UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:

BIOQUÍMICA

# ALTERAÇÕES BIOQUÍMICAS, MOLECULARES, HISTOLÓGICAS E COMPORTAMENTAIS NA PROLE DE RATAS WISTAR SUBMETIDAS À HIPERMETIONINEMIA GESTACIONAL

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ORIENTADORA

Prof ª Drª Angela Terezinha de Souza Wyse

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito para a obtenção do título de Doutora em Bioquímica.

**Porto Alegre, 2017**

Dedico este trabalho às pessoas mais importantes da minha vida, que com muito amor me deram a base necessária para meu crescimento pessoal e profissional

Aos meus pais, Nara e Geraldo,

Às minhas irmãs, Carla e Cristiane.

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“Apesar dos nossos defeitos, precisamos enxergar que somos pérolas únicas no teatro da vida e entender que não existem pessoas de sucesso ou pessoas fracassadas. O que existe são pessoas que lutam pelos seus sonhos ou desistem deles.”

Augusto Cury

**SUMÁRIO**

**RESUMO**

A hipermetioninemia é uma condição caracterizada por altos níveis de metionina no sangue e em outros tecidos, podendo causar danos neurológicos, hepáticos e musculares. Considerando que a placenta transfere a metionina do sangue materno para a circulação fetal e que pouco se sabe sobre o efeito da hipermetioninemia gestacional sobre o feto em desenvolvimento, o principal objetivo deste trabalho foi desenvolver um modelo animal de hipermetioninemia materna quimicamente induzido em ratas e utilizar o mesmo para investigar parâmetros bioquímicos (estresse oxidativo, atividade da $Mg^{2+}$-ATPase, atividade e imunoconteúdo da $Na^+,K^+$-ATPase, número de neurônios, níveis de neurotrofinas, metabolismo energético, inflamação e apoptose), moleculares (expressão gênica da $Na^+,K^+$-ATPase) e histológicos (microscopia eletrônica) nos encéfalos da prole, bem como avaliar tarefas comportamentais (campo aberto, esquiva inibitória e reconhecimento de objetos). Também analisamos parâmetros de estresse oxidativo/nitrosativo no músculo esquelético e parâmetros de dano muscular e inflamação no soro da prole. A hipermetioninemia foi induzida em ratas através de duas injeções subcutâneas diárias de metionina durante todo o período gestacional. Um grupo de ratas recebeu a dose 1 (1,34 µmol/g peso corporal) e outro recebeu a dose 2 (2,68 µmol/g peso corporal). O grupo controle recebeu salina. Após o nascimento, um grupo de filhotes foi eutanasiado no sétimo dia de vida e outro grupo foi eutanasiado aos 21 dias. Ambas as doses aumentaram os níveis encefálicos de metionina das mães e a dose 2 aumentou os níveis de metionina nos encéfalos da prole. Após estabelecer o modelo, a dose 2 de metionina foi escolhida para estudar os efeitos do tratamento sobre a prole. Os testes bioquímicos subsequentes foram realizados nos filhotes de 21 dias, a histologia foi realizada na prole de 21 e 30 dias e os testes comportamentais foram realizados em filhotes de 30 dias. Os resultados demonstraram que a hipermetioninemia materna reduziu a atividade da $Na^+,K^+$-ATPase, $Mg^{2+}$-ATPase, catalase e complexo II/succinato desidrogenase, o conteúdo de sulfidrilas, número de neurônios e níveis de NGF e BDNF, bem como aumentou os níveis de RNAm e imunoconteúdo da $Na^+,K^+$-ATPase nos encéfalos dos filhotes. Foram observados também alterações morfológicas, indicativas de degeneração celular nos neurônios da prole, e os testes comportamentais indicaram deficit de memória. Com relação aos danos musculares, houve um aumento na produção de espécies reativas de oxigênio e lipoperoxidação e uma redução do conteúdo de sulfidrilas, atividades das enzimas antioxidantes e nos níveis de nitritos no músculo esquelético da prole. A atividade da creatina cinase foi reduzida e os níveis de ureia e proteína C reativa foram aumentados no soro. Esses resultados foram acompanhados por perda de massa muscular. Tais achados mostraram que a hipermetioninemia gestacional induziu alterações bioquímicas, moleculares e histológicas no encéfalo e bioquímicas no músculo esquelético e soro dos filhotes, as quais podem contribuir para o entendimento dos mecanismos fisiopatológicos envolvidos nos danos neurológicos e musculares causados por essa condição. Ressaltamos a importância do desenvolvimento do referido modelo de hipermetioninemia

gestacional que além de ampliar o entendimento da toxicidade de altos níveis metionina, também abriu perspectivas para novos estudos a respeito dos efeitos ocasionados pela exposição ao excesso de metionina devido a uma condição genética ou uma dieta rica em proteína durante a vida pré-natal.

**Palavras-chaves:** Hipermetioninemia Gestacional, Status Oxidativo e Inflamatório, Metabolismo Energético, Ultraestrutura Cerebral, Memória, Fatores Neurotróficos.

**ABSTRACT**

Hypermethioninemia is a condition characterized by elevated levels of methionine in blood and other tissues and may cause neurological, hepatic and muscular damages. Considering that placenta transfers methionine from maternal blood to the fetal circulation and little is known about the effect of gestational hypermetioninemia on the developing fetus, the main objective of this work was to develop a chemically induced animal model of maternal hypermethioninemia in rats and to use it to investigate biochemical (oxidative stress, activity of $Mg^{2+}$-ATPase, activity and immunocontent of $Na^+,K^+$-ATPase, number of neurons, neurotrophins levels, energy metabolism, inflammation, and apoptosis), molecular (gene expression of $Na^+,K^+$-ATPase) and histological parameters (electron microscopy) in encephalon of the offspring, as well as evaluate behavioral tasks (open field, inhibitory avoidance and object recognition). We also analyzed oxidative/nitrosative stress parameters in skeletal muscle and parameters of muscle damage and inflammation in serum of the offspring. Hypermethioninemia was induced in rats through two daily subcutaneous injections of methionine throughout the gestational period. A group of pregnant rats received dose 1 (1.34 µmol/g body weight) and the other received dose 2 (2.68 µmol/g body weight). The control group received saline. After birth, a first group of pups was euthanized at the 7$^{th}$ day of life and the second group at the 21$^{st}$ day of life. Both doses 1 and 2 increased methionine levels in the brain of the mother rats and dose 2 increased methionine levels in encephalon of the offspring. After establishing the experimental model, the highest dose of methionine was chosen to study the effects of treatment on offspring. The subsequent biochemical tests were performed on 21-day-old pups, histological analyses were performed on offspring of 21 and 30 days of age, and behavioral tests were performed on 30-day-old pups. The results demonstrated that maternal hypermethioninemia reduced $Na^+,K^+$-ATPase, $Mg^{2+}$-ATPase, catalase and complex II/succinate dehydrogenase activities, sulfhydryl content, number of neurons and levels of NGF and BDNF, as well as increased levels of mRNA and immunocontent of $Na^+,K^+$-ATPase in the brains of the pups. Morphological changes indicative of cellular degeneration were also observed in offspring neurons, and behavioral tests indicated memory deficit. With regard to muscle damage, there was an increase in the production of reactive oxygen species and lipoperoxidation, and a reduction of the sulfhydryl content, antioxidant enzymes activities and in the levels of nitrites in skeletal muscle of the offspring. Creatine kinase activity was reduced and urea and C-reactive protein levels were increased in serum. These results were accompanied by loss of muscle mass. These findings showed that gestational hypermethioninemia induced biochemical, molecular and histological changes in the brain and biochemical changes in skeletal muscle and serum of pups, which may contribute to the understanding of the pathophysiological mechanisms involved in the neurological and muscular damages caused by this condition. We emphasize the importance of the development of this model of gestational hypermetioninemia that, in addition to increasing the understanding of toxicity of high methionine levels, also opened perspectives for new studies

regarding the effects caused by exposure to excess methionine due to a genetic condition or a diet rich in protein during prenatal life.

**Keywords:** Gestational Hypermethioninemia, Oxidative and Inflammatory Status, Energy Metabolism, Cerebral Ultrastructure, Memory, Neurotrophic Factors.

## LISTA DE FIGURAS

## LISTA DE ABREVIATURAS

5-MTHF – 5-metil tetrahidrofolato

5,10-MTHR – 5, 10-metilenotetra-hidrofolato redutase

ATP – trifosfato de adenosina

BDNF – fator neurotrófico derivado do encéfalo

BHMT – betaína-homocisteína-metiltransferase

CAT – catalase

CBS – cistationina β-sintase

DCF – diclorofluoresceína

DMG – N,N-dimetilglicina

ERO – espécies reativas de oxigênio

GNMT – glicina N-metiltransferase

$H_2O_2$ – peróxido de hidrogênio

IL-6 – interleucina 6

MAT – metionina adenosiltransferase

MS – metionina sintase

NGF – fator de crescimento neural

NO – óxido nítrico

SAHH – S-adenosilhomocisteína hidrolase

SAH – *S*-adenosil homocisteína

SAM – *S*-adenosil metionina

SDH – succinato desidrogenase

SOD – superóxido dismutase

TBARS – substâncias reativas ao ácido tiobarbitúrico

TNF-alfa – fator de necrose tumoral alfa

# 1. INTRODUÇÃO

## 1.1 Metionina e suas funções

A metionina (figura 1) é um aminoácido sulfurado essencial e, portanto, é obtido somente através da dieta e da degradação de proteínas endógenas. A metionina é importante para diversas funções em nosso organismo, incluindo a síntese proteica, uma vez que compõe proteínas e peptídeos e é o aminoácido iniciador no processo de tradução proteica. Além disso, a metionina é precursora de moléculas como a cisteína, glutationa, carnitina, taurina e creatina, além de doar seu grupamento metila para a biossíntese de DNA, RNA, proteínas, fosfolipídios, entre outros. Ainda, a literatura aponta que a metionina é capaz de conferir proteção antioxidante às células, uma vez que pode estar presente na superfície de proteínas protegendo os outros resíduos. As espécies reativas oxidam a metionina que está exposta, formando metionina sulfóxido, a qual pode ser reduzida novamente pela metionina sulfóxido redutase (Fontecave et al., 2004; Belalcázar et al., 2014; Kim et al., 2014).

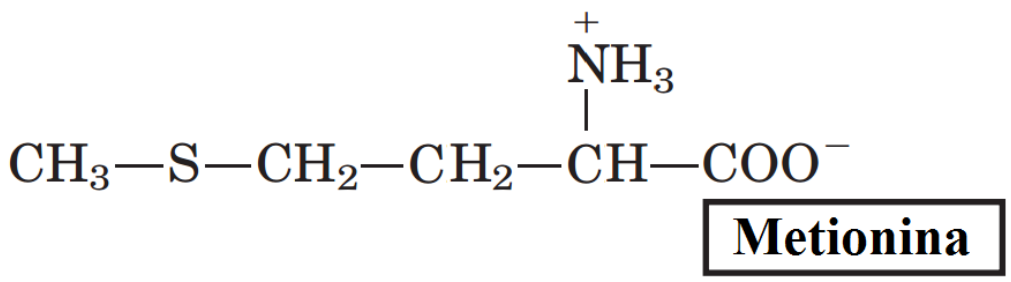


**Figura 1.** Estrutura química da metionina (Nelson & Cox, 2004).

## 1.2 Metabolismo da metionina no fígado

O fígado é o principal órgão responsável pelo metabolismo da metionina e utiliza mais de 70% da metionina da dieta. A metionina adenosiltransferase (MAT, EC 2.5.1.6) catalisa o primeiro passo da via metabólica e apresenta três isoformas. O gene MAT1A codifica as isoformas MAT I e III, que predominam no fígado. A MAT II prevalece em tecidos extra-hepáticos, fígado fetal e carcinoma hepático. A função dessa enzima é transferir o grupo adenosil do ATP para a metionina, formando tripolifosfato e S-adenosilmetionina (SAM), que doa seu grupamento metila em diversas reações de metilação, formando S-adenosilhomocisteína (SAH). A enzima SAH hidrolase (SAHH, EC 3.3.1.1) hidrolisa a SAH e forma homocisteína, que pode ser metabolizada por duas vias diferentes: remetilação ou transulfuração (De La Haba & Cantoni, 1959; Fontecave et al., 2004).

Na remetilação, a homocisteína recebe um grupamento metila proveniente do 5-metiltetrahidrofolato (5-metil-THF) (oriundo do metabolismo do ácido fólico) através de uma reação dependente de vitamina $B_{12}$ que é catalisada pela enzima metionina sintase (MS, EC 2.1.1.13). Uma vez que a homocisteína é remetilada, a metionina é regenerada. Quando toxinas comprometem a ação da MS, outra enzima pode atuar na via de remetilação. Tal enzima é denominada betaína-homocisteína-metiltransferase (BHMT) e transfere o grupo metila da betaína para a homocisteína, formando metionina e N,N-dimetilglicina (DMG) (Finkelstein et al., 1972; Guo et al., 2012).

Na via de transulfuração, uma enzima chamada cistationina *β*-sintase (CBS, EC 4.2.1.22), que usa a vitamina $B_6$ como cofator, atua condensando a homocisteína com a serina através de uma reação que gera cistationina, a qual é então convertida em alfa-cetobutirato e cisteína pela ação da cistationina γ-liase (também dependente de vitamina $B_6$). Dessa forma, a via de transulfuração é considerada uma importante provedora de glutationa, uma vez que esta é formada a partir da cisteína. A glutationa é um tripeptídeo hidrossolúvel que está envolvida em aspectos da homeostase celular e possui um papel essencial na defesa celular contra o estresse oxidativo (Selhub, 1999). O ciclo da metionina/homocisteína é mostrado na figura 2.

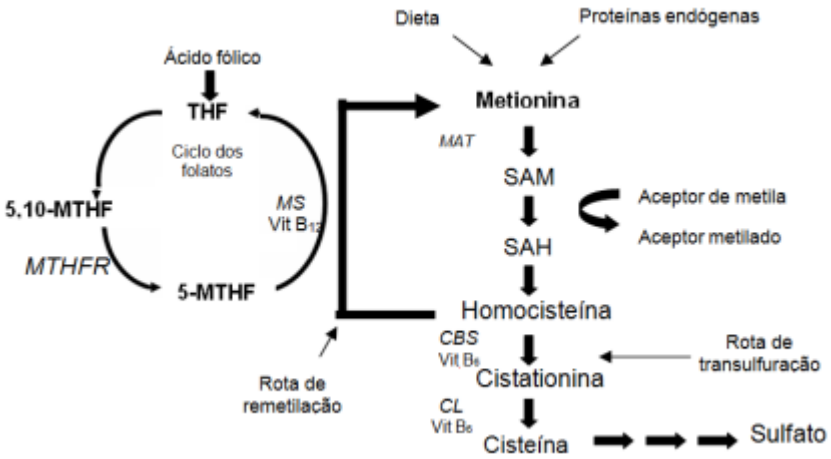


**Figura 2.** Metabolismo da metionina (adaptado de Mudd et al., 2001).

MAT – metionina adenosil transferase; CBS – cistationina β-sintase; CL – cistationina γ-liase; MS – metionina-sintase; MTHFR – metileno tetrahidrofolato redutase; SAM – *S*-adenosil metionina; SAH – *S*-adenosil homocisteína; THF – tetrahidrofolato; 5,10-MTHF – 5,10-metileno-tetrahidrofolato.

### 1.3 Metabolismo da metionina no cérebro

A literatura aponta que a via de remetilação é a principal responsável pelo metabolismo da metionina no tecido cerebral. Até a década de 90, acreditava-se que a via de transulfuração era incompleta no encéfalo devido à falta da enzima cistationina γ-liase, ocasionando o acúmulo de cistationina nesse órgão (Finkelstein, 1998). Porém, no ano de 2006, Vitvitsky e colaboradores publicaram dados demonstrando a existência de uma via de transulfuração funcional em neurônios e astrócitos humanos e em cérebro de rato.

### 1.4 Hipermetioninemia

Os níveis plasmáticos de metionina considerados normais variam de 13 a 45 μM (Stabler et al., 2002) e a hipermetioninemia é caracterizada quando os níveis sanguíneos ultrapassam o limite superior. Essa condição clínica pode ser consequência de fatores não genéticos ou de fatores hereditários. As causas não genéticas incluem: 1) ingestão excessiva de metionina através de uma dieta hiperproteica; 2) doenças hepáticas que levam ao mau funcionamento da enzima MAT I/III; 3) nascimento prematuro devido à maturação tardia da MAT I/III, sendo que a hipermetioninemia é frequentemente transitória nesse caso (Mudd, 2011).

Os fatores hereditários incluem mutações nos genes codificadores das seguintes enzimas envolvidas no metabolismo da metionina: 1) MAT I/III: a metionina acumula, pois não é convertida eficientemente em SAM; 2) SAHH: leva ao acúmulo de SAH, que por sua vez inibe reações de metilação causando o acúmulo de SAM e consequentemente hipermetioninemia; 3) CBS (homocistinúria clássica): aumenta os níveis de homocisteína, cujo excesso é desviado para a via de remetilação, aumentando a regeneração de metionina. Deficiências genéticas das enzimas glicina N-metiltransferase (GNMT) e fumarilacetoacetato hidrolase (tirosinemia tipo I) também são causas de elevação plasmática de metionina (Cacciari & Salardi, 1989; Baric et al., 2004; Mudd, 2011; Chien et al., 2015).

A deficiência de MAT I/III eleva os níveis plasmáticos de metionina para cerca de 600-2.541 μM em pacientes homozigotos e se distingue das demais porque causa hipermetioninemia isolada, ou seja, não está associada à elevação dos níveis plasmáticos dos metabólitos da metionina (SAM, SAH, homocisteína e cistationina), uma vez que se trata da primeira enzima da via de degradação desse aminoácido. Excepcionalmente, níveis levemente elevados de homocisteína podem ser encontrados nas deficiências severas da MAT I/III, mas os mecanismos envolvidos nesse efeito não são conhecidos. Outra característica que diferencia a baixa atividade da MAT I/III é que essa condição causa níveis reduzidos de SAM uma vez que impede sua formação, enquanto que outras causas de hipermetioninemia, como o aumento de metionina na dieta, frequentemente elevam os níveis dessa molécula, a qual pode causar

efeitos patológicos tanto em alta quanto em baixa concentração (Mudd et al., 1995; Chamberlin et al., 1996; Nagao & Oyanagi, 1997).

Embora a metionina seja indispensável para um desenvolvimento normal e exerça importantes funções no organismo, o seu excesso pode ser nocivo e causar os seguintes efeitos patológicos: hemossiderose esplênica, dismorfismo facial, distúrbio digestivos, danos hepáticos, miopatias e problemas neurológicos caracterizados por problemas de aprendizagem e perda de memória (Gout et al., 1977; Guízar et al., 1980; Gaull et al., 1981; Higashi, 1982; Benevenga & Steele, 1984; Lynch & Strain, 1989, Labrune et al., 1990; Chamberlin et al., 1997; Mudd et al., 2001). O presente trabalho enfatizará os danos neurológicos e musculares, os quais foram alvos das pesquisas realizadas neste estudo.

### 1.5 Efeitos neurológicos da hipermetioninemia

Em casos severos, a hipermetioninemia pode causar retardo mental, déficit cognitivo, edema cerebral e problemas no desenvolvimento psicomotor. Esses efeitos foram observados em diferentes condições clínicas, incluindo as deficiências da MAT I/III, CBS e SAHH e em casos de ingestão excessiva de metionina devido a uma dieta hiperproteica. No caso específico da deficiência da MAT I/III, também ocorre desmielinização do sistema nervoso central, uma vez que reduz os níveis de SAM, a qual é utilizada para metilar proteínas que compõem a mielina, estabilizando-a (Mudd et al., 2001; Harvey Mudd et al.,

2003; Baric et al., 2004; Braverman et al., 2005). Embora os mecanismos responsáveis pelos efeitos na aprendizagem e memória não estejam bem esclarecidos, estudos *in vitro* e *in vivo* têm mostrado evidências que a redução da atividade da $Na^+,K^+$-ATPase associada ao aumento do estresse oxidativo local, pode contribuir para os danos neurológicos observados em alguns pacientes hipermetioninêmicos (Streck et al., 2002; Stefanello et al., 2005, 2007a, 2007b, 2007c; Viggiano et al., 2012).

A $Na^+,K^+$-ATPase (EC 3.6.3.9) é uma enzima presente na membrana celular que tem a função de transportar íons $Na^+$ para fora da célula, enquanto transporta íons $K^+$ para o meio intracelular. Tal transporte ocorre contra o gradiente de concentração, sendo que a energia necessária para esse processo é oriunda das moléculas de ATP.

Uma vez que a $Na^+,K^+$-ATPase tem um papel crucial na manutenção do gradiente iônico celular e, portanto, é essencial para a excitabilidade neuronal, sua inibição pode ser extremamente prejudicial para o sistema nervoso. Dentre os danos decorrentes da inativação dessa enzima se encontram edema, morte neuronal, problemas de aprendizado e prejuízo à memória. A redução da atividade da $Na^+,K^+$-ATPase está também envolvida na fisiopatologia de diferentes doenças neurológicas, como por exemplo doença de Alzheimer, desordem bipolar e depressão (Banerjee et al., 2012; de Lores Arnaiz & Ordieres, 2014; Graham et al., 2015).

Alguns estudos têm correlacionado a inibição da $Na^+,K^+$-ATPase cerebral durante a hipermetioninemia com o estresse oxidativo, o qual é

caracterizado por uma condição biológica em que ocorre desequilíbrio entre a produção de espécies reativas de oxigênio (ERO) e a sua detoxificação através de antioxidantes enzimáticos e/ou não-enzimáticos, favorecendo o acúmulo de espécies reativas como os radicais superóxido e hidroxil, e o peróxido de hidrogênio ($H_2O_2$). As ERO podem causar lipoperoxidação, processo no qual ocorre o dano oxidativo aos lipídios insaturados presentes nas membranas celulares. Além disso, as ERO podem levar à inativação de proteínas ao oxidar seus grupamentos sulfidrilas. O excesso de tais moléculas pode ser combatido por defesas antioxidantes enzimáticas, como a superóxido dismutase (SOD) e a catalase (CAT). A SOD tem a função de catalisar a dismutação do radical superóxido em oxigênio e $H_2O_2$. O $H_2O_2$ pode ser então decomposto pela ação da CAT, formando água e oxigênio (Halliwell & Gutteridge, 2007).

Tanto experimentos *in vitro* como estudos em ratos mostraram que a exposição à metionina leva a alterações no estado redox celular do sistema nervoso causando lipoperoxidação e alteração na atividade de algumas enzimas antioxidantes. Uma vez que a $Na^+,K^+$-ATPase está presente na membrana celular, o processo de peroxidação dos lipídios presentes na membrana poderia levar a alterações na sua fluidez e em outras propriedades, causando a redução da atividade dessa enzima. Além disso, o ataque de espécies reativas aos grupamentos sulfidrilas dessa ATPase também poderia prejudicar seu funcionamento. Em concordância, demonstrou-se que a administração de antioxidantes reverte a inibição da enzima *in vitro* (Streck et al., 2002; Stefanello et al., 2005, 2007a, 2007b, 2007c; Viggiano et al., 2012).

A $Mg^{2+}$-ATPase é uma enzima de membrana que também pode ter sua atividade alterada devido ao ataque de ERO aos seus grupamentos sulfidrilas e aos lipídios presentes nas membranas celulares (Shimizu, 1979). Essa enzima participa da manutenção de níveis intracelulares adequados de $Mg^{2+}$, o qual atua como cofator para inúmeras enzimas, incluindo enzimas envolvidas no metabolismo energético, na síntese de proteínas e ácidos nucleicos (Saris et al., 2000). Dessa forma, um prejuízo na atividade da $Mg^{2+}$-ATPase induzido pelo aumento de ERO no cérebro, também pode comprometer o funcionamento das células do sistema nervoso central.

Vale ressaltar que o estresse oxidativo pode ainda levar ao desenvolvimento de quadros inflamatórios agudos. Nestas situações, ocorre a liberação de citocinas, as quais são proteínas que regulam a resposta inflamatória. Dentre as citocinas pró-inflamatórias, se destacam o fator de necrose tumoral alfa (TNF-alfa) e a interleucina 6 (IL-6). No cérebro, o aumento dessas citocinas pode causar apoptose das células neuronais e infiltração leucocitária (Tarkowski et al., 1999). Porém, existem evidências de que os neurônios não são somente alvos do processo inflamatório, mas também podem participar da regulação da resposta imunológica através de uma família de proteínas chamadas de neurotrofinas. O fator neurotrófico derivado do encéfalo (BDNF) e o fator de crescimento neural (NGF) representam umas das principais neurotrofinas, as quais podem ser produzidas por células do sistema nervoso como uma resposta protetora, estando envolvidas na neurogênese,

sobrevivência e maturação dos neurônios durante o desenvolvimento fetal e pós-natal (Jiang et al., 2010).

O aumento da atividade da acetilcolinesterase (EC 3.1.1.7) também parece contribuir para os danos neurológicos durante a hipermetioninemia. Tal enzima está presente em junções neuromusculares e sinapses colinérgicas e tem a função de catalisar a hidrólise do neurotransmissor acetilcolina em colina e ácido acético. Essa reação é necessária para que o neurônio colinérgico retorne ao estado de repouso, evitando assim, a ação excessiva da acetilcolina (Taylor & Radić, 1994).

No ano de 2006, Schulpis e colaboradores demonstraram que a metionina é capaz de induzir a atividade hipocampal da acetilcolinesterase *in vitro*. No ano seguinte, Stefanello e colegas (2007d) submeteram ratos a um modelo de hipermetioninemia e os animais apresentaram aumento na atividade de acetilcolinesterase no córtex cerebral associado a um déficit cognitivo. Em concordância, verificou-se que a exposição à metionina também aumenta a atividade da acetilcolinesterase cerebral em *zebrafish* (Vuaden et al., 2012). Uma vez que essa enzima é responsável pela hidrólise de acetilcolina, a sua estimulação excessiva poderia reduzir os níveis desse neurotransmissor, o qual é crucial para o desenvolvimento das funções cognitivas (memória e aprendizado). Além disso, tem sido relatado que a acetilcolina também pode gerar uma resposta anti-inflamatória relevante e alguns estudos têm correlacionado o aumento da atividade da acetilcolinesterase com o desenvolvimento de neuroinflamação (Scherer et al., 2014; Suzuki, 2016).

Dessa forma, tais efeitos poderiam estar contribuindo para os danos neuropatológicos encontrados em alguns pacientes que apresentam hipermetioninemia.

Excetuando-se em casos de deficiência da MAT I/III, a hipermetioninemia pode levar ao aumento dos níveis cerebrais de SAM, cujo excesso poderia causar hipermetilação do promotor do gene codificador da glicoproteína Relina, reduzindo seus níveis. Tal proteína é de extrema importância, pois estimula o desenvolvimento de espinhos dendríticos, os quais são necessários para a retenção da memória. Em concordância, estudos demonstraram que o tratamento com metionina reduz a densidade de espinhos dendríticos em neurônios piramidais no córtex de camundongos (Grayson et al., 2009).

### 1.6 Efeitos musculares da hipermetioninemia

Embora os efeitos neurológicos sejam os mais estudados em pesquisas científicas, a literatura também aponta que alguns pacientes que apresentam hipermetioninemia podem desenvolver miopatias, apresentando fraqueza e debilidade muscular. Também há relatos de indivíduos que sofrem de redução do tônus muscular (hipotonia), movimentos caracterizados por contrações involuntárias, espasmos (distonia) e tremores, causando uma importante perda na qualidade de vida desses pacientes (Mudd, 2011). Em portadores da deficiência da SAHH, a histologia do músculo ainda evidenciou miopatia

destrutiva lentamente progressiva (Baric, 2009). Porém, ainda há carência de estudos que busquem explicar os mecanismos fisiopatológicos envolvidos nesses efeitos.

### 1.7 Tratamento

A maioria dos pacientes com hipermetioninemia não apresenta sintomas e muitas vezes não necessita de tratamento. Entretanto, em casos severos o tratamento é de extrema importância para evitar danos ao sistema nervoso, fígado e músculo. Em geral, a terapia consiste de restrição de metionina na dieta, porém algumas considerações devem ser levadas em conta no caso da deficiência da MAT I/III. Uma vez que essa condição é detectada em testes de triagem neonatal, o prognóstico dos pacientes ainda é incerto nessa fase. Além disso, os estudos mostram que a medida da atividade da enzima não é o suficiente para determinar se o paciente vai desenvolver ou não retardo mental. Além da dificuldade em saber se há necessidade de tratamento, existem limitações na terapia, pois muitos sintomas dessa desordem genética são consequência da redução nos níveis de SAM e a restrição de metionina poderia diminuir ainda mais seu conteúdo. Devido a isso, recomenda-se também suplementação com SAM em alguns casos (Furujo et al., 2012; Hirabayashi et al., 2013).

