotection against HI injury in the developing brain. More studies are clearly needed to identify the mechanisms which cause reduction of mitochondrial number in cells of females and whether this selective mitochondrial depletion irreversibly commits cells to die or is acting as a cell repair mechanism.

Acknowledgments

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5. CAPÍTULO 3

Manuscrito: Sex and tissue-specific effects of autophagy cell death in neonates following hypoxia-ischemia – A ser submetido ao periódico *Neurobiology of Disease*

SEX AND TISSUE-SPECIFIC EFFECTS OF AUTOPHAGY CELL DEATH IN NEONATES FOLLOWING HYPOXIA-ISCHEMIA

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Abstract

Autophagy is the catabolic process responsible for the bulk degradation of cytoplasmic materials including organelles through the lysosomal machinery. Brain damage induced by neonatal hypoxia-ischemia (HI) causes cell death by either a caspase dependent or independent pathway. Ischemic insults also increase the formation of autophagosomes and activate autophagy. This study assessed the possible sex-specific differences of autophagy activity in neonates submitted to HI brain injury. Males exposed to HI had a small decrease in lysosome numbers and enhancement of caspases 3/7 activity in cortex. In contrast, females showed a slight decrease in autolysosomal number that was accompanied by a decreased number of lysosomes, an increased expression of LC3B-II levels and a substantial elevation of caspase activities. While cell death due to autophagy was not observed in cortex from males, females had induction of autophagy activity. However, downstream steps from autophagosome formation appeared to be inhibited, resulting in decreased autolysosomal degradation of LC3B-II. In hippocampus HI males had increased numbers of autolysosomes with no effect on lysosomes, Beclin-1 or LC3B levels. Females had an overall enhancement of autolysosomes in both sham and HI groups followed by large increases of caspase activities in the HI group. Decreased expression of Beclin-1 levels was also observed in sham females compared to males. Decreased Beclin-1 levels might be due to its inhibitory binding complex with Bcl-2 since BcL-2 is up regulated in female neonates following HI. The induction of autophagy activity in neonates could represent a survival role by supporting apoptosis. However, since evidence of apoptosis was also observed, cells may be irreversibly damaged by prolonged over activation of autophagy and therefore may progress to apoptosis or necrosis in both males and females. Taken together these data indicate that autophagy participates of cell death in neonates submitted to HI following region and sex-specific patterns.

Keywords: Hypoxia, ischemia, autophagy, lysosomes, apoptosis, cell death, sex effect.

Introduction

Perinatal asphyxia is still a common cause of severe damage to fetal and neonatal brain that in most cases is linked to death or subsequent neurological complications such as cerebral palsy, seizure disorders and mental retardation (Volpe, 2001; Ferriero, 2004; Blomgren et al., 2007). It has recently been suggested that the encephalopathy that results from hypoxia-ischemia (HI) in neonates may occur as a result of somewhat different cell death pathways in males and females. Accordingly, recent studies suggest that brain injury in male neonates is more caspase independent, whereas females have more caspase dependent brain injury (Zhu et al., 2006; Renolleau et al., 2007; 2008). Additionally, a sexual dimorphic pattern was observed in mitochondrial function, in which respiratory chain activities were found inhibited in both sexes with increased mitochondrial depolarization but that was accompanied by loss of mitochondrial mass only in neonatal brain of females (Weis et al., 2012).

The autophagy cell biology pathway, also known as a non-apoptotic form of programmed cell death (PCD or type II PCD) (Puissant et al., 2010), has gained attention since it seems to play an important role in cell death after neonatal HI insult. Macroautophagy (hereafter referred to as autophagy), the most prevalent form of autophagy, is an evolutionary mechanism highly conserved in eukaryotic cells involving the sequestration of cytoplasmic macromolecules and organelles by a double or multimembranous structure, known as autophagosome which then delivers the enclosed material to a lysosome for degradation (Shintani et al., 2004; Carloni et al., 2008; Uchiyama et al., 2008; Goldman et al., 2010; Mizushima et al., 2010). Its execution involves a set of autophagy-related genes (Atg) that encode proteins needed for process induction, generation, maturation and recycling of autophagosomes. Autophagy can be up regulated under starvation, differentiation and normal growth control to maintain cellular turnover and the intracellular pool of amino acids which are required for protein synthesis and glyconeogenesis (Uchiyama, 2001) ensuring cell homeostasis and survival (Kuma et al., 2004; Shintani and Klionsky, 2004; Komatsu et al., 2005). However, there is a growing evidence this self-eating process can act as both pro-survival and as a pro-death mechanism. In fact, autophagy has been implicated in

several diseases, including neurodegenerative disorders, such as Huntington's, Parkinson's and Alzheimer's disease (Koike et al., 2005; Levine and Yuan, 2005; Chu, 2006; Nixon, 2006; Zhu et al., 2007) and also cancer (Liang et al., 1999; Furuya et al., 2004).

Despite the growing interest in autophagy during HI brain damage, it is not clear whether autophagic activity may represent a protective mechanism or could promote ischemic damage. Ischemic insults have been shown to increase autophagosomes and to induce autophagic activity in neurons of neonatal and adult mouse cortex, hippocampus and striatum after HI injury (Zhu et al., 2005; 2006; Adhami et al., 2006; 2007; Koike et al., 2008; Uchiyama et al., 2008; Ginet et al., 2009) and focal cerebral ischemia (Rami et al., 2008; Wen et al., 2008). Such increase on autophagy is largely prevented by autophagy inhibitors (3-methyladenine and bafliomycin) (Wen et al., 2008) or by brain-specific deletion of Atg7 which blocked autophagy (Uchiyama et al., 2008). However, no study has addressed the possible differences between males and females in autophagy following ischemic neuronal injury.

The aim of present study was to determine whether induction of autophagy activity showed a sexual dimorphic pattern in neonates submitted to HI brain injury. Since autophagy is a mechanism that can either precede apoptosis exhibiting a protective role in the early stages of programmed cell death or in some circumstances can also promote apoptosis, therefore, we predict that, as males and females have been shown to have different apoptotic pathways after HI injury, autophagic activity might also be sexually dimorphic.

Materials and Methods

Animals

All experiments were carried out in accordance with National Institutes of Health guidelines and were reviewed and approved by the IACUC (Institutional Animal Care and Use) committee of the University of California at Davis. Fifteen pregnant Sprague–Dawley rats (Charles River Labs, Hollister, CA, USA) were used in this study. They were allowed to give birth and the litters were culled within 48h of birth to a maximum of eight pups per litter (totaling 120 rat pups). All rats had food and water

available *ad libitum* on a 12 hour light/dark cycle, and were acclimated to the animal room for at least one week before the experiment.

Hypoxia–Ischemia

Neonatal hypoxic-ischemic brain injury was conducted as described by Levine (1960) and modified by Rice and colleagues (1981). It produced damage in the brain hemisphere ipsilateral to the common carotid artery occlusion and affected the cerebral cortex, subcortical and periventricular white matter, striatum and hippocampus. Cell death is not usually observed in the contralateral hemisphere and is never present in pups rendered hypoxic without previous carotid artery ligation (Towfighi et al., 1995; Vannucci and Vannucci, 1997). Rat pups were maintained undisturbed with their dams until surgery at postnatal day 7. Then, half of the rats in each litter were subjected to HI, and the remaining animals had sham surgery. Neonates were anesthetized with 3% isoflurane and maintained with 2% isoflurane in 100% oxygen. The right common carotid artery was exposed, isolated from the nerve and vein and permanently occluded with surgical silk. Animals were then allowed to recover in a heated chamber and returned to their dams. After 120 minutes, pups were exposed to hypoxia (8% oxygen and 92% nitrogen) for 90 min in a temperature controlled chamber (37°C) (BioSpherix, NY, USA) and then returned to room air in their home cages until sacrifice (18 hours after HI procedure). Sham operated controls were submitted to anesthesia and the neck incision, but received neither artery occlusion nor hypoxia. Mortality rate was less than 10% of all operated animals. Eighteen hours after HI procedure, pups were killed by decapitation, their brains were quickly removed and cerebral cortex and hippocampus from the ipsilateral hemisphere were rapidly isolated on ice and kept refrigerated or frozen at -70°C until processed.

