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Júlia Pramio

O SULFITO CAUSA DISFUNÇÃO BIOENERGÉTICA E REDOX EM CÉREBRO DE ANIMAIS NEONATOS E CULTURAS DE ASTRÓCITOS

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2020

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

Orientador: Prof. Dr. Guilhian Leipnitz
Coorientador: Dr. Mateus Grings

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PARTE I

RESUMO

A deficiência isolada da sulfito oxidase (ISOD) e a deficiência do cofator molibdênio (MoCD) são doenças metabólicas hereditárias que afetam principalmente o sistema nervoso central (SNC). Grande parte dos pacientes apresentam sintomas graves de início precoce, incluindo crises convulsivas neonatais e encefalopatia de rápida progressão, geralmente levando a óbito nos primeiros meses de vida. Estudos vêm sendo realizados a fim de elucidar os mecanismos fisiopatológicos dessas doenças e indicam que o sulfito, principal metabólito acumulado nos pacientes, é neurotóxico. Com o intuito de avaliar a toxicidade do sulfito no período neonatal, ratos Wistar receberam uma injeção intracerebroventricular (icv) de sulfito (0,5 µmol/g) ou veículo (PBS) no 1º dia de vida, sendo eutanasiados 30 min após a administração do metabólito para a avaliação de parâmetros em córtex cerebral. Além disso, avaliamos os efeitos do sulfito (100 – 1.000 µM), *in vitro*, em culturas primárias de astrócitos corticais após um período de incubação de 6 e 24 h. Primeiramente, observamos que a administração icv de sulfito alterou a homeostase redox, pois diminuiu os níveis de glutationa reduzida (GSH) e a atividade da enzima glutationa S-transferase (GST) e aumentou o imunoconteúdo de heme oxigenase-1 (HO-1). Com relação aos parâmetros de metabolismo energético, o sulfito diminuiu a atividade das enzimas succinato desidrogenase (SDH) e creatina cinase (CK), bem como dos complexos II e II-III da cadeia respiratória. Por fim, também observamos que o sulfito aumentou o imunoconteúdo da cinase regulada por sinalização extracelular 1/2 (ERK 1/2) e da cinase p38, apesar de não alterar a fosforilação dessas proteínas. Em culturas de astrócitos, o sulfito aumentou a oxidação de 2’,7’- diclorofluorescina (DCFH) e os níveis de lactato extracelular após 6 e 24 h de incubação, respectivamente. Analisados em conjunto, esses achados indicam que o sulfito causa um desequilíbrio redox e altera o metabolismo energético, o que pode estar contribuindo para as anormalidades cerebrais observadas em pacientes recém-nascidos com a ISOD e a MoCD.

Palavras chave: Deficiência isolada da sulfito oxidase; Deficiência do cofator molibdênio; Sulfito; Homeostase redox; Metabolismo energético; Período neonatal; Cérebro.

ABSTRACT

Isolated sulfite oxidase deficiency (ISOD) and molybdenum cofactor deficiency (MoCD) are inherited metabolic diseases that affect mainly the central nervous system (CNS). Most patients have severe early onset symptoms, that include neonatal seizures and rapidly progressive encephalopathy, usually leading to death in the first months of life. Studies have been carried out in order to elucidate the pathophysiology of these diseases and have shown that sulfite, the main metabolite accumulated in patients, is neurotoxic. In order to assess the toxicity of the sulfite in the neonatal period, Wistar rats received an intracerebroventricular (icv) injection of sulfite (0.5 µmol / g) or vehicle (PBS) on the first day of life, being euthanized 30 min after the administration of the metabolite for the evaluation of parameters in the cerebral cortex. In addition, we evaluated the *in vitro* effects of sulfite (100 - 1,000 µM) in primary cultures of cortical astrocytes after an incubation period of 6 and 24 h. First, we observed that the icv administration of sulfite altered redox homeostasis, as verified by the decreased levels of reduced glutathione (GSH) and activity of the enzyme glutathione S-transferase (GST) and increased immunocontent of heme oxygenase-1 (HO-1). Regarding the parameters of energy metabolism, sulfite decreased the activity of the enzymes succinate dehydrogenase (SDH) and creatine kinase (CK), as well as of complexes II and II-III of the respiratory chain. Finally, we observed that sulfite increased the immunocontent of extracellular signal-regulated kinase 1/2 (ERK 1/2) and p38 kinase despite not altering the phosphorylation of these proteins. In astrocyte cultures, sulfite increased 2',7'- dichlorofluorescin (DCFH) oxidation and the extracellular lactate levels after 6 and 24 h of incubation, respectively. Taken together, these findings indicate that sulfite causes a redox imbalance and alters energy metabolism, which may contribute to the onset of brain abnormalities seen in neonates with ISOD and MoCD.

Keywords: Isolated sulfite oxidase deficiency; Molybdenum cofactor deficiency; Sulfite; Redox homeostasis; Energy metabolism; Neonatal period; Brain.

LISTA DE ABREVIATURAS

AO – aldeído oxidase

CAC – Ciclo do ácido cítrico

CAT – Catalase

CK – Creatina cinase

CLAE – Cromatografia líquida de alta eficiência

cPMP – Piranopterina cíclica monofosfato

CS – Citrato sintase

CTE – Cadeia transportadora de elétrons

DCFH – Diclorofluorescina

DRP1 – Proteína relacionada à dinamina 1

ERK 1/2 – Cinase regulada por sinalização extracelular 1/2

ERNs – Espécies reativas de nitrogênio

EROs – Espécies reativas de oxigênio

G6PDH – Glicose-6-fosfato desidrogenase

GDH – Glutamato desidrogenase

GFAP – Proteína fibrilar glial ácida

GPx – Glutationa peroxidase

GR – Glutationa redutase

GSH – Glutationa reduzida

GSSG – Glutationa oxidada

GST – Glutationa S-transferase

HO-1 – Heme oxigenase-1

Iba1 – Molécula adaptadora de ligação ao cálcio ionizado 1

ISOD – Deficiência isolada da sulfito oxidase

JNK – Cinase C-Jun N-terminal

MAPKs – Proteínas cinases ativadas por mitógenos

MAPs – Proteínas associadas a microtúbulos

mARC1 e 2 – Componente redutor da amidoxima mitocondrial 1 e 2

MCTs – Transportadores de monocarboxilato

MDH – Malato desidrogenase

MFN1 e 2 – Mitofusina 1 e 2

MKPs – MAPK fosfatases

MME – Membrana mitocondrial externa

MMI – Membrana mitocondrial interna

MoCD – Deficiência do cofator molibdênio

MoCo – Cofator molibdênio

MTT – Metiltiazolildifenil-tetrazólio

NeuN – Proteína nuclear específica de neurônios

OPA1 – Proteína atrófica óptica 1

PINK1 – Cinase 1 induzida por PTEN

SDH – Succinato desidrogenase

SNC – Sistema nervoso central

SO – Sulfito oxidase

SOD – Superóxido dismutase

XOR – Xantina oxidoredutase

$\Delta\Psi m$ – Potencial de membrana mitocondrial

I. 1. INTRODUÇÃO

I. 1.1. SULFITO OXIDASE (SO)

A SO está localizada no espaço intermembranas mitocondrial (Tan et al., 2005; Hoffmann et al., 2007; Claerhout et al., 2017) de diversos tecidos, sendo que fígado, rim, músculo esquelético, coração, placenta e cérebro estão entre os que mais expressam o gene desta enzima em seres humanos (Woo et al., 2003; Tan et al., 2005). A função da SO é catalisar a última etapa da via de degradação de aminoácidos sulfurados, cisteína e metionina, oxidando sulfito a sulfato (Johnson, 2003; Relinque et al., 2015; Atwal e Scaglia, 2016; Lee et al., 2017). Além disso, a SO também é responsável por metabolizar o sulfito derivado da exposição ambiental ao dióxido de enxofre (SO_2) e ingerido por meio de alimentos e fármacos (Mendel e Bittner, 2006; Pundir e Rawal, 2013; Kappler e Enemark, 2014; Velayutham et al., 2016), mantendo sua concentração sérica em até 10 µM (Ji, Savon e Jacobsen, 1995; Pundir e Rawal, 2013).