## 1.8 Hipermetioninemia gestacional

Durante a gestação, a placenta tem a função de transferir nutrientes para o feto, incluindo aminoácidos, sendo que suas concentrações no sangue fetal humano são mais elevadas do que no sangue materno. A transferência dos aminoácidos envolve transportes mediados nos microvilos e na membrana basal. A metionina, mais especificamente, parece ser transferida por um processo de transporte ativo contra um gradiente químico de concentração (Gaull et al., 1973).

Uma vez que a metionina é transferida através da placenta e é capaz de causar diversos danos ao organismo quando em excesso, a hipermetioninemia materna (uma condição clínica caracterizada por níveis sanguíneos elevados de metionina durante a gestação) poderia causar sérias consequências para o feto. Embora os mecanismos ainda não estejam bem elucidados, estudos em ratos têm sugerido que a ingestão excessiva de metionina por gestantes poderia causar prejuízo ao crescimento fetal, danos a diferentes órgãos e dismorfismo facial na prole (Römer et al., 2012).

A identificação de processos patológicos durante o período gestacional é de suma importância, uma vez que podem causar prejuízos ao desenvolvimento adequado no meio intrauterino. Dessa forma, são necessárias mais pesquisas científicas que busquem estudar os efeitos da hipermetioninemia gestacional e sua capacidade de causar danos ao desenvolvimento fetal com o objetivo de minimizar os possíveis efeitos

adversos tanto na mãe como no filho, como também auxiliar na identificação do risco aumentado de processos patológicos futuros na prole.

### 1.9 Modelos experimentais de hipermetioninemia

Os estudos acerca dos efeitos patogênicos da metionina sobre o organismo podem ser realizados através de testes *in vitro*. Na literatura, há estudos sobre a toxicidade da metionina que foram realizados em homogeneizados de fígado (Costa et al., 2013) e em homogeneizados de hipocampo de ratos Wistar (Streck et al., 2002, Stefanello et al., 2005; Schulpis et al., 2006). Ensaios *in vitro* possuem a vantagem de terem execução mais rápida e simples. Entretanto, inúmeros fatores podem influenciar na toxicidade de uma determinada substância, como por exemplo, a capacidade do composto em se solubilizar nos fluídos orgânicos e a sua afinidade ao tecido alvo do estudo. Uma vez que tais influências não podem ser avaliadas nos testes *in vitro*, os testes *in vivo* não podem ser substituídos completamente. Dessa forma, os testes *in vitro* geralmente servem como um estudo precedente aos experimentos realizados em animais.

A maioria dos modelos experimentais animais publicados na literatura que estudam os efeitos da hipermetioninemia foram realizados em roedores. Os principais modelos desenvolvidos incluem: 1) *knockout* do gene MAT1A que codifica a enzima MAT I/III, a qual está envolvida no metabolismo da metionina (Lu et al., 2001), 2) suplementação de metionina na dieta (Earle et al., 1942;

Lynch & Strain, 1989; Toborek et al., 1996; Mori & Hirayama, 2000; Yalçinkaya et al., 2007; Yalçinkaya et al., 2009; Viggiano et al., 2012), 3) modelos quimicamente induzidos através de administrações crônicas do aminoácido nos animais (Stefanello et al., 2007a).

Vale ressaltar que nos modelos em que se faz uso de animais *knockout* obtidos pela deleção do gene MAT1A, ocorre o desenvolvimento de hipermetioninemia isolada, associada à redução dos níveis de SAM, uma vez que a primeira etapa da via de degradação da metionina está inibida. Já nos modelos experimentais em que se faz um enriquecimento de metionina na dieta ou em que se faz a administração crônica de metionina através de injeções subcutâneas nos animais, pode ocorrer um aumento dos níveis dos metabólitos da metionina, como a SAM e a homocisteína, uma vez que a via metabólica de degradação da metionina está íntegra nesses casos. Tais metabólitos, quando em excesso, também podem participar dos efeitos patológicos decorrentes da hipermetioninemia.

Embora existam alguns modelos animais que visem investigar os efeitos da hipermetioninemia, há carência de metodologias adequadas para se avaliar as consequências que a hipermetioninemia materna poderia causar à prole. O único trabalho encontrado na literatura que se propôs a investigar os efeitos da hipermetioninemia materna foi um estudo desenvolvido por Römer e colaboradores (2012), em que se induziu hipermetioninemia em ratas gestantes através de uma dieta rica em metionina e se verificou um prejuízo ao crescimento craniano nos ratos neonatos. Entretanto, o desenvolvimento de um

modelo experimental animal de hipermetioninemia gestacional induzido através de injeções subcutâneas diárias de metionina em ratas durante o período gestacional seria bastante vantajoso, pois dessa forma, é possível padronizar a quantidade de metionina que o animal recebe, reduzindo a variabilidade que ocorre durante a administração oral. Uma vez estabelecido um modelo experimental de hipermetioninemia gestacional adequado, é possível avaliar os danos e mecanismos que essa condição poderia causar à prole em diferentes estágios de desenvolvimento da vida pós-natal.

# 2. OBJETIVOS

## *2.1 Objetivos gerais*

O presente trabalho busca ampliar o conhecimento referente às possíveis alterações bioquímicas, moleculares, histológicas e comportamentais causadas na prole devido ao excesso de metionina plasmática durante o período gestacional.

## *2.2 Objetivos específicos*

Os objetivos específicos do presente trabalho serão subdivididos em cinco capítulos, os quais correspondem a artigos científicos, como segue:

### *2.2.1 Capítulo I*

- Desenvolver um modelo experimental quimicamente induzido para hipermetioninemia gestacional em ratas;
- Avaliar na prole as atividades encefálicas das enzimas $Na^+,K^+$-ATPase e $Mg^{2+}$-ATPase;
- Determinar os seguintes parâmetros de estresse oxidativo: conteúdo de grupamentos sulfidrilas, lipoperoxidação e as atividades das enzimas antioxidantes SOD e CAT nos encéfalos dos filhotes;
- Medir os níveis séricos e encefálicos de metionina e homocisteína nas mães e na prole.

*2.2.2 Capítulo II*

- Verificar os efeitos da hipermetioninemia gestacional sobre o número de neurônios (anti-NeuN), parâmetros apoptóticos (Bax, Bcl-2, Bcl-xL e p53), níveis de NGF e de BDNF, parâmetros de metabolismo energético (succinato desidrogenase, complexo II e citocromo c oxidase), imunoconteúdo e expressão da $Na^+,K^+$-ATPase, formação de edema, marcadores inflamatórios (TNF-alfa e IL-6) e níveis mitocondriais de $H_2O_2$ nos encéfalos da prole de ratos.

*2.2.3 Capítulo III*

- Verificar a capacidade de locomoção, ansiedade, memória e comportamento exploratório da prole através dos seguintes testes comportamentais: Campo Aberto, Esquiva inibitória e Reconhecimento de Objetos;
- Realizar a análise histológica do tecido cerebral dos filhotes.

*2.2.4 Capítulo IV*

- Avaliar parâmetros de estresse oxidativo/nitrosativo (ERO, lipoperoxidação, conteúdo de grupamentos sulfidrilas, SOD, CAT e nitritos), bem como o conteúdo total de proteínas no músculo gastrocnêmico da prole de ratas submetidas à hipermetioninemia gestacional;
- Verificar a ocorrência de dano muscular e inflamação pela medida da

atividade da enzima creatina cinase, níveis de creatinina, ureia e proteína C reativa e pela presença de troponina I no soro.

### *2.2.5 Capítulo V*

- Reunir o conhecimento já publicado na literatura a respeito da toxicidade induzida pela metionina aos tecidos cerebral e hepático, focando em resultados obtidos de pacientes, experimentos *in vitro* e modelos experimentais animais.

# 3. METODOLOGIA E RESULTADOS

### 3.1 Modelo experimental de hipermetioninemia gestacional

Os capítulos I, II, III e IV correspondem a artigos científicos de pesquisa experimental. O capítulo I se refere ao artigo científico no qual se desenvolveu o modelo experimental de hipermetioninemia gestacional. Neste modelo, ratas Wistar receberam duas injeções subcutâneas diárias de metionina durante o período gestacional. Um grupo de ratas recebeu a dose 1 (1,34 µmol/g peso corporal) e outro grupo recebeu a dose 2 (2,68 µmol/g peso corporal). As doses foram escolhidas baseadas em um estudo anterior em que se verificou que a administração de 1,34 µmol/g peso corporal em ratos em desenvolvimento, eleva os níveis sanguíneos de metionina para cerca de 1,4 mM. Já a dose de 2,68 µmol/g peso corporal, eleva os níveis de metionina no sangue para cerca de 2 mM (Stefanello et al., 2007a). As doses utilizadas neste estudo induzem a níveis plasmáticos de metionina similares àqueles encontrados em pacientes hipermetioninêmicos (Mudd et al., 1995). Nos capítulos II, III e IV, as ratas Wistar foram tratadas apenas com a dose 2 de metionina. O grupo controle recebeu solução salina. Os filhotes foram decapitados aos 7, 21 ou 30 dias de vida, dependendo do experimento.

## 3.2 Capítulo I

### MANUSCRITO 1

**Development of an animal model for gestational hypermethioninemia in rat and its effect on brain $Na^+,K^+$-ATPase/$Mg^{2+}$-ATPase activity and oxidative status of the offspring**

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ORIGINAL PAPER

# Development of an animal model for gestational hypermethioninemia in rat and its effect on brain $Na^+,K^+$-ATPase/$Mg^{2+}$-ATPase activity and oxidative status of the offspring

**Bruna M. Schweinberger · Lígia Schwieder · Emilene Scherer · Angela Sitta · Carmem R. Vargas · Angela T. S. Wyse**

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**Abstract** In the present study we developed a chemically induced experimental model for gestational hypermethioninemia in rats and evaluated in the offspring the activities of $Na^+,K^+$-ATPase and $Mg^{2+}$-ATPase, as well as oxidative stress parameters, namely sulfhydryl content, thiobarbituric acid-reactive substances and the antioxidant enzymes superoxide dismutase and catalase in encephalon. Serum and encephalon levels of methionine and total homocysteine were also evaluated in mother rats and in the offspring. Pregnant Wistar rats received two daily subcutaneous injections of methionine throughout the gestational period (21 days). During the treatment, a group of pregnant rats received dose 1 (1.34 μmol methionine/g body weight) and the other one received dose 2 (2.68 μmol methionine/g body weight). Control group received saline. After the rats give birth, a first group of pups was killed at the 7th day of life and the second group at the 21th day of life for removal of serum and encephalon. Mother rats were killed at the 21th day postpartum for removal of serum and encephalon. Both doses 1 and 2 increased methionine levels in encephalon of the mother rats and dose 2 increased methionine levels in encephalon of the offspring. Maternal hypermethioninemia also decreased the activities of $Na^+,K^+$-ATPase, $Mg^{2+}$-ATPase and catalase, as well as reduced total sulfhydryl content in the encephalon of the pups. This chemical model seems to be appropriate for studies aiming to investigate the effect of maternal hypermethioninemia on the developing brain during gestation in order to clarify possible neurochemical changes in the offspring.

**Keyword** Animal model · Encephalon · Gestational hypermethioninemia · $Na^+,K^+$-ATPase · $Mg^{2+}$-ATPase · Oxidative stress

B. M. Schweinberger · L. Schwieder · E. Scherer · A. T. S. Wyse
Laboratório de Neuroproteção e Doenças Metabólicas,
Porto Alegre, Brazil

B. M. Schweinberger · E. Scherer · A. T. S. Wyse (✉)
Programa de Pós-Graduação em Ciências Biológicas – Bioquímica.
Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003 Porto Alegre, RS, Brazil
e-mail: wyse@ufrgs.br

A. Sitta · C. R. Vargas
Serviço de Genética Médica, HCPA, Rua Ramiro Barcelos 2350,
Porto Alegre, RS CEP 90035-003, Brazil

## Introduction

Hypermethioninemia is a condition characterized by elevated plasma Methionine (Met) levels and may occur in a variety of metabolic disorders. The most common genetic cause for isolated hypermethioninemia is the deficiency of Met adenosyltransferase (MAT) I/III, an enzyme that catalyzes the synthesis of S-adenosylmethionine (AdoMet) from Met and ATP. MAT I and MATIII are expressed predominantly in liver and are encoded by the MAT1A gene (Mudd 2011). MAT1A R264H in heterozygosis has been shown to be one of the most frequent mutations and may lead to mild hypermethioninemia (Couce et al. 2013). Other causes for hypermethioninemia include classical homocystinuria (due to cystathionine beta-synthase deficiency), deficiencies of citrin, glycine N-methyltransferase, S-adenosylhomocysteine hydrolase, and fumarylacetoacetate hydrolase (tyrosinemia type I) (Mudd et al. 2001).

The clinical consequences of MAT I/III deficiency may include neurological disorders, such as cognitive deficits, cerebral edema and demyelination. However, despite a great deal of works on the neurotoxic effects of Met, the mechanisms involved in these alterations are still not well-understood (Chamberlin et al. 1996; Mudd et al. 2000, 2001).

In a previous study, a chronic experimental model of hypermethioninemia was induced in developing rats (6th to the 28th postpartum day). The results of such study suggest that the brain toxicity mediated by Met may be a consequence of a reduction in $Na^+,K^+$-ATPase activity (Stefanello et al. 2011), an integral membrane protein responsible for the maintenance of intra and extracellular electrolyte balance (Lees 1991). Studies show that $Na^+,K^+$-ATPase can be inhibited by reactive oxygen species (ROS) (Lees 1993), lipid peroxidation (Mishra et al. 1989; Viani et al. 1991) and oxidation of the sulfhydryl (SH) group (Yufu et al. 1993). Evidences also show that administration of antioxidants were able to partially prevent the induced Met-inhibition of this enzyme in rat hippocampus (Stefanello et al. 2011), suggesting that oxidative stress is involved in the inhibition of $Na^+,K^+$-ATPase during hypermethioninemia.

Although it is known that elevated blood levels of certain amino acids can cause severe neuronal damage to the fetus during pregnancy (Mabry et al. 1963; Huether et al. 1992; de Franceschi et al. 2013), the effect of maternal hypermethioninemia on the developing brain during intrauterine life is poor studied. Therefore, the objective of this study was to develop a chemically induced experimental model for gestational hypermethioninemia. The serum and encephalon levels of Met and its metabolite homocysteine (Hcy) were evaluated in the offspring of rats exposed to Met during pregnancy. We also evaluated the activities of $Na^+,K^+$-ATPase and $Mg^{2+}$-ATPase, as well as oxidative stress parameters, namely sulfhydryl content, thiobarbituric acid-reactive substances (TBARS) and the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) in encephalon.

## Materials and methods

### Animals and reagents

Female Wistar rats were obtained from the Central Animal House of the Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22±1 °C) colony room. Rats had free access to a 20 % (w/w) protein commercial chow and water. The NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80–23, revised 1996) and the official governmental guidelines in compliance with the Federação das Sociedades Brasileiras de Biologia Experimental were followed in all experiments. All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

### Chronic methionine treatment

After mating the female rats with males of the same strain, pregnancy was confirmed by the presence of sperm in the vaginal smear. The pregnant rats (70–90 days of age) received two daily subcutaneous injections of Met (at intervals of 12 h) throughout the gestational period (21 days). During the treatment, a group of pregnant rats received 1.34 μmol Met/g body weight and the other one received 2.68 μmol Met/g body weight. These doses were calculated based on a previous work that induced elevated concentrations of Met in the blood by injecting subcutaneously Met (1.34–2.68 μmol/g of body weight) to developing animals of various ages (Stefanello et al. 2006). Control rats received saline. After birth, a first group of pups was killed at the 7th day of life and the second group at the 21th day of life. Mother rats were killed 21 days after the last injection.

### Tissue preparation and serum obtainment

Animals were killed by decapitation without anesthesia followed by the removal of encephalon and blood. Encephalon was divided into two parts. The first part was homogenized in 10 volumes (1:10, w/v) of Medium buffer for determining the activities of $Na^+,K^+$-ATPase and $Mg^{2+}$-ATPase. The second part was homogenized in 10 volumes (1:10, w/v) of buffer solution (sodium phosphate 20 mM, KCl 140 mM, pH 7.4) for determining oxidative stress parameters. To obtain serum, blood was collected and centrifuged at 1000xg (3, 000 rpm) for 10 min at 4 °C. After, serum was removed by suction and stored at −80 °C for subsequent determination of serum Met and total Hcy (tHcy) levels.

### Methionine levels determination

The concentrations of Met in serum and encephalon were determined by high-performance liquid chromatography (HPLC) according to Joseph and Marsden (1986). The analysis was performed using a reverse phase column (ODS 25 cm×4.6 mm×5 μm) and fluorescent detection after precolumn derivatization with OPAplus mercaptoethanol. The flow rate was adjusted to 1.4 mL/min in a gradient of the mobile phase of methanol and 0.5 M sodium phosphate buffer pH 5.5 (buffer A, 80 % methanol; buffer B, 20 % methanol). Each sample run lasts 45 min. Met was identified by its retention time and was quantitatively determined by using its chromatographic peak area and correlating with the internal standard peak area (homocysteic acid).

### Total homocysteine levels determination

tHcy levels in serum and encephalon were determined as described by Magera et al. (1999), using liquid chromatography electrospray tandem mass spectrometry (LC–MS/MS). After samples reduction and deproteinization, the tHcy concentration was detected through the transition from the precursor to the product ion (m/z 136 to m/z 90). Homocysteine-d (8) was added as an internal standard.

### $Na^+,K^+$-ATPase activity assay

The reaction mixture for $Na^+,K^+$-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 170 μL. The reaction was initiated by the addition of ATP. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. The activity was calculated by the difference between the two assays, as previously described (Wyse et al. 2000). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein. All samples were run in duplicate.

### $Mg^{2+}$-ATPase activity assay

Total ATPase activity was assayed by the addition of ATP at the mixture containing 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4. Pi released was then measured. The activity of $Mg^{2+}$-ATPase was calculated by the difference between the total ATPase activity and $Na^+,K^+$-ATPase activity. Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein. All samples were run in duplicate.

### Thiobarbituric acid-reactive substances

TBARS were measured according to Ohkawa et al. (1979). Briefly, the following reagents were added (in this order) to glass tubes: 200 μL of tissue supernatant; 20 μL of sodium dodecyl sulfate (SDS) 8.1 %; 600 μL of 20 % acetic acid in aqueous solution (v/v) pH 3.5; 600 μL of 0.8 % thiobarbituric acid. The mixture was vortexed and the reaction was carried out in a boiling water bath for 1 h. The tube was then allowed to cool on water for 5 min, and was centrifuged at 1,000*g* for 10 min. The resulting pink stained TBARS were determined spectrophotometrically at 535 nm in a Beckman DU® 800 (Beckman Coulter, Inc., Fullerton, CA, USA). A calibration curve was generated using 1,1,3,3-tetramethoxypropane as a standard. TBARS were calculated as nmol TBARS/mg protein.

### Sulfhydryl content

This assay is based on the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) by thiols, which in turn become oxidized (disulfide), generating the yellow derivative thionitrobenzoic acid (TNB) whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery 2001). Briefly, 50 μL of homogenate were added to 1 mL of PBS buffer pH 7.4 containing 1 mM EDTA. Then 30 μL of 10 mM DTNB, prepared in a 0.2 M potassium phosphate solution pH 8.0, were added. Subsequently, 30 min incubation at room temperature in a dark room was performed. Absorption was measured at 412 nm using a Beckman DU1 640 spectrophotometer. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB/mg protein.

### Superoxide dismutase assay

SOD activity assay is based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide, which is the substrate for SOD. The inhibition of the autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed at 420 nm using the SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA) (Marklund 1985). A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. The results are reported as units/mg protein.

### Catalase assay

CAT activity was assayed using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA). The method used is based on the disappearance of hydrogen peroxide ($H_2O_2$) at 240 nm in a reaction medium containing 20 mM $H_2O_2$, 0.1 % Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1–0.3 mg protein/mL (Aebi 1984). One CAT unit is defined as 1 μmol of $H_2O_2$ consumed per minute and the specific activity is calculated as pmol/mg protein.

### Protein determination

Protein concentration was measured by the method of Lowry et al. (1951) and Bradford (1976) using bovine serum albumin as standard.

### Statistical determination

Data were analyzed by One-way ANOVA followed by the Tukey test, when F-test was significant. All analyses were

performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. Differences were considered statistically significant if $p<0.05$.

## Results

### Methionine and total homocysteine levels in serum and encephalon of the mother rats

At the 21th day after giving birth, mother rats were decapitated followed by the removal of serum and encephalon for evaluation of Met levels. Since Hcy is formed during Met metabolism, tHcy levels were also evaluated. As can be observed in Table 1, results showed that female rats that received Met during pregnancy, had no difference in serum Met [$F(2,9)=0.10$; $p>0.05$] and tHcy levels [$F(2,9)=0.52$; $p>0.05$] when compared to the control group. On the other hand, encephalon Met levels were significantly increased in female rats treated with dose 1 (~45 %) [$F(2,9)=11.61$; $p<0.05$] and dose 2 (~59 %) [$F(2,9)=11.61$; $p<0.01$]. Encephalon tHcy levels of treated-rats were not different from the control [$F(2,9)=5.12$; $p>0.05$].

### Methionine and total homocysteine levels in serum of the offspring

Table 2 shows that serum Met levels of 21 days-of-age pups from Met-treated mothers did not differ from pups whose mothers were treated with saline [$F(2,9)=0.27$; $p>0.05$]. Our findings also demonstrated that animals submitted to the model had no difference in tHcy serum levels when compared to the control [$F(2,9)=1.14$; $p>0.05$]. Met and tHcy serum levels were not evaluated in 7 days-of-age pups due to the low volume of samples.

**Table 1** Methionine and total homocysteine levels in serum and encephalon of the mother rats

| Group | Serum Met levels (μM) | Serum tHcy levels (μM) | Encephalon Met levels (μM) | Encephalon tHcy levels (μM) |
|---|---|---|---|---|
| Saline | 23.50±4.10 | 4.02±1.98 | 7.40±0.50 | 1.14±0.13 |
| Dose 1 | 23.35±1.77 | 5.87±2.50 | 10.73±1.80* | 1.43±0.13 |
| Dose 2 | 20.65±11.38 | 5.20±2.27 | 11.80±0.10** | 1.98±0.45 |

At the 21th day after giving birth, serum and encephalon of the mother rats were collected. Data are expressed as mean ± S.D. for 4 rats in each group. Different from control, * $p<0.05$; ** $p<0.01$ (One-way ANOVA and Tukey test)

**Table 2** Methionine and total homocysteine levels in serum of the offspring

| Group | Serum Met levels (μM) | Serum tHcy levels (μM) |
|---|---|---|
| Saline | 54.27±3.80 | 4.10±1.63 |
| Dose 1 | 57.66±13.33 | 6.29±3.24 |
| Dose 2 | 59.60±7.50 | 5.65±0.88 |

At the 21th day after birth, serum of the offspring was collected. Data are expressed as mean ± S.D. for 4 rats in each group (One-way ANOVA and Tukey test)

### Methionine and total homocysteine levels in encephalon of the offspring

Table 3 shows that encephalon Met levels were significantly higher in 21 days-of-age pups whose mothers were treated with dose 2 (~230 %) [$F(2,9)=11.65$; $p<0.01$] but not with dose 1 [$F(2,9)=11.65$; $p>0.05$]. Dose 2 also increased Met levels in encephalon of 7 days-of age pups (~129 %) [$F(2,9)=3.77$; $p<0.01$], while dose 1 did not alter Met levels [$F(2,9)=3.77$; $p>0.05$]. Encephalon tHcy levels were also evaluated and it was observed no difference between the groups in pups of 21 [$F(2,9)=1.27$; $p>0.05$] and 7 days of age [$F(2,9)$: $7.80=p>0.05$].

### Effect of gestational hypermethioninemia on $Na^+K^+$-ATPase activity in encephalon of the offspring

Figure 1 shows that maternal hypermethioninemia significantly decreased $Na^+K^+$-ATPase activity in encephalon of 21 days-of-age pups whose mothers where treated with dose 1 [$F(2,15)=p<0.001$] and dose 2 [$F(2,15)=p<0.001$]. This parameter was not altered in 7 days-of-age pups (control: 24.20±8.16; dose 1: 19.48±5.87; dose 2: 23.69±6.57; $p>0.05$) (data not shown).

### Effect of gestational hypermethioninemia on $Mg^{2+}$-ATPase activity in encephalon of the offspring

Figure 2 indicates that gestational hypermethioninemia significantly reduced $Mg^{2+}$-ATPase activity in encephalon of 21 days-of-age pups whose mothers where treated with dose 1 [$F(2,15)=p<0.001$] and dose 2 [$F(2,15)=p<0.01$]. This parameter was not altered in 7 days-of-age pups (control: 285.88±3.14; dose 1: 275.19±38.65; dose 2: 241.61±30.71; $p>0.05$) (data not shown).

### Effect of gestational hypermethioninemia on parameters of oxidative stress in the encephalon of the offspring

Encephalon lipid damage was measured by TBARS levels and we observed that gestational hypermethioninemia did not change this parameter neither in 7 (control: 6.34±1.2; dose 1:

**Table 3** Methionine and total homocysteine levels in encephalon of the offspring

At the 7th and the 21th day after birth, encephalon of the offspring was collected. Data are expressed as mean ± S.D. for 4 rats in each group. Different from control, ** $p<0.01$ (One-way ANOVA and Tukey test)

| | Group | Encephalon Met levels (μM) | Encephalon tHcy levels (μM) |
|---|---|---|---|
| 21 days-of-age pups | Saline | 2.53±0.76 | 1.48±0.42 |
| | Dose 1 | 3.54±1.46 | 1.27±0.65 |
| | Dose 2 | 8.35±2.32** | 1.83±0.35 |
| 7 days-of-age pups | Saline | 3.50±0.08 | 0.94±0.65 |
| | Dose 1 | 6.25±1.85 | 0.89±0.10 |
| | Dose 2 | 8.02±2.14** | 0.90±0.51 |

7.50±1.27; dose 2: 7.20±2.41; $p>0.05$) and 21 days-of-age pups (control: 4.97±0.66; dose 1: 4.38±0.31; dose 2: 5.77±0.57; $p>0.05$) (data not shown). On the other hand, we observed that proteins were affected by the Met treatment since SH content was significantly decreased in encephalon of 21 days-of-age pups whose mothers were treated with dose 1 [$F(2,15)=5.76$; $p<0.05$] and dose 2 [$F(2,15)=5.76$; $p<0.05$] (Fig. 3). Met treatment did not alter SH content in 7 days-of-age pups (control: 67.09±1.05; dose 1: 65.29±10.87; dose 2: 52.92±8.99; $p>0.05$) (data not shown).

Antioxidant enzymes were also evaluated, and we observed that the treatment did not change SOD activity in pups of both 7 (control: 5.46±1.17; dose 1: 4.18±1.10; dose 2: 4.46±1.69; $p>0.05$) and 21 days of age (control: 3.26±0,40; dose 1: 3.20±0.49; dose 2: 3.12±0.46; $p>0.05$) (data not shown). Aversely, CAT activity was significantly reduced in 21 days-of-age pups whose mothers were treated with dose 2 [$F(2,15)=7.63$; $p<0.05$], but not with dose 1 [$F(2,15)=7.63$; $p>0.05$] (Fig. 4). Pups of 7 days of age did not present changes in CAT activity in encephalon (control: 2.12±0.75; dose 1: 2.86±0.50; dose 2: 2.40±0.79; $p>0.05$) (data not shown).

## Discussion

In face of the importance of identifying factors that may cause damage to the structures and functions of the developing brain during the prenatal period and since hypermethioninemia may be associated with neurological disorders (Mudd et al. 2000, 2001), the main objective of the present study was to develop an experimental model for gestational hypermethioninemia in rats.

In our study, Wistar rats received daily subcutaneous injection of Met in two different doses (1.34 or 2.68 μmol Met/g body weight) during all gestational period. Serum Met and tHcy levels of the treated-mother rats and their pups demonstrated no significant difference when compared to the control, probably because Met levels return back to the control values 12 h after the injection of this amino acid (Stefanello et al. 2006). Enhanced Met levels in encephalon, on the other hand, persisted 21 days after the interruption of the treatment in mother rats treated with doses 1 and 2, as well as in pups whose mothers were treated with dose 2.