Live-Cell Staining

The Cyto-ID Autophagy Detection Kit (Enzo Life Sciences) was used for the autophagy flux analysis. The kit employs a 488 nm-excitable green-emitting fluorescent probe to highlight the various vacuolar components (selective marker of autolysosomes and earlier autophagic compartments) of the autophagy pathway. Briefly, tissue samples

were mechanically dissociated with PBS containing collagenase and subsequently filtered through 100 µm nylon cell strainer (Cell Filter Strainer – BD Biosciences). Samples were centrifuged at 400 x *g* for 5 minutes and each live cell sample pellet was re-suspended in 0.5 mL of freshly diluted Cyto-ID® Green Detection reagent obtaining a density of about 1 x 10^5 to 1 x 10^6 cells per mL. Cells were incubated for 30 minutes at 37°C in the dark.

To label the acidic organelles in live cells, LysoTracker Red (LTR) DND-99 (Invitrogen-Molecular Probes) was employed. LTR is a fluorophore in the form of a conjugated multi-pyrrole ring structure containing a weakly basic amine that selectively accumulates in acidic compartments and exhibits red fluorescence. It is commonly used as a lysosomal marker. Dissociated cells, previously filtered and centrifuged, were re-suspended and stained in a 60nM LTR solution. Samples were incubated for 30 minutes at 37°C in the dark.

Analysis was carried out using LSR II flow cytometer (BD Biosciences, San Jose, CA). Cyto-ID® Green reagent was measured in the FITC channel (515-45 nm band pass filters with excitation at 488 nm) and LTR in the PE channel (562–588 nm band pass filters with excitation at 488 nm).

Flow Cytometry Analysis

The experimental design, acquisition and data analysis on LSR II flow cytometer were performed using BD FACSDiva™ (BD Biosciences) and FlowJo analysis software (TreeStar; Ashland, OR). Negative controls (samples without stain) were included for normalization. Controls stained with a single dye were used to set compensation of FITC and PE channels. Fluorescence emissions were collected using logarithmic amplification. Briefly, data from 10.000 events were acquired and mean relative fluorescence intensity was determined after exclusion of debris events from the data set. Data from samples stained with Cyto-ID® Green reagent and LTR dyes were plotted as a histogram of the number of events (live cells) by the relative fluorescence for each sample and dye. The histogram was divided in two halves, named cells with low and high fluorescence, based on the controls peak for both dyes, and this evaluation was applied to all data. By this method, analyses of cells resulted in two populations, with different number of autolysosomes and lysosomes. The first population represents

cells with low number of autolysosomes and lysosomes and the second, autolysosomes and lysosomes numbers.

Western Blot Analysis

Samples were homogenized in ice-cold lysis buffer (Cell Signaling Technology) containing 1mM phenylmethanesulfonyl fluoride (Sigma-Aldrich). Lysates were centrifuged at 10 000 x *g* for 10 minutes at 4°C and the supernatant containing total proteins was used to detect the expression of LC3B-I, LC3B-II and Beclin-1. Protein concentrations were determined with a BCA detection kit (Pierce, 23225) using bovine serum albumin standards and adjusted to equal concentrations across different samples. Aliquots of 40µg protein per lane were separated onto 12% Tris-Glycine gels (Life Technologies) and transferred to nitrocellulose membranes (Invitrogen). After blocking in 5% non fat dry milk (Santa Cruz Biotechnology), membranes were incubated overnight at 4°C with the following primary antibodies: rabbit anti-LC3B (1:700, Cell Signaling Technology, USA) and rabbit anti-Beclin-1 (1:1000, Cell Signaling Technology, USA). Membranes were washed with TBS-T and incubated with peroxidase-conjugated goat anti-rabbit IgG (1:2000, Cell Signaling Technology, USA) for 2h at room temperature. The blots were visualized by enhanced chemiluminescence (SuperSignal® West Pico Complete Rabbit IgG Detection, ThermoFischer Scientific). Protein levels were normalized to β-actin (1:4000, Cell Signaling Technology, USA) as a loading control. The density of bands was determined with ImageJ software (ImageJ 1.45s, National Institutes of Health, USA).

Caspase 3/7 Activity

Activities of caspases 3/7 were measured using a luminescent assay (Caspase-Glo 3/7 Assay, Promega, Madison, WI, USA). For this assay the caspase-3/7 cleaves luminogenic substrate containing the tetrapeptide sequence DEVD (asp-glu-val-asp). This reaction liberates free aminoluciferin, which can be used as a substrate by luciferase to generate light. The luminescent signal is proportional to the amount of caspase activity present in the cells (Riss and Moravec, 2004). Cortex and hippocampus were homogenized in PBS, centrifuged at 4000 x *g* and a sample was incubated for 1h

at 37°C, followed by adding of 0.1mL of Caspase-Glo 3/7 reagent to each sample. After a 30 min incubation at room temperature, the luminescence intensity was measured using a luminometer Plate Reader.

Statistical Analysis

All data are presented as mean ± S.E.M. Two-way analysis of variance (ANOVA) was used considering injury (sham x HI) and sex (male x female) as the factors followed by a post hoc two-tailed independent Student's *t*-test ($p<0.05$). Statistical analysis was conducted on the Statistical Package for the Social Sciences software (SPSS).

Results

Live-Cell Staining

No statistical differences were found in cortex cells stained with the Cyto-ID® Green reagent (Figure 1A). In contrast, HI produced an overall increase in the autophagic activity in hippocampus as manifested by the greater number of cells with high fluorescence ($F(1,20)=6.335$, $p=0.020$ – *injury* effect) and a decrease at low fluorescence ($F(1,20)=8.562$, $p=0.008$) (Figure 1B). Also, females presented an overall increased number of cells with high fluorescence ($F(1,20)=12.667$, $p=0.002$ – *sex* effect) with a consequent decrease at low fluorescence ($F(1,20)=10.183$, $p=0.005$) (Figure 1B). Student's *t*-test showed significant differences between sham male x sham female and between sham male x HI male in hippocampus (Figure 1B).

Concerning cells stained for acidic organelles with LTR, a decreased number of cells with high fluorescence were observed in cortex ($F(1,12)=5.411$, $p=0.038$) with no effect on low fluorescence (Figure 2A). Although the ANOVA showed no effect on hippocampus cells (Figure 2B), a Student's *t*-test revealed a significant difference between sham male x sham female at both high and low fluorescence, with males presenting less acidic organelles than females.

Western Blot Analysis

ANOVA revealed an increase in LC3B-II expression in cerebral cortex (F(1,12)=7,467, p=0,018 – *injury* x *sex* interaction) in HI females (Figure 3C), but no differences between males and females in Beclin-1 (Figure 3A) or LC3B-I (Figure 3C) expression. No changes were observed in hippocampus in either sex (Figures 3B and 3D). However, by analyzing the groups separately, a Student's t-test showed some sex specific differences. Sham females had decreased LC3B-II expression compared to sham males in cortex (Figure 3C) and decreased Beclin-1 expression in hippocampus (Figure 3B).