A SO é um homodímero, tendo monômeros constituídos de três domínios: o domínio heme citocromo b_5 (N-terminal); o domínio catalítico central, que abriga o cofator molibdênio (MoCo); e o domínio C-terminal, responsável pela dimerização (Schwarz, 2016; Bender et al., 2019). O MoCo, que consiste em uma molécula de molibdopterina ligada a um molibdênio, deve ser previamente sintetizado pelo organismo a partir de GTP (Figura 1) (Johnson, 2003; Atwal e Scaglia, 2016; Schwarz, 2016). Durante a reação enzimática, o molibdênio (VI) é reduzido a molibdênio (IV) por dois elétrons provenientes do sulfito, resultando na formação de sulfato. A reoxidação do molibdênio (IV) se dá primeiramente pela transferência intramolecular dos elétrons para o heme citocromo b_5, que, em seguida, é oxidado pelo citocromo *c* da cadeia respiratória (Garrett et al., 1998; Belaidi et al., 2015; Bender et al., 2019).

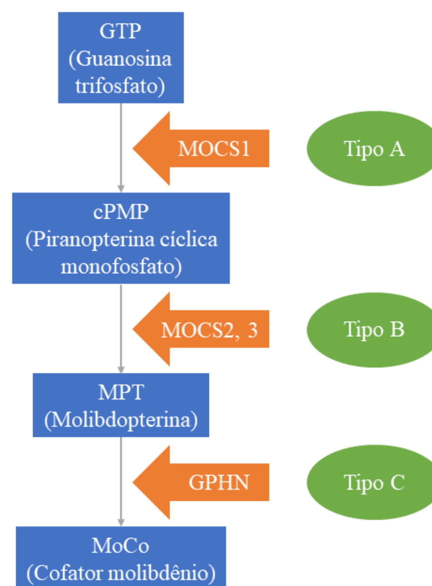


Figura 1. Biossíntese do cofator molibdênio (MoCo) e classificação da doença de acordo com a deficiência enzimática (Adaptado de Alonzo Martínez et al., 2020).

I. 1.2. DEFICIÊNCIA DA SO

A deficiência da SO pode ocorrer de duas formas: deficiência isolada da SO (ISOD) ou deficiência do cofator molibdênio (MoCD) (Kisker et al., 1997; Garrett et al., 1998; Edwards et al., 1999; Basheer et al., 2007; Westerlinck et al., 2014; Relinque et al., 2015; Sharawat et al., 2020). Ambas são doenças neurometabólicas genéticas raras (Tan et al., 2005; Relinque et al., 2015; Durmaz e Özbakir, 2018; Sharawat et al., 2020) e possuem padrão de herança autossômico recessivo (Johnson, 2003; Rocha et al., 2014; Relinque et al., 2015; Cornet, Sands e Cilio, 2018), logo, heterozigotos não manifestam a doença (Edwards et al., 1999; Johnson, 2003). Enquanto os pacientes portadores da ISOD apresentam mutações no gene *SUOX*, que codifica a SO (Claerhout et al., 2017; Lee et al., 2017), indivíduos com a MoCD apresentam mutações nos genes *MOCS1, MOCS2, MOCS3* ou *GPHN*, que codificam enzimas envolvidas na biossíntese do MoCo (Sass et al., 2010; Westerlinck et al., 2014; Zaki et al., 2016; Durmaz

e Özbakir, 2018; Alonzo Martínez et al., 2020). Dois terços dos casos de MoCD são causados por mutações no gene *MOCS1* (Belaidi et al., 2015; Atwal e Scaglia, 2016; Zaki et al., 2016; Cornet, Sands e Cilio, 2018) e apenas dois pacientes são descritos com mutações no gene *GPHN* (c.65-?_102+?del, resultando em Exon2/3del, e c.1739A>C, resultando na substituição p.D580A) (Reiss e Hahnewald, 2010; Bayram et al., 2013; Atwal e Scaglia, 2016; Scelsa et al., 2019). Atwal e Scaglia (2016) relataram haver mais de 60 variantes patogênicas em *MOCS1* e *MOCS2*. Quanto ao gene *MOCS2*, Arican et al. (2019) contabilizaram vinte e nove diferentes mutações descritas, incluindo deleções, duplicações, variações missense, nonsense e frameshift. As mutações 956G>A (R319Q), 418+1G>A e 1523del2 em *MOCS1* parecem frequentes, sendo, cada uma, já identificada em pelo menos dez famílias, segundo Johnson (2003). Recentemente, um paciente com mutação missense no gene *MOCS3* também foi identificado (c.769G>A; substituição p.Ala257Thr) (Huijmans et al., 2017). Já no gene *SUOX*, mutações missense, nonsense e frameshift foram observadas. Mutações missense podem estar relacionadas a alterações na conformação do sítio ativo da SO, na sua dimerização e em resíduos de ligação do MoCo (Johnson et al., 2002; Karakas e Kisker, 2005). De acordo com Tan et al. (2005), a mutação 479G>A (substituição R160Q) ocorreu em pelo menos quatro pacientes.

Aproximadamente cinquenta indivíduos com ISOD e cento e cinquenta com MoCD são relatados na literatura. A prevalência dessas doenças é desconhecida, mas estima-se que 1 a cada 100.000 a 200.000 nascidos vivos sejam portadores da MoCD (Zaki et al., 2016; Bindu et al., 2017; Alonzo Martínez et al., 2020). Devido à similaridade fenotípica com outras patologias, deve ser destacado que a ISOD e a MoCD são possivelmente subdiagnosticadas (Reiss e Hahnewald, 2010; Atwal e Scaglia, 2016; Bindu et al., 2017; Durmaz e Özbakir, 2018).

I. 1.2.1. Apresentação Clínica

O MoCo é necessário para a atividade não apenas da SO, mas também da xantina oxidoredutase (XOR), da aldeído oxidase (AO) e do componente redutor da amidoxima mitocondrial (mARC1 e mARC2). Apesar de pacientes com a MoCD também apresentarem deficiência dessas três outras enzimas (Reiss e Hahnewald, 2010; Belaidi e Schwarz, 2013; Atwal e Scaglia, 2016; Schwarz, 2016; Alonzo Martínez et al., 2020), sabe-se que os sintomas observados são decorrentes da atividade deficiente da SO (Kisker et al., 1997; Edwards et al., 1999; Johnson, 2003; Schwarz, 2016), já que portadores de xantinúria tipo 1 (deficiência de XOR) ou xantinúria tipo 2 (deficiência de XOR e AO) não manifestam problemas neurológicos e são, muitas vezes, assintomáticos (Tan et al., 2005; Schwarz, 2016; Claerhout et al., 2017). Até o presente momento, também não foram descritas na literatura patologias decorrentes da deficiência isolada da AO ou do mARC (Tan et al., 2005; Claerhout et al., 2017; Alonzo Martínez et al., 2020).

Dessa forma, a ISOD e a MoCD são clinicamente indistinguíveis (Kisker et al., 1997; Reiss e Hahnewald, 2010; Belaidi e Schwarz, 2013; Belaidi et al., 2015; Atwal e Scaglia, 2016; Zaki et al., 2016), manifestando-se na maioria das vezes de forma grave e ainda no período neonatal, apesar de formas mais leves e de início tardio também já terem sido relatadas (Johnson, 2003; Hoffmann et al., 2007; Rocha et al., 2014; Relinque et al., 2015; Bindu et al., 2017; Claerhout et al., 2017; Alonzo Martínez et al., 2020; Sharawat et al., 2020). O tipo de mutação e a consequente atividade residual enzimática parecem estar associados à gravidade da doença (Claerhout et al., 2017). Os pacientes acometidos pela forma clássica (grave e precoce) da doença apresentam crises convulsivas refratárias à terapia, retardo psicomotor grave, dificuldades alimentares, face com características dismórficas, microcefalia, hipotonia axial e hipertonia periférica (Hoffmann et al., 2007; Reiss e Hahnewald, 2010; Rocha et al., 2014; Relinque et al., 2015; Schwarz, 2016; Bindu et al., 2017; Claerhout et al., 2017; Alonzo

Martínez et al., 2020; Sharawat et al., 2020). Além disso, *ectopia lentis* é comumente observada em pacientes que sobrevivem ao período neonatal (Johnson, 2003; Reiss e Hahnewald, 2010; Bindu et al., 2017; Lee et al., 2017).