Since cerebral dysfunction may be observed in patients with hypermethioninemia and changes in the activity of the enzyme $Na^+,K^+$-ATPase seem to be associated with neurological diseases (Cannon 2004; de Carvalho et al. 2004; Zhang et al. 2013; Banerjee et al. 2012), the next step of this study was to investigate the effect of maternal hypermethioninemia on encephalon $Na^+,K^+$-ATPase activity of the offspring. The results demonstrated a significant decrease in the activity of this enzyme in 21 days-of-age pups, corroborating with other work described in literature which shows that acute and chronic hypermethioninemia reduce

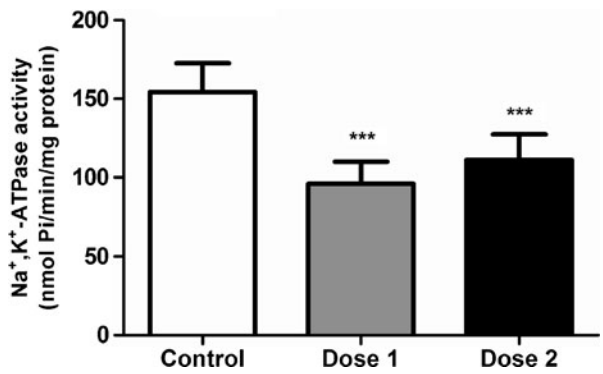


**Fig. 1** Effect of gestational hypermethioninemia on encephalon $Na^+,K^+$-ATPase activity of 21 days-of-age rat pups. Results are expressed as means ± SD for six animals in each group. Different from control, *** $p<0.001$ (One-way ANOVA and Tukey test)

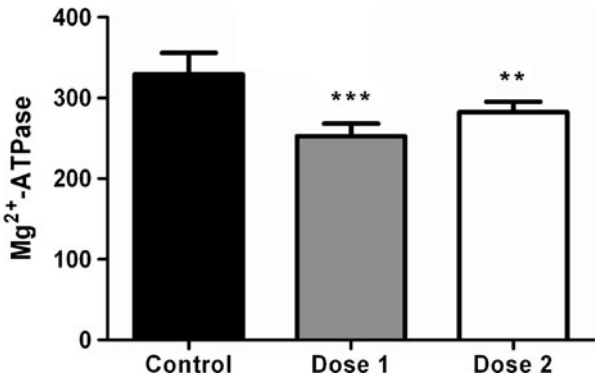


**Fig. 2** Effect of gestational hypermethioninemia on encephalon $Mg^{2+}$-ATPase activity of 21 days-of-age rat pups. Results are expressed as means ± SD for six animals in each group. Different from control, ** $p<0.01$; *** $p<0.001$ (One-way ANOVA and Tukey test)

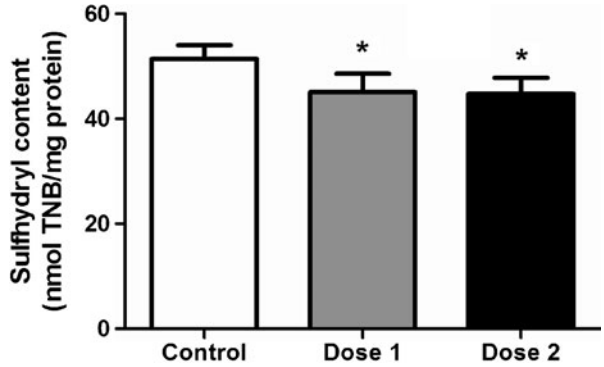


**Fig. 3** Effect of gestational hypermethioninemia on encephalon SH content of 21 days-of-age rat pups. Results are expressed as means ± SD for six animals in each group. Different from control, $^{*}p<0.05$ (One-way ANOVA and Tukey test)

$Na^{+},K^{+}$-ATPase activity in rat hippocampus (Stefanello et al. 2011). Such inhibition may lead to an impairment of sodium and potassium membrane transport with a consequent intracellular accumulation of sodium and water, which could explain the cerebral edema sometimes observed during hypermethioninemia (Mudd et al. 2003). Besides, it has been reported that administration of $Na^{+},K^{+}$-ATPase inhibitors alters neuronal firing (Johnson et al. 1992; Vaillend et al. 2002) and impairs learning process (Mizumori et al. 1987; Sato et al. 2004; Zhan et al. 2004).

$Mg^{2+}$-ATPase is the main enzyme in maintenance of high brain intracellular $Mg^{2+}$ concentrations, which is involved in controlling protein synthesis and cell growth (Sanui and Rubin 1982). In the present study, $Mg^{2+}$-ATPase activity was analyzed and it was found a decrease in the encephalon activity of this enzyme in 21 days-of-age pups. Reduced $Mg^{2+}$-ATPase activity has been correlated with reduced learning performance (Carageorgiou et al. 2008), what could elucidate, at least partially, the cognitive deficits found in some patients with hypermethioninemia.

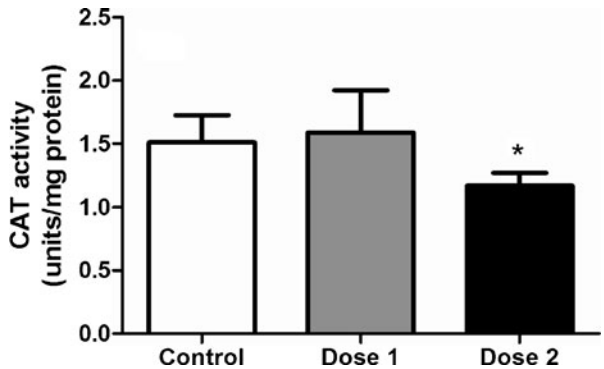


**Fig. 4** Effect of gestational hypermethioninemia on encephalon catalase activity of 21 days-of-age rat pups. Results are expressed as means ± SD for six animals in each group. Different from control, $^{*}p<0.05$ (One-way ANOVA and Tukey test)

Given that previous studies suggest a link between hypermethioninemia and the induction of oxidative stress in hippocampus of rats (Stefanello et al. 2007) and that SH groups of $Na,^{+}K^{+}$-ATPase and $Mg^{2+}$-ATPase are susceptible to oxidative damage, we also evaluated SH content in the encephalon of the offspring. Met treatment significantly reduced this parameter in 21 days-of-age pups, what may possibly indicate that hypermethioninemia leads to an increased superoxide radical production, which can combine with nitric oxide (NO) to form $ONOO^{-}$ or can be dismutated to $H_2O_2$, being that both may oxidize proteins bound SH (Winterbourn and Hampton 2008) and might explain the reduced activities of the ATPase enzymes observed in this study.

In addition, because the enzymes $Na,^{+}K^{+}$-ATPase and $Mg^{2+}$-ATPase are embedded in cellular membrane and reactive species may lead to peroxidation of membrane lipids, TBARS levels were measured to identify lipid damage. Maternal hypermethioninemia did not alter this parameter in the encephalon of the offspring, suggesting that lipoperoxidation is not involved in the alterations of ATPase enzymes activities. In accordance, a recent study demonstrated that chronic administration of Met does not change TBARS levels in liver of rats (Stefanello et al. 2009).

In order to evaluate whether Met induces alterations in the behavior of antioxidant enzymes, we studied the effect of this amino acid on the activities of SOD and CAT, which represent an efficient system responsible for removing ROS (Halliwell 2001; Halliwell and Gutteridge 2007). Results showed that maternal hypermethioninemia did not alter cerebral SOD activity; however CAT activity was significantly reduced in encephalon of 21 days-of-age pups whose mothers were treated with dose 2. These findings are in agreement with a previous work, which shows that hypermethioninemia provokes a significant decrease in CAT activity in liver of rats, but does not affect SOD activity (Stefanello et al. 2009). This condition can make the cellular environment more susceptible to the formation of $H_2O_2$ and consequently could lead to oxidative stress generation.

However, it should be emphasized that Hcy is formed from Met metabolism. Hyperhomocysteinemia has been reported to inhibit $Na^{+},K^{+}$-ATPase in brain, to decrease CAT activity in brain, lung and heart and to reduce total thiol content in liver of rats (Streck et al. 2002; da Cunha et al. 2011; Kolling et al. 2011). Although we did not observe increased tHcy levels in serum and encephalon of the pups, we cannot discard the possibility that Hcy is involved in the changes occurred in the encephalon of the offspring observed in the present study. It is also important to note that interestingly only 21 days-of-age pups presented alterations on the parameters evaluated. During the gestational period, placenta exerts a maternal-fetal transference of antioxidants, such as vitamin A, maintaining adequate supply to the fetus (Underwood 1994; Dimenstein et al. 1996). On this basis, it is possible that younger pups have

a more efficient antioxidant protection, which is derived from their mothers.

In summary, our data show that gestational Met-treatment promotes, in the encephalon of the offspring, a reduction in the activities of $Na^+$,$K^+$-ATPase and $Mg^{2+}$-ATPase as well as alters the oxidative status, reducing CAT activity and total SH content. In the present study, the largest number of altered parameters occurred in 21-days-of-age pups whose mothers were treated with dose 2. Therefore, this chemical model seems to be appropriate for futures studies aiming to investigate the effect of maternal hypermethioninemia on the developing brain during gestation in order to clarify possible neurochemical and/or behavioral changes in the offspring.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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## 3.3 Capítulo II

### MANUSCRITO 2

**Maternal hypermethioninemia affects neurons number, neurotrophins levels, energy metabolism and $Na^+,K^+$-ATPase expression/content in brain of rat offspring**

Schweinberger BM, Rodrigues AF, Turcatel E, Pierozan P, Pettenuzzo LF, Grings M, Scaini G, Parisi MM, Leipnitz G, Streck EL, Barbé-Tuana FM, Wyse ATS

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# Maternal Hypermethioninemia Affects Neurons Number, Neurotrophins Levels, Energy Metabolism, and $Na^+,K^+$-ATPase Expression/Content in Brain of Rat Offspring

**Bruna M. Schweinberger[1] · André F. Rodrigues[1] · Elias Turcatel[1] · Paula Pierozan[1] · Leticia F. Pettenuzzo[1] · Mateus Grings[1] · Giselli Scaini[2] · Mariana M. Parisi[1] · Guilhian Leipnitz[1] · Emilio L. Streck[2] · Florencia M. Barbé-Tuana[1] · Angela T. S. Wyse[1]**

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**Abstract** In the current study, we verified the effects of maternal hypermethioninemia on the number of neurons, apoptosis, nerve growth factor, and brain-derived neurotrophic factor levels, energy metabolism parameters (succinate dehydrogenase, complex II, and cytochrome c oxidase), expression and immunocontent of $Na^+,K^+$-ATPase, edema formation, inflammatory markers (tumor necrosis factor-alpha and interleukin-6), and mitochondrial hydrogen peroxide levels in the encephalon from the offspring. Pregnant Wistar rats were divided into two groups: the first one received saline (control) and the second group received 2.68 μmol methionine/g body weight by subcutaneous injections twice a day during gestation (approximately 21 days). After parturition, pups were killed at the 21st day of life for removal of encephalon. Neuronal staining (anti-NeuN) revealed a reduction in number of neurons, which was associated to decreased nerve growth factor and brain-derived neurotrophic factor levels. Maternal hypermethioninemia also reduced succinate dehydrogenase and complex II activities and increased expression and immunocontent of $Na^+,K^+$-ATPase alpha subunits. These results indicate that maternal hypermethioninemia may be a predisposing factor for damage to the brain during the intrauterine life.

**Keywords** Brain · Maternal hypermethioninemia · Energy metabolism · $Na^+,K^+$-ATPase · Neurons number · Neurotrophins

## Introduction

Elevation in plasma methionine (Met) levels is defined as hypermethioninemia and may occur in some genetic abnormalities. Deficiency of methionine adenosyltransferase I/III is the most usual cause for isolated hypermethioniemia. Other hereditary causes for this condition include classical homocystinuria, deficiencies of citrin, glycine *N*-methyltransferase, *S*-adenosylhomocysteine hydrolase, and fumarylacetoacetate hydrolase. Hypermethioninemia of non-genetic origin occurs during liver disease and excessive consumption of proteins [1].

It is well known that patients with severe hypermethioninemia may present a variable degree of neurological pathology, including mental retardation, cognitive deficit, and cerebral edema [1, 2]. However, little is known about the effect of maternal hypermethioninemia on the neurodevelopment during intrauterine life. In this context, we have recently shown that increased Met levels during rat gestation reduces $Na^+,K^+$-ATPase activity and induces oxidative stress in the encephalon of the pups [3].

As a consequence of these alterations, neuronal necrosis may occur [4, 5]. Therefore, evaluation of programmed cell death and quantification of neurons are very important to test this hypothesis and determine Met toxicity. In addition to

✉ Angela T. S. Wyse
wyse@ufrgs.br

[1] Postgraduate Program in Biological Sciences—Biochemistry, Department of Biochemistry, Institute of Health Basic Sciences, Federal University of Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP, Porto Alegre, RS 90035-003, Brazil

[2] Postgraduate program in Health Sciences, Laboratory of Bioenergetics, University of Southern Santa Catarina, Criciúma, SC, Brazil

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these parameters, the measurement of neurotrophic factors levels could be helpful to identify another potential mechanism that may contribute to neurological damage during maternal hypermethioninemia since the reduction in these molecules levels may impair growth, survival, and/or differentiation of neurons [6].

Met induces oxidative stress and decreases $Na^+,K^+$-ATPase activity, an enzyme highly dependent on an adequate supply of ATP [7]. An increased reactive oxygen species production may cause energy metabolism impairment and also contribute to inflammatory responses [8].

This work aimed to evaluate in brain of pups born to hypermethioninemic rats: neurons number; apoptosis-related proteins (Bax, Bcl-2, Bcl-xL, p53); nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) content; succinate dehydrogenase (SDH), complex II, and cytochrome c oxidase (COX) activities; $Na^+,K^+$-ATPase expression/immunocontent; edema; tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), and hydrogen peroxide ($H_2O_2$) levels.

## Materials and Methods

### Animals and Reagents

Wistar rats were acquired from the Central Animal House of the Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12:12 h light/dark cycle in constant temperature (22 ± 1 °C) and had free access to a 20% (*w*/*w*) protein commercial chow and water. Experiments followed the NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80–23, revised 1996) and the official governmental guidelines in compliance with the Federação das Sociedades Brasileiras de Biologia Experimental. This research project was approved by the Ethics Committee in Research of Universidade Federal do Rio Grande do Sul (protocol number 25913).

Chemicals were acquired from Sigma Chemical Co., St. Louis, MO, USA.

### Chronic Methionine Treatment

After mating the female rats with males, pregnancy was presumed by the presence of sperm in the vaginal smear. During the gestational period (around 21 days), the pregnant rats (70 to 90 days of age) received two daily subcutaneous injections of 2.68 μmol Met/g body weight. Control rats received saline. After birth, pups were killed at the 21st day of life [3]. After decapitation, encephalon was immediately removed and kept chilled until homogenization.

### Number of Neurons

Tissues were dissociated with PBS/Collagenase, washed with PBS, and then suspended in PBS/collagenase. After, the cell was permeabilized with 0.2% PBS Triton X-100 at room temperature for 10 min and blocked with BSA 5% for 15 min. Cells were incubated in blocking solution containing the monoclonal antibodies anti-NeuN (clone A60) diluted 1:100 during 2 h. The cells were washed with PBS and incubated for 1 h in blocking solution containing Alexa Fluor 488-anti-rabbit IgG diluted 1:200. The levels of positive NeuN cells were determined by flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA). Alexa Fluor 488 was excited at 488 nm using an air-cooled argon laser. Samples with the secondary antibody (negative controls) 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 filter green (FL-1; 530 nm/30). Fluorescence emissions were collected using logarithmic amplification. Data from 10,000 events were acquired, and the mean relative fluorescence intensity was determined after exclusion of debris events from the data set. Flow cytometric acquisitions and analyzes were performed through Flow Jo software 7.6.3 (Treestar, Ashland, OR). The proportion of cells stained with NeuN was expressed as percentage of control.

### Apoptosis

Samples were homogenized in Laemmli-sample buffer (62.5 mM Tris–HCl, pH 6.8, 1% (*w*/*v*) SDS, 10% (*v*/*v*) glycerol). The same quantities of protein (30 μg/well) were fractionated by 10–15% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes. Electro-blotting efficiency and protein loading were verified through Ponceau S staining. Membranes were blocked in Tween-Tris buffered saline (100 mM Tris-HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20) containing 5% albumin. Then, membranes were incubated overnight at 4 °C with rabbit polyclonal antibody against Bcl-2 (Cell Signaling – 2876), Bax (Cell Signaling – 2772), Bcl-xL (Cell Signaling – 2762), and p53 (Cell Signaling – 9282). The primary antibody was removed, and membranes were washed four times during 15 min. Then, an anti-rabbit IgG peroxidase-linked secondary antibody was incubated with the membranes for 1 h (diluted 1:10,000), and membranes were washed once more. Lastly, the immunoreactivity was verified through an enhanced chemiluminescence ECL Plus kit. After exposure, membranes were stripped and incubated with a mouse monoclonal antibody to β-Actin (Sigma – A2228) in the presence of 5% milk. An anti-mouse IgG peroxidase-linked secondary antibody was incubated with the membranes during 1 h (diluted 1:10,000), and the membranes were washed once more. Immunoreactivity

was verified through an enhanced chemiluminescence ECL Plus kit. Densitometry was executed through Image J v.1.34 software, and SeeBlue ® Plus2 Prestained Standard (Invitrogen) was utilized as a molecular weight marker to provide certainty that the right bands were analyzed for proteins.

### Nerve Growth Factor Levels

Brain tissue was homogenized in PBS (Laborclin, Paraná, Brazil) with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). NGF levels were determined using a sandwich-ELISA assay with monoclonal antibodies for NGF (Millipore, USA and Canada). Microtitre plates (96-well flat-bottom) were coated during 24 h with the samples (diluted 1:2) and a standard curve (15.6 to 1000 pg/ml of NGF). The plates were washed four times with the sample diluent. Next, a monoclonal anti-NGF mouse antibody (diluted 1:1000) was added to each well and incubated during 2 h at room temperature. Then, a peroxidase-conjugated anti-rabbit antibody (diluted 1:1000) was added to each well and incubated for 2 h at room temperature. After addition of streptavidin enzyme, substrate, and stop solution, NGF levels were determined by measuring the absorbance at 450 nm. The standard curve indicated a relationship between optical density and NGF levels.

### Energy Metabolism

Encephalon was homogenized (1:20, *w*/*v*) in SETH (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 UI $mL^{-1}$ heparin) buffer, pH 7.4. The homogenates were centrifuged at 800×*g* for 10 min, and the supernatants were kept frozen until determinations.

### Succinate Dehydrogenase Activity

SDH activity was measured as described by Fischer and collaborators [9]. Samples were frozen and thawed three times to break mitochondrial membranes. The enzymatic activity was determined following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol at 600 nm with 700 nm as reference wavelength ($\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$) in the presence of phenazine methasulfate. The reaction mixture containing 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8 μM 2,6-dichloroindophenol was preincubated with 40–80 μg homogenate protein for 20 min at 30 °C. Then, 4 mM sodium azide, 7 μM rotenone, and 40 μM 2,6-dichloroindophenol were added. After adding 1 mM phenazine methasulfate, the reaction initiated and was monitored for 5 min.

### Complex II (Succinate: 2,6-Dichloroindophenol Oxireductase) Activity

Homogenates are following the decrement in absorbance due to the reduction of 2,6-dichloroindophenol at 600 nm with 700 nm as reference wavelength ($\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$), in accordance to Fischer et al. [9]. The reaction mixture containing 40 mM potassium phosphate, pH 7.4, 16 mM succinate, and 8 μM 2,6-dichloroindophenol was pre-incubated with 40–80 μg homogenate protein for 20 min at 30 °C. After, 4 mM sodium azide and 7 μM rotenone were added. After adding 40 μM 2,6-dichloroindophenol, the reaction initiated and was monitored for 5 min.

### Cytochrome c Oxidase

COX activity was measured according to Rustin and colleagues [10]. The activity of this enzyme was determined at 25 °C for 10 min by following the decrease in absorbance due to oxidation of previously reduced cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\varepsilon = 19.1\ mM^{-1} \times cm^{-1}$). The reaction buffer consisted of 10 mM potassium phosphate, pH 7.0, 0.6 mM *n*-dodecyl-β-D-maltoside, 2–4 μg homogenate protein. Reaction initiated after addition of 0.7 μg reduced cytochrome *c*.

### Gene Expression Analyzes

The analysis of ATPase isoforms alpha1 (Atp1a1), alpha2 (Atp1a2), and alpha3 (Atp1a3) expression were performed by quantitative real-time PCR using SYBR Green (Molecular Probes) as the fluorescent detector and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. Gene sequences available from free databanks (www.ncbi.nlm.nih.gov and www.ensembl.org) were used for primers design with a free software (www.idtdna.com) (Table 1).

Animals were euthanized, and cerebral tissue was immediately frozen in liquid nitrogen and subsequently stored at −80 °C. mRNA was extracted using TRIZOL reagent. RNA was measured in a biophotometer (Eppendorf) at 260/280 nm, and the integrity was confirmed by electrophoresis in a 1% formamide-agarose gel. Complementary DNA (cDNA) was synthesized with M-MLV reverse transcriptase enzyme (Sigma) from 2 μg of total RNA. Three μl of diluted cDNA (1:10) were used as template for PCR reactions with Platinum® Taq Polymerase (Invitrogen) in a final volume of 20 μl. The thermal cycling profile was an initial denaturation at 94 °C for 10 min followed by 40 cycles of 15 s at 94 °C, 15 s at 60 °C, 15 s at 72 °C for data acquisition. The specificity of amplification and absence of primer-dimer was confirmed using melting curve analysis at the end of each run. We also confirmed the amplification of a single amplicon of the

**Table 1** Primers sequence of Atp1a1, Atp1a2, ATP1a3, and GAPDH

| Name | RefSeq (mRNA) | Ensembl ID | Primer sequence | Amplicon size | |
|---|---|---|---|---|---|
| Atp1a1 | NM_ 012504 | ENSRNOG00000030019 | Forward | CTGCTTTCCTGTCC TACTGC | 125 bp |
| | | | Reverse | CTTCCGCACCTCGT CATAC | |
| Atp1a 2 | NM_ 012505 | ENSRNOG00000007290 | Forward | GAGGACGAACCATC CAATGAC | 133 bp |
| | | | Reverse | CTAGGCACCATGTT CTTGAAGG | |
| Atp1a 3 | NM_ 012506 | ENSRNOG00000020263 | Forward | TTAAGTGCATCGAG CTGTCC | 142 bp |
| | | | Reverse | AGGTATCGGTTGTC ATTGGG | |
| GAPDH | NM_ 017008 | ENSRNOG00000018630 | Forward | GGTGATGCTGGTGC TGAGTA | 272 bp |
| | | | Reverse | ACTGTGGTCATGAG CCCTTC | |

expected size by agarose gel electrophoresis. All reactions were carried out in a StepOnePlus® real-time PCR system (Applied Biosystems). For ΔΔCT analysis [11], samples were normalized by the constitutive gene (GAPDH) and calibrated by the average of the ΔCT of the group itself. Similar specific gene reaction efficiencies were confirmed before the ΔΔCT analysis was done.

### Immunocontent of $Na^+$,$K^+$-ATPase and Brain-Derived Neurotrophic Factor

Tissues from the brain were homogenized in lysis solution (Tris-HCl 20 Mm). For electrophoresis, samples were dissolved in Laemmli buffer 2× (4% SDS, 20% glycerol, 120 mM Tris-HCl, pH 6.8) and boiled during 3 min. Total protein homogenate was analyzed in 10% SDS-PAGE (30 μg total protein/lane) and transferred (Trans-blot SD semi-dry transfer cell; Bio-Rad, Hercules, CA) to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS). Blots were incubated in blocking solution (TBS plus 5% bovine serum albumin) during 2 h. Subsequently, blots were washed twice with TBS 0.05% Tween-20 (T-TBS) for 5 min and incubated overnight at 4 °C in blocking solution containing one of the following antibodies: monoclonal anti-$Na^+$,$K^+$-ATPase (alpha1 subunit) clone M8-P1-A3 obtained from Sigma, $Na^+$,$K^+$-ATPase alpha2-isoform from Millipore (Billerica, MA, USA), monoclonal anti-$Na^+$-$K^+$-ATPase (alpha3 subunit) clone XVIF9-G10 obtained from Sigma diluted 1:5000, and polyclonal anti-BDNF obtained from Abcam (Cambridge, MA, USA). Membranes were washed twice during 5 min with T-TBS and incubated for 3 h in a solution containing polyclonal peroxidase-conjugated rabbit anti-mouse IgG (1:5000) or polyclonal peroxidase-conjugated anti-rabbit IgG (1:5000). Membranes were washed twice with T-TBS during 5 min and twice with TBS for 5 min. Membranes were developed with the chemiluminescence ECL kit (Amersham, Oakville, Ontario).

### Cerebral Edema

After decapitation, brains were removed and immediately weighted. Each sample was dehydrated during 24 h at 110 °C. Then, the weight was measured again and its water content was calculated through the following formula: ((wet weight) − (dry weight)/wet weight) × 100 [12].

### Inflammatory Parameters

TNF-alpha and IL-6 levels were quantified by a high-sensitivity ELISA with commercial kits (Invitrogen®). The amounts of these cytokines were measured through an optical densitometry at 450 nm in SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

### Mitochondrial Hydrogen Peroxide Release

Forebrain mitochondria were isolated from the pups as described by Rosenthal and collaborators [13] with slight modifications. After decapitation, brains were rapidly removed and put into ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1% bovine serum albumin (free of fatty acids), and 10 mM HEPES, pH 7.2. The forebrain was cut into small pieces, extensively washed, and homogenized 1:10 in a Dounce homogenizer using both a loose-fitting and a tight-fitting pestle. The homogenate was centrifuged during 3 min at 2000*g*. The supernatant was centrifuged during 8 min at 12,000 *g*. The pellet was suspended in isolation buffer

containing 10 μL of 10% digitonin and centrifuged during 8 min at 12,000*g*. The final pellet was washed and suspended in isolation buffer devoid of EGTA. This preparation results in a mixture of synaptosomal and non-synaptosomal mitochondria similar to the brain composition. The mitochondrial preparations (0.5 mg protein $mL^{-1}$) supported by 2.5 mM glutamate plus 2.5 mM malate were incubated in standard reaction medium in the presence of 10 μM Amplex red and 1 U $mL^{-1}$ horseradish peroxidase. The fluorescence was verified over time on a Hitachi F-4500 spectrofluorometer operated at excitation and emission wavelengths of 563 and 587 nm, respectively, and slit width of 5 nm. Antimycin A (0.1 μg $mL^{-1}$) was added at the end of the measurement.

### Protein Determination

Protein concentration was measured by the method of Lowry and colleagues [14] using bovine serum albumin as standard.

### Statistical Determination

Data were analyzed by Student's *t* test. Analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. Differences were considered statistically significant if $p < 0.05$.

## Results

### Effect of Gestational Hypermethioninemia on Number of Neurons of the Offspring

As can be seen in Fig. 1, the average labeled neurons was lower in the group of pups whose mothers were treated with Met during gestational period ($T = 4.74$; $p < 0.01$).

### Effect of Gestational Hypermethioninemia on Apoptosis in Encephalon of the Offspring

High Met levels during pregnancy had no effect on Bax ($T = 0.49$; $p > 0.05$), Bcl-2 ($T = 2.08$; $p > 0.05$), Bcl-xL ($T = 1.10$; $p > 0.05$), and p53 content ($T = 0.43$; $p > 0.05$) from the encephalon of the offspring (Fig. 2).

### Effect of Gestational Hypermethioninemia on Nerve Growth Factor and Brain-Derived Neurotrophic Factor Levels in Encephalon of the Offspring

NGF concentration was determined in the brain, and as can be observed in Fig. 3a, there was a significant reduction in this parameter in pups born to hypermethioninemic mothers ($T = 3.07$; $p < 0.05$). BDNF immunocontent was also decreased ($T = 3.05$; $p < 0.05$) (Fig. 3b).

### Effect of Gestational Hypermethioninemia on Energy Metabolism in Encephalon of the Offspring

Figure 4a, b respectively shows that gestational hypermethioninemia significantly decreased SDH ($T = 4.27$; $p < 0.01$) and complex II ($T = 4.98$; $p < 0.01$) activities in the encephalon of the offspring. COX activity was not altered (control: 116.87 ± 23.81; Met: 139.51 ± 42.36).