Caspase 3/7 Activity

Two-way ANOVA revealed an *injury* effect in cortex (F(1,12)=10.079, p=0.009), a *sex* effect (F(1,12)=6.661, p=0.026) and an *injury* x *sex* interaction (F(1,12)=8.186, p=0.015) with HI females having higher caspase activities than males (Figure 4).

The same pattern of changes was seen on hippocampus in which an *injury* effect (F(1,12)=12.981, p=0.004), a *sex* effect (F(1,12)=12.584, p=0.004) and an *injury* x *sex* interaction (F(1,12)=13.288, p=0.003) was observed. A Student's *t*-test also showed significant differences between sham female x HI female and HI male x HI female in both cortex and hippocampus with HI females showing increased caspase activities even when compared to injured males (Figure 4).

Discussion

It was previously shown that HI causes early oxidative damage to the neonatal brain (Weis et al., 2011) and this damage was partly due in part to mitochondrial dysfunction that differed in male and female brains (Weis et al., 2012). In the present study, it is shown that the autophagy pathway may be activated following neonatal HI and that it is sexually dimorphic.

Autophagy is the major mechanism by which cells degrade long-lived proteins and the only known pathway for degrading organelles (Klionsky and Emr,

2000; Levine and Klionsky, 2004). This degradation generates nucleotides, amino acids and free fatty acids that could be recycled for macromolecular synthesis and ATP generation. Autophagy can not only be cytoprotective but, under some conditions can also lead to cell death (Meijer and Codogno, 2009). Autophagy activity was measure using an assay that utilizes a reagent (Cyto-ID Green) that specifically labels autolysosomes (Mizushima et al., 2010; Warenius et al., 2011). No significant changes were observed in cortex in any groups except for a slight decrease in fluorescence intensity in HI female neonates. Though there was no effect on Cyto fluorescence, analysis of cells labeled with LTR showed a minor decrease in lysosomes at both HI males and females. In contrast, a large increase in Cyto fluorescence occurred in hippocampus of HI males compared to the sham controls. Surprisingly, females had an overall increase of autolysosomes numbers in both sham and HI groups. No changes were observed in LTR labeled hippocampal cells.

The autolysosome is a hybrid organelle. It is generated by an autophagosome or an endosome that contains cytoplasmatic materials which then fuses to a lysosome where both autophagosome membrane and its content are degraded by hydrolases (Mizushima et al., 2010). Though not statistically significant, the modest decrease in autolysosomal number in HI female cortex could be due to a defect in the fusion process between autophagosome and lysosome or to a loss of its degradative functions, since lysosomal numbers also decreased. On the other hand, an activation of autophagy activity on hippocampus was observed in injured males and females manifested by increased autolysosome numbers but without changes in lysosomal numbers. Lysosomes are acidic vacuoles containing hydrolytic enzymes that degrade unneeded intra and extracellular materials (de Duve, 1963; Kornfeld and Mellman, 1989; Winchester, 1992). Although a lysosomal rupture has been found to be an early event in apoptosis after exposure to a variety of damaging agents such as oxidative stress and oxidized lipids (Brunk et al., 1997; Yuan et al., 2000; Kagedal et al., 2001; Stoka et al., 2001; Yuan et al., 2002), minor leakage as seen in cortex from both sexes might be compatible with cell survival that is mediated by autophagy (Brunk et al., 2001).

Western blot analysis from cerebral cortex revealed no changes on Beclin-1 expression in any groups and a small increase in LC3B-II levels only in HI females. In contrast, sham females had lower basal levels of hippocampal Beclin-1 compared to males, with no effect on LC3B-II levels. The mammalian autophagy protein LC3

(microtubule-associated protein light chain 3) is considered to be an autophagosomal marker. The cytoplasmic LC3 is posttranslationally modified to LC3-I and subsequently conjugated with phosphatidylethanolamine (PE) to become LC3-II (LC3-PE). The LC3-I form remains in cytoplasm whereas LC3-II is found in the inner and outer membranes of autophagosomes (Kelekar, 2005; Meijer and Codogno, 2009; Mizushima et al., 2010). The inner membrane LC3-II is degraded by lysosomal enzymes after the fusion of autophagosomes with lysosomes, and the outer membrane LC3-II is de-conjugated and returns to the cytosol (Meijer and Codogno, 2009). Of the several isoforms, LC3B is the most widely used (Mizushima et al., 2010) and its elevated levels are correlated to elevated levels of autophagic vesicles in response to autophagy-inducing stress (Klionsky et al., 2008). The elevated levels of LC3B-II seen in HI female cortex imply increased numbers of autophagosomes and induction of the autophagy pathway. However, as the number of autolysosomes seems to be slightly decreased, an accumulation of autophagosomes could have taken place possibly by suppression of steps in the autophagy pathway downstream of autophagosome formation (Mizushima et al., 2010), resulting in decreased autolysosomal degradation of LC3B-II. In contrast, HI had no effect on either Beclin-1 or LC3B levels in hippocampus while decreased basal levels of Beclin-1 were observed in sham females when compared to sham males. It is known that LC3-II increases transiently upon induction of autophagy but can be rapidly cleaved and degraded by lysosomal proteases (Hu et al., 2011). This makes it difficult to detect increased levels and thus the increased autolysosomal number found might be explained by the lack of effect on LC3B-II levels.

Beclin-1, a BH3 (Bcl-2 homology domain) protein, is a key regulator of autophagy that directly interacts with the anti-apoptotic proteins Bcl-2/BcL-xL (Sinha and Levine, 2008; Chipuk et al., 2010; Djavaheri-Mergny et al., 2010; Funderburk et al., 2010). The inhibitory binding complex of Bcl-2 with Beclin-1 prevents activation of autophagy by Beclin-1 (Meijer and Codogno, 2009). Decreased basal levels of Beclin-1 in females compared to males might be related to the up-regulation of Bcl-2 through extracellular signal-related kinase (ERK) phosphorylation (Subramanian and Shaha, 2007) possibly related to the female estrogen microenvironment. Therefore, the increased levels of Bcl-2 would bind more Beclin-1 and serve as a constitutive mechanism to prevent excessive autophagy activation in females.

Conversely, lower levels of Beclin-1 also may have resulted because of partial caspase-3 cleavage producing a truncated protein that reduces its ability to promote autophagy (Luo and Rubinsztein, 2010) even though higher numbers of autophagosomes observed. Activity of caspase 3/7 confirmed that female brains following neonatal HI show higher cleavage and activation of caspase-3 in both cortex and hippocampus, representing a caspase-dependent cell death pathway. Indeed, neurons from immature male brains after HI has previously been shown to have pronounced translocation of apoptosis-inducing factor (AIF) from mitochondria to nucleus to activate poly(ADP-ribose) polymerase-1 (PARP-1), whereas females had greater cleavage and activation of caspase-3 activity (Zhu et al., 2006; Renolleau et al., 2007; 2008).

It is becoming more clear that autophagy and apoptosis are linked processes in which autophagy could control apoptosis by making it more or less probable and apoptosis might also modulate autophagy (Gump and Thorburn, 2011). However, under some circumstances it can promote death and can be a mechanism of cell death by itself (Debnath, et al., 2005; Yu et al., 2006; Kroemer and Levine, 2008). Autophagy could degrade cytoplasmic components so that the cell eventually activates the apoptosis machinery or can even non-selectively degrade cellular components to the point that the cell can no longer survive (Gump and Thorburn, 2011). Based on the present results we believe that brain starvation and stress caused by neonatal HI can activate autophagy and apoptosis pathways, prompting cells to die by both processes.