I. 1.2.2. Achados Neurológicos

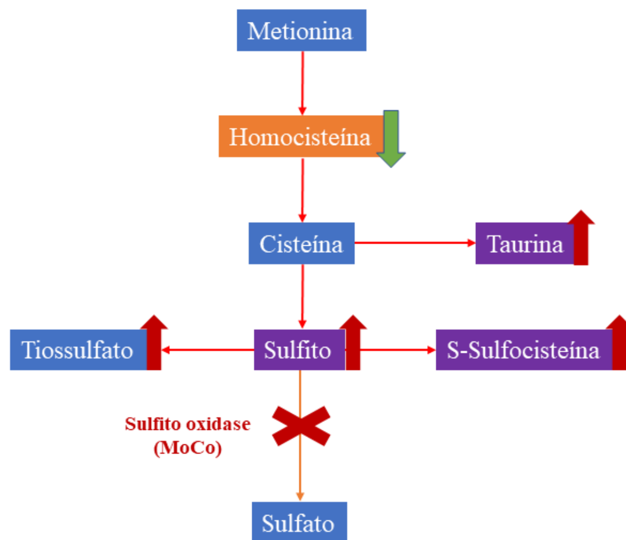
Exames de neuroimagem e neuropatológicos realizados em pacientes com a ISOD e a MoCD já evidenciaram alterações como encefalomalácia cística, atrofia cerebral e cerebelar progressiva, edema difuso, dilatação dos ventrículos, hipoplasia do corpo caloso, gânglios basais e tronco cerebral, perda neuronal massiva, desmielinização, gliose e alterações focais ou bilaterais do globo pálido e regiões subtalâmicas (Tan et al., 2005; Schwarz, 2016; Zaki et al., 2016; Bindu et al., 2017; Lee et al., 2017; Yoganathan et al., 2018).

I. 1.2.3. Achados Laboratoriais

Os indivíduos portadores da ISOD e da MoCD apresentam elevados níveis de sulfito nos tecidos e líquidos biológicos, além de níveis aumentados de tiossulfato e S-sulfocisteína, produtos da conjugação do sulfito ao sulfeto de hidrogênio (H_2S) e à cist(e)ina, respectivamente (Figura 2A) (Mills et al., 2012; Atwal e Scaglia, 2016; Zaki et al., 2016; Lee et al., 2017; Wyse et al., 2018). O sulfito é facilmente detectado na urina utilizando-se fitas indicadoras, mas por ser uma molécula muito instável, podendo ser oxidado em poucas horas, deve ser mensurado em amostra fresca a fim de evitar falsos-negativos. Resultados falsos-positivos podem ocorrer pelo aumento de ácido succínico, ácido benzoico, ácido 2-hidroxiglutárico e uracil na urina decorrentes de degradação bacteriana, pela presença de certos antibióticos (cefotaxima, cefuroxima, ampicilina e benzilpenicilina) ou por drogas que contenham grupo sulfidril alifático livre (N-acetilcisteína, mercaptamina, dimercaprol e 2-mercaptoetano sulfonato) (Hobson et al., 2005; Tan et al., 2005; Basheer et al., 2007; Reiss e Hahnewald, 2010; Sass et

al., 2010; Bindu et al., 2017; Claerhout et al., 2017; Alonzo Martínez et al., 2020). Já o tiossulfato e a S-sulfocisteína são mais estáveis e podem ser úteis para reforçar o resultado (Tan et al., 2005; Sass et al., 2010; Bindu et al., 2017). O tiossulfato pode ser determinado na urina por espectrofotometria, e a S-sulfocisteína, no plasma ou na urina, por espectrometria de massas em tandem ou cromatografia líquida de alta eficiência (CLAE) (Tan et al., 2005; Relinque et al., 2015; Bindu et al., 2017; Alonzo Martínez et al., 2020). O aumento de taurina também é observado em plasma e urina de pacientes devido ao aumento da degradação de cisteína-sulfinato (Mills et al., 2012; Claerhout et al., 2017; Wyse et al., 2018).

Por outro lado, os pacientes apresentam diminuição de cistina e homocisteína total no plasma, decorrentes da reação do sulfito a seus grupos tiois livres (Figura 2A). A homocisteína total plasmática torna-se praticamente indetectável, representando um indicador sensível e específico dessas patologias (Sass et al., 2004; Tan et al., 2005; Basheer et al., 2007; Sass et al., 2010; Bindu et al., 2011; Rocha et al., 2014; Bindu et al., 2017). Nos casos de MoCD, devido ao comprometimento da atividade da XOR, também são observados aumentos na excreção urinária de xantina e hipoxantina e diminuição de ácido úrico em plasma e urina (Figura 2B) (Tan et al., 2005; Reiss e Hahnewald, 2010; Sass et al., 2010; Atwal e Scaglia, 2016). A partir desses achados bioquímicos, a confirmação do diagnóstico é então realizada por análise mutacional e determinação da atividade da SO em cultura de fibroblastos (Sass et al., 2010; Salas e Arca, 2018; Alonzo Martínez et al., 2020).



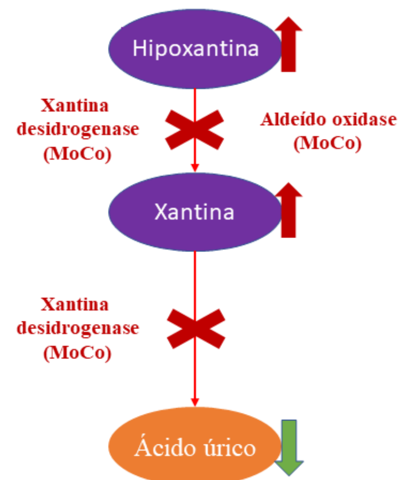


Figura 2. Vias metabólicas de enzimas dependentes do MoCo: o metabolismo de aminoácidos sulfurados (A) e o metabolismo de purinas (B) (Adaptado de Alonzo Martínez et al., 2020).

I. 1.2.4. Diagnóstico Pré-Natal

Na literatura, há poucos casos diagnosticados no período pré-natal, que geralmente ocorrem após uma primeira gravidez, quando se tem irmãos previamente afetados (Alonzo Martínez et al., 2020). A atividade da SO pode ser mensurada a partir de biópsia das vilosidades coriônicas, enquanto que o líquido amniótico pode ser usado para a dosagem de metabólitos. O diagnóstico por análise mutacional é geralmente realizado quando o gene afetado já é conhecido (Edwards et al., 1999; Johnson, 2003; Carmi-Nawi et al., 2011; Alonzo Martínez et al., 2020).

Avaliações neurológicas já mostraram o surgimento de alterações durante a gestação, indicando que a ISOD e a MoCD podem levar à interrupção do neurodesenvolvimento no feto (Carmi-Nawi et al., 2011; Lee et al., 2017). Carmi-Nawi et al. (2011) relataram dano cerebral difuso com ventriculomegalia, mega cisterna magna, disgenesia do corpo caloso, múltiplos cistos subcorticais e cerebelo hipoplásico em um feto a partir da 35ª semana de gestação. Já Alonzo Martínez et al. (2020) apresentaram exames de ultrassom de um feto a partir da 26ª

semana de gestação com mega cisterna magna, cavum vergae e dilatação ventricular, enquanto Lee et al. (2017) relataram baixa rotação e diferenciação das camadas corticais, por ressonância magnética cerebral, na 21ª semana de idade gestacional. Chen et al. (2014) também observaram achados como mega cisterna magna e leucoencefalopatia multicística em um recém-nascido com 14 horas de vida, sugerindo início pré-natal.

I. 1.2.5. Tratamento

A fim de melhorar a qualidade e perspectiva de vida dos pacientes, é feita a implementação de uma dieta com restrição de aminoácidos sulfurados que, apesar de levar à uma redução dos níveis de sulfito, promove melhora clínica especialmente em casos mais leves da doença (Reiss e Hahnewald, 2010; Rocha et al., 2014; Relinque et al., 2015; Schwarz, 2016; Bindu et al., 2017; Claerhout et al., 2017). Alguns compostos já foram testados sem sucesso clínico, como é o caso da betaína (usada para ocasionar a remetilação da homocisteína à metionina, diminuindo a formação de cisteína e sulfito) e da D-penicilamina (com o intuito de ligar sulfito a seus grupos tiois eliminando S-sulfopenicilamina), por exemplo (Tan et al., 2005; Bindu et al., 2017; Alonzo Martínez et al., 2020).