### Effect of Gestational Hypermethioninemia on Expression and Immunocontent of $Na^+$,$K^+$-ATPase in Encephalon of the Offspring

Expression of $Na^+$,$K^+$-ATPase subunits was higher in the encephalon of the pups whose mothers received Met: alpha1 ($T = 2.89$; $p < 0.05$), alpha2 ($T = 3.27$; $p < 0.01$), and alpha3 ($T = 3.30$; $p < 0.01$) (Fig. 5a). Examination of $Na^+$,$K^+$-ATPase alpha subunits by immunoblot also revealed that alpha1 ($T = 2.75$; $p < 0.05$), alpha2 ($T = 3.23$; $p < 0.01$), and alpha3

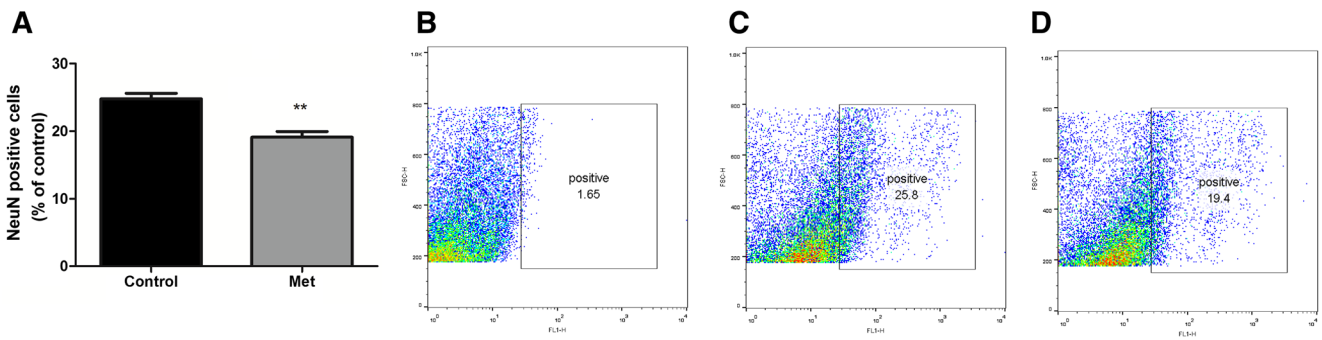


**Fig. 1** **a** Graph representing the effect of gestational hypermethioninemia on the number of neurons in the rat pups. Results are expressed as means ± SD for six animals in each group. Different from control, $^{**}p < 0.01$ (Student's *t* test). **b** Representative plot from negative control sample processed without NeuN antibody, just the secondary antibody. **c** Representative plot from control rat. **d** Representative plot from treated rat

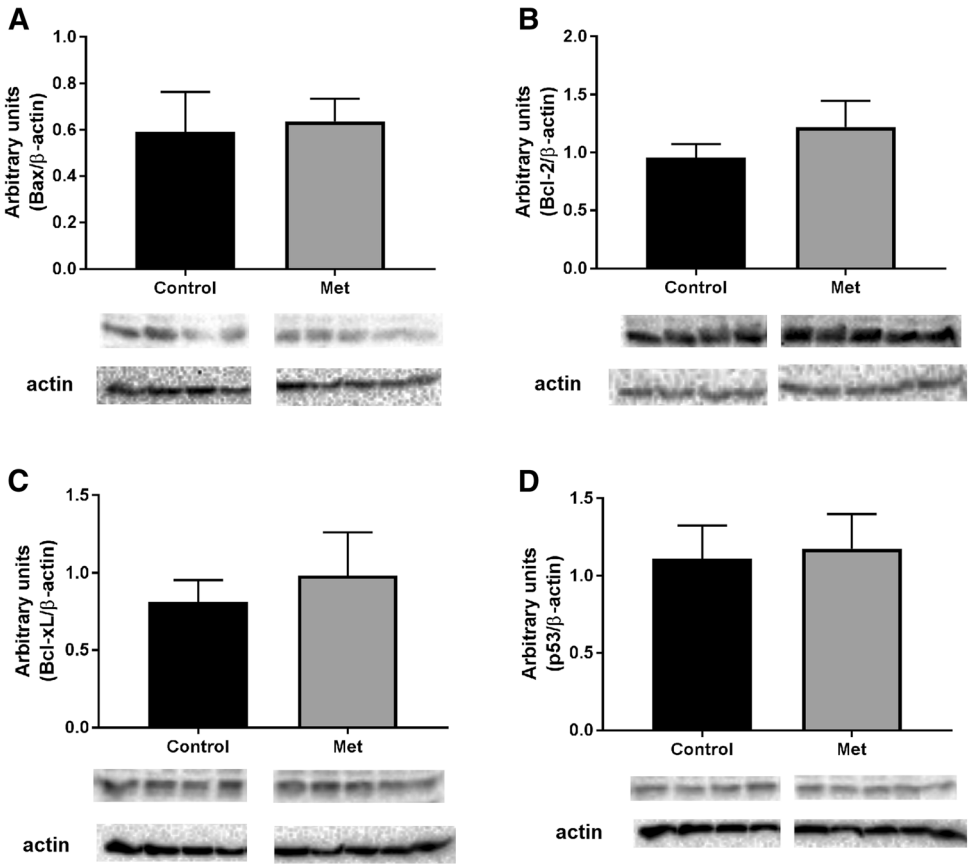


**Fig. 2** Effect of gestational hypermethioninemia on Bax (**a**), Bcl-2 (**b**), Bcl-xL (**c**), and p53 (**d**) content in encephalon of the rat pups. Results are expressed as means ± SD for six animals in each group, $p > 0.05$ (Student's $t$ test)

($T = 3.33$; $p < 0.01$) protein content was increased in the encephalon of pups whose mothers were treated with Met, as shown in Fig. 5B.

### Effect of Gestational Hypermethioninemia on Cerebral Edema Formation in the Offspring

Determination of tissue water content revealed that maternal hypermethioninemia did not cause cerebral edema in the offspring (control: 77.03 ± 0.65; Met: 76.77 ± 1.41).

### Effect of Gestational Hypermethioninemia on Biomarkers of Inflammation in the Encephalon of the Offspring

Gestational hypermethioninemia was not able to significantly alter the levels of TNF-alpha (control: 6.81 ± 1.31; Met: 5.52 ± 0.99) and IL-6 (control:

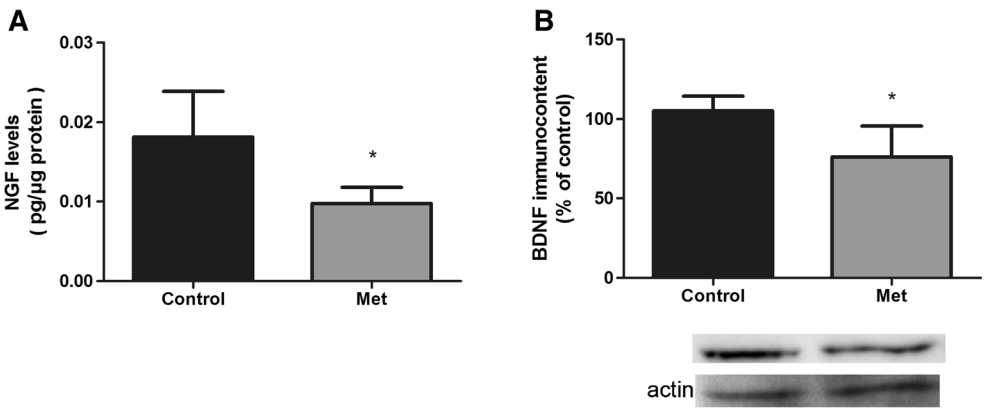


**Fig. 3** Effect of gestational hypermethioninemia on NGF (**a**) and BDNF (**b**) levels in encephalon of the rat pups. Results are expressed as means ± SD for six animals in each group. Different from control, $^{*}p < 0.05$ (Student's $t$ test)

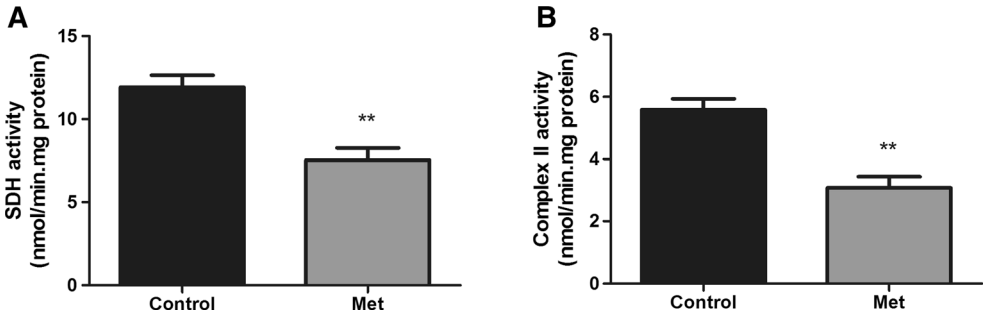


**Fig. 4** Effect of gestational hypermethioninemia on SDH (**a**) and complex II (**b**) activities in encephalon of the rat pups. Results are expressed as means ± SD for six animals in each group. Different from control, $^{**}p < 0.01$ (Student's *t* test)

140.67 ± 34.42; Met: 177.33 ± 31.60) in the brain of the offspring.

### Effect of Gestational Hypermethioninemia on Mitochondrial Hydrogen Peroxide Levels in the Encephalon of the Offspring

The levels of $H_2O_2$ were quantified in the encephalon of pups but no difference between the groups was observed (control: 1.82 ± 0.10; Met: 1.85 ± 0.06).

## Discussion

Patients with severe hypermethioninemia may present neurological dysfunction manifested by cognitive deficit and mental retardation [1]. However, the effect of the maternal hypermethioninemia on the developing brain during intrauterine life is still poorly studied. Therefore, in the present study, we chemically induced hypermethioninemia in pregnant rats and evaluated the number of neurons, thorough monoclonal antibody anti-NeuN, in the encephalon of the offspring. There was a significant difference between the groups, indicating that maternal hypermethioninemia reduced neurons number. Although we have observed a loss of neuronal cells, neither the pro-apoptotic proteins (Bax and p53) nor the anti-apoptotic proteins (Bcl-2 and Bcl-xL) were altered. This result may suggest that the Met treatment induced apoptosis by a mechanism not dependent on p53 or Bcl-2 family members. Besides, the neuronal loss observed is probably related to the decreased NGF and BDNF content. These neurotrophins have important role in the generation of neurons, as well as in the neuronal survival. These results are very important since decreased number of neurons during brain development can impair synaptic responses and lead to learning problems in the offspring. Besides, NGF and BDNF play a crucial role during the process of memory formation [15, 16].

Next, we evaluated brain energy metabolism. Results showed that SDH and complex II activities were significantly reduced in the encephalon of pups whose mothers were treated with Met, suggesting an impaired respiratory chain function. It was previously reported that maternal hypermethioninemia induces oxidative stress in brain of the offspring [3], and it is well known that the complexes of electron transport chain are susceptible to injury by free radicals [17], which could explain these results. Since complex II/SDH plays a key role in the respiratory chain and the tricarboxylic acid cycle [18, 19] and since the brain is highly dependent on a

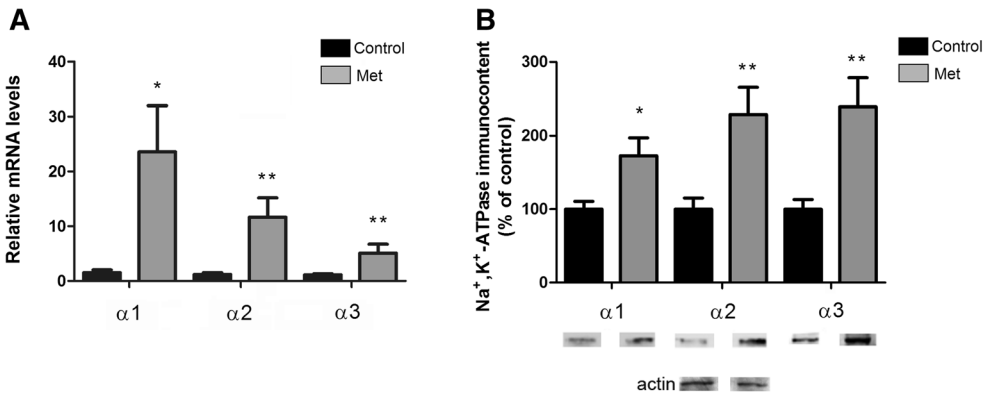


**Fig. 5** Effect of gestational hypermethioninemia on the expression (**a**) and immunocontent (**b**) of $Na^+,K^+$-ATPase alpha1, alpha2, and alpha3 subunits in encephalon of the rat pups. Results are expressed as means ± SD for six animals in each group. Different from control, $^{*}p < 0.05$; $^{**}p < 0.01$ (Student's *t* test)

continuous supply of energy, this condition could cause neurological damage [20]. Studies have shown that reduced energy demand is associated with various neurodegenerative disorders, such as Alzheimer's, Parkinson's, and Huntington's diseases, as well as Friedreich's ataxia [21–23].

It is also important to underline that in our previous work, brain $Na^+,K^+$-ATPase activity was reduced in pups due to maternal hypermethioninemia [3]. Once this enzyme consumes ATP at a high rate, the reduced complex II/SDH activity could reduce energy supply and consequently contribute to the diminish in the activity of $Na^+,K^+$-ATPase. To better understand the mechanisms involved in the reduced activity of this enzyme during gestational hypermethioninemia, we also evaluated the expression and immunocontent of $Na^+,K^+$-ATPase. Results showed that the inhibitory effect of Met on $Na^+,K^+$-ATPase activity observed in our previous study was inversely correlated to the mRNA levels and immunocontent of the catalytic alpha subunits of $Na^+,K^+$-ATPase. This result suggests that the Met-induced decrease in $Na^+,K^+$-ATPase activity does not occur by altering gene expression or the total number of enzyme molecules, but is a post-translational inhibition probably due to reduced energy metabolism and/or oxidative damage to SH groups of $Na^+,K^+$-ATPase. Besides, the up-regulation in transcription/translation with consequent increase in the amount of the enzyme probably indicates the development of an adaptive compensatory mechanism.

Previous studies have demonstrated that reduced $Na^+,K^+$-ATPase activity in the brain is able to increase the intracellular $Na^+$ concentration, contributing to the physiopathological mechanisms involved in the formation of cerebral edema [24]. On this basis, we also evaluated the content of water in the brain of the offspring, but no difference was observed when compared to control. Therefore, although patients with severe hypermethioninemia may present cerebral edema [1], this condition during gestation does not seem to affect the offspring. Studies show that the inactivation of $Na^+$-$K^+$-ATPase activity does not necessarily cause an increase in cell volume, since inhibition of $Na^+$ exit may be rapidly compensated by a reduction in apical $Na^+$ entry and an improve in basolateral $Cl^-$ conductance [25].

Neuroinflammation has been identified as a factor that contributes to development of neurological diseases, such as Alzheimer's disease [26], Parkinson's disease [27], Huntington's disease [28], and multiple sclerosis [29]. Once enhanced reactive oxygen species production may up-regulate pro-inflammatory process [30] and we demonstrated in our previous work that Met treatment during gestation induces oxidative stress in brain of the offspring [3], we evaluated the effect of this treatment on brain inflammation of rats pups. TNF-alpha is a cell signaling protein that induces the migration of leukocytes to the inflamed tissue and promotes apoptosis [31], while IL-6 is considered an activator of acute phase responses as well as a lymphocyte stimulatory factor [32]. In the present work, we did not observe important alterations in these parameters, suggesting that neuroinflammation is not involved in the pathophysiological process of maternal hypermethioninemia. In agreement, previous studies showed that diet rich in Met does not alter TNF-alpha and IL-6 levels in plasma of mice [33].

We have previously demonstrated that maternal hypermethioninemia decreased catalase activity in the encephalon of the offspring [3]. Once this antioxidant enzyme decomposes $H_2O_2$, we believed that this condition could increase $H_2O_2$ levels. However, we measured this reactive oxygen species in the present study and no alteration was observed. It is possible that the action of other peroxidases responsible for $H_2O_2$ detoxification could have been enough to eliminate this molecule.

In conclusion, we demonstrated for the first time that gestational hypermethioninemia decreases the number of neurons associated to decreased NGF and BDNF levels in brain of the offspring. Maternal hypermethioninemia also reduced SDH and complex II activities and increased gene expression and immunocontent of $Na^+,K^+$-ATPase. Cerebral edema and neuroinflammation were not observed. These results indicate that maternal hypermethioninemia may be a predisposing factor for damage to the brain during the intrauterine life. Neurological injury during this period could prejudice the developing of central nervous system and cause behavior alterations to the offspring.

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**Compliance with Ethical Standards**

**Declaration of Interest** The authors declare that they have no conflict of interest.

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## 3.4 Capítulo III

### MANUSCRITO 3

**Methionine administration in pregnant rats causes memory deficit in the offspring and alters ultrastructure in brain tissue**

Schweinberger BM, Rodrigues AF, dos Santos TM, Rohden F, Barbosa S, da Luz Soster PR, Partata WA, Faccioni-Heuser MC, Wyse ATS

**Artigo a ser submetido.**

**Methionine administration in pregnant rats causes memory deficit in the offspring and alters ultrastructure in brain tissue**

Bruna M. Schweinberger[a], André F. Rodrigues[a], Tiago Marcon dos Santos[a], Francieli Rohden[a], Silvia Barbosa[c], Paula Rigon da Luz Soster[c], Wania Aparecida Partata[d], Maria Cristina Faccioni-Heuser[d] and Angela T. S. Wyse[a,b]

[a]Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[b]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[c]Departamento de Ciências Morfológicas, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[d]Departamento de Fisiologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

**Address reprint requests to:** Dra. Angela T. S. Wyse, Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil, Phone: 55 51 3308 5573, Fax: 55 51 3308 5535, E-mail: wyse@ufrgs.br

**Abstract**

In the present work we evaluated the effect of gestational hypermethioninemia on locomotor activity, anxiety, memory, and exploratory behavior of rat offspring through the following behavior tests: open field, object recognition, and inhibitory avoidance. Histological analysis was also done in the brain tissue of the pups. Wistar female rats received methionine (2.68 μmol/g body weight) by subcutaneous injections during pregnancy. Control rats received saline. Histological analyses were made in brain tissue from 21 and 30 days-of-age pups. Another group was left to recover until the 30$^{th}$ day of life to perform behavior tests. Results from open field task showed that pups exposed to methionine during intrauterine development spent more time in the center of the arena. In the object recognition memory task, we observed that methionine administration during pregnancy significantly reduced the total exploration time of rat offspring during training session. The test session showed that the methionine reduced the Recognition Index. Regarding to inhibitory avoidance task, the decrease in the step-down latency at 1 and 24 h after the training demonstrated that maternal hypermethioninemia impaired short-term and long-term memories of rat offspring. Electron microscopy revealed alterations in the ultrastructure of neurons at 21 and 30 days of age. Our findings suggest that the cell morphological changes caused by maternal hypermethioninemia may be, at least in part, associated to the memory deficit of rat offspring.

**Keywords:** Gestation; Hypermethioninemia; Histology; Memory.

## Introduction

The fetal brain development is susceptible to several adverse conditions within the mother's environment. Neurotoxicity during prenatal period may lead to biochemical, histological and behavior alterations to the offspring, following toxic substances exposure of the mother during gestation. Therefore, it is very important to recognize pathological conditions that may impair embryo brain formation.

We have published data about the effects of maternal hypermethioninemia on the offspring (Schweinberger et al., 2014; 2017). Hypermethioninemia may be caused by the hereditary deficiencies in enzymes involved in methionine (Met) metabolism, such as Met adenosyltransferase I/III and Cystathionine β-synthase. Diet rich in proteins and liver diseases may also increase blood Met levels (Mudd, 2011). In our previous works, we demonstrated that this condition induces oxidative stress, inhibits $Na^+$ ,$K^+$ -ATPase and $Mg^{2+}$ -ATPase activities, impairs energy metabolism, and decreases neurons number and neurotrophins in the encephalon of 21 days-of-age rat pups (Schweinberger et al., 2014; 2017).

All these alterations together could impair neuronal cells and cause cognitive deficit. Oxidative stress may cause cell death by damaging biomolecules, including the enzyme $Na^+$ ,$K^+$ -ATPase. The inefficient functioning of brain $Na^+$ ,$K^+$ -ATPase may affect neurotransmitter signaling and neural activity (de Lores Arnaiz and Ordieres, 2014). Besides, cerebral oxidative stress may impair enzymes and complexes involved in energy metabolism, which may cause additional brain damage since this tissue depends on a high energy demand (Howarth et al., 2012). Decreased neurotrophins may negatively impact on growth, survival and/or differentiation of neurons, and

consequently also affect memory formation (Gómez-Palacio-Schjetnan and Escobar, 2013).

Considering what has been exposed above, the objective of the present study was to evaluate the effect of gestational hypermethioninemia on locomotor activity, anxiety, memory, and exploratory behavior of rat offspring (at 30$^{th}$ day of life) through the following behavior tests: open field, object recognition, and inhibitory avoidance. Brain tissue from 21 and 30 days-of-age pups were analyzed by electron microscopy. Our hypothesis is that the cerebral biochemical alterations induced by gestational hypermethioninemia observed in our earlier studies could impair the memory of the offspring and cause morphological alterations in neuronal cells.

## Materials and Methods

### *Animals and reagents*

The Wistar rats used in this study were obtained from the Central Animal House of the Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in a constant temperature (22±1$^{o}$C) room and had free access to a 20% (w/w) protein commercial chow and water. All the experimental protocol followed the official governmental guidelines issued by the Brazilian Federation of Societies for Experimental Biology, following the Guide for the Care and Use of Laboratory Animals (No. 80-23, revised 1996) and Arouca Law (Law no. 11.794/2008). This research project was approved by the Ethics Committee on the Use of Animals of Universidade Federal do Rio Grande do Sul (registration number:

25913). Chemicals were acquired from Sigma Chemical Co., St. Louis, MO, USA.

*Chronic Methionine treatment*

After mating the female Wistar rats with males, we verified the presence of sperm in the vaginal smear to confirm pregnancy. Then, the pregnant rats (70 to 90 days of age) received two daily subcutaneous injections of 2.68 µmol Met/g body weight during all gestation (approximately 21 days) (Schweinberger et al., 2014). Such dose was chosen based on a previous study that verified that this treatment induces plasma levels of Met close to 30-fold higher (around 2 mM) than normal levels (Stefanello et al., 2007a). Control animals received saline. Female and males pups were killed at the 21$^{st}$ and 30$^{th}$ day of life. Another group was left to recover until the 30$^{th}$ day of life to perform behavior tests.

*Open-field task*

This test was used as a standard test of general activity. Animals were monitored during 5 min in a 50 cm × 50 cm × 50 cm open field. The traveled distance, mobility time, lines crossing, time spent in the center of the box, and the mean speed were determined to evaluate locomotion and anxiety. A video camera connected to a computer was located above the arena with the aim to record the test for subsequent analysis using the ANY-Maze software (Netto et al., 1986).

*Object recognition task*

This task was based on Ennaceur and Delacour (1988) and adapted from the task by Ouchi et al. (2013) with some modifications. The test consists of three phases:

habituation, familiarization, and test. In the habituation session, the animal freely explored a 50 cm × 50 cm × 50 cm box in the absence of objects. In the familiarization session, the animal was placed in the arena containing two identical objects (A1 and A2) for 5 min and the time spent in the exploration of each object was recorded. After 24 h, the test session was performed and the animal was returned to the arena for 5 min with two objects: the familiar object (A1) and a novel object (B). The time devoted to each object was registered. *Recognition Index* (RI) was determined through the division of the time devoted to the novel object by the total time spent exploring both objects.

*Step-down inhibitory avoidance task*

The animals were placed on a 2.5 cm high, 7.0 cm wide, 25 cm long platform at a 50×25×25 cm apparatus. The floor contained a series of parallel 0.1 cm caliber stainless steel bars. In the training session, after the rats stepped down placing the four paws on the grid, they received 3×0.4 s, 0.6 mA foot shock. Short-term and long-term memories were tested 1 and 24 h after training, respectively. The test session procedure was identical to the training, except that no foot shock was given, and the step-down latency was determined (cutoff at 180 s) (Izquierdo et al., 1997; Wyse et al., 2004).

*Electron microscopy*

Cardiac perfusion was first performed with 0.9% saline solution and then with paraformoldehyde 4% plus glutaraldehyde 2.5% in 0.1M phosphate buffer (PB) at room temperature. The brains were sectioned in vibratomo (1000 μm), the slices were immersed again in the same fixative solution. After, they were washed in PB and fixed in 1% osmium tetroxide, OsO4 (Sigma) in PB, pH 7.4 for one hour at room

temperature. They were washed again with PB and then dehydrated gradually with acetone (MERCK) and soaked in epon resin. The polymerization was carried out for 48 hours at 60° C. Semi-thin sections were made in the thickness of 1 μm using ultramicrotome and stained with 1% toluidine blue. Ultra-fine cuts were obtained in ultramicrotome for the assembly of copper grids (200 mesh). The samples were counterstained with 1% uranyl acetate (MERCK) and then with 1% lead citrate (MERCK) and examined in transmission electron micrometer (JEM 1200 EXII, Japan). 246 images were analyzed.

*Statistical determination*

Data from inhibitory avoidance task were assessed by individual (two tailed) Mann-Whitney U tests. Other behavior tests were analyzed by Student's *t*-test. Statistical Package for the Social Sciences (SPSS) software was used in a PC-compatible computer. Differences were considered statistically significant if $p<0.05$.

**Results**

*Effect of gestational hypermethioninemia on the open field task*

No differences were found in traveled distance, mobility time, lines crossing, and mean speed, when compared to the control group. However, the time spent in the center was higher in the pups exposed to Met during intrauterine development [$T = 2.96$; $p<0.05$] (table 1).

*Effect of gestational hypermethioninemia on object recognition task*

In the object recognition memory task, Met administration during pregnancy significantly reduced total exploration time of the rat pups in training session [$T = 5.47$; $p<0.001$], but not in test session [$T = 0.74$; $p>0.05$] (figure 1A). Figure 1B demonstrates that control [$T = 1.27$; $p>0.05$] and treated pups [$T = 0.37$; $p>0.05$] had no difference in the exploration of both objects in training session. In test session, control animals distinguished the new object from the familiar one [$T = 2.18$; $p<0.05$], whereas pups born to hypermethioninemic rats did not recognize the familiar object [$T = 1.62$; $p>0.05$] (figure 1C). Besides, the test session (24 h after familiarization) showed that the treatment reduced the Recognition Index [$T = 2.82$; $p<0.05$] (figure 1D).

*Effect of gestational hypermethioninemia on the inhibitory avoidance task*

In the training session, no difference in latency was observed between control and treated rats [$U = 24$; $p>0.05$, data not shown]. In the test session, the decrease in the step-down latency at 1 and 24 h after the training showed that maternal hypermethioninemia significantly impaired short-term [$U = 0$; $p<0.001$] and long-term [$U = 0$; $p<0.001$] memories of the offspring, as can be seen in figures 2A and 2B, respectively.

*Electron microscopy*

Examination of the brain tissue from 21 days-of-age rat pups whose mothers received Met showed neurons with few organelles, scarce endoplasmic reticulum, and large number of mitochondria. The nucleus presented very condensed chromatin. At 30 days of age, the treatment caused deformed neurons and vacuolated neuropile.

Cytoplasm of neurons presented mitochondria with deranged architecture, scarce endoplasmic reticulum and remnants of myelin sheath (figures 3 to 6).

**Discussion**

Hypermethioninemia is a pathological condition characterized by increased Met levels in blood and other tissues. Such condition may result from a hereditary deficiency in the enzyme that metabolizes Met, which is denominated Met adenosyltransferase. Hypermethioninemia has been also identified in classical homocystinuria, tyrosinemia, and galactosemia. Hypermethioninemia from non-genetic origin principally occurs in liver injury and during excessive protein intake (Mudd et al., 2011). In severe cases, affected individuals may present neurobehavioral deficits, such as mental retardation and cognitive disorders (Mudd et al., 2001, Schweinberger at al., 2016). However, the effect of gestational hypermethioninemia on the postnatal development of the offspring is still poorly studied.

Therefore, the present study extended the investigations on this theme and determined the effect of Met exposure during the prenatal period on some behavior parameters, such as locomotion, anxiety, memory, and exploratory activity. First, the rat pups were subjected to the open field task, but we did not observe significant changes in traveled distance, mobility time, lines crossing, and mean speed, indicating that locomotor activity was not altered by the treatment. However, we observed an increase in the time spent in the center of the open field by pups exposed to Met, which may indicate an anxiolytic-like behavior. Rats normally spend greater amount of time exploring the periphery once the center is considered an unprotected area. Data from literature demonstrated that *S*-adenosyl-methionine (SAM), an

intermediate metabolite of Met formed by Met adenosyltransferase reaction, present anxiolytic effects and may be used as adjunctive treatment for psychiatric disorders (Bressa, 1994; Papakostas, 2009; Di Pierro et al., 2015). Therefore, increased SAM levels induced by hypermethioninemia could explain this result. However, it should be noted that young animals normally show an innate unconditioned fear and anxiety. Therefore, this result may be harmful, once the basal anxiety participates of the mechanisms by which aversive memories are formed. Besides, decreased basal anxiety may impair the ability in recognizing fear and so decrease alertness or attention of the animals.