Several recent studies suggest autophagy is involved in neuronal cell death induced by HI. Most have found that it is highly induced in brain of neonates subjected to HI and that this mechanism may contribute to ischemic neuronal injury (Zhu et al., 2005; Adhami et al., 2006; Uchiyama et al., 2008; Ginet et al., 2009; Puyal et al., 2009) whereas others showed that autophagy induction can have a protective role and prevent neuronal cell death (Carloni et al., 2008). However, no previous study has considered the differences between males and females. The sex-specific changes of autophagy found in the present study may explain some of the discrepancies between previous studies.

In conclusion, the present results indicate that HI in female cerebral cortex increased autophagosomes number by reducing its turnover, probably by a defect in fusion of autophagosome with lysosome. This blockade of autophagy seems to commit

cells to die either by apoptosis or necrosis, a fact verified by the activated caspase 3/7 activity. On the other hand, in hippocampus, a great induction of autophagy was seen in both HI males and all females. The activation of autophagy could represent a survival role by supporting apoptosis, however, as apoptosis features was also found (i.e. increased caspases activities) we believe that cells may be irreversibly damage by prolonged overactivation of autophagy and therefore may progress towards both apoptosis and necrosis in males (Balduini et al., 2009). In females, autophagy seems display an important role in rescuing mitochondria. In a recent study, our group demonstrated that 18h after HI a substantial decrease in mitochondrial mass was followed by decreased membrane potential and respiratory chain activity whereas males did not present any changes in mitochondrial mass (Weis et al., 2012). Therefore, we suggest that increased basal levels of autophagy that remained on females submitted to HI contributed to the removal of defective mitochondria with the intention of intensify mitochondrial turnover. Actually, it is well recognized that during starvation, mitochondrial membrane depolarization has been shown to precede autophagy induction (Elmore et al., 2001) that in turn accelerate mitochondrial turnover by the process called mitophagy (Lemasters, 2005). Specific investigations into the sexual dimorphic pattern and tissue-specific signaling pathway of neuron cell death after neonatal HI has to be done to find out the role of mitophagy in females brain damage.

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Conflicts of Interest

The authors have indicated that they have no conflicts of interest regarding the content of this article.

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Legends to figures:

1. Autophagy activity in cells of cerebral cortex and hippocampus. Cyto-ID green reagent incorporation in autolysosomes and earlier autophagic compartments was detected by flow cytometry in cerebral cortex (A) and hippocampus (B) cells from male and female neonates at 18h after HI injury. Data are expressed as the mean of percentage of cells (mean±SEM) from six animals in each group and were plotted as a histogram of two populations with high and low autophagy activity. Significant differences revealed by two-way ANOVA between sham/HI versus male/female are illustrated as: **sex* main effect and $^{\#}$*injury* main effect ($p \leq 0.05$).

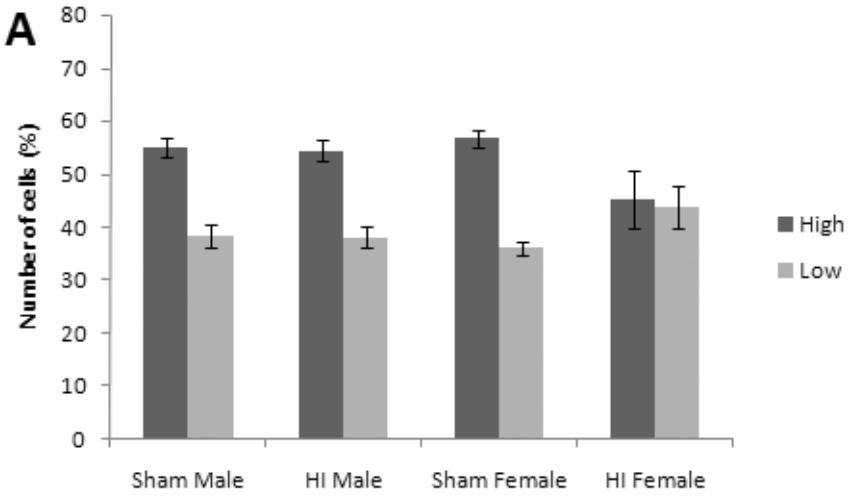
2. Acidic organelles fluorescence intensity in cells of cerebral cortex and hippocampus. Lysotracker Red incorporation in acidic organelles was detected by flow cytometry in cerebral cortex (A) and hippocampus (B) cells from male and female neonates at 18h after HI injury. Data are expressed as the mean of percentage of cells (mean±SEM) from four animals in each group and were plotted as a histogram of two populations with high and low acidic organelles. Significant differences revealed by two-way ANOVA are illustrated as: $^{\#}$*injury* main effect ($p \leq 0.05$).

3. Western blot analysis showing Beclin-1 (60 kDa) expression in cerebral cortex (A) and hippocampus (B), LC3B-I (16 kDa) and LC3B-II (14 kDa) expression in cerebral cortex (C) and hippocampus (D) 18h after HI injury. Equal amounts of proteins (40µg per lane) were loaded and β-actin was used as an internal control to normalize the amount of protein applied in each lane. Quantitative analysis of protein levels were performed with ImageJ software (ImageJ 1.45s,

National Institutes of Health, USA). Data are expressed as mean±SEM from four animals in each group. Significant differences and interactions revealed by two-way ANOVA are illustrated as: $^{\&}$*injury* x *sex* interactions ($p \leq 0.05$).

4. Caspase 3/7 activities in cerebral cortex and hippocampus from male and female neonates at 18h after HI injury. Data are expressed as the number of relative light units (RLU) per mg of protein (mean±SEM) from three to four animals in each group. Significant differences and interactions revealed by two-way ANOVA are illustrated as: **sex* main effect, $^{\#}$*injury* main effect and $^{\&}$*injury* x *sex* interactions ($p \leq 0.05$).

Figure 1


A
Number of cells (%)
80
70
60
50
40
30
20
10
0
Sham Male
HI Male
Sham Female
HI Female
High
Low