Recentemente, a terapia de reposição com piranopterina cíclica monofosfato (cPMP), intermediário da biossíntese do MoCo, tem se mostrado eficaz para pacientes com mutações em *MOCS1* (MoCD tipo A) (Figura 1), que apresentaram normalização dos índices metabólicos, melhora das convulsões e controle da deterioração neurológica. Contudo, como não há reversão dos danos cerebrais observados antes da suplementação com cPMP, o diagnóstico precoce é fundamental para um resultado favorável. Pacientes tratados precocemente podem apresentar um neurodesenvolvimento praticamente normal (Reiss e Hahnewald, 2010; Veldman et al., 2010; Schwarz, 2016; Cornet, Sands e Cilio, 2018; Alonzo Martínez et al., 2020).

Diferentemente da MoCD tipo A, a ISOD e a MoCD tipo B e C (Figura 1) não possuem um tratamento efetivo, o qual se limita apenas à restrição da dieta e ao manejo dos sintomas, com antiepilépticos para tentar controlar as convulsões e sonda de gastrostomia para facilitar a deglutição. Nestes casos, o prognóstico da doença é frequentemente ruim e os pacientes geralmente vão a óbito nos primeiros meses de vida (Sass et al., 2010; Veldman et al., 2010; Carmi-Nawi et al., 2011; Belaidi et al., 2015; Relinque et al., 2015; Atwal e Scaglia, 2016; Bindu et al., 2017; Claerhout et al., 2017).

I. 1.2.6. Fisiopatologia

Diversos estudos vêm sendo desenvolvidos para elucidar mecanismos de toxicidade do sulfito que possam levar à encefalopatia grave observada nos pacientes com deficiência da SO (Hoffmann et al., 2007; Relinque et al., 2015; Claerhout et al., 2017). Nesse sentido, já foi evidenciado que o sulfito pode se auto-oxidar na presença de metais ou sofrer oxidação enzimática por peroxidases e formar radicais de enxofre, além de ser capaz de produzir peróxido de hidrogênio (H_2O_2) e superóxido ($O_2^{\bullet-}$) diretamente (Izgüt-Uysal et al., 2005; Kocamaz et al., 2012). Um aumento da oxidação de 2′,7′- diclorofluorescina (DCFH) ocasionado pelo sulfito já foi observado em hipocampo e córtex cerebral de ratos *in vitro*, bem como o aumento de H_2O_2 em hipocampo e em mitocôndrias isoladas de cérebro (Grings et al., 2013; de Moura Alvorcem et al., 2017). Além disso, estudos já demonstraram que o sulfito induz peroxidação lipídica em cérebro, eritrócitos e plasma de ratos deficientes para a SO (Küçükatay et al., 2006; Herken et al., 2008; Ozturk et al., 2010), sendo que no plasma também foram observados aumento do estado oxidante total e diminuição do conteúdo de sulfidrilas livres (Herken et al., 2008). O aumento da peroxidação lipídica, além da diminuição da atividade da catalase (CAT) também ocorreu *in vitro* em córtex cerebral, hipocampo e estriado de ratos de 10 e 60 dias de idade (Chiarani et al., 2007). Kucukatay et al. (2007) observaram aumento da atividade de

enzimas antioxidantes em hipocampo de ratos normais após administração de sulfito, enquanto que Kocamaz et al. (2012) mostraram que o sulfito leva à perda de neurônios hipocampais tanto em ratos normais como em deficientes para a SO. Em linhagens de células neuronais (Neuro-2a e PC12) e hepáticas (HepG2 e células fetais humanas), o sulfito aumentou a produção de espécies reativas de oxigênio (EROs) e provocou depleção do ATP intracelular (Zhang et al., 2004). Já em mitocôndrias isoladas de cérebro de ratos, o sulfito diminuiu o consumo de oxigênio (O_2), a biossíntese de ATP e a atividade das enzimas glutamato desidrogenase (GDH) e malato desidrogenase (MDH) (Zhang et al., 2004; Grings et al., 2014). Além disso, Grings et al. (2014) constataram que, na presença de cálcio exógeno, o sulfito induz inchamento mitocondrial e reduz o potencial de membrana mitocondrial ($\Delta\Psi m$), a capacidade de retenção de cálcio, o conteúdo de NADPH e de citocromo c devido a abertura do poro de transição da permeabilidade mitocondrial em mitocôndrias de cérebro de ratos. Em outro trabalho, Grings et al. (2017) verificaram que o acúmulo de sulfito leva à disfunção mitocondrial, diminui as defesas antioxidantes, induz reatividade glial e dano neuronal em estriado de ratos jovens. Por fim, o sulfito também diminui a atividade da creatina cinase (CK) *in vitro* em córtex cerebral, hipocampo, cerebelo e estriado (Grings et al., 2013; de Moura Alvorcem et al., 2017) e *in vivo* em estriado de ratos jovens (Grings et al., 2017). Embora muito já se saiba a respeito da toxicidade do sulfito, é importante ressaltar que não há trabalhos na literatura visando estabelecer a fisiopatologia neonatal da deficiência da SO, período em que grande parte dos pacientes são acometidos pela doença.

I. 1.3. HOMEOSTASE REDOX

EROs compreendem um grupo de moléculas (radicais e não radicais) que derivam do O_2, abrangendo espécies iniciais formadas pela sua redução ($O_2^{\bullet-}$ e H_2O_2) e também produtos reativos secundários (Turrens, 2003; Winterbourn, 2008; Lambert e Brand, 2009; Pisoschi e

Pop, 2015). A redução do O_2 por um elétron resulta na formação de $O_2^{\bullet-}$. A principal fonte desse radical é a respiração mitocondrial, devido ao vazamento de elétrons de centros redox da cadeia transportadora de elétrons (CTE). Além disso, o $O_2^{\bullet-}$ também pode ser gerado por enzimas como NADPH oxidases, oxigenases dependentes de citocromo P450, ciclooxigenases e xantina oxidases, bem como pela redução direta do O_2 por coenzimas e xenobióticos reduzidos, por exemplo.

A formação desse radical pode mediar reações oxidativas em cadeia. O $O_2^{\bullet-}$ pode sofrer dismutação e gerar H_2O_2, o qual também pode ser formado diretamente a partir da transferência de dois elétrons para o O_2 por urato oxidases, glicose oxidases e D-aminoácido oxidases. O H_2O_2 pode reagir com metais de transição reduzidos e levar à formação do radical hidroxila ($OH^{\bullet}$) (Turrens, 2003; Pisoschi e Pop, 2015) ou ser convertido por peroxidases e resultar na formação de ácido hipocloroso (HOCl), oxigênio *singlet* ($^1O_2$) e outras espécies (Winterbourn, 2008; Brieger et al., 2012).

O $O_2^{\bullet-}$ também pode reagir com o óxido nítrico ($NO^{\bullet}$) e gerar peroxinitrito ($ONOO^-$) (espécie reativa de nitrogênio - ERN). As ERNs são derivadas de $NO^{\bullet}$, gerado a partir de L-arginina pela óxido nítrico sintase ou através da redução de nitrato inorgânico (Turrens, 2003; Winterbourn, 2008; Pisoschi e Pop, 2015).

As espécies reativas são continuamente produzidas pelo organismo como resultado do metabolismo celular normal. Em níveis baixos e regulados, desempenham funções fisiológicas essenciais, estando envolvidas tanto na sinalização celular (proliferação, diferenciação, crescimento, sobrevivência, apoptose, etc.) como em processos biossintéticos (produção de hormônio tireoidiano, por exemplo). Além disso, também são importantes na defesa do hospedeiro contra microrganismos. No entanto, em altas concentrações podem causar dano oxidativo e nitrosativo a biomoléculas, como ácidos nucleicos, proteínas, carboidratos e

lipídeos (Turrens, 2003; Winterbourn, 2008; Lambert e Brand, 2009; Birben et al., 2012; Brieger et al., 2012; Atashi, Modarressi e Pepper, 2015).