Next, we performed the object recognition task, which demonstrated that the long-term memory of the rat pups was impaired. Control rats spent more time exploring the novel object during the test phase. However, the pups born to hypermethioninemic rats devoted similar time in both objects, suggesting that these animals did not recognize the familiar object, as indicated by the decreased Recognition Index. In agreement with these results, adult zebrafish exposed to Met had memory impairment on inhibitory avoidance task (Vuaden et al., 2012). Besides, developing rats treated with injections of Met from the $6^{th}$ to the $28^{th}$ day of age, presented impaired working memory performance, as observed in the Morris water maze task (Stefanello et al., 2007b).

However, it should be noted that Met treatment during pregnancy significantly reduced the total exploration time of the offspring in training session. Therefore, it is possible that a reduction of interest in exploring may be contributing to the lower performance in the object recognition test, since less exploration during training session may influence the non-recognition of familiar object in the test session due to

acquisition deficits. Stefanello and collaborators (2007b) have previously shown that the induction of chronic hypermethioninemia in developing rats leads to a significant increase of acetylcholinesterase activity in cerebral cortex. As a consequence of this alteration, excessive destruction of acetylcholine may occur. Therefore, this mechanism could explain the altered exploratory behavior observed in this study once it has been reported that the cholinergic mechanisms contribute to the exploratory motivation (Lamprea et al., 2003).

We also performed step-down inhibitory avoidance task to evaluate aversive memory. In this test, longer latencies in the test session indicate that the animal remembers the shock, suggesting a better memory. The results showed a reduced step-down latency at 1 and 24 h after the training, when compared to control group, indicating that gestational hypermethioninemia impaired short-term and long-term memories, respectively.

Electron microscopy revealed neuronal nucleus with very condensed chromatin, disorganized cytoplasm and loss of integrity of most organelles, except for the mitochondria, which were more numerous in the brain tissue of the Met treated 21-days-of-age pups. We suggest that the large number of mitochondria may be a compensatory mechanism since mitochondrial biogenesis is able to contribute to cellular recuperation from impairment caused by different pathophysiological events. Besides, we have previously demonstrated that maternal hypermethioninemia affects energy metabolism in encephalon of the offspring (Schweinberger et al., 2017). Therefore, it is possible that the increase in the amount of mitochondria represents an attempt to enhance cellular bioenergy capacity.

At the 30$^{th}$ day of life, morphological changes were more severe. At this age, we

observed disorganized cytoplasm and loss of organelles, including mitochondria. Cytoplasm and neuropile presented prominent vacuoles in the brain tissue from rats whose mothers were treated with Met. Remnants of myelin sheath was also seen. The alterations verified in the cells structure suggest a degenerative process of neurons.

In summary, our findings demonstrated that gestational hypermethioninemia seems to decrease basal anxiety and affects the memory and exploratory activity of the offspring. Besides, electron microscopy revealed alterations in ultrastructure of neurons from cerebral tissue. Therefore, the data identified maternal hypermethioninemia as a condition that may cause neurological injury to the offspring even during postnatal life. Identification of pathological processes during pregnancy that may impair the brain development during the intrauterine life is important since this knowledge offers the possibility to avoid or minimize potential adverse effects that could prejudice life quality of the offspring.

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**Table and Figures**

**Table 1** Effect of gestational hypermethioninemia on distance traveled, mobility time, lines crossing, time spent in center, and mean speed of the rat pups in the open field task. Results are expressed as means ± SD for eight animals in each group. Different from control, $^{*}p<0.05$ (Student's t-test).

| Group | Distance (m) | Mobility time (s) | Lines croosing | Center time (s) | Speed |
|---|---|---|---|---|---|
| Saline | 21.57±3.81 | 256.5±39.66 | 263.3±36.46 | 15.07±4.49 | 0.35±0.09 |
| Met | 21.98±3.47 | 283.1±14.59 | 279.2±40.88 | 24.02±5.56* | 0.37±0.06 |

**Figure 1** Effect of gestational hypermethioninemia on total time spent in exploration (A), time spent in each object (A1 and A2) during training session (B), time spent in familiar (A1) and novel object (B) during the test session (C), and object recognition index (D). Results are expressed as means ± SD for eight animals in each group. Different from control, $^{*}p<0.05$; $^{***}p<0.001$ (Student's t-test).

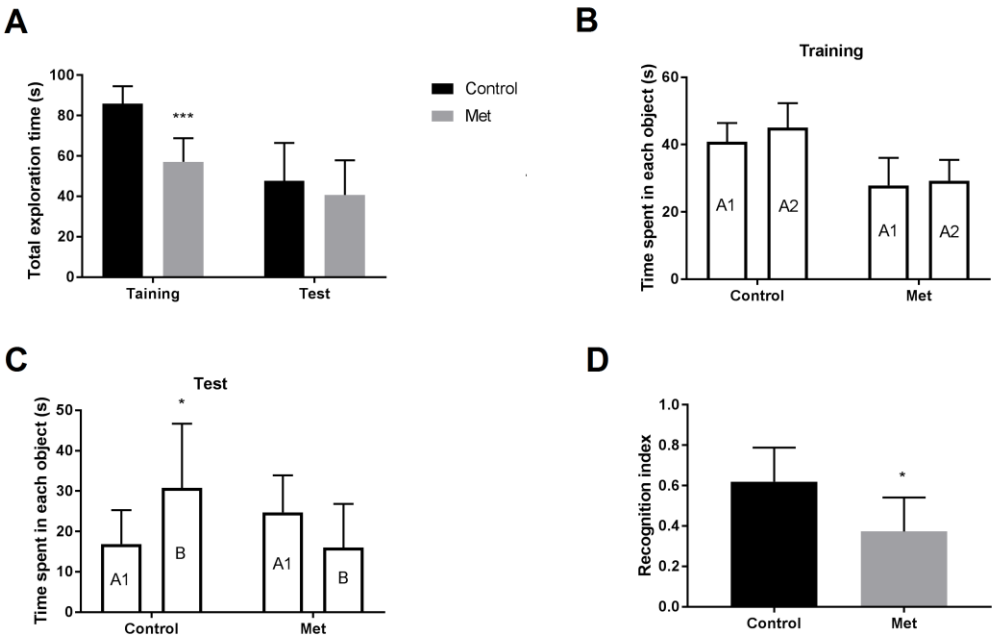


**Figure 2** Effect of gestational hypermethioninemia on latency time in test session of step-down inhibitory avoidance task at 1 hour (short-term memory) (A) and 24 hours (long-term memory) (B) after training rat pups. Data are median (interquartile range) for eight animals in each group. Different from control, $^{###}p<0.001$ (Mann-Whitney).

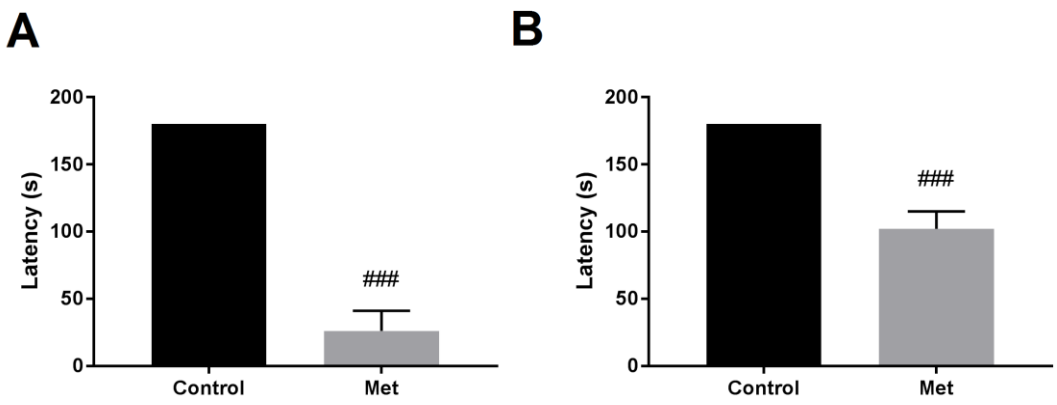


**Fig. 3** Representative images of brain tissue samples from 21-day-old pups from saline-treated mothers (n = 3). A) shows an intact neuron, with its well preserved dendrite, several organelles, and well-designed plasma membrane. The characteristic nucleus presenting a nucleolus rather electrodensis (arrow). The absence of extracellular space is remarkable, since the elements of the neuropile (#) make intimate contact with each other. B and C) show a perikaryon rich in intact organelles and free ribosomes in the form of rosettes, surrounded by visible plasma membrane. N: nucleus, BV: blood vessel, RER: rough endoplasmic reticulum, GC: Golgi complex, M: mitochondria, SD: synaptic density, R: ribosome rosettes. PM: plasma membrane, C: caryotheca. Magnifications 4,000, 25,000 and 50,000X respectively.

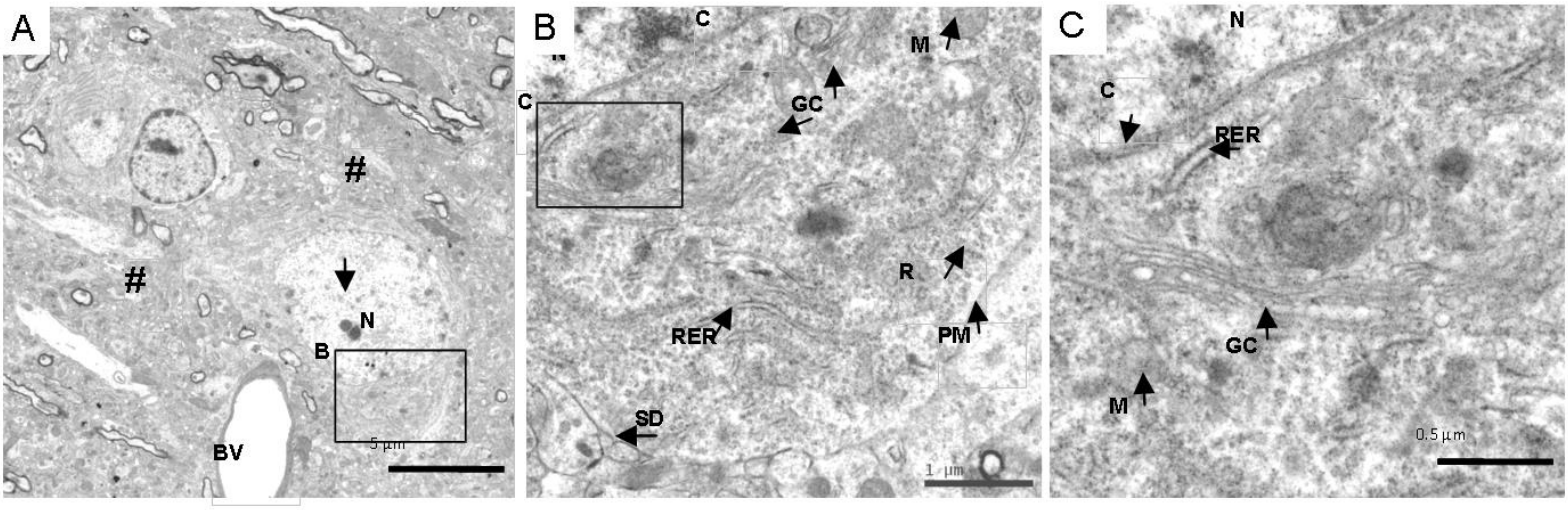


**Fig. 4** Representative images of brain tissue samples from 21-day-old pups from Met treated mothers (n = 3). A) It presents two neuronal cells, with concentrated neuropile (#). B) It shows an intact neuron, with its conserved cellular body, but with few organelles and large number of mitochondria. The characteristic nucleus well delimited, with very condensed chromatin next to the caryotheca. C) Evidence the cell body of another neuron, where we can also observe large numbers of mitochondria and a scarce endoplasmic reticulum, where the concentrated presence of ribosomes is well visible. It also shows free ribosomes in the form of rosettes. N: nucleus, RER: rough endoplasmic reticulum, GC: Golgi complex, M: mitochondria, R: ribosome rosettes, SD: synaptic density. Magnifications 12,000 and 25,000X respectively.

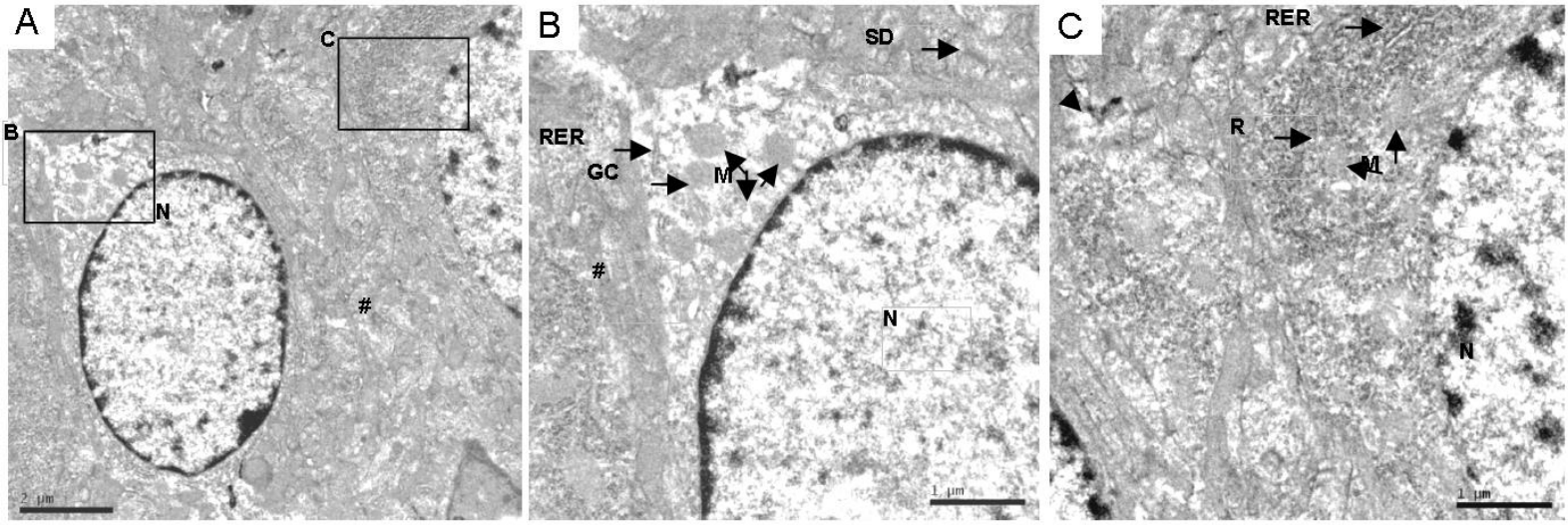


**Fig. 5** Representative images of brain tissue samples from 30-day-old pups from saline treated mothers (n = 3). A) Shows an intact neuron, with its well preserved dendrite, several organelles, and well-designed plasma membrane. The characteristic nucleus presenting a nucleolus (*) rather electrodensing. The absence of extracellular space is remarkable, since the elements of the neuropile (#) make intimate contact with each other. B and C) show a perikaryon rich in intact organelles and free ribosomes in the form of rosettes, surrounded by visible plasma membrane. N: nucleus, BV: Blood vessel, RER: rough endoplasmic reticulum, GC: Golgi complex, M: mitochondria, SD: synaptic density, R: ribosome rosettes, PM: plasma membrane, C: caryotheca. Magnifications 12,000, 25,000 and 50,000X respectively.

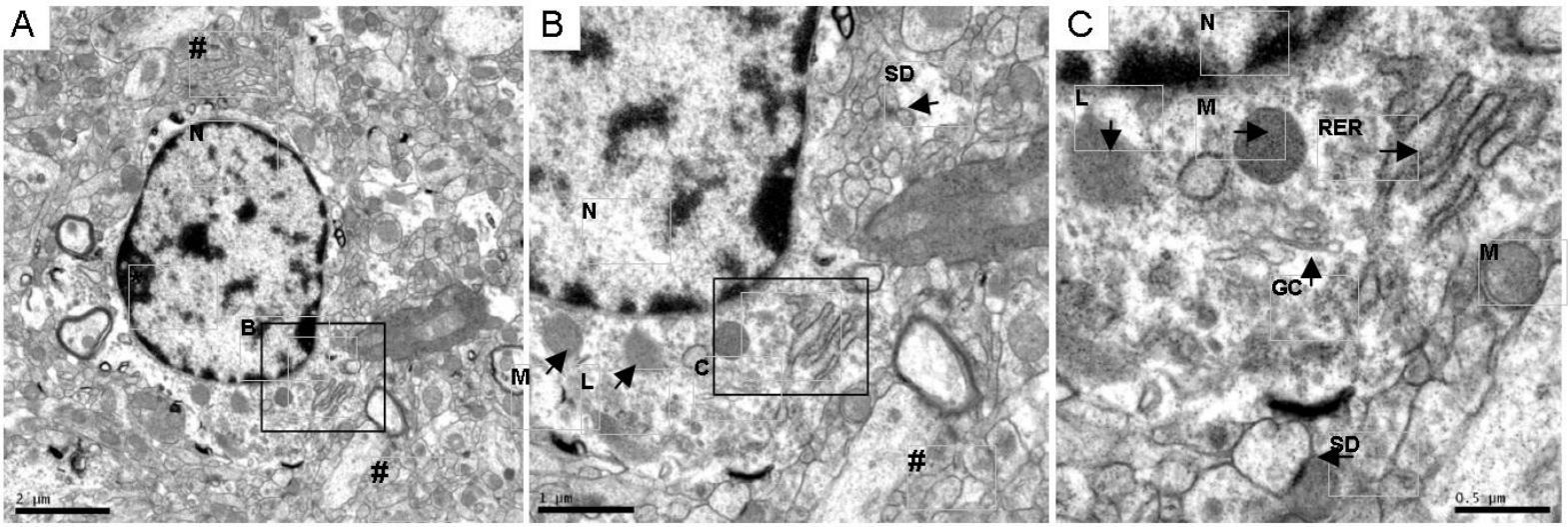
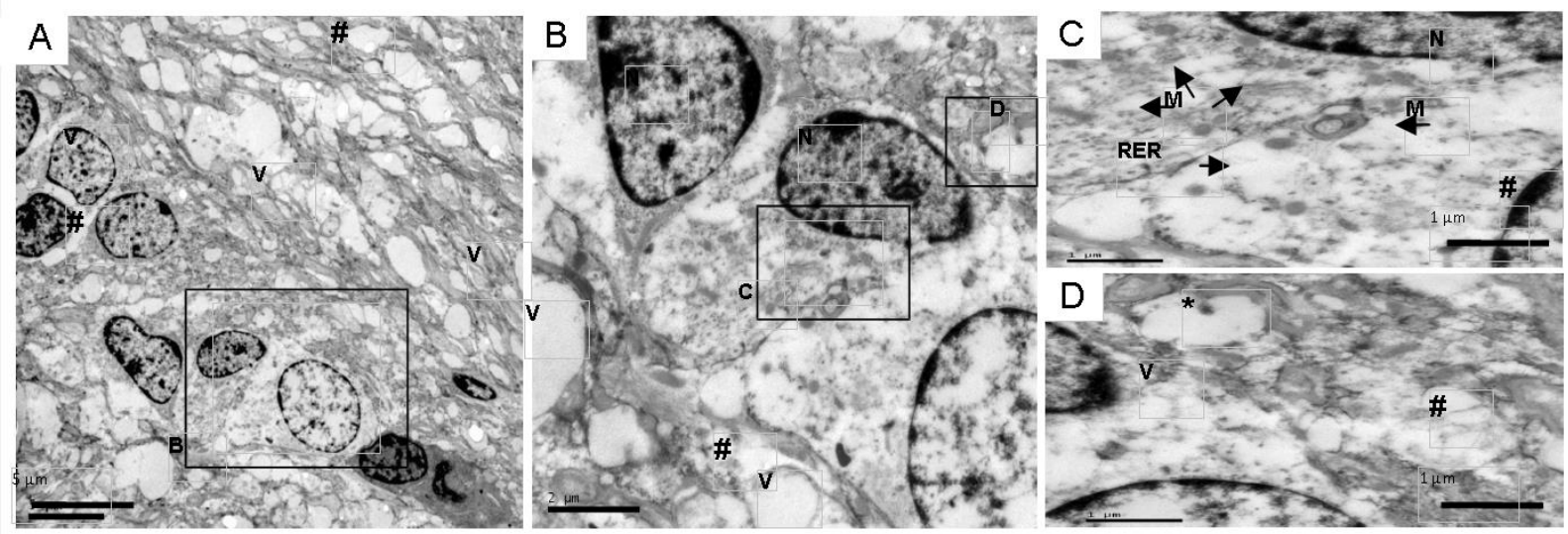


**Fig. 6** Representative images of brain tissue samples from 30-day-old pups from Met treated mothers (n = 3). A and B) Deformed neurons and neuropile (#) are observed with prominent vacuoles. C) Detail of the cytoplasm of one of the neurons, showing mitochondria with deranged architecture and a scarce endoplasmic reticulum. D) Shows the completely deformed and vacuolated neuropile and also remnants of myelin sheath (*). N: nucleus, RER: rough endoplasmic reticulum, M: mitochondria, V: vacuole. Magnifications 4,000, 12,000 and 25,000X respectively.



## 3.5 Capítulo IV

### MANUSCRITO 4

**Gestational hypermethioninaemia alters oxidative/nitrative status in skeletal muscle and biomarkers of muscular injury and inflammation in serum of rat offspring**

Schweinberger BM, Turcatel E, Rodrigues AF, Wyse AT.

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ORIGINAL ARTICLE

# Gestational hypermethioninaemia alters oxidative/nitrative status in skeletal muscle and biomarkers of muscular injury and inflammation in serum of rat offspring

Bruna M. Schweinberger*,†, Elias Turcatel*,†, André F. Rodrigues*,† and Angela T. S. Wyse*,†

*Laboratório de Neuroproteção e Doenças Neurometabólicas, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil and †Programa de Pós-Graduação em Ciências Biológicas – Bioquímica. Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

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**Correspondence:**
Angela T. S. Wyse
Departamento de Bioquímica, ICBS
Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos 2600-Anexo
CEP 90035-003
Porto Alegre, RS
Brazil
Tel.: 55 51 3308 5573
Fax: 55 51 3308 5535
E-mail: wyse@ufrgs.br

## SUMMARY

In this study we evaluated oxidative/nitrative stress parameters (reactive oxygen species production, lipid peroxidation, sulfhydryl content, superoxide dismutase, catalase and nitrite levels), as well as total protein content in the gastrocnemius skeletal muscle of the offspring of rats that had been subjected to gestational hypermethioninaemia. The occurrence of muscular injury and inflammation was also measured by creatine kinase activity, levels of creatinine, urea and C-reactive protein and the presence of cardiac troponin I in serum. Wistar female rats (70–90 days of age) received methionine (2.68 μmol/g body weight) or saline (control) twice a day by subcutaneous injections during the gestational period (21 days). After the rats gave birth, pups were killed at the twenty-first day of life for removal of muscle and serum. Methionine treatment increased reactive oxygen species production and lipid peroxidation and decreased sulfhydryl content, antioxidant enzymes activities and nitrite levels, as well as total protein content in skeletal muscle of the offspring. Creatine kinase activity was reduced and urea and C-reactive protein levels were increased in serum of pups. These results were accompanied by reduced muscle mass. Our findings showed that maternal gestational hypermethioninaemia induced changes in oxidative/nitrative status in gastrocnemius skeletal muscle of the offspring. This may represent a mechanism which can contribute to the myopathies and loss of muscular mass that is found in some hypermethioninaemic patients. In addition, we believe that these results may be relevant as gestational hypermethioninaemia could cause damage to the skeletal muscle during intrauterine life.

**Keywords**
gestational hypermethioninaemia, inflammation, muscle damage, nitrite levels, oxidative stress, skeletal muscle

Methionine (Met) is an essential sulphur-containing amino acid, which is metabolized by the enzyme Met adenosyltransferase (MAT). MAT I/III predominates in the liver and catalyses the transfer of the adenosyl group from ATP to Met, resulting in S-adenosylmethionine. This is a methyl-donating compound used by different methyltransferase reactions that form S-adenosylhomocysteine, which is hydrolysed to generate homocysteine (Cantoni 1953; Stipanuk 2004; Reytor *et al.* 2009).

Hypermethioninaemia occurs when plasma Met exceeds the normal levels that range from 13 to 45 μM (Stabler *et al.* 2002). This condition may be of non-genetic origin, such as in liver diseases, premature birth and where the diet is rich in proteins. However, inherited deficiency of MAT I/III is the most common hereditary cause for isolated hypermethioninaemia (Mudd 2011). Although this disease can be clinically benign, some patients can present facial dysmorphy; neurological dysfunction, such as cerebral oedema and

cognitive impairments; and liver damage (Labrune *et al.* 1990; Moss *et al.* 1999; Mudd *et al.* 2001). Besides, it has been reported that genetic disorders involving Met metabolism may cause muscle damage, including hypotonia and destructive myopathy (Barić 2009), whose mechanisms are still not well elucidated.

As we have previously shown that high Met levels during pregnancy induce oxidative stress in the encephalon of the offspring (Schweinberger *et al.* 2014) and the oxidative stress is associated with different muscular damages (Tidball & Wehling-Henricks 2007; Arbogast *et al.* 2009; Turki *et al.* 2012; Sullivan-Gunn & Lewandowski 2013), in the present study we evaluated the effect of maternal hypermethioninaemia on some oxidative/nitrative stress parameters, namely 2′,7′-dichlorofluorescein fluorescence assay (DCF), sulfhydryl content, thiobarbituric acid-reactive substances (TBARS), the activities of superoxide dismutase (SOD) and catalase (CAT), nitrite levels, and total protein content in gastrocnemius muscle homogenates from offspring of rats with hypermethioninaemia. The occurrence of muscular injury and inflammation was evaluated by specific biomarkers in serum, such as creatine kinase (CK) activity, the levels of creatinine, urea and C-reactive protein (CRP) and the presence of cardiac troponin I (cTnI).

## Materials and methods

### *Animals and reagents*

Wistar rats were obtained from the Central Animal House of the Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12-h light/dark cycle in an air-conditioned constant temperature (22 ± 1°C) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. The experimental protocol followed the NIH 'Guide for the Care and Use of Laboratory Animals' (NIH publication No. 80-23, revised 1996). All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

### *Ethical approval*

This research project was approved by the Ethics Committee in Research of Universidade Federal do Rio Grande do Sul under protocol number 25913.

### *Chronic Methionine treatment*

After mating the female Wistar rats with males, pregnancy was verified by the presence of sperm in the vaginal smear. Pregnant rats (70 to 90 days of age) received two daily subcutaneous injections of Met (2.68 μmol Met/g body weight) during the gestational period (around 21 days). This dose was chosen based on previous work, which demonstrated that this protocol induces plasma levels of Met around 30-fold higher (approximately 2 mM) than normal levels (Stefanello *et al.* 2007). Control rats received saline. After birth, pups of both sexes were killed at the 21st day of life (Schweinberger *et al.* 2014).

### *Tissue preparation and serum obtainment*

Animals were killed by decapitation without anaesthesia followed by the removal of gastrocnemius skeletal muscle and blood. The muscles from the left and right hindlimbs were individually weighed, pooled for analysis and homogenized in 10 volumes (1:10, w/v) of buffer solution (sodium phosphate 20 mM, KCl 140 mM, pH 7.4). Blood was collected and centrifuged at 1000 ***g*** for 10 min at 4°C, and serum was removed by suction.

### *2′,7′-Dichlorofluorescein fluorescence assay*

This assay is based on the cleavage of 2′,7′-dichlorofluorescein diacetate ($H_2$DCF-DA) to 2′,7′-dichlorofluorescein ($H_2$DCF), which is oxidized by reactive species present in the samples. The last reaction produces the fluorescent compound DCF, which was quantified following 488-nm excitation and 525-nm emission. Results were expressed as nmol DCF/mg protein (LeBel *et al.* 1990).

### *Sulfhydryl content*

This method is based on the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) by thiols, which become oxidized (disulphide), generating the yellow derivative thionitrobenzoic acid (TNB) whose absorption was determined at 412 nm (Aksenov & Markesbery 2001) using a Beckman DU1 640 spectrophotometer. The sulfhydryl content is inversely correlated with oxidative impairment to proteins. Results were expressed as nmol TNB/mg protein.

### *Thiobarbituric acid-reactive substances*

Samples were incubated in a medium containing 8.1% SDS; 20% acetic acid in aqueous solution pH 3.5; and 0.8% thiobarbituric acid. The reaction was performed in a boiling water bath. After centrifugation, the resulting pink stained TBARS were measured at 535 nm in a Beckman DU® 800 (Beckman Coulter, Inc., Fullerton, CA, USA). A calibration curve was generated using 1,1,3,3-tetramethoxypropane as standard. Results were expressed as nmol TBARS/mg protein (Ohkawa *et al.* 1979).

### *Superoxide dismutase assay*

SOD activity measurement is based on the capacity of pyrogallol to auto-oxidize, a process dependent on superoxide (substrate for SOD). The inhibition of the auto-oxidation of this compound occurs in the presence of SOD, whose activity can be indirectly measured at 420 nm using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA, USA) (Marklund

1985). SOD activity was calculated using a calibration curve performed with purified SOD as standard.