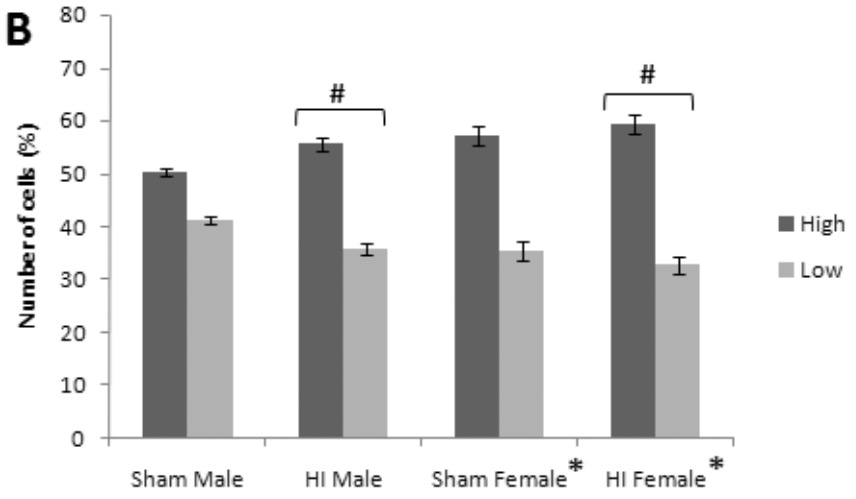
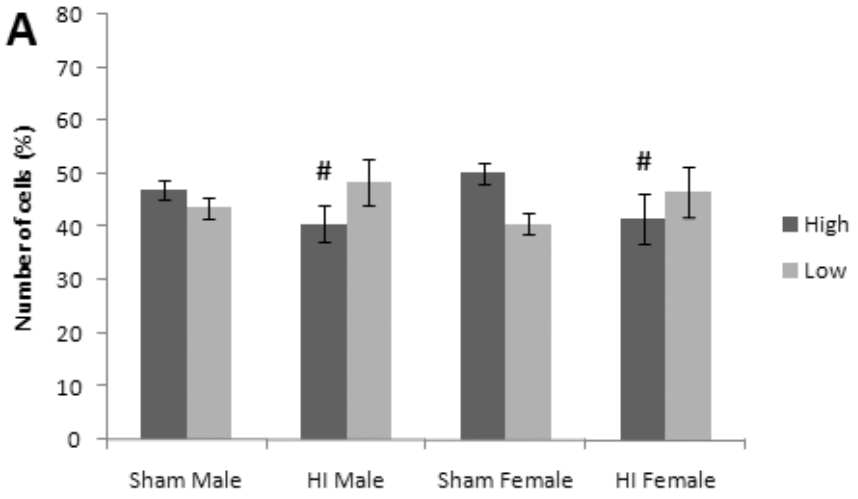

B
Number of cells (%)
80
70
60
50
40
30
20
10
0
#
#
Sham Male
HI Male
Sham Female*
HI Female*
High
Low

Figure 2


A
Number of cells (%)
80
70
60
50
40
30
20
10
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#
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Sham Male
HI Male
Sham Female
HI Female
High
Low

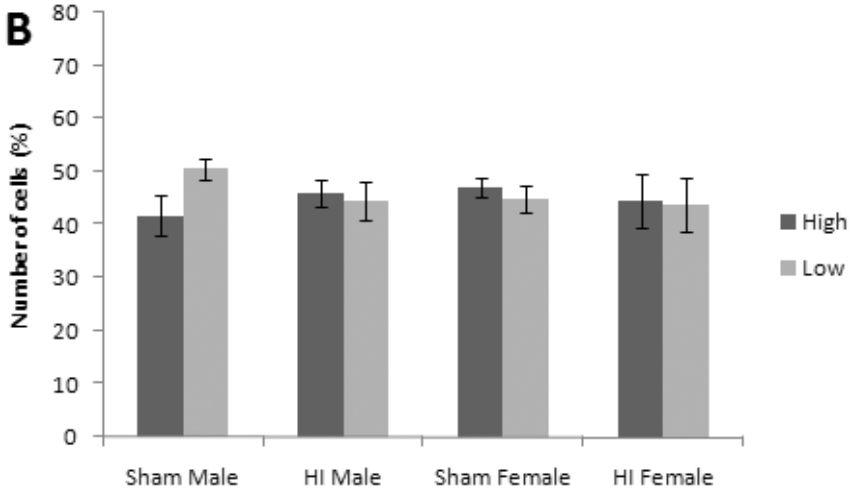
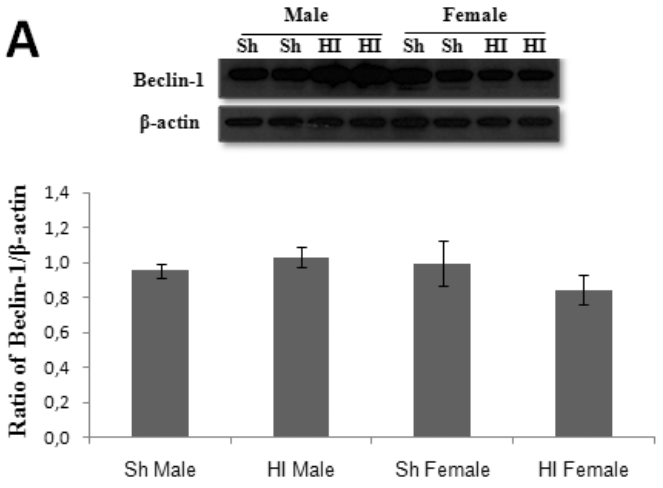
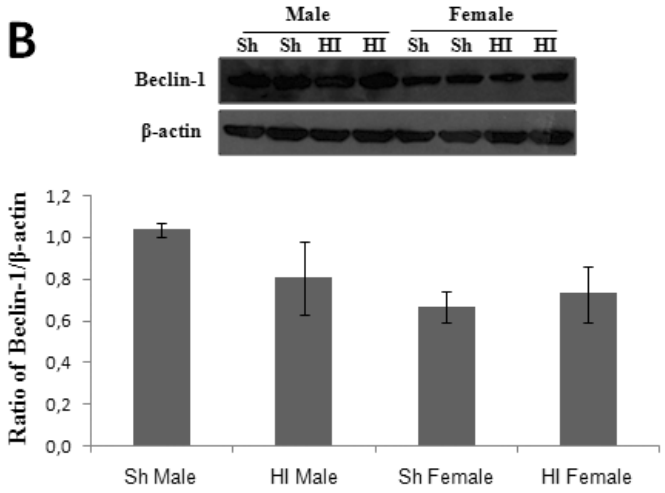
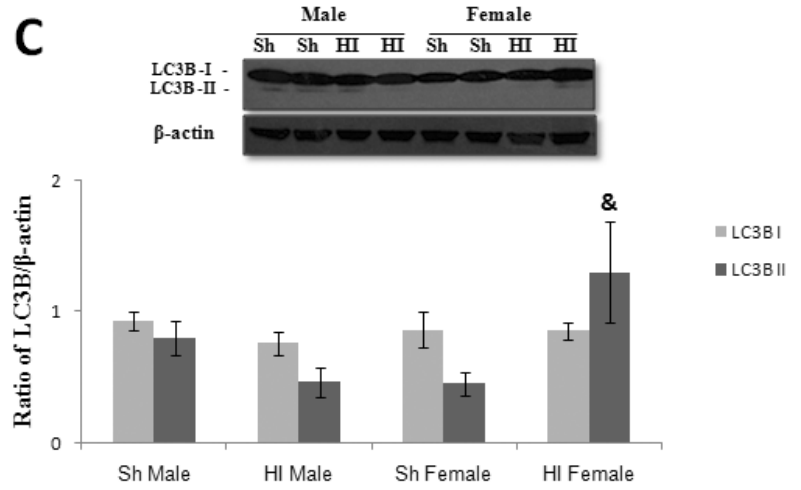

B
Number of cells (%)
80
70
60
50
40
30
20
10
0
Sham Male
HI Male
Sham Female
HI Female
High
Low

Figure 3


A
Male
Female
Sh Sh HI HI Sh Sh HI HI
Beclin-1
β-actin
Ratio of Beclin-1/β-actin
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
Sh Male
HI Male
Sh Female
HI Female


B
Male
Female
Sh Sh HI HI Sh Sh HI HI
Beclin-1
β-actin
Ratio of Beclin-1/β-actin
1,2
1,0
0,8
0,6
0,4
0,2
0,0
Sh Male
HI Male
Sh Female
HI Female


C
Male
Female
Sh Sh HI HI Sh Sh HI HI
LC3B-I -
LC3B-II -
β-actin
Ratio of LC3B/β-actin
2
1
0
&
LC3B I
LC3B II
Sh Male
HI Male
Sh Female
HI Female