A regulação do estado redox ocorre pelo equilíbrio entre a geração de espécies reativas e sua neutralização por sistemas antioxidantes (Atashi, Modarressi e Pepper, 2015). Os sistemas de defesa antioxidantes podem tanto impedir a produção como bloquear espécies reativas já formadas por meio de processos enzimáticos e não-enzimáticos. Além disso, também estão envolvidos no reparo de biomoléculas danificadas (Sies, 1997; Winterbourn, 2008; Pisoschi e Pop, 2015). Certas condições patológicas podem levar ao desequilíbrio entre produção e remoção de espécies reativas, seja pelo aumento da produção ou pela diminuição da capacidade antioxidante, causando estresse oxidativo e podendo acarretar em morte celular por apoptose (Turrens, 2003; Lambert e Brand, 2009; Ray, Huang e Tsuji, 2012; Pisoschi e Pop, 2015).

I. 1.4. METABOLISMO ENERGÉTICO CEREBRAL

O cérebro possui alta demanda metabólica para exercer suas funções e requer um influxo contínuo de substratos sanguíneos, já que apresenta baixa capacidade de reserva energética (Bélanger, Allaman e Magistretti, 2011; Camandola e Mattson, 2017). Em condições normais, a glicose é o principal substrato para o cérebro adulto. No entanto, durante o período neonatal, devido ao alto consumo de gordura durante a lactação, os corpos cetônicos também são usados como fonte energética (Brekke, Morken e Sonnewald, 2015; Magistretti e Allaman, 2015). Transportadores de monocarboxilato (MCTs), que promovem a entrada de corpos cetônicos no cérebro, são muito mais abundantes durante o desenvolvimento, uma vez que esses nutrientes podem fornecer de 30 a 70% da energia necessária nesse período (Brekke, Morken e Sonnewald, 2015; Camandola e Mattson, 2017).

Apesar de o cérebro possuir outras fontes de energia, a glicose é indispensável para a síntese de nucleotídeos e produção de NADPH através da via das pentoses fosfato. O

equivalente redutor NADPH é usado tanto para a síntese lipídica como para manter as defesas antioxidantes cerebrais, regenerando glutationa reduzida (GSH) (Brekke, Morken e Sonnewald, 2015; Camandola e Mattson, 2017). A via das pentoses fosfato representa de 5 a 15% do metabolismo da glicose no cérebro de ratos de 7 dias e essa porcentagem é ainda mais significativa em recém-nascidos (Brekke, Morken e Sonnewald, 2015).

O ciclo do ácido cítrico (CAC) é a via final comum da oxidação de aminoácidos, triacilgliceróis, carboidratos e corpos cetônicos. Esse processo ocorre na matriz mitocondrial e produz, dentre outras moléculas, coenzimas reduzidas (NADH e $FADH_2$) que doam elétrons para complexos da CTE incorporados na membrana mitocondrial interna (MMI). A transferência de elétrons entre os complexos, com o consumo de O_2, permite a formação de um gradiente eletroquímico de prótons que impulsiona a síntese de ATP pela ATP sintase (Akram, 2013; Nelson e Cox, 2017; Van der Bliek, Sedensky e Morgan, 2017). O acoplamento entre a CTE e a fosforilação oxidativa gera grande parte do ATP necessário para o cérebro (Erecińska e Silver, 1994; Nelson e Cox, 2017).

I. 1.4.1. Creatina cinase (CK)

A CK está presente em tecidos com alta demanda energética, como o cérebro, e catalisa a transferência reversível de um fosfato do ATP para a creatina, produzindo ADP e fosfocreatina (Wendt, Schlattner e Wallimann, 2002). Essa enzima apresenta diferentes isoformas, três citosólicas e duas mitocondriais (Wang et al., 2001; Wallimann, Tokarska-Schlattner e Schlattner, 2011), sendo que, nos tecidos, uma isoforma citosólica é sempre co-expressada com uma isoforma mitocondrial. Assim, a CK participa da homeostase energética celular, podendo regenerar ATP prontamente e transportar energia intracelular de sítios de produção de ATP (fosforilação oxidativa mitocondrial) para sítios de consumo (por proteínas

motoras, bomba de íons, etc.) (Wendt, Schlattner e Wallimann, 2002; Wallimann, Tokarska-Schlattner e Schlattner, 2011; Schlattner et al., 2016).

I. 1.5. DINÂMICA MITOCONDRIAL E MITOFAGIA

As mitocôndrias são organelas altamente dinâmicas, capazes de se fragmentar e de formar redes hipertubulares através de processos de fissão e fusão, respectivamente, para atender as necessidades metabólicas das células (Bertholet et al., 2016; Lee e Yoon, 2016; Wai e Langer, 2016; Yoo e Jung, 2018). A morfologia das mitocôndrias está diretamente associada às suas funções: produção de ATP, biossíntese de esteroides, regulação da morte celular programada, homeostase do cálcio, geração e controle de EROs, dentre outras (Lee e Yoon, 2016; Wai e Langer, 2016). Enquanto redes mitocondriais parecem ser mais eficientes na geração de ATP, a fragmentação mitocondrial pode permitir a distribuição de mitocôndrias na célula antes da divisão celular, facilitar a apoptose através da liberação de citocromo *c* e isolar mitocôndrias disfuncionais para posterior eliminação via mitofagia. Por outro lado, uma mitocôndria danificada também pode ser recuperada através da fusão com uma mitocôndria viável, tendo seus componentes danificados ou mtDNA mutado compensados por componentes funcionais (Bertholet et al., 2016; Wai e Langer, 2016; Meyer, Leuthner e Luz, 2017).

As principais proteínas envolvidas nos processos de dinâmica mitocondrial são GTPases da família da dinamina. A proteína relacionada à dinamina 1 (DRP1) medeia a fissão mitocondrial ao ser recrutada do citosol para a superfície mitocondrial, onde se oligomeriza formando anéis de constrição em locais de divisão. Já as mitofusinas, em suas duas isoformas (MFN1 e MFN2), encontram-se ancoradas à membrana mitocondrial externa (MME) e realizam a fusão destas membranas por meio de interações homo (MFN1-MFN1 e MFN2-MFN2) ou heterotípicas (MFN1-MFN2). A proteína atrófica óptica 1 (OPA1), por sua vez,

realiza a fusão da MMI (Chan, 2012; Lee e Yoon, 2016; Pernas e Scorrano, 2016; Wai e Langer, 2016).

Assim como a fissão e a fusão, a mitofagia também age para manter a homeostase mitocondrial e celular (Vásquez-Trincado et al., 2016). Esse processo tem um importante papel no controle de qualidade mitocondrial, sendo responsável pelo reconhecimento e remoção de mitocôndrias danificadas (Um e Yun, 2017; Guan et al., 2018; Yoo e Jung, 2018). Uma das vias de reconhecimento de mitocôndrias danificadas para posterior degradação é a da cinase 1 induzida por PTEN (PINK1) - Parkin.

Em condições normais, a PINK1 é direcionada para as mitocôndrias, onde é rapidamente degradada por peptidases e proteases. Todavia, a despolarização da membrana mitocondrial que ocorre em mitocôndrias danificadas impede a importação de PINK1 através da MMI, levando ao seu acúmulo na MME e consequente recrutamento de Parkin do citosol. A PINK1 ativa então a Parkin por fosforilação, a qual ubiquitina proteínas mitocondriais, promovendo a degradação proteossomal e o recrutamento de receptores de autofagia que direcionam as mitocôndrias danificadas para os fagóforos (Ashrafi e Schwarz, 2012; Durcan e Fon, 2015; Vásquez-Trincado et al., 2016; Um e Yun, 2017; Yoo e Jung, 2018). O fagóforo envolve a organela formando uma estrutura de membrana dupla, chamada autofagossomo. Por fim, o autofagossomo se funde ao lisossomo para formar um autolisossomo, onde ocorre a degradação hidrolítica do conteúdo do autofagossomo (Ashrafi e Schwarz, 2012; Vásquez-Trincado et al., 2016; Um e Yun, 2017; Guan et al., 2018).