### *Catalase assay*

CAT activity was measured using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies). This assay is based on the disappearance of hydrogen peroxide ($H_2O_2$) at 240 nm in a medium containing 20 mM $H_2O_2$, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0 and 0.1–0.3 mg protein/ml (Aebi 1984). One CAT unit was defined as one μmol of $H_2O_2$ consumed per min.

### *Nitrite assay*

Nitric oxide (NO) was indirectly measured by nitrite levels. Samples were mixed with Griess reagent (1:1 mixture of 1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water), and absorbance was determined on a microplate reader (SpectraMax M5/M5 Microplate Reader; Molecular Devices, MDS Analytical Technologies) at 543 nm. Nitrite concentration was calculated using sodium nitrite standards (Green *et al.* 1982).

### *Protein determination*

Protein concentration was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

### *Biomarkers of muscle damage and inflammation measurement*

Serum CK activity and serum creatinine, urea and CRP levels were assayed using the Cobas Mira Plus analysis system and the specific kits (Labtest Diagnóstica SA).

The detection of cTnI in serum was performed using the One Step Troponin I Test (Bioeasy Diagnóstica Ltda), a rapid chromatographic immunoassay for the qualitative detection of cTnI.

### *Statistical determination*

Data were analysed by Student's *t*-test. Analyses were performed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) software in a PC-compatible computer. Differences were considered statistically significant if $P < 0.05$.

## Results

### *Effect of gestational hypermethioninaemia on oxidative stress parameters in gastrocnemius skeletal muscle*

Maternal hypermethioninaemia increased DCF [$T(6) = 7.16$; $P < 0.001$] and TBARS [$T(6) = 2.97$; $P < 0.05$] levels (Figure 1a and c), suggesting an increase in reactive oxygen species (ROS) production and lipoperoxidation in gastrocnemius skeletal muscle of the pups. Sulfhydryl content [$T(6) = 3.86$; $P < 0.01$] was reduced (Figure 1b).

Met treatment also decreased the activities of SOD [$T(6) = 3.04$; $P < 0.05$] and CAT [$T(6) = 3.05$; $P < 0.05$] (Figure 2a and b respectively).

### *Effect of gestational hypermethioninaemia on nitrite levels in gastrocnemius skeletal muscle*

Maternal hypermethioninaemia significantly decreased NO levels in skeletal muscle of pups [$T(6) = 4.72$; $P < 0.01$] (Figure 3).

### *Effect of gestational hypermethioninaemia on total protein content and weight of gastrocnemius skeletal muscle*

We observed an important decrease (around 20%) in the muscle protein content [$T(6) = 3.35$; $P < 0.05$] (Figure 4a) accompanied by a significant reduction (around 35%) in gastrocnemius weight [$T(6) = 2.75$; $P < 0.05$] (Figure 4b) of the offspring as a result of hypermethioninaemia in mother rats.

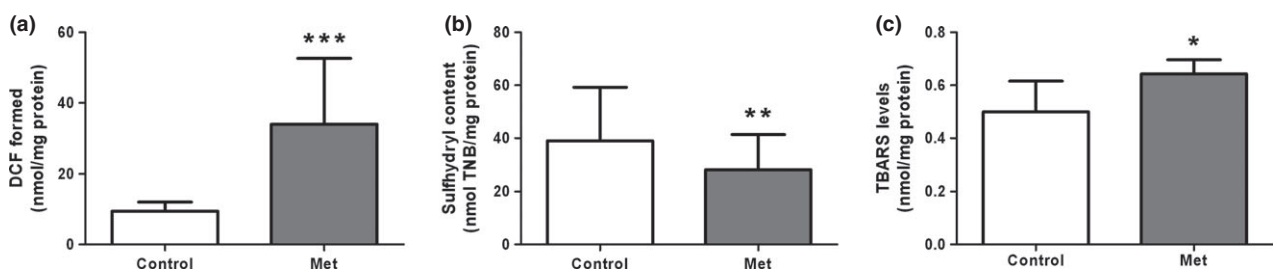


**Figure 1** Effect of gestational hypermethioninaemia on DCF levels (a), sulfhydryl content (b) and TBARS levels (c) in gastrocnemius skeletal muscle of the rat pups. Results are expressed as means ± SD for six animals in each group. Different from control, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (Student's *t*-test).

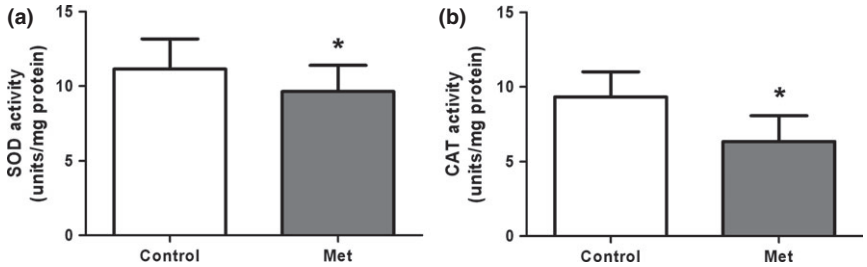


**Figure 2** Effect of gestational hypermethioninaemia on SOD (a) and CAT (b) activities in gastrocnemius skeletal muscle of the rat pups. Results are expressed as means ± SD for six animals in each group. Different from control, $*P < 0.05$ (Student's *t*-test).

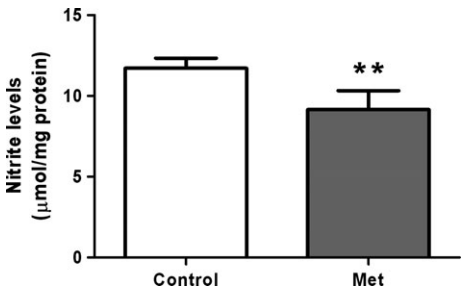


**Figure 3** Effect of gestational hypermethioninaemia on nitrite levels in gastrocnemius skeletal muscle of the rat pups. Results are expressed as means ± SD for six animals in each group. Different from control, $**P < 0.01$ (Student's *t*-test).

*Effect of gestational hypermethioninaemia on biomarkers of muscle damage and inflammation in serum*

Hypermethioninaemia reduced CK activity [$T(6) = 2.23$; $P < 0.05$] (Figure 5a), but did not alter creatinine levels [$T(6) = 1.0$; $P > 0.05$] in serum of pups (Figure 5b). Urea levels were significantly increased by Met [$T(6) = 3.61$; $P < 0.05$] (Figure 5c).

Regarding cardiac damage and inflammation, results show that gestational hypermethioninaemia did not alter cTnI (data not shown), but increased CRP levels in serum [$T(6) = 8.21$; $P < 0.001$] (Figure 6).

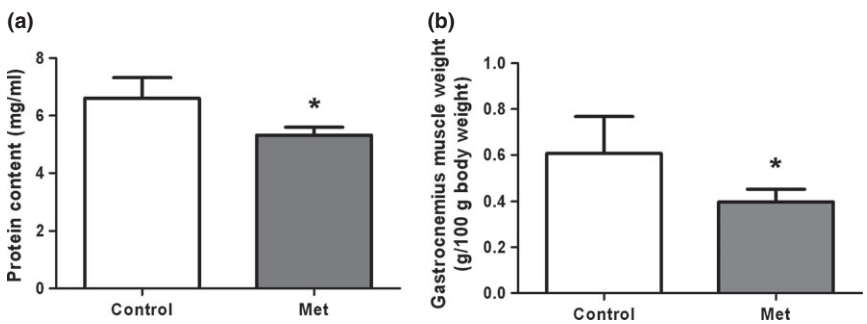


**Figure 4** Effect of gestational hypermethioninaemia on total muscle protein content (a) and gastrocnemius weight (b) of the rat pups. Results are expressed as means ± SD for six animals in each group. Different from control, $*P < 0.05$ (Student's *t*-test).

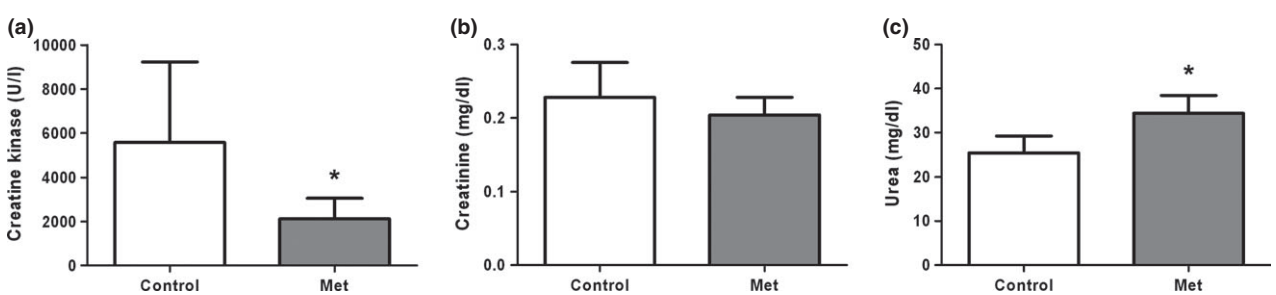


**Figure 5** Effect of gestational hypermethioninaemia on serum CK (a), creatinine (b) and urea levels (c) of the rat pups. Results are expressed as means ± SD for six animals in each group. Different from control, $*P < 0.05$ (Student's *t*-test).

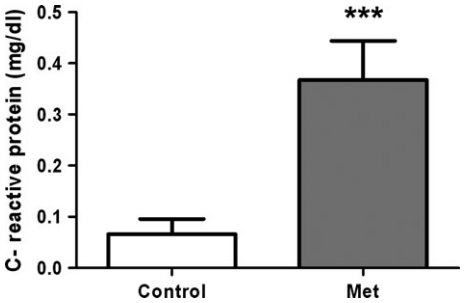


**Figure 6** Effect of gestational hypermethioninaemia on serum CRP levels of the rat pups. Results are expressed as means ± SD for six animals in each group. Different from control, ***$P < 0.001$ (Student's *t*-test).

### *Effect of gestational hypermethioninaemia on body weight of mother rats and pups*

At the 20th day of pregnancy, body weight of pregnant rats treated with Met was not significantly different from control (control: 305.40 ± 10.36; Met: 300.60 ± 12.70). Body weight of 21-day-old pups also was not altered by maternal hypermethioninaemia (control: 60.25 ± 4.27; Met: 59.50 ± 4.44).

## Discussion

As we have recently developed an experimental model of gestational hypermethioninaemia, which induces oxidative stress in encephalon of rat offspring (Schweinberger *et al.* 2014) and this oxidative stress is correlated with sarcopaenia and other muscle diseases (Tidball & Wehling-Henricks 2007; Arbogast *et al.* 2009; Turki *et al.* 2012; Sullivan-Gunn & Lewandowski 2013), we extended the studies evaluating the effect of this model on gastrocnemius skeletal muscle damage. We chose to carry out the analysis on day 21 after birth because in our previous work we investigated the effects of maternal hypermethioninaemia on 7- and 21-day-old pups and only the latter pups showed alterations in the parameters evaluated (Schweinberger *et al.* 2014). As the placenta exerts a maternal–foetal transfer of anti-oxidants during gestation, we believe that younger pups have anti-oxidant protection provided by their mothers. Gastrocnemius was chosen because it has a substantial number of fast-twitch fibres and, therefore, it is more susceptible to the oxidative stress.

The first step of the present study was to evaluate the effect of increased blood Met levels during gestation on parameters of oxidative stress in gastrocnemius skeletal muscle of pups. Results showed that DCF levels were significantly increased in muscle homogenates of pups whose mothers received Met, suggesting an increase in ROS production.

We also verified the effect of gestational hypermethioninaemia on protein and lipid damage in muscle of pups. Our results showed that protein-bound sulfhydryl status was significantly decreased by hypermethioninaemia, indicating that sulfhydryl-containing amino acid residues in proteins were targets to ROS. In agreement with these results, we have previously shown that Met treatment during pregnancy reduces sulfhydryl content in brain of rat pups (Schweinberger *et al.* 2014). In addition, Stefanello *et al.* (2009) demonstrated that chronic administration of Met in developing rats causes protein oxidative injury in liver. Concerning the effect of hypermethioninaemia during gestation on muscle lipid damage of the offspring, our results showed a significant increase in TBARS levels, which reflects an increase in malondialdehyde levels, a product of lipid peroxidation (Ohkawa *et al.* 1979). The induction of lipid peroxidation suggests an injury of the cell membrane initiated by ROS, indicating an oxidative damage to the skeletal muscle.

Enzymatic antioxidant defences were also evaluated to better establish oxidative status in the muscle of the offspring. Results showed that Met treatment during pregnancy significantly decreased SOD and CAT activities in pups' muscle. These results reflect a decrease in protection against cellular oxidative damage and may be responsible, at least partially, for the elevation in ROS production as well as in protein and lipid oxidative damage observed in this study. In agreement, we have previously reported, with the same animal model, that maternal hypermethioninaemia reduces CAT activity in encephalon of pups (Schweinberger *et al.* 2014).

We also observed a reduction of nitrite levels in muscle of the offspring. As explained previously, we observed decreased muscular SOD activity in this work, a condition that may lead to increased superoxide radical formation. Once superoxide anion can react with NO to yield peroxynitrite (Huie & Padmaja 1993), our hypothesis is that a high rate of this reaction decreased the bioavailability of NO. Furthermore, it has been reported that formation of peroxynitrite in muscle fibres, as an effect of lack of SOD, may contribute to fibre loss in mice (Sakellariou *et al.* 2011).

As studies suggest that the oxidative process may contribute to cell injury, we also studied the effect of maternal hypermethioninaemia on biomarkers of muscle damage in serum. We initially investigated the activity of CK, an enzyme that catalyses the transfer of the N-phosphoryl group from phosphocreatine to ADP regenerating ATP and contributing to energy homoeostasis (Wallimann *et al.* 1992). Once CK is primarily expressed in muscle, sarcolemma disruption causes enzyme release to the bloodstream, increasing its levels in serum (Jones *et al.* 1986). Surprisingly, serum CK activity of the pups was reduced by gestational hypermethioninaemia, which probably indicates an inhibition of this enzyme. The consequences of CK inactivation may include the following: increased mitochondrial volume and altered glycogenolytic/glycolytic potential; abnormal contractions; tubular aggregates of sarcoplasmic reticulum membranes; and accumulation of ADP (van Deursen *et al.* 1993; Steeghs *et al.* 1997; Saupe *et al.* 1998).

Furthermore, it should be noted that CK activity is highly susceptible to impairment by ROS (Aksenov *et al.* 2000), probably because of the oxidation of cysteinyl residue, which is critical for substrate binding (Kenyon 1996). Thus, whereas CK is a thiol-containing enzyme and we verified that maternal hypermethioninaemia significantly increased ROS production associated with a reduced content of sulfhydryl groups in muscle of pups, we propose that oxidative stress could be, at least partially, correlated with the decreased CK activity observed in serum.

To investigate the effect of Met treatment on the loss of muscle mass, the total protein concentration was measured and the gastrocnemius weight was determined. We observed a significant reduction in both parameters, suggesting augmented muscular protein degradation, what might result in the increase in urea production as it is the main metabolite derived from tissue protein turnover. Besides, skeletal muscle mass is the primary determinant of creatinine levels in blood as this metabolite is derived from muscular creatine metabolism (Refsum & Strömme 1974; Andersson *et al.* 2008). Based on these points, in the present study we also evaluated these parameters in the serum of the offspring. Gestational hypermethioninaemia did not alter creatinine values but increased urea levels. We suggest that the increase in urea can have occurred in consequence of the damage to muscle tissue caused by oxidative stress, as the action of free radicals on protein thiol groups may cause proteolysis.

Nevertheless, it should be noted that once Met metabolism results in the production of $H_2S0_4$, the ingestion of this amino acid may produce a metabolic acidosis (Hood & LaGrange 1988), which in turn seems to increase protein degradation resulting in muscle loss (Bailey *et al.* 1996). In addition, during several pathological conditions, skeletal muscle provides amino acids for acute-phase protein synthesis through increased proteolysis, resulting in decreased muscle mass. However, different responses to diseases have been described between slow-twitch and fast-twitch muscles. It has been demonstrated that during inflammatory processes, the increase in myofibrillar proteolysis and the decrease in protein synthesis are more pronounced in fast-twitch muscle. Moreover, studies showed that inhibition of proteasome decreased proteolytic events in both types of muscles, suggesting that ubiquitin–proteasome system mediates a considerable part of proteolysis in skeletal muscle (Kadlcíková *et al.* 2004; Muthny *et al.* 2008). Gastrocnemius muscle is made up of a substantial part of fast-twitch muscle fibres and may be affected during pathological events. Further studies will be performed in the future to explore the points discussed above.

As a complementary study about the effects of maternal hypermethioninaemia on the offspring, the next step of this work was the qualitative detection of serum cTnI, a large globular protein size that regulates heart muscle contraction and is considered an indicator of myocardial necrosis when detectable in blood (Adams *et al.* 1993). Results demonstrated that cTnI was not altered by hypermethioninaemia, suggesting that the Met treatment was not able to induce myocardial injury in the offspring.

Finally, we also measured serum levels of CRP, which is an acute-phase protein produced in the liver and is considered one of the most sensitive and systemic biomarker of inflammation (Pepys & Hirschfield 2003). Serum CRP levels were increased in the offspring, which may represent a cause and/or consequence of oxidative stress as the stimulation of immune system leads to release of ROS by neutrophils and macrophages and increased ROS levels lead to further stimulation of immune responses and inflammation (Geronikaki & Gavalas 2006). In addition, studies with animals and humans have demonstrated an association between inflammation and low muscle mass (Goodman 1991, 1994; van Hall *et al.* 2008). More specifically, Cesari *et al.* (2005) showed that CRP levels were inversely related to appendicular lean mass.

## Conclusions

In summary our findings show that gestational hypermethioninaemia promotes an increase in DCF and TBARS levels, as well as a decrease in sulfhydryl content, antioxidant enzymes activities, nitrite levels and total protein content in the skeletal muscle of the offspring. In serum, CK levels were diminished, while urea and CRP levels were enhanced. Gastrocnemius weight was reduced. These results may represent, at least in part, a mechanism able to contribute to myopathies and loss of muscular mass found in hypermethioninaemia. Nevertheless, more studies are necessary to better understand the pathological effects of maternal hypermethioninaemia on the offspring. After new approval from the ethics committee to obtain more animals, we intend to perform histological analysis to determine muscle fibre number and size, as well as to quantify regenerating fibres, necrotic fibres and inflammatory cell accumulation.

## Acknowledgements

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## Conflict of interest

The authors declare that they have no conflict of interest.

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## 3.6 Capítulo V

### MANUSCRITO 5

**Mechanistic basis of hypermethioninemia.**

Schweinberger BM, Wyse AT.

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REVIEW ARTICLE

# Mechanistic basis of hypermethioninemia

**Bruna M. Schweinberger[1] · Angela T. S. Wyse[1,2]**

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**Abstract** Hypermethioninemia is a condition defined as elevated plasma methionine levels and may be a consequence of different conditions that include non-genetic and genetic causes. In severe cases, hypermethioninemia may lead to development of neurological and hepatic impairments, but mechanisms are still not well elucidated. Therefore, this review aims to reunite the knowledge acquired about the methionine-induced brain and liver toxicity focusing on the results obtained by studies from patients, in vitro experiments, and in vivo animal models. In general, some studies have shown that methionine decreases $Na^+$,$K^+$-ATPase activity, induces oxidative stress, increases acetylcholinesterase activity, and leads to dendritic spine downregulation in brain. Concerning to liver, hypermethioninemia seems to provoke changes in cell morphology, lipid accumulation, oxidative stress, inflammation, and ATP depletion. It is possible to infer that oxidative damage is one of the most important mechanisms responsible for methionine toxicity, since different studies showed that this amino acid induces oxidative stress in brain and liver tissues. Besides, reactive oxygen species may mediate other alterations induced by methionine, such as the reduction in brain $Na^+$,$K^+$-ATPase activity, and liver inflammation.

**Keywords** Brain · Hypermethioninemia · Liver · Methionine · Oxidative stress

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✉ Angela T. S. Wyse
wyse@ufrgs.br

[1] Programa de Pós-Graduação em Ciências Biológicas, Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[2] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, Porto Alegre, RS CEP 90035-003, Brazil

## Roles of methionine

Methionine (Met) is an essential sulfur-containing amino acid obtained from diet or degradation of endogenous proteins. Some of the main functions of Met in organism include: production of its derivative molecules cysteine, glutathione, carnitine, taurine, and creatine (Wesseling et al. 2009; Wyss and Kaddurah-Daouk 2000; Crill and Helms 2007), protein synthesis since Met composes proteins and peptides and is the only natural initiating amino acid in the eukaryotic translation (Lucas-Lenard 1971), as well as donation of its methyl group to a variety of molecules such as nucleic acids, histones, amino acids, and lipid-derivatives (Chiang et al. 1996).

Besides, it has been reported that Met residues in proteins also provide antioxidant protection since they are often positioned so that they establish an interaction, through hydrophobic bond, between their sulfur atoms and the rings of aromatic amino acids (Valley et al. 2012), which are much susceptible to oxidation by reactive species (El Refaey et al. 2015). Furthermore, the oxidation of surface exposed Met protects the other residues because reactive species may oxidize Met to Met sulfoxide, which may be reduced back by the enzyme Met sulfoxide reductase (Brot et al. 1981).

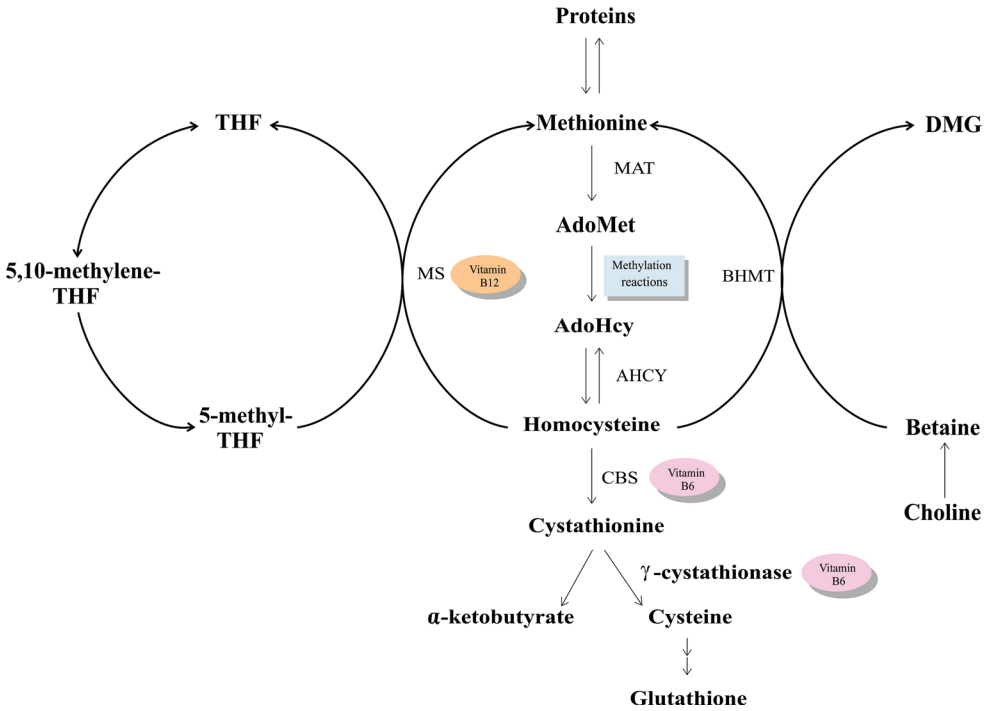


**Fig. 1** Pathways of Met metabolism in mammals. *MAT* Methionine adenosyltransferase, *AdoMet S*-adenosylmethionine, *AdoHcy S*-adenosylhomocysteine, *AHCY S*-adenosylhomocysteine hydrolase, *CBS* cystathionine *β*-synthase, *5,10-methylene-THF* 5,10-methylenetetrahydrofolate, *5-methyl-THF* 5-methyltetrahydrofolate, *THF* tetrahydrofolate, *BHMT* betaine-homocysteine-methyltransferase, *DMG N,N*-dimethylglycine

## Metabolism of methionine

Met is mainly metabolized in the liver by the enzyme Met adenosyltransferase (MAT, EC 2.5.1.6), which is present in three isoforms. MAT I and III are encoded by the same gene MAT1A and predominate in adult liver. MAT II activity is present at smaller amount in adult liver and its activity is predominant in non-hepatic tissues, fetal liver, and hepatocellular carcinoma (Frago et al. 1998; Horikawa et al. 1990, 1993; Okada et al. 1981; Gil et al. 1996; Cai et al. 1996). This enzyme transfers the adenosyl group from ATP to Met, forming *S*-adenosylmethionine (AdoMet) and tripolyphosphate. AdoMet is reacquired as a methyl donor in reactions that include methylation of nucleic acids, proteins, and lipids. The product of AdoMet transmethylation is the *S*-adenosylhomocysteine (AdoHcy), which is hydrolyzed by AdoHcy hydrolase (AHCY, EC 3.3.1.1), resulting in homocysteine (Hcy) formation (Mudd 1962; Cantoni 1953; Finkelstein 1990; de la Haba and Cantoni 1959).

Hcy can be metabolized by two different pathways: remethylation or transsulfuration. Remethylation is catalyzed by Met synthase (MS, EC 2.1.1.13), a vitamin $B_{12}$-dependent enzyme that regenerates Met by transferring a methyl group to Hcy. The methyl group is derived from the endogenous 5-methyltetrahydrofolate (5-methyl-THF), which is formed during the metabolism of folic acid. Additionally, betaine-Hcy-methyltransferase (BHMT) uses betaine derived from choline as a methyl donor for Hcy remethylation, which is considered a salvage pathway when toxins compromise the action of MS. BHMT transfers the methyl group from betaine to Hcy, forming Met and *N,N*-dimethylglycine (DMG). Transsulfuration pathway catalyzes the condensation of Hcy with serine to form cystathionine through the action of a vitamin $B_6$-dependent enzyme named cystathionine *β*-synthase (CBS, EC 4.2.1.22). Cystathionine is then converted to α-ketobutyrate and cysteine by the enzyme γ-cystathionase, which is also dependent of vitamin $B_6$. Therefore, transsulfuration pathway is a very important source of non-enzymatic antioxidant protection to the liver, since it forms cysteine, the precursor of glutathione (Finkelstein 2000; Selhub 1999; Beatty and Reed 1980; Mosharov et al. 2000). The Met/Hcy cycle is shown in Fig. 1.

In cerebral tissue, Met is primarily metabolized through remethylation pathway. Some years ago, data published in literature indicated that the transsulfuration was incomplete in the brain due to absence of the enzyme γ-cystathionase, leading to cystathionine accumulation in this organ (Finkelstein 1998). However, Vitvitsky et al. (2006) have demonstrated the existence of a functional transsulfuration pathway in human neurons and astrocytes and in mouse brain, suggesting that this may contribute to the protection under oxidative stress conditions through brain glutathione synthesis.

## Hypermethioninemia

Normal plasma concentration of Met range from 13 to 45 μM (Stabler et al. 2002). Hypermethioninemia occurs

when Met levels increase in blood, which may be a consequence of different conditions. Non-genetic causes for hypermethioninemia include liver disease, premature birth (frequently transient), and diet rich in proteins, which may increase plasma Met levels to 1206 μM when protein intake achieves 7 g/kg/day. On the other hand, hypermethioninemia from genetic causes (hereditary conditions) includes: MAT I/III deficiency, classical homocystinuria (due to CBS deficiency), deficiencies of glycine *N*-methyltransferase (GNMT, EC 2.1.1.49), AHCY, citrin, and fumarylacetoacetate hydrolase (tyrosinemia type I) (Mudd 2011; Levy et al. 1969).

A characteristic that distinguishes MAT I/III deficiency from GNMT, AHCY, and CBS deficiencies is that the first one leads to isolated hypermethioninemia, with plasma Met reaching levels from 600 to 2541 μM in patients with homozygous mutations (Mudd et al. 1995; Chamberlin et al. 1996; Nagao and Oyanagi 1997). The term isolated hypermethioninemia designates elevated plasma Met levels which are not associated with the increase in Met metabolites, including AdoMet, AdoHcy, Hcy, and cystathionine. As exception, patients with severe MAT I/III deficiency may have plasma Hcy slightly elevated, but the mechanisms involving this effect are still not well understood (Stabler et al. 2002; Lagler et al. 2000). Besides, MAT I/III deficiency may lead to decreased AdoMet, while the other causes of hypermethioninemia often enhance AdoMet levels (Mudd 2011). Therefore, the reader should be clarified that the effects of hypermethioninemia may differ depending on the cause, since AdoMet may be involved in the pathological effects either when increased or decreased.