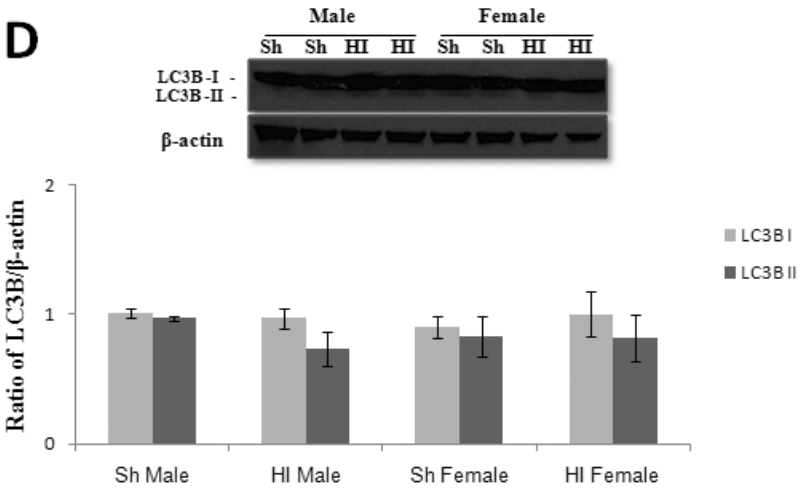
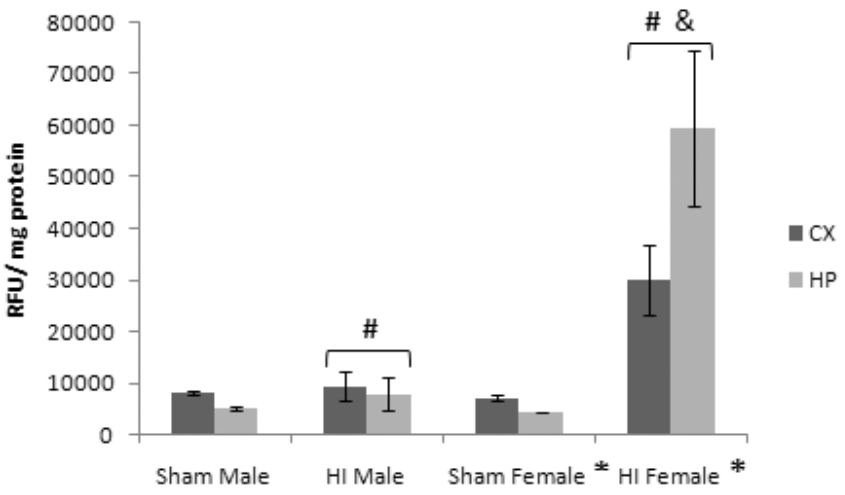

D
Male
Female
Sh Sh HI HI Sh Sh HI HI
LC3B-I -
LC3B-II -
β-actin
Ratio of LC3B/β-actin
2
1
0
LC3B I
LC3B II
Sh Male
HI Male
Sh Female
HI Female

Figure 4


80000
70000
60000
50000
40000
30000
20000
10000
0
RFU/ mg protein
&
#
CX
HP
Sham Male
HI Male
Sham Female
HI Female
*
*

6. DISCUSSÃO

A presente tese teve por objetivo investigar os efeitos do dano encefálico causado pela HI em ratos neonatos sobre possíveis alterações bioquímicas e celulares. Mais especificamente, as diferenças nas respostas ao insulto entre machos e fêmeas foram analisadas e comparadas para obtermos um melhor entendimento sobre os mecanismos de lesão da HI.

A patogênese da HI neonatal é multifatorial e extremamente complexa. Diversos fatores contribuem para a lesão encefálica, incluindo danos à barreira hematoencefálica, falha energética, perda da homeostase iônica e osmótica da célula, aumento do Ca^{2+} e Na^{+} intracelular, excitotoxicidade, deficiência nos níveis dos fatores de crescimento, ativação das cascatas de mediadores inflamatórios e toxicidade celular mediada por radicais livres (CORNETTE & LEVENE, 2009). Os radicais livres são moléculas altamente reativas geradas predominantemente durante a respiração celular e no metabolismo normal. Um desequilíbrio entre a produção de radicais livres e a capacidade de detoxificação das mesmas pelas células é conhecido como estresse oxidativo (VASILJEVIC *et al.*, 2011). Durante as últimas décadas, vários estudos com modelos experimentais de HI neonatal vêm demonstrando que a EHI está intimamente relacionada à produção exacerbada de radicais livres (MAULIK *et al.*, 1998; CHAN, 2001; OHSAWA *et al.*, 2007; KUMAR *et al.*, 2008).

O **Capítulo 1** desta tese teve por objetivo investigar o dano oxidativo e as alterações na atividade da enzima Na^{+}, K^{+}-ATPase causadas pela HI neonatal logo após o insulto. Para tal, os animais foram submetidos ao procedimento experimental da HI e imediatamente, 1 ou 2h após o insulto foram sacrificados e o córtex e o hipocampo analisados. A formação de espécies reativas, peroxidação lipídica e ativação da SOD tanto em córtex quanto em hipocampo foram detectadas a partir de 1h após a lesão. Já a

atividade da Na^+, K^+-ATPase respondeu imediatamente à lesão encefálica no hipocampo. Estas alterações corroboram com os resultados encontrados na literatura, que utilizam diferentes cobaias e modelos experimentais de lesão hipóxico-isquêmica e acrescentam novas informações sobre o desenvolvimento do dano imediatamente após a lesão.

Dois tipos de lesões compõem o modelo experimental da HI em neonatos: isquemia e hipóxia. Durante a isquemia, a interrupção do fluxo sanguíneo leva à diminuição do suprimento de glicose e oxigênio para as estruturas afetadas. Como conseqüência, ocorre uma diminuição geral do metabolismo energético a fim de manter a homeostase celular, fato que também é visto durante a hipóxia sistêmica. Entretanto, no período imediatamente após o final da hipóxia, ou seja, na reoxigenação, o encéfalo dos neonatos experimenta uma situação de hiperoxigenação, responsável pela grande produção de radicais livres através da cadeia transportadora de elétrons. Os radicais livres formados reagem com os lipídios de membrana, causando uma desestruturação da bicamada lipídica com conseqüente inibição da atividade da Na^+, K^+-ATPase (KAKO *et al.*, 1988; RAZDAN *et al.*, 1993; MATTÉ *et al.*, 2006).

Como pode ser observado no presente estudo, a disfunção na atividade da enzima Na^+, K^+-ATPase está intimamente relacionada com a produção de radicais livres imediatamente após a lesão. Ainda, a inibição dessa enzima observada no hipocampo, mas não no córtex pode ser devido à distribuição das diferentes isoformas presentes em cada estrutura. Enquanto no hipocampo e no estriado a isoforma predominante é a subunidade α1, o córtex cerebral apresenta uma grande expressão da subunidade α3 da Na^+, K^+-ATPase (HIEBER *et al.*, 1991). Esta mesma sensibilidade hipocampal já foi relatada em trabalhos do nosso grupo de pesquisa (BAVARESCO *et al.*, 2005; 2006),

levando-nos a sugerir uma grande suscetibilidade ao estresse oxidativo da subunidade α1 quando comparada com as outras isoformas.