I. 1.6. PROTEÍNAS CINASES ATIVADAS POR MITÓGENOS (MAPKs)

As MAPKs compreendem uma família de serina/treonina cinases que, frente a diversos estímulos extracelulares, são ativadas por uma cascata fosforilativa de transdução de sinal e passam a fosforilar alvos específicos (fatores de transcrição, fosfolipases, proteínas associadas

a microtúbulos - MAPs, proteínas pró e anti-apoptóticas, etc.), levando a uma resposta adaptativa celular (Chico, Van Eldik e Watterson, 2009; Danquah et al., 2014; Kim e Choi, 2015; Sun e Nan, 2016). Elas estão envolvidas em diversos processos celulares como proliferação, diferenciação, sobrevivência, apoptose, inflamação e imunidade inata (Kim e Choi, 2015). Três subfamílias de MAPKs são mais estudadas: a cinase regulada por sinalização extracelular 1/2 (ERK1/2), a cinase C-Jun N-terminal (JNK) e a cinase p38 (Sun e Nan, 2016; Du et al., 2019). Enquanto a via de sinalização da ERK 1/2 vem sendo principalmente relacionada à proliferação e diferenciação celular, as vias da p38 e da JNK são principalmente relacionadas ao estresse e à apoptose celular (Du et al., 2019; Guo et al., 2020).

I. 1.7. PROTEÍNA TAU

A Tau é uma fosfoproteína pertencente à família de MAPs, expressa principalmente em neurônios. Está envolvida na montagem e estabilização dos microtúbulos, constituintes do citoesqueleto que são fundamentais para a manutenção da forma celular, mitose e transporte axonal. Além disso, a Tau também promove ligações entre microtúbulos e outros elementos do citoesqueleto, organelas e membrana plasmática. A interação da Tau com os microtúbulos é altamente dinâmica e regulada negativamente por fosforilação. Alterações na quantidade ou na estrutura dessa proteína podem afetar a organização celular e provocar danos patológicos (Buée et al., 2000; Avila et al., 2004; Mandelkow e Mandelkow, 2012; Ferrari e Rüdiger, 2018; Goedert, 2018).

I. 2. OBJETIVOS

I. 2.1. OBJETIVO GERAL

Visando contribuir para o esclarecimento dos mecanismos neurotóxicos do sulfito na ISOD e na MoCD, o objetivo deste trabalho foi investigar os efeitos *in vivo* desse metabólito sobre a homeostase redox e mitocondrial e sobre proteínas de sinalização celular em córtex cerebral de ratos neonatos (1 dia de vida), bem como os efeitos *in vitro* sobre a homeostase redox, metabolismo energético e viabilidade celular em culturas primárias de astrócitos corticais.

I. 2.2. OBJETIVOS ESPECÍFICOS

Foram avaliados os efeitos do sulfito em córtex cerebral de ratos neonatos sobre:

a) Os níveis de GSH e atividade das enzimas antioxidantes superóxido dismutase (SOD), glutationa peroxidase (GPx), glutationa S-transferase (GST), glicose-6-fosfato desidrogenase (G6PDH) e glutationa redutase (GR);

b) A atividade da CK, de enzimas do CAC (citrato sintase – CS, succinato desidrogenase - SDH e MDH) e dos complexos II, II-III e IV da CTE;

c) O imunoconteúdo de heme oxigenase-1 (HO-1), MFN1, DRP1 e PINK1;

d) O imunoconteúdo e grau de fosforilação de ERK1/2, p38, JNK e Tau;

Também foram estudados os efeitos do sulfito sobre a oxidação de DCFH, a concentração de lactato extracelular e a redução de brometo de metiltiazolildifenil-tetrazólio (MTT) em culturas primárias de astrócitos corticais.

PARTE II

II. CAPÍTULO I

High levels of sulfite induce bioenergetic impairment and redox imbalance in brain of neonatal rats and primary astrocytes

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PARTE III

III.1. DISCUSSÃO

A ISOD e a MoCD são doenças metabólicas hereditárias que afetam principalmente o sistema nervoso central (SNC) (Tan et al., 2005; Relinque et al., 2015; Atwal e Scaglia, 2016; Schwarz, 2016; Durmaz e Özbakir, 2018). Geralmente se manifestam de forma grave e precoce, com crises convulsivas intratáveis logo nas primeiras horas ou dias de vida e encefalopatia de rápida progressão (Johnson, 2003; Basheer et al., 2007; Rocha et al., 2014; Bindu et al., 2017; Cornet, Sands e Cilio, 2018; Durmaz e Özbakir, 2018). Exames neurológicos em pacientes evidenciam alterações em diferentes regiões cerebrais, incluindo anormalidades corticais como lesões císticas, aumento dos sulcos decorrente de atrofia, edema, perda neuronal massiva e gliose (Rocha et al., 2014; Zaki et al., 2016; Bindu et al., 2017; Lee et al., 2017; Cornet, Sands e Cilio, 2018; Durmaz e Özbakir, 2018; Alonzo Martínez et al., 2020).

Essas doenças são raras e pouco diagnosticadas, o que contribui para a dificuldade no entendimento de seus mecanismos fisiopatológicos (Bindu et al., 2017; Durmaz e Özbakir, 2018; Alonzo Martínez et al., 2020). Contudo, diversos estudos apontam para uma ação neurotóxica do sulfito (Zhang et al., 2004; Chiarani et al., 2007; Kocamaz et al.; 2012; Grings et al., 2013; Grings et al., 2014; Relinque et al., 2015; de Moura Alvorcem et al., 2017; Grings et al., 2017). No entanto, embora a maioria dos pacientes apresente os sintomas neurológicos graves logo após o nascimento, até onde sabemos, não foram realizados estudos com o objetivo de elucidar os patomecanismos nesse período da vida. Desse modo, no presente trabalho, avaliamos primeiramente os efeitos da administração de sulfito sobre a homeostase redox e mitocondrial, bem como sobre proteínas de sinalização celular em córtex cerebral de ratos neonatos. O córtex, por apresentar maior expressão de SO do que outras regiões cerebrais, pode desempenhar um papel crítico no metabolismo de sulfito cerebral e, consequentemente, ser mais suscetível ao seu acúmulo (Woo et al., 2003; Tan et al., 2005).

Inicialmente, observamos que o sulfito diminuiu a concentração de GSH cortical, o tiol de baixo peso molecular mais abundante nas células. Devido ao seu resíduo de cisteína, a GSH é prontamente oxidada por substâncias eletrofílicas, sendo considerada a principal defesa antioxidante do cérebro (Wu et al., 2004; Forman, Zhang e Rinna, 2009; Bélanger, Allaman e Magistretti, 2011; Schwarz, 2016). Dessa forma, uma diminuição de seus níveis pode indicar uma inabilidade do córtex cerebral para combater o aumento de espécies reativas induzido pelo sulfito (Izgüt-Uysal et al., 2005; Kocamaz et al., 2012; Grings et al., 2013; de Moura Alvorcem et al., 2017). Além disso, a formação de S-sulfocisteína a partir de sulfito poderia estar diminuindo a disponibilidade de cistina e impactando a síntese de GSH, exacerbando o desequilíbrio redox (Schwarz, 2016). Nosso achado corrobora com outros estudos *in vivo* em cérebro de ratos, onde o sulfito diminuiu os níveis de GSH em córtex cerebral de ratos deficientes para SO (Grings et al., 2016) e em estriado de ratos normais (Grings et al., 2017).

Em seguida, ao avaliarmos a atividade das enzimas antioxidantes, verificamos que o sulfito diminuiu a atividade da GST, enzima que catalisa a conjugação de compostos eletrofílicos, como peróxidos orgânicos, à GSH (Lu, 2013; Espinosa-Diez et al., 2015; Ruszkiewicz e Albrecht, 2015). Levando em consideração que, além de atuar na remoção de espécies reativas de forma direta, a GSH também serve como substrato para a GST (Bélanger, Allaman e Magistretti, 2011), a diminuição de seus níveis induzida por sulfito poderia contribuir ainda mais para a diminuição observada na atividade dessa enzima. Nesse sentido, Shen et al. (1993) demonstraram que o H_2O_2 pode reagir com resíduos de cisteína da GST, levando à modificação de sua estrutura e consequente inativação, ao passo que, Grings et al. (2013) e de Moura Alvorcem et al. (2017) evidenciaram que o sulfito induz a geração dessa ERO. Por outro lado, o sulfito poderia estar ocasionando a lise de glutationa oxidada (GSSG), que resulta na formação de glutationa S-sulfonato, um potente inibidor da atividade desta enzima (Menzel, Keller e Leung, 1986). Uma diminuição na atividade da GST pelo sulfito

também foi evidenciada *in vivo* em estriado de ratos normais (Grings et al., 2017) e em córtex e cerebelo de ratos deficientes para a SO (Grings et al., 2016), bem como *in vitro* em diversas estruturas cerebrais de ratos (Parmeggiani et al., 2015; de Moura Alvorcem et al., 2017). Por outro lado, a atividade das enzimas SOD, GPx, G6PDH e GR não foram alteradas no nosso modelo.