## Pathological effects of hypermethioninemia

Met is crucial for normal growth and development, but when this amino acid and/or its metabolites are present at abnormally elevated plasma levels, potentially toxic events may occur. Although it may be asymptomatic, hypermethioninemia can cause the following pathological effects: myopathy, hypotonia, altered erythrocyte morphology with consequent splenic hemosiderosis, facial dysmorphia associated to abnormal teeth and hair, anorexia and digestive disturbances, development of neurological problems (tremor, dystonia, and cognitive deficit), and/or liver diseases (Chamberlin et al. 1996; Gaull et al. 1981a; Guízar Vázquez et al. 1980; Benevenga and Steele 1984; Higashi 1982; Lynch and Strain 1989; Labrune et al. 1990; Gout et al. 1977; Chamberlin et al. 1997; Harvey Mudd et al. 2003; Mudd et al. 2001). In view of severity of the symptoms, this review will empathize the neurological and hepatic effects of hypermethioninemia.

### Neurological effects

The increase in Met levels can be toxic to the brain regardless of the cause. In general, patients with severe hypermethioninemia may present neurological dysfunction, including mental retardation and cognitive deficit. It has been also reported that cerebral edema may be observed during CBS and MAT I/III deficiencies and during excessive Met diet when plasma Met achieves levels extremely elevated (Harvey Mudd et al. 2003; Mudd et al. 2001; Braverman et al. 2005). However, the mechanisms involved in these alterations are still not well elucidated. In the attempt to understand such mechanisms, some studies have been developed.

#### $Na^+,K^+$-ATPase activity and oxidative stress

$Na^+,K^+$-ATPase plays a crucial role in maintaining the ionic gradient required for neuronal excitability and regulation of neuronal cell volume through the transport of $Na^+$ and $K^+$ ions in the nervous system (Glynn 1985). Inhibition of this enzyme may induce brain edema, neuronal death, and impairment of learning and memory (Wyse et al. 2004; de Lores Arnaiz and Ordieres 2014). In this context, the decrease in brain $Na^+,K^+$-ATPase activity seems to be involved in neurological diseases, such as dystonia (Cannon 2004), Alzheimer disease (Zhang et al. 2013), bipolar affective disorder (Mynett-Johnson et al. 1998), ischemia (de Souza Wyse et al. 2000), epilepsy (Grisar et al. 1992), depressive disorders in rats (Gamaro et al. 2003; Acker et al. 2009), hyperprolinemia (Ferreira et al. 2011), and phenylketonuria (Wyse et al. 1999).

Oxidative stress is characterized by an imbalance between reactive oxygen species (ROS) and the cellular antioxidant defenses that include non-enzymatic protection, such as vitamins C and E and reduced glutathione, and enzymatic protection, such as glutathione peroxidase, superoxide dismutase (SOD), and catalase (CAT) (Apel and Hirt 2004). Increased ROS production can directly cause tissue damage and lead to inflammation process (Geronikaki and Gavalas 2006). Besides, $Na^+,K^+$-ATPase activity may be affected by ROS through lipid peroxidation and sulfhydryl groups oxidation.

In this context, an in vitro study showed that Met inhibits $Na^+,K^+$-ATPase in synaptic plasma membrane from hippocampus of rats (Streck et al. 2002a). Posteriorly, Stefanello et al. (2005) verified that the preincubation of hippocampal homogenates with antioxidants (glutathione and tocopherol) prevented the inhibitory action of Met on $Na^+,K^+$-ATPase. In the same work, the evaluation about the in vitro effects of Met on some parameters of oxidative stress demonstrated that this amino acid caused lipoperoxidation and reduced non-enzymatic antioxidant capacity in rat hippocampus. Together, these results suggest that

Met-induced $Na^+,K^+$-ATPase inhibition is possibly mediated by free radical formation.

Therefore, Stefanello et al. (2007a) extended the investigations and developed an in vivo model for hypermethioninemia in which developing Wistar rats receive injections of Met leading to concentrations approximately 30-fold the control levels. Using this experimental model, it was demonstrated that both chronic and acute administration of Met lead to lipoperoxidation and decreased $Na^+,K^+$-ATPase activity in Wistar rat hippocampus. Since $Na^+,K^+$-ATPase is embedded in cellular membrane, it is possible that peroxidative process could provoke changes of fluidity or other membrane properties, prejudicing the enzyme functioning and decreasing its activity (Stefanello et al. 2007b).

In a further study, Stefanello et al. (2007c) also demonstrated that chronic injections of Met significantly reduced $Na^+,K^+$-ATPase activity in rat cerebral cortex accompanied by reduced amount of gangliosides (GM1, GD1a, GD1b, and GT1b), phospholipids (sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine) and cholesterol. Lipoperoxidative process was also observed, strengthening the hypothesis that oxidative damage of the cellular membrane lipids could provoke changes in lateral assembly of glycosphingolipids, unsaturated glycerophospholipids and cholesterol, leading to alteration in $Na^+,K^+$-ATPase activity.

The neurotoxic effects of Met were also demonstrated in Sprague–Dawley rats submitted to a Met-enriched diet during 8 weeks. The results from this study showed an enhance in the activity of the antioxidant enzyme SOD in cerebral cortex of the rats fed on 1 and 5 % Met, suggesting a metabolic adjustment to combat a possible augment in ROS production. This alteration was accompanied by apparent impairment of locomotor skills and synaptic plasticity in rats fed on 5 % Met (Viggiano et al. 2012).

More recently, an animal model for maternal hypermethioninemia was developed. In this study, pregnant Wistar rats received injections of Met during gestational period. The administration of 2.68 μmol Met/g body weight increased encephalon Met levels (without Hcy elevation) in the offspring. Decrease in the activities of $Na^+,K^+$-ATPase, $Mg^{2+}$-ATPase, and CAT, as well as in total sulfhydryl content was also found. However, cerebral lipoperoxidation was not observed and in this case, the reduction in $Na^+,K^+$-ATPase activity may be associated to attack of reactive species to the sulfhydryl groups present in the enzyme (Schweinberger et al. 2014).

### Acetylcholinesterase activity

Schulpis et al. (2006) published data showing that Met is able to increase hippocampal acetylcholinesterase (AChE) activity in vitro. At the following year, Stefanello et al. (2007d) showed that chronic subcutaneous injections of Met in developing Wistar rats increased AChE activity in cerebral cortex associated to an impaired working memory performance. Since AChE acts into the synapse by rapid hydrolysis of the acetylcholine (Ach), a neurotransmitter whose adequate maintenance has been associated with cognitive manifestations (learning and memory) (Bartus et al. 1982), the stimulation of this enzyme activity could lead to a decrease in cerebral Ach levels and provide an explanation for the memory deficit found in the hypermethioninemic rats. In agreement, studies showed that long-term Met exposure caused an important increase in brain AChE activity and memory deficit in zebrafish (Vuaden et al. 2012). Since Ach has a role as an anti-inflammatory molecule, some studies have correlated increased AChE activity with neuroinflammation (Scherer et al. 2014), what could be also related to the pathogenic effects found in hypermethioninemia.

### Dendritic spine downregulation

In 1952, Osmond and Smythies (1952) proposed the ‘‘transmethylation theory’’ of schizophrenia, suggesting that this psychotic disease is a result of a disturbance in methylation. In 2009, Grayson et al. also reported that Met treatment could worsen schizophrenia symptoms, possibly because it increases brain levels of AdoMet. More specifically, excessive AdoMet could provoke hypermethylation of Reelin gene promoter. Since Reelin is a glycoprotein secreted by GABAergic neurons that stimulates dendritic spines development, this process could be impaired by Met (Levenson et al. 2008).

Indeed, it has been demonstrated that the treatment with Met causes a decrease in dendritic spine density of layer III pyramidal neurons in frontal cortex of mice, a pathological alteration similar to the dendritic spine downregulation found in brain during schizophrenia (Tueting et al. 2010). In agreement, clinical studies have demonstrated that patients with psychotic disorders present increased Met levels in cerebrospinal fluid (Regland et al. 2004).

Besides, it should be noted that that learning and novel sensory experiences lead to spine formation and the new spines that are preserved seem to provide a structural basis for memory retention (Yang et al. 2009). Thus, when hypermethioninemia is associated with enhanced AdoMet levels, the reduction in dendritic spine density may occur and cause lifelong memory impairment.

### Hepatic effects

Since Met is primarily metabolized in the liver (Finkelstein 1990), it has been suggested that excess of Met may cause

liver injury, but mechanisms are still not well elucidated. In this context, several studies have been performed to figure it out.

### Liver cell alterations

In humans, electron microscopy revealed augmented smooth endoplasmic reticulum, reduced rough endoplasmic reticulum, enhanced lysosomes, and short breaks in the outer membranes of liver from patients with persistent hypermethioninemia (MAT activity ranged from 7.8 to 17.5 %) and with no abnormalities in other sulfur amino acid concentrations (Gaull et al. 1981b). In rats, excess dietary Met (10–12.4 % dl-Met) caused atrophy of liver cells and changes in the distribution of the chromatin, which was condensed and deposited at the periphery of the nucleus (Earle et al. 1942).

### Hepatic lipid accumulation

Whereas the liver is the organ directly related to lipid metabolism, fatty accumulation (steatosis) may be observed during some pathological conditions. Steatosis is associated with hepatocyte damage and consequently can cause cirrhosis, inflammation, and liver failure leading to end-stage disease (Angulo 2010). In this context, histological examinations of liver tissues from patients with persistent and transient hypermethioninemia showed moderate fatty degeneration, wherein the condition improved after low Met diet (Tsuchiyama et al. 1982).

Furthermore, Lu et al. (2001) evaluated the effect of MAT1A knockout in mice and observed, at 3 months, an increase of 776 % in plasma Met levels and reduction of liver AdoMet content. At 8 months, development of spontaneous macrovesicular steatosis and predominantly periportal mononuclear cell infiltration occurred. These changes were accompanied by augmented expression of acute phase-response/inflammatory markers (orosomucoid, amyloid, metallothionein, Fas antigen) and growth-related genes (early growth response 1 and proliferating cell nuclear antigen), as well as increased liver weights. Posteriorly, Martínez-Chantar et al. (2002) also demonstrated that knockout in MAT1A gene leads to abnormal expression of genes involved in the metabolism of lipids and carbohydrates associated with hyperglycemia and increased hepatic triglyceride levels in mice.

Met diet supplementation was also able to induce hepatic damage by stimulating cholesterol synthesis in liver cells (probably through increased hepatic expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase) (Hirche et al. 2006), augmenting accumulation of hepatic total lipids and phospholipids (Yang and Kadowaki 2011), and inducing microvesicular steatosis, hepatocyte degeneration, and inflammatory reactions in liver of rats (Yalçinkaya et al. 2009). Met diet restriction, on the other hand, seems to be advantageous as described in a previous study, which demonstrated that rats submitted to restrictive Met intake presented reduced visceral fat associated to a decrease in basal insulin, glucose, and leptin, and increased adiponectin and triiodothyronine. Besides, Met restriction prevented age-associated increase in serum lipids (Malloy et al. 2006). In 2013, Malloy et al. also demonstrated that Met restriction was able to reverse the severity of steatosis in obese mice accompanied by reduced hepatic triglycerides levels, increased VLDL secretion, and increased mRNA levels of apolipoprotein B and microsomal triglyceride transfer protein. The expression of inflammatory markers (Tnf-α and Ccr2) was also attenuated by Met restriction in this study.

It is important to note that excessive lipids in liver may cause lipid peroxidation, which can increase the production of pro-inflammatory cytokines (Bradbury 2006). Besides, the increase in lipids can exceed mitochondrial beta-oxidation further enhancing oxidative stress and inflammation (Schreuder et al. 2008). On this basis, Met-induced lipid accumulation in liver could lead to oxidative stress, which may have a role in hepatic damage during hypermethioninemia.

### Oxidative stress

The role of oxidative stress on the hepatic toxicity caused by Met has been shown in different animal studies: enriched Met diet increased lipid peroxidation in liver of rats and rabbits, as well as, altered antioxidant enzyme activities and induced inflammatory infiltration of portal triads in liver of rabbits (Lynch and Strain 1989; Mori and Hirayama 2000; Toborek et al. 1996); high Met diet also increased hepatotoxicity and oxidative stress in the liver of chronically ethanol-treated rats (Yalçinkaya et al. 2007); MAT1A knockout increased susceptibility to oxidative stress and reduced glutathione content in mice liver (Lu et al. 2001; Martínez-Chantar et al. 2002).

To further the knowledge about these mechanisms, Stefanello et al. (2009) evaluated the toxic effects of chronic Met injections in rats. The treatment decreased non-enzymatic antioxidant defenses, increased protein carbonylation, and altered the activities of the antioxidant enzymes glutathione peroxidase and CAT in the liver, indicating oxidative stress. These alterations were accompanied by morphological alterations in liver.

In addition, rats fed with a high Met diet (2 %, w/w) during 6 months presented hepatic oxidative and nitrosative stress characterized by increased lipid peroxide and nitrotyrosine levels, as well as decreased non-enzymatic and enzymatic antioxidant defenses in liver. Increased levels of

alanine transaminase and aspartate transaminase in blood and altered apoptotic parameters in liver indicated that the hepatic tissue was disrupted. These alterations were accompanied by enhanced Hcy levels in blood (Yalçinkaya et al. 2009).

Gomez et al. (2009) also demonstrated that Wistar rats fed a Met supplemented diet (2.5 g/100 g) for 7 weeks had increased mitochondrial ROS generation and oxidative damage to mitochondrial DNA in liver. In agreement, Caro et al. (2008) showed that lowered Met ingestion has the exactly opposite effects, decreasing mitochondrial ROS production and DNA oxidative damage in liver of rats. More recently, a swine model was used to determine if a methionine-restricted diet for 2 weeks could reduce oxidative stress in hepatic mitochondria. The results showed that methionine restriction decreased markers of oxidative damage to DNA and proteins in liver mitochondria of pigs, being that effects probably were consequence of attenuated ROS production since a reduction in $H_2O_2$ generation and in free radical leak was also observed. The authors suggest that the decrease in ROS generation possibly occurred due to reduced complex I activity, which was associated with decreased levels of the apoptosis inducing factor, a protein related to complex I function (Ying et al. 2015).

Besides, excessive Met intake by γ-cystathionase-deficient mice led to the development of acute hepatitis attended by serum and hepatic lipoperoxidation (Yamada et al. 2012). It has been previously described that peroxidized fatty acids (arachidonic and linolenic) stimulate interleukin-8 production by peripheral blood monocytes in liver (Jayatilleke and Shaw 1998). Interleukin-8, in turn, has been associated with hepatic neutrophil infiltration and to activation of hepatic profibrogenic cells (Bird 1994; Zimmermann et al. 2011; Taïeb et al. 2000; Dong and Zheng 2015; Tachibana et al. 2007).

More recently, Costa et al. (2013) performed in vitro and in vivo studies about the toxic effects of Met in liver. For in vitro studies, liver homogenates were incubated with Met and results showed changes in CAT and SOD activities, as well as in ROS production. For in vivo studies, the animals received injections of Met (0.4 g/kg) and were euthanized after 1 and 3 h. Results showed that Met enhanced carbonyl content at 1 h, as well as decreased CAT activity 1 and 3 h after administration. Data indicated that Met modifies liver homeostasis by altering the redox cellular state both in vivo and in vitro.

### Cholestasis

Cholestasis is a pathological condition defined as an impairment of bile flow that causes the accumulation of toxic compounds, which induce liver damage, biliary fibrosis, cirrhosis, and finally end-stage liver disease. Studies performed in rabbits by Moss et al. (1999) showed that intravenous administration of Met (121 mg $kg^{-1}$ $d^{-1}$) leads to decreased bile flow. The excretion of a bilirubin analog (bromosulfophthalein) tended to be delayed by Met treatment. It was also verified histological liver injury, balloon degeneration, and inflammation characterized by infiltration of the portal triads with eosinophils. Therefore, these results suggest that excessive Met may lead to cholestasis.

In addition, four cases of human neonates positive for hypermethioninemia and two for both hypermethioninemia and hypergalactosemia have been described, which presented severe intrahepatic cholestasis of unknown origin (Ohura et al. 2003). Cholestasis induced by hypermethioninemia may be a consequence of the inflammatory process induced by Met since the cytokines produced under this condition may impair the hepatocellular transport systems that mediate biliary excretion of bile salts and non-bile salt organic anions (Trauner et al. 1999).

### ATP depletion

Since Met transmethylation initiates through the ATP-dependent conversion of Met to AdoMet (Finkelstein 1990), ATP depletion from excessive AdoMet formation may induce or augment hepatotoxicity during hypermethioninemia (Hardwick et al. 1970). In accordance with this hypothesis, injections of Met in guinea pigs led to accumulation of AdoMet with concomitant ATP deficiency and nucleolar disaggregation in liver (Shinozuka et al. 1971). Besides, Regina et al. (1993) performed an experiment in which the feeding of toxic levels of Met led to a pronounced accumulation of AdoMet in liver of rats.

### Met transamination

Met transamination consists of an alternative pathway for Met metabolism and results in the formation of 2-keto-4-methylthiobutyric acid, which is oxidatively decarboxylated to form 3-methylthiopropionic acid (3-MTP) (Cooper 1989; Scislowski and Pickard 1993; Steele and Benevenga 1978). 3-MTP is then metabolized to highly toxic molecules, including methanethiol, a compound that inhibits enzymes involved in protection against peroxidative damage (Finkelstein and Benevenga 1986).

In this context, Dever and Elfarra (2008) demonstrated that Met is hepatotoxic through an experiment in which freshly isolated male mouse hepatocytes were incubated with different doses of this amino acid, leading to cell disruption and glutathione depletion. The exposure of hepatocytes to 3-MTP resulted in similar effects. Besides, the addition of aminooxyacetic acid, an inhibitor of Met transamination, partially blocked Met-induced cytotoxicity,

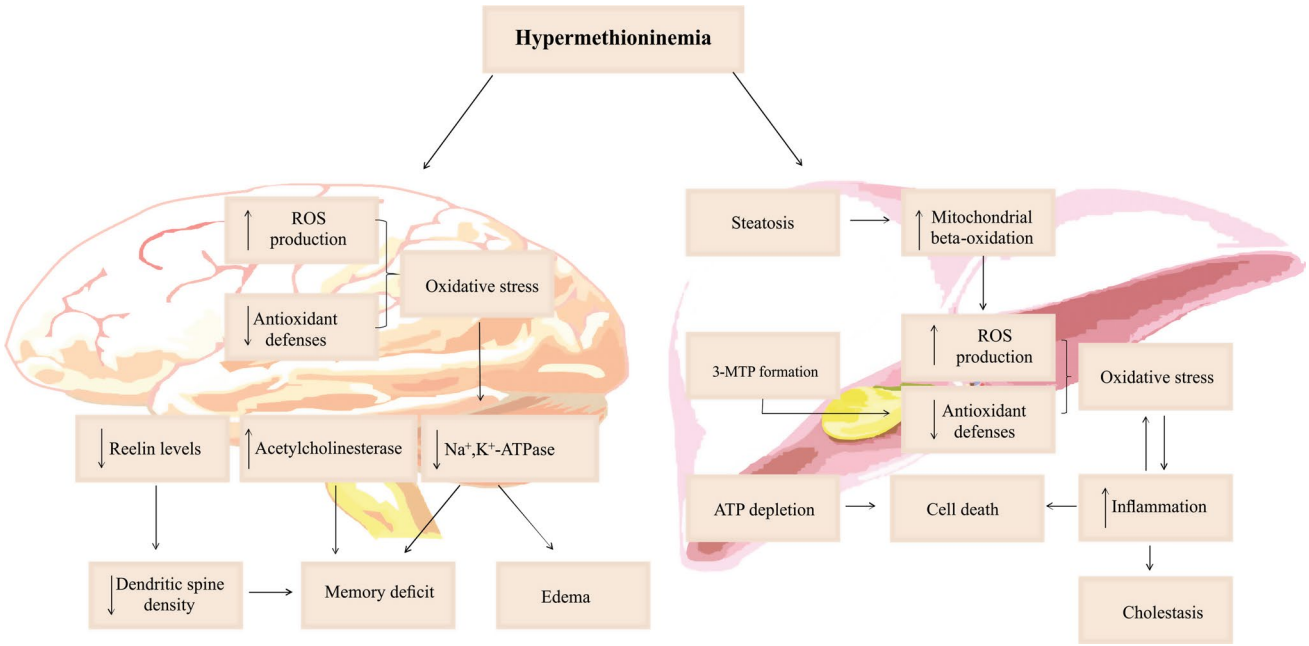


**Fig. 2** Schematic representation of Met effects reported in the literature up to now. In brain, hypermethioninemia increases ROS production and decreases antioxidant defenses, leading to oxidative stress, which in turn may reduce $Na^+,K^+$-ATPase activity. $Na^+,K^+$-ATPase inhibition is related to cerebral edema and memory deficit. Increased AChE activity and dendritic spine downregulation (induced by decreased Reelin levels) may also impair memory during hypermethioninemia. In liver, hypermethioninemia induces steatosis that increases mitochondrial beta-oxidation, leading to increased ROS production. Hypermethioninemia also induces 3-MTP formation which reduces antioxidant defenses. This imbalance between ROS and antioxidants induces oxidative stress. Inflammation is both consequence and cause of oxidative stress and is able to lead to cholestasis. Inflammation and Met-induced ATP depletion causes cell death

indicating that the toxicity was at least partially mediated by Met transamination.

## Final considerations

Based on the information presented above, it is possible to infer that oxidative damage is one of the main mechanisms responsible for toxicity caused by Met, since oxidative stress was induced in brain and liver tissues in different studies that includes in vitro experiments or in vivo animal models by injecting Met, enriching Met in diet and/or knocking MAT1A gene. Besides, oxidative stress seems to mediate, at least partially, other alterations induced by Met, such as the reduction of brain $Na^+,K^+$-ATPase activity and liver inflammation.

Some Met metabolites, such as Hcy, may induce oxidative stress and alter AChE and $Na^+,K^+$-ATPase activities in brain and liver, contributing to the toxic effects of Met in some cases (Streck et al. 2002b; Scherer et al. 2011, 2013, 2014; Machado et al. 2011; Matté et al. 2004; 2009a, b). However, this review described different in vitro studies and animal models that induced isolated hypermethioninemia, which caused pathological effects, suggesting that Met per se is able to elicit important hepatic and neurological toxicity.

In conclusion, Met may be extremely toxic to brain by inducing oxidative stress, decreasing $Na^+,K^+$-ATPase activity and dendritic spine density, as well as increasing AChE activity. In liver, hypermethioninemia seems to induce histological changes, liver lipid accumulation, oxidative stress, inflammation, and ATP depletion. Schematic representations of Met effects in brain and liver are shown in Fig. 2.

## Dedication

This review is dedicated to the memory of Dr. S. Harvey Mudd, who developed a superb work on diseases involving disturbances of sulfur amino acid metabolism The studies performed by Dr. Mudd motivated us to develop experimental models of hypermethioninemia and hyperhomocysteinemia in the attempt to better understand the underlying mechanisms involved in the pathophysiology of these conditions We express our gratitude to this eminent scientist for his scientific contribution and for the opportunity to have exchanged ideas about our research.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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# 4. DISCUSSÃO

O desenvolvimento cerebral adequado durante o período pré-natal é de suma importância uma vez que evita danos às estruturas e funções do cérebro que podem levar a disfunções neurológicas na vida pós-natal. Embora a placenta tenha a função de proteger o feto, algumas substâncias podem penetrá-la e causar efeitos tóxicos levando a disfunções bioquímicas, fisiológicas e/ou comportamentais. Sabe-se que determinadas influências hereditárias e nutricionais podem afetar adversamente o desenvolvimento embrionário prejudicando processos celulares fundamentais.

A hipermetioninemia consiste em uma condição patológica em que os níveis de metionina se encontram elevados no sangue e nos tecidos. Essa condição pode ser oriunda de alterações genéticas, como por exemplo, a deficiência da MAT I/III e a homocistinúria clássica, ou pode ser adquirida através de uma dieta hiperproteica. Estudos mostram que a hipermetioninemia severa está associada a disfunções neurológicas graves e a algumas alterações musculares (Mudd et al., 2001). Porém o efeito da hipermetioninemia gestacional sobre o feto ainda é pouco estudado. Dessa forma, o objetivo deste estudo foi desenvolver um modelo experimental para hipermetioninemia gestacional em ratos e avaliar os danos na prole, dando enfoque às disfunções cerebrais e musculares através de análises bioquímicas, moleculares e histológicas, bem como testes comportamentais.

Primeiramente, padronizou-se um modelo experimental de hipermetioninemia materna quimicamente induzido em ratas gestantes, as quais foram divididas em três grupos: o primeiro grupo (controle) recebeu

solução salina, o segundo grupo recebeu 1,34 µmol metionina/g peso corporal e o terceiro grupo recebeu 2,68 µmol metionina/g peso corporal durante todo o período gestacional, que se estende por cerca de 21 dias. Um grupo de filhotes foi eutanasiado aos sete dias de vida e o outro grupo aos 21 dias junto a suas mães. Os níveis séricos de metionina e homocisteína total foram medidos nas mães e nos filhotes de 21 dias, mas não foi observada diferença significativa quando comparados ao grupo controle, provavelmente porque os níveis sanguíneos de metionina retornam aos níveis normais 12 horas após a última injeção desse aminoácido (Stefanello et al., 2007a). Entretanto, os níveis encefálicos de metionina das mães se mantiveram elevados 21 dias após a interrupção do tratamento com ambas as doses. Os níveis de metionina também se encontravam elevados nos encéfalos dos filhotes de 7 e 21 dias cujas progenitoras receberam a dose mais alta de metionina.

O próximo passo deste trabalho foi determinar a atividade da $Na^+,K^+$-ATPase nos encéfalos dos filhotes uma vez que pacientes com hipermetioninemia severa apresentam disfunções cognitivas que podem estar associadas a alterações na atividade dessa enzima (Stefanello et al., 2011). Os resultados provenientes dos experimentos mostraram uma significativa redução na atividade da $Na^+,K^+$-ATPase em filhotes de 21 dias de idade. Uma vez que essa enzima é essencial para a condução de impulsos nervosos, sua inibição poderia causar prejuízos no processo de formação de memória e de aprendizado, levando a um retardo mental (Sahu et al., 2013).

A atividade da $Mg^{2+}$-ATPase também foi determinada no tecido cerebral

da prole devido à sua importância para a manutenção de altos níveis intracelulares de $Mg^{2+}$, estando envolvida na síntese proteica e crescimento celular. A atividade dessa enzima também estava significativamente reduzida em filhotes de 21 dias. Dados da literatura mostram que a inibição da $Mg^{2+}$-ATPase também está associada a alterações na capacidade aprendizagem (Carageorgiou et al., 2008). Portanto, a redução concomitante das duas ATPases analisadas neste estudo poderiam levar ao déficit cognitivo.

A hipermetioninemia induz estresse oxidativo em hipocampo de ratos (Stefanello et al., 2007b) e sabe-se que os grupamentos sulfidrilas das enzimas $Na,^{+}K^{+}$-ATPase e $Mg^{2+}$-ATPase são suscetíveis a danos oxidativos. Dessa forma, o conteúdo de grupamentos sulfidrilas também foi avaliado e verificamos que este parâmetro estava reduzido em encéfalos de filhotes de 21 dias. É possível que a exposição à metionina tenha levado ao aumento dos níveis do radical superóxido, o qual pode reagir com óxido nítrico (NO) e formar peroxinitrito ou pode ser convertido em $H_2O_2$. Ambas as espécies reativas citadas podem danificar os grupamentos sulfidrilas das proteínas (Winterbourn & Hampton, 2008), o que poderia explicar a redução da atividade das ATPases observada neste estudo.

Visto que as enzimas $Na,^{+}K^{+}$-ATPase e $Mg^{2+}$-ATPase consistem em proteínas membranares e as espécies reativas são capazes de causar a peroxidação dos lipídios presentes nas membranas celulares, sugeriu-se a hipótese de que o dano oxidativo aos lipídios poderia causar alterações na fluidez da membrana e contribuir para a redução de tal atividade enzimática.

Dessa forma, mediram-se os níveis encefálicos de TBARS, os quais consistem em uma medida indireta do produto final das reações de lipoperoxidação chamado malondialdeído. A hipermetioninemia materna não alterou esse parâmetro, sugerindo que a lipoperoxidação não tem participação nas alterações da atividade das ATPases observadas nos filhotes. Em concordância com o presente estudo, um trabalho recente mostrou que a administração crônica de metionina em ratos, não causa mudança nos níveis hepáticos de TBARS (Stefanello et al., 2009).