A falha da bomba de Na^+ e K^+ determina a rápida despolarização neuronal, causando fluxo excessivo de Ca^{2+} e Na^+ para o meio intracelular, edema e lise celular. Ao mesmo tempo, uma vez que o transporte de glutamato é dependente de Na^+ (SZATKOWSKI *et al.*, 1990), a remoção deste do meio extracelular e/ou a inativação da Na^+, K^+-ATPase causam acúmulo de glutamato na fenda sináptica, superestimulação dos seus receptores ionotrópicos e assim, excitotoxicidade neuronal. Estes eventos levam a uma maior produção de radicais livres que por sua vez causam peroxidação lipídica e desequilíbrio na homeostase gerando um círculo vicioso que resulta em morte celular.

Para obtermos uma melhor compreensão do dano oxidativo exposto no primeiro trabalho, no **Capítulo 2** nós decidimos avaliar os efeitos da lesão hipóxico-isquêmica sobre a função mitocondrial. Neste capítulo, a função mitocondrial foi avaliada de acordo com o sexo do animal, a fim de detectar uma possível presença de dimorfismo sexual no dano celular causado pela HI neonatal.

Um grande número de estudos sugere que a morte celular induzida pela HI em neonatos possui duas fases, uma fase inicial e rápida, induzida pela depleção de ATP intracelular, chamada de morte celular necrótica e outra mais tardia, onde a célula já restabeleceu grande parte da produção de ATP, denominada morte celular apoptótica (NITATORI *et al.*, 1995; RENOLLEAU *et al.*, 1997; 1998; ZHU *et al.*, 2005; PUYAL *et al.*, 2009). No presente estudo, as alterações na função mitocondrial foram avaliadas em dois períodos distintos - 2h e 18h após a lesão causada pela HI – a fim de se obter uma avaliação da lesão inicial e outra mais tardia. Em 2h, foi observada uma fraca diminuição da atividade do complexo II da cadeia respiratória no hipocampo, sendo

acompanhada de diminuição da massa e do $\Delta\psi$ mitocondrial em ambos os sexos e estruturas cerebrais. Entretanto, quando analisados 18h após o insulto, machos e fêmeas mostraram diferentes respostas ao dano. Enquanto as fêmeas apresentaram uma inibição da atividade dos complexos (I-III, II e IV) da cadeia respiratória, diminuição da massa e $\Delta\psi$ mitocondriais, nos machos neonatos as mudanças na atividade mitocondrial e no $\Delta\psi$ não foram acompanhadas de alterações na massa mitocondrial. Ainda, os resultados obtidos evidenciam a presença de dimorfismo sexual na função mitocondrial. As fêmeas, além de possuírem uma atividade *per se* da cadeia respiratória maior do que os machos, ainda mostraram-se mais vulneráveis ao dano causado pela HI.

O dimorfismo sexual caracteriza-se por diferenças morfológicas, comportamentais, cognitivas e bioquímicas entre machos e fêmeas de uma mesma espécie (STEFANOVA & OVTSCHAROFF, 2000). As diferenças entre os sexos surgem em resposta a diversos sinais sexo-específicos provenientes de diferenças que são inerentes ao genoma e envolvem mecanismos celulares que são específicos para cada tecido ou mesmo para as diversas estruturas do encéfalo. Mais especificamente em relação ao encéfalo, as estruturas encefálicas respondem de maneira distinta à sinalização celular em machos e fêmeas, como por exemplo no número de células responsivas em uma determinada área nervosa, na conectividade sináptica entre as estruturas, na comunicação célula-célula, nos efeitos mediados por receptores hormonais nucleares e de membrana e na síntese local de esteróides, entre outros (MCCARTHY & ARNOLD, 2011). Além das diferenças fisiológicas entre os sexos, a susceptibilidade à doenças ou à disfunções no encéfalo podem apresentar-se de 2 a 5 vezes maior dependendo do sexo afetado. Neste contexto, estudos mostram uma maior tendência a transtornos neuropsiquiátricos e de aprendizagem nos indivíduos do sexo masculino e uma elevada taxa de algumas doenças neurodegenerativas relacionadas ao

envelhecimento, como por exemplo a doença de Alzheimer, em representantes do sexo feminino (SWAAB & HOFMAN, 1995; MCCARTHY et al., 2009; VOSKUHL, 2011).

A disfunção mitocondrial encontrada neste estudo corrobora com os resultados obtidos no Capítulo 1, mostrando que a formação de espécies reativas bem como a peroxidação lipídica parecem ocorrer devido à inibição das atividades dos complexos da cadeia respiratória. A inibição dos complexos, além de diminuir a produção de ATP, pode provocar a desorganização da cadeia respiratória, permitindo com isso que os elétrons escapem mais facilmente, levando à redução incompleta do O_2 à H_2O e por consequência, maior formação de $O_2^{\bullet-}$ (HALLIWELL & GUTTERIDGE, 2000; MILATOVIC *et al.*, 2001). Desta forma, o aumento da atividade da SOD encontrada no trabalho anterior também parece corroborar com estes resultados, mostrando um possível efeito compensatório da atividade dessa enzima em resposta aos altos níveis de $O_2^{\bullet-}$ produzidos. A diminuição do fluxo de elétrons através dos complexos também diminui o bombeamento de prótons para o espaço intermembranas, desfazendo o gradiente eletroquímico e por consequência o $\Delta\psi$ mitocondrial. Com isso, ocorre um aumento da permeabilidade da membrana externa da mitocôndria e liberação de proteínas pró-apoptóticas para o citoplasma, desencadeando o processo de morte celular.

Dentre as proteínas liberadas pela mitocôndria está o AIF que, quando liberado no citoplasma, migra para o núcleo ativando a PARP-1. Em condições fisiológicas, a PARP-1 atua nas vias de reparo da quebra da fita do DNA via reparo por excisão de bases (BOULTON *et al.*, 1999) catalisando a transferência intracelular de unidades de ADP-ribose a partir da nicotinamida adenina dinucleotídeo (NAD^+) para as proteínas nucleares, levando à formação de polímeros de ADP-ribose (PENNING *et al.*, 2009). Entretanto, quando superativada, a atividade da PARP-1 leva a um uso exacerbado de

NAD^+ e ATP afetando desta forma a homeostase energética; além de enviar um sinal nuclear para a mitocôndria o que desencadeia a liberação de mais AIF no citoplasma (HONG *et al.*, 2004). Este círculo de eventos gera o chamado dano mitocondrial secundário, mais tardio à lesão primária induzida pela HI. Essas evidências explicariam o fato dos machos não apresentarem perda de massa mitocondrial 18h após a lesão, já que está bem documentado que, após a lesão da HI, eles apresentam uma maior liberação de AIF e por conseqüência ativação da PARP-1 quando comparados com as fêmeas tendo, portanto, uma morte celular independente da ativação de caspases (ZHU *et al.*, 2006; RENOLLEAU *et al.*, 2007).

Ao contrário dos machos, as fêmeas apresentaram uma perda significativa de massa mitocondrial 18h após o evento hipóxico-isquêmico. Esta perda de massa mitocondrial poderia estar relacionada a uma ativação do processo de autofagia nas fêmeas, mais especificamente da mitofagia, o processo de eliminação e *turnover* seletivo das mitocôndrias danificadas. Não se sabe ainda ao certo quais sinais seriam os responsáveis por ativar este processo, mas algumas evidências recentes sugerem que a permeabilidade transitória da membrana mitocondrial acompanhada de despolarização mitocondrial induziria a ativação da autofagia (LEMASTERS *et al.*, 2002). A indução da mitofagia teria o intuito de proteger a célula da liberação de proteínas pró-apoptóticas para o meio intracelular como um mecanismo de reparo ao dano da HI. Além da permeabilidade transitória mitocondrial, alguns estudos sugerem que a formação de EROs pela inibição dos complexos mitocondriais (CHEN *et al.*, 2007) seria capaz de ativar este processo e, em particular, o $O_2^{\bullet-}$ formado pela atividade dos complexos mitocondriais seria a principal espécie reativa a ativar a autofagia (CHEN *et al.*, 2009).