A HO-1 é considerada uma defesa antioxidante pois, ao degradar heme livre, impede que ele participe de reações pró-oxidantes. Assim, por gerar EROs, o heme livre é potencialmente tóxico e sua concentração deve ser controlada para a manutenção da homeostase celular. A HO-1 é a isoforma induzível da enzima, que responde a estímulos relacionados ao estresse. Além do heme, seu indutor prototípico, a HO-1 também é estimulada por oxidantes, mediadores inflamatórios, metais pesados, radiação UV, endotoxinas, hormônios, entre outros. O heme é um complexo formado por ferro e protoporfirina IX que serve como grupo prostético para diversas hemoproteínas (hemoglobina, mioglobina, citocromos, CAT, GPx, óxido nítrico sintases, etc.) (Choi e Alam, 1996; Ryter e Tyrrell, 2000; Chiang, Chen e Chang, 2018; Szabo et al., 2018; Vijayan, Wagener e Immenschuh, 2018). Dessa forma, sabendo que o aumento de grupamentos heme livres pode ocorrer pelo ataque oxidativo à estrutura de hemoproteínas (Ryter e Tyrrell, 2000), acreditamos que o aumento no conteúdo de HO-1 induzido pelo sulfito nesse estudo possa representar uma estratégia de citoproteção como consequência do desequilíbrio redox gerado por esse metabólito.

Considerando que a SO é uma enzima mitocondrial, pode-se presumir que sua deficiência gere altos níveis de sulfito nessa organela (Rupar et al., 1996). Logo, avaliamos os efeitos desse composto sobre a atividade de enzimas do CAC e complexos da cadeia respiratória, a principal maquinaria energética da célula. Observamos que o sulfito diminuiu a atividade da SDH e dos complexos II e II-III da CTE. Esses resultados indicam um possível prejuízo mitocondrial na produção de coenzimas reduzidas e na transferência de elétrons através

da cadeia respiratória, que poderiam estar acarretando em redução do $\Delta\Psi m$, do consumo de O_2 e da produção de ATP, observados em outros estudos (Zhang et al., 2004; Grings et al., 2014; Grings et al., 2019). De acordo com isso, um aumento do metabolismo glicolítico como mecanismo compensatório da disfunção mitocondrial poderia explicar o aumento de lactato observado em pacientes com a deficiência da SO (Teksam, Yurdakok e Coskun, 2005; Basheer et al., 2007; Holder et al., 2014; Lee et al., 2017; Alonzo Martínez et al., 2020). As atividades da CS, MDH e do complexo IV não foram modificadas nesse estudo.

Outro componente importante para a homeostase energética celular é a atividade da CK. Foi demonstrado que o sulfito diminuiu essa atividade enzimática, o que também já havia sido observado *in vitro* em diversas estruturas cerebrais (Grings et al., 2013; de Moura Alvorcem et al., 2017) e *in vivo* em estriado de ratos (Grings et al., 2017), indicando que esse metabólito prejudica a transferência e o tamponamento de ATP celular. Acreditamos que a produção de espécies reativas desencadeada pelo sulfito possa estar contribuindo para a oxidação de resíduos de aminoácidos da CK, como a cisteína do sítio ativo da enzima, que é bastante vulnerável a oxidantes (Wendt, Schlattner e Wallimann, 2002; Li et al., 2011).

O comprometimento da fosforilação oxidativa e a produção de EROs vem sendo associados a alterações na dinâmica mitocondrial. Tanto um prejuízo no processo de fusão como um aumento na taxa de fissão favorecem a fragmentação da rede mitocondrial. A fragmentação mitocondrial, dentre outras funções, facilita a remoção de mitocôndrias danificadas, isolando-as para posterior depuração autofágica (mitofagia) (Wai e Langer, 2016; Sprenger e Langer, 2019). Nesse sentido, considerando o distúrbio energético e redox ocasionado pelo sulfito, decidimos avaliar o imunoconteúdo de proteínas relacionadas à dinâmica mitocondrial (MFN1 e DRP1) e mitofagia (PINK1), porém não observamos alterações. Apesar disso, experimentos anteriores realizados em fibroblastos de um paciente com a MoCD constataram a diminuição de MFN1 e MFN2 e o aumento de DRP1 (Grings et

al., 2019), mostrando o envolvimento desses mecanismos na doença. Desse modo, acreditamos que o fato de não termos observado nenhuma alteração nesses parâmetros possa estar relacionado à janela temporal avaliada no nosso modelo. Pretendemos dar seguimento a esses estudos, verificando o conteúdo dessas proteínas em diferentes tempos de exposição ao sulfito.

Alterações nas vias das MAPKs têm sido relacionadas à patogênese de diversas doenças, uma vez que estas cinases respondem à uma grande variedade de estímulos como neurotransmissores, hormônios, fatores de crescimento, fatores inflamatórios e condições de estresse, estando envolvidas na regulação de muitas funções celulares (Kim e Choi, 2015; Sun e Nan, 2016). Nesse sentido, buscamos elucidar se o sulfito altera a sinalização das MAPKs, determinando o imunoconteúdo e grau de fosforilação de ERK1/2, p38 e JNK. Nossos dados mostram que a administração de sulfito induziu o aumento do imunoconteúdo de ERK1/2 e p38, possivelmente por aumentar a expressão gênica dessas proteínas. Apesar disso, não observamos uma maior atividade nem de ERK1/2 nem de p38, pois a fosforilação de ambas não foi alterada. Especulamos que a maior expressão de MAPK fosfatases (MKPs) ou a inibição de cinases relacionadas a essas vias possam estar mantendo a fosforilação dessas proteínas normais.

Exames neurológicos de pacientes com a ISOD e a MoCD revelam, dentre outros achados, perda neuronal massiva (Hobson et al., 2005; Bindu et al., 2011; Carmi-Nawi et al., 2011; Lee et al., 2017) e estudos em modelos animais mostram que o sulfito leva à perda de neurônios em estriado e hipocampo de ratos (Kocamaz et al., 2012; Grings et al., 2017). Devido ao envolvimento da Tau com o dano neuronal em diversas doenças neurodegenerativas (Buée et al., 2000; Avila et al., 2004; Ferrari e Rüdiger, 2018) , buscamos verificar se alterações nessa proteína poderiam estar relacionadas com a morte neuronal observada em córtex cerebral de pacientes afetados pela ISOD e MoCD (Rupar et al., 1996; Lee et al., 2017). Contudo, ao determinarmos seu imunoconteúdo e grau de fosforilação, não vimos mudanças significativas.

Apesar de a Tau não ter sido modificada pelo sulfito, outros marcadores de dano neuronal, tais como marcação da proteína nuclear específica de neurônios (NeuN), conteúdo de sinaptofisina e de caspases, além de experimentos em culturas primárias de neurônios, devem ainda ser realizados.

Os astrócitos são células gliais importantes para o metabolismo energético, defesa antioxidante, resposta inflamatória, homeostase de íons, excitabilidade neuronal, plasticidade sináptica, dentre outras funções no SNC (Bélanger, Allaman e Magistretti, 2011; Sun et al., 2017). Visto que indivíduos com deficiência da SO também apresentam gliose (Zaki et al., 2016; Lee et al., 2017; Durmaz e Özbakir, 2018) e a fim de melhor avaliar os efeitos do sulfito sobre a homeostase redox e energética cerebral, submetemos culturas primárias de astrócitos corticais a incubações de 6 e 24 h com sulfito (100, 500 ou 1.000 µM) e investigamos a oxidação de DCFH, os níveis de lactato extracelular e a redução de MTT.