No intuito de avaliar se a via da autofagia poderia estar relacionada com as diferentes alterações mitocondriais encontradas em machos e fêmeas, no **Capítulo 3** a atividade autofágica foi avaliada em neonatos machos e fêmeas expostos à HI. Os resultados mostraram uma resposta sexo-específica além de diferenças regionais no encéfalo dos animais afetados. No córtex cerebral, nenhuma alteração foi encontrada nos machos ao passo que as fêmeas apresentaram aumento na atividade autofágica demonstrado pelo aumento da expressão da proteína LC3B-II. O LC3B na sua forma citosólica, LC3B-I, é conjugado à PE, formando o LC3B-II presente somente na membrana dos autofagossomos. Quando estes se fundem aos lisossomos, originando os autolissosomos, o LC3B-II é parcialmente degradado e a PE é desligada do LC3B que retorna ao citoplasma (MEIJER & CODOGNO, 2009). Apesar do aumento visto nos níveis da proteína LC3B-II, evidenciando o aumento no número de autofagossomos, a concentração de autolisossomos não foi alterada, apesar de fracamente diminuída nas fêmeas. Estes dados mostram uma indução da autofagia nas fêmeas, porém a diminuição dos autolisossomos demonstra que possivelmente alguns passos desta via poderiam estar bloqueados ou inibidos como, por exemplo, a fusão dos autofagossomos aos lisossomos, impedindo a finalização do processo. Esta idéia corrobora com a diminuição do número de lisossomos encontrada nos neonatos submetidos à HI. A falha ao completar o processo de autofagia compromete a tentativa de restabelecer a homeostase celular levando as células a entrar em processo de apoptose, fato comprovado pelo aumento da atividade das caspases encontrado em ambos os sexos, porém de forma mais severa nas fêmeas.

No hipocampo, apesar da falta de alterações nos níveis de LC3B-II, o aumento de autolisossomos detectados evidencia a ativação da autofagia tanto nos machos quanto nas fêmeas. Esta ausência de efeito na expressão da proteína LC3B-II pode ser

explicada pela rápida degradação desta pela via lisossomal. Como o aumento dos níveis de LC3B-II pode ser rápido e transitório sob condições severas de dano, é possível que a lesão causada pela HI tenha sido severa o suficiente para levar à rápida degradação e *turnover* do LC3B. Surpreendentemente, somente os machos submetidos à HI responderam à ativação da autofagia uma vez que o número de autolisossomos nas fêmeas apresentava-se elevado *per se* tanto nos animais controle quanto nos HI. Este efeito pode representar um mecanismo intrínseco de proteção por renovação celular presente nas fêmeas. Em ambos os casos, entretanto, a indução da autofagia pode ter sido tão intensa a ponto de comprometer a viabilidade celular levando as células a entrar em processo de apoptose, uma vez que machos e fêmeas apresentaram aumento da atividade das caspases. Tanto no córtex quanto no hipocampo as fêmeas apresentaram um aumento muito maior na atividade das caspases quando comparadas aos machos submetidos à HI. Estes resultados corroboram com os dados encontrados na literatura, mostrando que a via de morte celular preferencial nas fêmeas é dependente da ativação das caspases (RENOLLEAU *et al.*, 2007).

É possível que o aumento das EROs pela cadeia respiratória mitocondrial e a perda de $\Delta\psi$ tenham induzido a autofagia após o dano causado pela HI no encéfalo dos neonatos. Entretanto, apenas nas fêmeas parece ter ocorrido um aumento no *turnover* de mitocôndrias a fim de retirar as organelas danificadas do meio, por mecanismos ainda a serem estudados.

Em linhas gerais, foi observado que a HI é capaz de induzir estresse oxidativo no encéfalo dos neonatos em um curto período após o dano, aumentando os níveis de peroxidação lipídica, de radicais livres e causando distúrbios tanto nas atividades das enzimas antioxidantes quanto na atividade da enzima Na^+, K^+-ATPase. Observou-se que a disfunção mitocondrial parece ser um fator que contribuiu de maneira significativa

para estes eventos. Tanto no córtex quanto no hipocampo, a atividade da cadeia respiratória, a massa e o $\Delta\psi$ mitocondriais apresentavam-se diminuídos em ambos os sexos, 2h após o insulto. Em 18h, as alterações encontradas em 2h repetiram-se, exceto pelo fato dos machos não apresentarem perda de massa mitocondrial. Estes dados mostram a relevância da função mitocondrial frente ao dano gerado pela HI no encéfalo dos neonatos; além disso, mas não menos importante, os resultados apontam a presença de dimorfismo sexual para as alterações mitocondriais analisadas. A HI neonatal demonstrou ainda ativar a via da autofagia de forma sexo e região cerebral específicas. Estas diferenças sexo-específicas são importantes não somente para entendermos o mecanismo de dano causado pelo insulto, mas também para direcionarmos os estudos sobre as estratégias terapêuticas de acordo com o sexo do indivíduo afetado.

7. CONCLUSÕES

Os resultados obtidos nestes estudos nos permitem concluir que:

1. A lesão causada pela HI no encéfalo dos neonatos gera estresse oxidativo e altera a homeostase celular por aumentar a formação de radicais livres, induzir peroxidação lipídica e por ativar a enzima SOD 1h após a lesão. Estes efeitos provavelmente levaram à inibição da atividade da enzima Na^+, K^+-ATPase imediatamente após o insulto.

2. A HI promoveu a disfunção mitocondrial que se apresentou de maneira sexo-dependente em dois momentos distintos, 2h e 18h após a lesão, em córtex e hipocampo. Pelos dados obtidos nós podemos concluir que as fêmeas além de apresentarem maior atividade mitocondrial, também parecem ser mais vulneráveis do que os machos quando submetidas à HI neonatal.

3. A HI induziu a autofagia a qual foi provavelmente causada pelo estresse oxidativo e pela disfunção mitocondrial. A autofagia apresentou-se de forma distinta em córtex e hipocampo e também de maneira diferente em machos e fêmeas. Independente da situação, nós sugerimos que esta ativação da via da autofagia invariavelmente leva à morte celular quando os animais são submetidos à HI neonatal por mostrar-se incapaz de restabelecer a homeostase através do *turnover* celular.

8. PERSPECTIVAS

1. Verificar o envolvimento do processo de degradação seletiva das mitocôndrias, a mitofagia, através da avaliação dos níveis de proteínas específicas deste processo, como BNIP3, NIX e Parkin, em machos e fêmeas submetidos à HI neonatal.

2. Avaliar se os processos de fusão e fissão mitocondrial estão envolvidos na indução de autofagia celular pela análise de algumas proteínas relacionadas a estes processos como a *Mitofusina* (Mfn 1 e 2) e a *Fissão 1* (Fis1).

3. Investigar se agentes neuroprotetores, como os ácidos eicosapentaenóico (EPA) e docosaexaenóico (DHA), produtos do metabolismo dos ácidos graxos da série ômega-3, são capazes de proteger ou, pelo menos, diminuir a disfunção mitocondrial, o estresse oxidativo e a morte celular causada pela HI em neonatos machos e fêmeas.

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