Primeiramente, observamos que o sulfito, na concentração de 1.000 µM, aumentou a geração de EROs após 6 h de exposição. No cérebro, os astrócitos apresentam uma capacidade antioxidante significativamente maior em relação aos neurônios, apesar de ser nos neurônios que ocorra grande parte do metabolismo oxidativo cerebral, que é uma importante fonte de EROs (Bélanger, Allaman e Magistretti, 2011). Sabendo que astrócitos são mais resistentes ao dano celular por pró-oxidantes (Bélanger, Allaman e Magistretti, 2011), nosso achado sugere que o sulfito é altamente tóxico para o córtex cerebral. Avaliamos ainda a oxidação de DCFH em um período maior de incubação (24 h), porém não observamos mais alterações, o que pode ter ocorrido devido à metabolização do sulfito ou à indução de genes antioxidantes nos astrócitos.

Em seguida, observamos que o sulfito (1.000 µM) aumentou a liberação de lactato após 24 h de exposição, ou seja, acelerou o metabolismo glicolítico astrocitário. Em condições fisiológicas, devido aos padrões de expressão gênica, os astrócitos apresentam um perfil

predominantemente glicolítico e grande parte da glicose utilizada por essas células é liberada no espaço extracelular na forma de lactato. Por outro lado, os neurônios têm um metabolismo predominantemente oxidativo e usam o lactato para suprir suas necessidades energéticas, alimentando o CAC e a CTE, levando à produção de ATP via fosforilação oxidativa. Além de atender as demandas energéticas neuronais, visto a complementariedade metabólica existente entre astrócitos e neurônios, o lactato também age como uma molécula sinalizadora, sendo capaz de modular processos como excitabilidade e plasticidade neuronal e neuroproteção. É importante ressaltar ainda que, apesar do metabolismo glicolítico predominar em astrócitos, eles também são capazes de oxidar totalmente a glicose, do mesmo modo que neurônios também realizam glicólise, porém em um grau menor (Bélanger, Allaman e Magistretti, 2011; Magistretti e Allaman, 2015; Magistretti e Allaman, 2018). Nesse sentido, acreditamos que o aumento nos níveis de lactato astrocitário possa ser reflexo do prejuízo que o sulfito exerce no metabolismo mitocondrial, como previamente demonstrado neste trabalho.

Quanto à redução de MTT, não foram vistas alterações após 24 h de exposição, indicando que o sulfito não altera a viabilidade celular de astrócitos corticais. Do mesmo modo, Parmeggiani et al. (2015) também não observaram alterações na redução de MTT em fatias de córtex cerebral após 1 h de incubação com sulfito.

Se analisados em conjunto os resultados *in vivo* e *in vitro* desse trabalho, pode-se concluir que eles são complementares. Enquanto verificamos que o sulfito alterou as defesas antioxidantes *in vivo*, o aumento da geração de EROs foi demonstrado *in vitro*. Do mesmo modo, a inibição da atividade de enzimas do metabolismo oxidativo *in vivo* corrobora com a aceleração do metabolismo glicolítico *in vitro*. Esses achados indicam que o sulfito causa um desequilíbrio redox e energético, o que pode estar contribuindo para as anormalidades corticais observadas em recém-nascidos com deficiência da SO.

III.2. CONCLUSÕES

- O sulfito diminuiu os níveis de GSH e a atividade da enzima GST, mas aumentou o imunoconteúdo de HO-1, indicando alteração nas defesas antioxidantes em córtex cerebral de ratos neonatos;
- O sulfito diminuiu a atividade da enzima SDH e dos complexos II e II-III da CTE, indicando que prejudica a produção mitocondrial de coenzimas reduzidas e a transferência de elétrons através da CTE em córtex cerebral de ratos neonatos;
- O sulfito diminuiu a atividade da enzima CK, sugerindo um dano na transferência e no tamponamento de ATP celular em córtex cerebral de ratos neonatos;
- O sulfito aumentou o imunoconteúdo de ERK 1/2 e p38, indicando que induz maior expressão gênica dessas proteínas em córtex cerebral de ratos neonatos;
- O sulfito, na concentração de 1.000 μM, aumentou a oxidação de DCFH em cultura primária de astrócitos corticais após 6 h de incubação, indicativo de aumento na produção de EROs;
- O sulfito, na concentração de 1.000 μM, aumentou os níveis de lactato extracelular em cultura primária de astrócitos corticais após 24 h de incubação, indicando indução do metabolismo glicolítico astrocitário;
- Nossos dados indicam que alterações na homeostase redox e energética estão envolvidas na disfunção neurológica encontrada no período neonatal em doenças com acúmulo de sulfito.

III.3. PERSPECTIVAS

- Avaliar o conteúdo de MFN1, DRP1 e PINK1, bem como de outras proteínas envolvidas na dinâmica mitocondrial e mitofagia em diferentes tempos de exposição dos animais neonatos ao sulfito administrado por via icv;
- Avaliar os efeitos da injeção icv de sulfito sobre a marcação de NeuN e o conteúdo de sinaptofisina e de caspases em ratos neonatos;
- Avaliar os efeitos *in vitro* do sulfito sobre a homeostase redox e energética em culturas de neurônios;
- Avaliar os efeitos da injeção icv de sulfito sobre a marcação da proteína glial fibrilar ácida (GFAP) e da molécula adaptadora de ligação ao cálcio ionizado 1 (Iba1) em ratos neonatos;
- Avaliar os efeitos da injeção icv de tiossulfato sobre a homeostase redox e mitocondrial e sobre a via das MAPKs em ratos neonatos;
- Avaliar os efeitos *in vitro* do tiossulfato sobre a homeostase redox e energética em culturas de astrócitos.

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ANEXO I

Molecular Neurobiology - Submission guidelines

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2. This result was later contradicted by Becker and Seligman [5].

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Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

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• Book

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• Book chapter

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• Images such as x rays, laparoscopic images, ultrasound images, brain scans, pathology slides unless there is a concern about identifying information in which case, authors should ensure that consent is obtained.

• Reuse of images: If images are being reused from prior publications, the Publisher will assume that the prior publication obtained the relevant information regarding consent. Authors should provide the appropriate attribution for republished images.

Consent and already available data and/or biologic material

Regardless of whether material is collected from living or dead patients, they (family or guardian if the deceased has not made a pre-mortem decision) must have given prior written consent. The aspect of confidentiality as well as any wishes from the deceased should be respected.

Data protection, confidentiality and privacy

When biological material is donated for or data is generated as part of a research project authors should ensure, as part of the informed consent procedure, that the participants are made aware what kind of (personal) data will be processed, how it will be used and for what purpose. In case of data acquired via a biobank/biorepository, it is possible they apply a broad consent which allows research participants to consent to a broad range of uses of their data and samples which is regarded by research ethics committees as specific enough to be considered "informed". However, authors should always check the specific biobank/biorepository policies or any other type of data provider policies (in case of non-bio research) to be sure that this is the case.

Consent to Participate

For all research involving human subjects, freely-given, informed consent to participate in the study must be obtained from participants (or their parent or legal guardian in the case of children under 16) and a statement to this effect should appear in the manuscript. In the case of articles describing human transplantation studies, authors must include a statement declaring that no organs/tissues were obtained from prisoners and must also name the institution(s)/clinic(s)/department(s) via which organs/tissues were obtained. For manuscripts reporting studies involving vulnerable groups where there is the potential for coercion or where consent may not have been fully informed, extra care will be taken by the editor and may be referred to the Springer Nature Research Integrity Group.

Consent to Publish

Individuals may consent to participate in a study, but object to having their data published in a journal article. Authors should make sure to also seek consent from individuals to publish their data prior to submitting their paper to a journal. This is in particular applicable to case studies. A consent to publish form can be found
here. (Download docx, 36 kB)

Summary of requirements

The above should be summarized in a statement and placed in a 'Declarations' section before the reference list under a heading of 'Consent to participate' and/or 'Consent to publish'. Other declarations include Funding, Conflicts of interest/competing interests, Ethics approval, Consent, Data and/or Code availability and Authors' contribution statements.

Please see the various examples of wording below and revise/customize the sample statements according to your own needs.

Sample statements for **"Consent to participate":**

Informed consent was obtained from all individual participants included in the study.

Informed consent was obtained from legal guardians.

Written informed consent was obtained from the parents.

Verbal informed consent was obtained prior to the interview.

Sample statements for **"Consent to publish":**

The authors affirm that human research participants provided informed consent for publication of the images in Figure(s) 1a, 1b and 1c.

The participant has consented to the submission of the case report to the journal.

Patients signed informed consent regarding publishing their data and photographs.

Sample statements if identifying information about participants is available in the article:

Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

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