Com o objetivo de verificar se a exposição à metionina durante o período pré-natal poderia levar a alterações na ação de enzimas antioxidantes, também foram avaliadas as atividades das enzimas SOD e CAT. A SOD catalisa a dismutação do radical superóxido em oxigênio e $H_2O_2$. A CAT, por sua vez, decompõe o $H_2O_2$, formando água e oxigênio. O superóxido e o $H_2O_2$ podem causar danos diretos a estruturas celulares ou podem levar à formação de outras ERO que, por sua vez, causam danos adicionais. Portanto, as defesas representadas pela SOD e pela CAT são essenciais para a adequada manutenção do estado redox e proteção antioxidante celular (Bastaki et al., 2006).

Os resultados obtidos neste estudo mostraram que a hipermetioninemia gestacional não causa modificações na atividade encefálica da SOD. A atividade da CAT, por outro lado, apresentou uma importante redução nos encéfalos dos filhotes de 21 dias cujas mães foram tratadas com a dose mais alta de metionina. Esses resultados estão em concordância com um estudo

anterior que mostrou que a hipermetioninemia leva à redução da atividade hepática da CAT, mas não é capaz de alterar a atividade da SOD (Stefanello et al., 2009). Tal condição pode tornar o ambiente celular mais suscetível à formação e ao acúmulo de $H_2O_2$, e consequentemente poderia levar aos danos causados por essa molécula.

Entretanto, é necessário enfatizar que a homocisteína é formada durante o metabolismo da metionina e níveis sanguíneos de homocisteína em excesso podem causar a inibição da $Na^+,K^+$-ATPase cerebral, reduzir a atividade da CAT em cérebro, pulmão e coração, e reduzir o conteúdo de grupamentos sulfidrilas em fígado de ratos (Streck et al., 2002; da Cunha et al., 2011; Kolling et al., 2011). Embora, no presente trabalho não tenhamos encontrado níveis elevados de homocisteína total em soro e encéfalos dos filhotes, não se pode descartar a possibilidade de que a homocisteína esteja envolvida nas alterações que ocorreram na prole de ratas hipermetiononêmicas.

Também é importante notar que os filhotes de sete dias não apresentaram alteração em nenhum dos parâmetros analisados. Apenas os filhotes de 21 dias de idade apresentaram alterações em decorrência da hipermetioninemia materna. Uma possível explicação para tal resultado é que durante o período gestacional, a placenta exerce uma transferência materno-fetal de substâncias antioxidantes, mantendo uma concentração adequada ao feto (Underwood, 1994; Dimenstein et al., 1996). Dessa forma, é possível que os filhotes mais novos ainda apresentem a proteção antioxidante oriunda de suas progenitoras. Com o passar do tempo, porém, é provável que essa

proteção seja depletada, tornando os filhotes expostos aos danos oxidativos da metionina.

Visto que o maior número de alterações patológicas observadas nesta primeira etapa do estudo, ocorreram nos filhotes de 21 dias de idade cujas mães receberam a dose 2 de metionina durante a gestação, os experimentos bioquímicos seguintes foram realizados apenas na prole de 21 dias cujas progenitoras receberam a dose mais alta do aminoácido. As investigações acerca dos danos cerebrais causados à prole pela hipermetioninemia materna durante o período gestacional foram ampliadas pesquisando-se outros possíveis mecanismos fisiopatológicos envolvidos nessa condição.

Utilizando-se o modelo experimental desenvolvido, as pesquisas foram estendidas avaliando-se o número de neurônios da prole através de anticorpos monoclonais anti-NeuN. Houve uma redução significativa desse parâmetro no grupo de filhotes cujas mães receberam metionina, indicando que a hipermetioninemia materna reduziu o número de neurônios da prole. Entretanto, observou-se também que nenhuma das proteínas relacionadas à apoptose foi alterada, nem as proteínas pró-apoptóticas (Bax e p53), nem as proteínas anti-apoptóticas (Bcl-2 e Bcl-xL). É possível que o tratamento tenha induzido apoptose de uma maneira independente de p53 e das proteínas da família Bcl-2. Além disso, a perda neuronal observada provavelmente está relacionada à redução do conteúdo de NGF e BDNF. NGF é uma neurotrofina envolvida na regulação do crescimento, proliferação e sobrevivência de neurônios. BDNF também consiste em uma neurotrofina essencial para o

sistema nervoso central uma vez que está envolvida na plasticidade sináptica, tendo importante papel na maturação e integridade neural. Os resultados que foram obtidos são muito importantes uma vez que a redução do número de neurônios durante o desenvolvimento cerebral pode prejudicar a resposta sináptica e levar a problemas de aprendizagem na prole. Além disso, a manutenção de níveis adequados de NGF e BDNF é fundamental durante o processo de formação de memória (Bramham & Messaoudi, 2005; Zhang et al., 2013).

Em seguida, o metabolismo energético encefálico foi avaliado. Os resultados mostraram que as atividades da succinato desidrogenase (SDH) e do complexo II foram significativamente reduzidas nos encéfalos dos filhotes de progenitoras hipermetioninêmicas. Os primeiros resultados do presente estudo mostraram que a hipermetioninemia materna induz estresse oxidativo cerebral nos filhotes e sabe-se que os complexos da cadeia transportadora de elétrons são suscetíveis aos danos causados por ERO, o que poderia explicar tais efeitos (Sverdlov et al., 2015). Uma vez que o complexo II/SDH tem um papel importante na cadeia respiratória e no ciclo do ácido tricarboxílico (Ackrell, 2000; Rustin et al., 2002) e uma vez que o cérebro é altamente dependente de uma fonte constante de energia, essa condição pode causar dano neurológico. Estudos têm demonstrado que uma redução na demanda energética está associada a diversas desordens neurodegenerativas como as doenças de Alzheimer, Parkinson e Huntington, bem como a ataxia de Friedreich (Schapira, 1999; Ebadi et al., 2001; Ferrer, 2009).

Também é importante ressaltar que, como mencionado anteriormente, encontrou-se uma diminuição da atividade cerebral da $Na^+,K^+$-ATPase na prole devido à hipermetioninemia gestacional. Uma vez que essa enzima consome altas taxas de ATP, a redução da atividade do complexo II/SDH poderia restringir o suprimento de energia e, consequentemente, contribuir para a diminuição da atividade da $Na^+,K^+$-ATPase que foi observada neste trabalho. Para melhor entender os mecanismos envolvidos na alteração da atividade dessa enzima durante a hipermetioninemia gestacional, também foram avaliados a expressão e o imunoconteúdo da $Na^+,K^+$-ATPase. Os resultados mostraram que o efeito inibitório da metionina sobre a atividade da $Na^+,K^+$-ATPase é inversamente correlacionado aos níveis de mRNA e imunoconteúdo das subunidades alfa (catalíticas) da $Na^+,K^+$-ATPase. Esse resultado sugere que o decaimento na atividade da $Na^+,K^+$-ATPase induzida pela metionina não ocorre pela alteração da expressão gênica ou do número de moléculas da enzima, mas trata-se de uma inibição pós-traducional provavelmente devido à redução do metabolismo energético e/ou dano oxidativo aos grupamentos sulfidrilas da $Na^+,K^+$-ATPase, como já discutido previamente. Além disso, o aumento da transcrição/tradução com consequente aumento no conteúdo de moléculas da enzima, provavelmente indica o desenvolvimento de um mecanismo adaptativo de compensação.

Estudos prévios têm demonstrado que a redução da atividade da $Na^+,K^+$-ATPase encefálica é capaz de levar ao aumento da concentração de $Na^+$ intracelular, contribuindo para os mecanismos fisiopatológicos envolvidos

na formação do edema cerebral (Kempski, 2001). Dessa forma, avaliou-se o conteúdo de água nos encéfalos da prole, mas nenhuma diferença foi observada quando comparado ao grupo controle. Portanto, embora pacientes com hipermetioninemia severa possam apresentar edema cerebral (Mudd et al., 2001), essa condição durante a gestação não parece afetar esse parâmetro na prole.

A neuroinflamação também é descrita na literatura como um importante fator que pode contribuir para o desenvolvimento de doenças neurodegenerativas, como as doenças de Alzheimer (Heneka et al., 2015), Parkinson (Stojkovska et al., 2015) e Huntington (Chang et al., 2015), bem como a esclerose múltipla (Frohman et al., 2006). Uma vez que o aumento da produção de ERO pode levar ao aumento do processo pró-inflamatório (Martinon, 2010) e foi demonstrado no presente estudo que o tratamento com metionina durante a gestação induz estresse oxidativo no cérebro da prole, avaliou-se também o efeito desse tratamento sobre os níveis de TNF-alfa e IL-6 nos encéfalos da prole. TNF-alfa é uma proteína sinalizadora que induz a migração de leucócitos no tecido inflamado e promove apoptose (Bradley, 2008), enquanto que a IL-6 é considerada um ativador da reposta de fase aguda bem como um fator estimulador de linfócitos (Rath et al., 2015). Nossos resultados não demonstraram alterações importantes nesses parâmetros, sugerindo que a neuroinflamação não participa dos processos fisiopatológicos induzidos pela metionina ao feto durante a gestação.

Na primeira etapa deste estudo, demonstrou-se que a

hipermetioninemia materna diminui a atividade da CAT encefálica da prole. Uma vez que essa enzima antioxidante tem a função de decompor o $H_2O_2$, surgiu a hipótese de que essa condição poderia levar ao aumento dos níveis cerebrais de $H_2O_2$. Entretanto, essa espécie reativa foi medida e nenhuma alteração foi observada. É possível que a ação de outras peroxidases que têm a função de detoxificar o $H_2O_2$ possam eficientemente eliminar o excesso dessa molécula.

Tendo em vista que no presente trabalho demonstrou-se que a hipermetioninemia gestacional reduz as atividades da $Na^+$, $K^+$-ATPase e $Mg^{2+}$-ATPase, número de neurônios, níveis de NFG e BDNF e atividade do complexo II/SDH, bem como induz estresse oxidativo nos encéfalos da prole, é possível que esses danos bioquímicos possam causar lesões celulares, as quais podem ser evidenciadas por alterações morfológicas. Portanto, realizou-se a avaliação da ultraestrutura de neurônios através de microscopia eletrônica, a qual revelou que no tecido cerebral dos filhotes de 21 dias havia uma redução geral do número de organelas, exceto pelo número de mitocôndrias, as quais se encontravam mais numerosas quando comparadas ao controle. É provável que o aumento no número de mitocôndrias represente um mecanismo compensatório uma vez que a biogênese mitocondrial é capaz de contribuir para a recuperação celular durante diferentes eventos fisiopatológicos. Aos 30 dias de vida, já não era mais possível identificar organelas intactas, nem mesmo mitocôndrias, em filhotes cujas mães foram tratadas com metionina. Além disso, observou-se vacuolização do citoplasma. Tanto aos 21 quanto aos

30 dias de vida o tecido cerebral dos animais apresentava o citoplasma desorganizado.

As alterações bioquímicas e morfológicas observadas nos tecidos cerebrais dos filhotes que foram expostos ao excesso de metionina na vida intrauterina, podem causar danos à memória e à capacidade de aprendizado. Dessa forma, verificou-se o efeito da exposição à metionina durante a vida pré-natal em alguns testes comportamentais. Primeiramente os filhotes foram submetidos à tarefa de campo aberto, a qual tem por objetivo medir respostas comportamentais como a atividade motora e o nível de ansiedade. No presente estudo, porém, não foram observadas mudanças na distância percorrida, tempo de mobilidade, número de cruzamentos das linhas, e velocidade média, quando comparado com o grupo controle, indicando que a atividade locomotora não foi alterada.

Entretanto, houve um aumento no tempo gasto no centro do aparato pelos filhotes que foram expostos à metionina. Os roedores normalmente gastam mais tempo explorando a periferia do aparato, já que o centro é considerado uma área desprotegida. Dessa forma, o acréscimo de tempo gasto no centro do campo aberto indica um efeito do tipo ansiolítico. Dados da literatura demonstram que a SAM, metabólito intermediário da metionina formado pela reação da MAT I/III, apresenta efeitos ansiolíticos, sendo inclusive usada como um tratamento adjuvante em transtornos psiquiátricos (Bressa, 1994; Papakostas, 2009; Di Pierro et al., 2015). Dessa forma, elevados níveis de SAM induzidos pela hipermetioninemia poderiam explicar esse resultado.

Realizou-se o teste comportamental de esquiva inibitória do tipo *step-down* para avaliar as memórias de curto e de longo prazo dos filhotes. Nesse teste, os animais aprendem que quando descem de uma plataforma e colocam as quatro patas sobre um piso formado por uma grade de metal, recebem um leve choque. Em uma segunda exposição à caixa de esquiva inibitória (sessão de teste), os animais que consolidam a memória evitam o ato de descer da plataforma para explorar a caixa. Cada filhote foi testado uma e 24 horas após o treino para avaliação das memórias de curta e longa duração, respectivamente. Os resultados mostraram que os animais cujas mães receberam metionina durante a gestação apresentaram uma importante redução na latência de descida da plataforma uma e 24 horas após o treino, indicando que a hipermetioninemia gestacional significativamente prejudicou as memórias de curta e longa duração.

Um segundo teste de memória, denominado teste de reconhecimento de objetos, foi realizado. De forma resumida, os animais foram colocados em uma caixa com dois objetos exatamente iguais e deixados por alguns minutos para explorarem tais objetos. O tempo de exploração foi cronometrado. Para testar a memória de longa duração, os animais foram recolocados na caixa 24 horas após a sessão de treino. Porém, agora a caixa possuía um objeto conhecido (o mesmo da sessão de treino) e um objeto totalmente distinto. O tempo de exploração de cada objeto foi novamente cronometrado. Essa tarefa se baseia no fato de que animais sem comprometimento de memória tendem a explorar mais um objeto novo do que um objeto familiar. Os resultados desse teste

confirmam que a memória de longa duração dos filhotes de ratos foi prejudicada pela hipermetioninemia gestacional, uma vez que os ratos controles gastaram mais tempo explorando o objeto novo na sessão de teste, enquanto que os filhotes nascidos de progenitoras hipermetioninêmicas gastaram tempos semelhantes em ambos os objetos, sugerindo que não reconheceram o objeto familiar. Em concordância, a exposição de *zebrafish* adulto à metionina induziu dano à memória no teste de esquiva inibitória. Ainda, a administração crônica de metionina em ratos do 6º ao 28º dia de vida, também causou perda de memória como observado no teste labirinto aquático de Morris (Stefanello et al., 2007d; Vuaden et al., 2012).

O teste de reconhecimento de objetos também demonstrou que o tratamento com metionina durante a gestação significativamente reduziu o tempo total de exploração dos objetos pela prole. Estudos anteriores mostram que a indução de hipermetioninemia crônica em ratos em desenvolvimento leva ao aumento da atividade da acetilcolinesterase em córtex cerebral (Stefanello et al., 2007d). Tal mecanismo poderia explicar a perda da capacidade exploratória pelos filhotes expostos à metionina uma vez que tem sido descrito que os mecanismos colinérgicos contribuem para a motivação exploratória (Lamprea et al., 2003).

A última etapa do presente estudo foi realizada em homogeneizados de músculo esquelético. Uma vez que foi demonstrado que a hipermetioninemia gestacional induz estresse oxidativo e sabe-se que ERO estão correlacionadas com o desenvolvimento de sarcopenia e outras doenças musculares (Tidball &

Wehling-Henricks, 2007; Arbogast et al., 2009; Turki et al., 2012; Sullivan-Gunn & Lewandowski, 2013), os efeitos desse modelo experimental também foram avaliados sobre o músculo gastrocnêmio da prole. O músculo gastrocnêmio foi escolhido uma vez que é composto por um número significativo de fibras de contração rápida, o que o torna mais suscetível aos possíveis danos oxidativos gerados pela exposição à metionina.

Primeiramente, avaliou-se o efeito dos níveis elevados de metionina durante a gestação sobre a geração de ERO. Tal avaliação foi realizada através da medida dos níveis de DCF, os quais se encontravam significativamente elevados em homogeneizados de músculos da prole cujas progenitoras receberam administração de metionina, indicando aumento da produção e/ou diminuição da detoxificação de ERO.

Também foi verificado o efeito da hipermetioninemia gestacional sobre os danos a proteínas e lipídios nos músculos dos filhotes. Os resultados demonstraram que o *status* de proteínas ligadas a grupamentos sulfidrilas estava significativamente reduzido em decorrência do excesso de metionina, sugerindo que os resíduos de aminoácidos contendo grupamentos sulfidrilas foram alvos de ERO. Em concordância com o que foi observado neste estudo, Stefanello e colaboradores (2009) demonstraram que a administração crônica de metionina em ratos em desenvolvimento causa danos oxidativos a proteínas hepáticas. Com relação ao efeito da hipermetioninemia durante a gestação sobre o dano lipídico nos músculos da prole, os resultados mostraram um aumento importante nos níveis de TBARS. A indução da peroxidação lipídica

sugere um dano à membrana celular muscular causado por ERO, reiterando a evidência de estresse oxidativo no músculo esquelético (Ohkawa et al., 1979).

As defesas antioxidantes enzimáticas também foram avaliadas para melhor estabelecer o *status* oxidativo no músculo da prole de ratas hipermetioninêmicas. Os resultados mostraram que o tratamento com metionina durante a gestação significativamente reduziu as atividades da SOD e da CAT, o que reflete um decaimento na proteção contra o dano celular oxidativo. Tal evento pode estar associado, ao menos parcialmente, com a elevação nos níveis de DCF e TBARS, bem como com a redução dos níveis de grupamentos sulfidrilas.

Além disso, foi observada uma redução nos níveis de nitritos no músculo da prole exposta à metionina, indicando decréscimo dos níveis de NO. Como visto acima, foi encontrada uma diminuição da atividade da SOD muscular, uma condição que torna o ambiente celular mais favorável à formação do radical superóxido. Uma vez que o ânion superóxido pode reagir com o NO, gerando peroxinitrito (Huie & Padmaja, 1993), é possível que uma taxa elevada dessa reação possa ter sido responsável pela redução da biodisponibilidade de NO. Além disso, tem sido descrito que a formação de peroxinitrito em células musculares, devido à deficiência da atividade da SOD, pode contribuir para a perda de massa muscular em camundongos (Sakellariou et al., 2011).

Uma vez que a literatura aponta que o processo oxidativo pode contribuir para o dano celular, também se avaliou o efeito da hipermetioninemia materna sobre biomarcadores séricos que indicam a presença de injúria

muscular. Inicialmente, mediu-se a atividade da creatinina cinase, uma enzima que catalisa a transferência do grupo fosforil da fosfocreatina ao ADP, regenerando ATP e contribuindo para a homeostasia energética (Wallimann et al., 1992). Uma vez que a creatina cinase é altamente expressa no músculo esquelético, o dano a este tecido leva a liberação dessa enzima para a circulação sanguínea periférica, elevando seus níveis séricos (Jones et al., 1986). Surpreendentemente, a atividade da creatina cinase sérica dos filhotes foi reduzida pela hipermetioninemia gestacional, o que provavelmente sugere que essa condição inibiu a atividade de tal enzima.

De fato, dados proveniente da literatura mostram que a creatina cinase é altamente suscetível aos danos causados por ERO (Aksenov et al., 2000), possivelmente devido à oxidação de resíduos de cisteína, os quais são críticos para a atividade dessa enzima (Kenyon, 1996). Dessa forma, uma vez que a creatina cinase é uma enzima que contém grupamentos tióis e verificou-se neste estudo que a hipermetioninemia materna significativamente aumenta a produção de ERO associada a uma redução de grupamentos sulfidrilas nos músculos dos filhotes, pode-se inferir que o estresse oxidativo possa estar, ao menos em parte, correlacionado com a redução sérica da creatina cinase. Como consequências da inativação da creatina cinase, o músculo pode sofrer as seguintes alterações patológicas: aumento do volume mitocondrial e alteração do potencial glicogenolítico/glicolítico, contrações anormais; agregados tubulares na membrana do retículo sarcoplasmático e acúmulo de ADP (van Deursen et al., 1993; Steeghs et al., 1997; Saupe et al., 1998).

Com o objetivo de se investigar se o tratamento com metionina é capaz de causar perda de massa muscular, a concentração de proteínas musculares totais foi medida e o peso do músculo gastrocnêmio foi determinado. Observou-se uma redução importante em ambos os parâmetros analisados, sugerindo um aumento da degradação proteica muscular, o que pode resultar no aumento da produção de ureia, a qual consiste no principal metabólito derivado do *turnover* proteico. Além disso, a massa do tecido muscular esquelético é a principal determinante dos níveis séricos de creatinina uma vez que esse metabólito é derivado do metabolismo muscular da creatina (Refsum & Strömme, 1974; Andersson et al., 2008). Baseado nessas observações, também se avaliou, no presente trabalho, os níveis séricos de ureia e creatinina na prole das ratas. A hipermetioninemia gestacional não alterou a concentração de creatinina, entretanto elevou significativamente os níveis de ureia. Sugere-se a hipótese de que o aumento da ureia sérica ocorreu como consequência do dano muscular causado pelo estresse oxidativo, uma vez que a ação de espécies reativas sobre os grupos tióis de proteínas musculares pode levar à proteólise.

Além disso, é importante notar que uma vez que o metabolismo da metionina resulta na produção de $H_2S0_4$, a ingestão excessiva desse aminoácido pode causar acidose metabólica (Hood & LaGrange, 1988), a qual parece induzir o aumento da degradação de proteínas, resultando em perda da massa muscular (Bailey et al., 1996). Ainda, durante diversas condições patológicas, o músculo esquelético proporciona aminoácidos para a produção

de proteínas de fase aguda através do aumento da proteólise, também resultando em perda de massa muscular. Entretanto, vale ressaltar que diferentes respostas a diferentes condições patológicas têm sido descritas entre músculos de contração rápida e de contração lenta. Tem-se demonstrado que durante o processo inflamatório, o aumento da proteólise e redução da síntese proteica são mais pronunciados em músculos de contração rápida. Além disso, estudos mostraram que a inibição do proteassoma reduziu os eventos proteolíticos em ambos os tipos de músculos, sugerindo que o sistema ubiquitina-proteassoma participa de uma parte considerável da proteólise no músculo esquelético (Kadlcíková et al., 2004; Muthny et al., 2008). O músculo gastrocnêmio é composto de uma grande quantidade de fibras de rápida contração e, portanto, pode ser afetado durante diversos eventos patológicos.

Com o intuito de complementar o estudo a respeito dos efeitos patológicos da hipermetioninemia materna sobre a prole, a detecção qualitativa de troponina I cardíaca foi realizada no soro. A troponina I cardíaca consiste em uma proteína que regula a contração do músculo cardíaco e é considerada um indicador muito sensível de necrose do miocárdio quando se torna detectável no sangue (Adams et al., 1993). Os resultados deste estudo demonstram que a troponina I cardíaca não se encontrava alterada devido à exposição à metionina, sugerindo que não houve lesão cardíaca aos filhotes.

Por fim, mediram-se ainda os níveis séricos de proteína C reativa, a qual consiste em uma proteína de fase aguda produzida no fígado e é considerada um dos mais sensíveis biomarcadores, se elevando em resposta a quase todos

os estímulos inflamatórios (Pepys & Hirschfield, 2003). Os níveis séricos de proteína C reativa estavam significativamente aumentados na prole oriunda de progenitoras hipermetioninêmicas, o que pode representar uma causa e/ou consequência do estresse oxidativo, visto que o estímulo do sistema imune leva ao aumento da produção de ERO por neutrófilos e macrófagos, e o aumento dos níveis de ERO intensifica ainda mais a resposta inflamatória pelo sistema imunológico (Geronikaki & Gavalas, 2006). Além disso, estudos realizados tanto em animais quanto em seres humanos demonstraram uma associação entre inflamação e perda de massa muscular (Goodman, 1991, 1994; van Hall et al., 2008). Cesari e colaboradores (2005) também demonstraram que os níveis de proteína C reativa são inversamente proporcionais à massa muscular.

De forma resumida, os resultados obtidos a partir dos encéfalos da prole mostraram que a hipermetioninemia gestacional inibe ATPases, altera o equilíbrio redox e metabolismo energético das células, e reduz o número de neurônios e níveis de neurotrofinas. A morfologia de neurônios também foi afetada. Essas alterações podem ser responsáveis pela redução da memória exibida pelos filhotes nos testes comportamentais. No músculo esquelético da prole, a hipermetioninemia materna promove estresse oxidativo/nitrosativo associado à perda de massa muscular. Observou-se, ainda, aumento de um parâmetro inflamatório sérico.

No presente trabalho desenvolvemos um novo modelo animal para hipermetioninemia gestacional, oferecendo uma ferramenta que possibilitará o

desenvolvimento de estudos que visem investigar os efeitos causados pelo excesso de metionina na corrente sanguínea materna durante o desenvolvimento pré-natal da prole. No presente estudo, esse modelo animal permitiu a avaliação de alterações bioquímicas, histológicas e moleculares em diferentes tecidos e, também alterações comportamentais dos filhotes de ratas submetidas ao modelo em questão. Tais estudos são de suma importância, uma vez que ao se identificar os mecanismos tóxicos pelos quais a hipermetioninemia materna causa danos à prole, torna-se possível minimizar os possíveis efeitos prejudiciais, como também possibilita a identificação do risco aumentado de processos patológicos futuros na prole. Dessa forma, ressaltamos a importância de termos desenvolvido um modelo de hipermetioninemia gestacional apropriado para o estudo a respeito dos efeitos ocasionados pela exposição ao excesso de metionina devido a uma condição genética ou uma dieta rica em proteína durante a vida pré-natal.

# 5. CONCLUSÕES

No presente estudo, foi desenvolvido um modelo de hipermetioninemia materna em ratas Wistar. A partir da utilização desse modelo, observamos que níveis elevados de metionina durante a gestação causaram as seguintes alterações na prole:

1. Inibição da atividade da $Na^+,K^+$-ATPase e da $Mg^{2+}$-ATPase e aumento da expressão gênica e imunoconteúdo da $Na^+,K^+$-ATPase em encéfalo;
2. Alteração do equilíbrio redox cerebral, observada pela redução da atividade da CAT e do conteúdo de grupamentos sulfidrilas;
3. Redução do número de neurônios;
4. Diminuição dos níveis de NGF e BDNF encefálicos;
5. Comprometimento do metabolismo energético cerebral, verificado pela redução da atividade do complexo II/SDH;
6. Alterações morfológicas dos neurônios, indicando degeneração celular;
7. Efeito do tipo ansiolítico, como observado no teste comportamental de campo aberto;
8. Perda de memória indicada pelos testes comportamentais de esquiva inibitória e reconhecimento de objetos;
9. Alteração do comportamento exploratório verificado através da redução do tempo de exploração total na tarefa de reconhecimento de objetos;
10. Estresse oxidativo/nitrosativo no músculo esquelético gastrocnêmio, indicado pelo aumento da produção de ERO e de peroxidação lipídica, bem como redução do conteúdo de grupamentos sulfidrilas, atividade de

certas enzimas antioxidantes e níveis de nitritos;

11. Redução da concentração proteica total no músculo esquelético gastrocnêmio;
12. Perda de peso do músculo esquelético gastrocnêmio;
13. Diminuição da atividade de creatina cinase e elevação dos níveis de ureia e proteína C reativa no soro.

Concluindo, nossos achados reforçam a importância do uso de modelos animais para investigar mecanismos fisiopatológicos envolvidos em diferentes condições fisiológicas ou patológicas. No presente estudo demonstramos que a hipermetioninemia durante o período gestacional pode ocasionar danos cerebrais e musculares na prole, podendo resultar em mudanças na ultraestrutura cerebral e déficit de memória na vida adulta. Cabe ressaltar que uma dieta hiperproteica durante a gestação pode aumentar os níveis de metionina e consequentemente causar os efeitos tóxicos à prole que foram observados neste estudo. Portanto, acreditamos que o desenvolvimento do modelo de hipermetioninemia gestacional em ratos possibilitará a realização de novos estudos a respeito dos danos causados por essa condição patológica à prole na vida pós-natal.

# 6. PERSPECTIVAS

Os resultados obtidos no presente estudo abrem perspectivas para darmos continuidade às nossas investigações acerca dos danos causados ao feto pela hipermetioninemia materna. Dessa forma, nossos objetivos futuros incluem os seguintes pontos:

- Avaliar se os efeitos patológicos devido à hipermetioninemia gestacional observados neste estudo se estendem durante a vida adulta da prole de ratas (cerca de 60 dias de idade);

- Determinar se o tratamento com metionina nas ratas mães durante a amamentação causaria efeitos tóxicos aos filhotes;

- Determinar os níveis encefálicos de glutamato da prole;

- Avaliar a atividade da acetilcolinesterase e os níveis de determinados aminoácidos no liquor dos filhotes;

- Investigar, na prole, as vias de sinalização ativadas pelas neurotrofinas através de receptores Trk, como as vias das proteínas cinases ativadas por mitógenos (MAPK) e as cinases reguladas por sinal extracelular (ERK), proteína fosfatidil-inositol-3 cinase ($PI_3K$) e a proteína cinase independente de cálcio (PKC);

- Determinar a densidade de espinhos dendríticos em neurônios corticais dos filhotes;

- Quantificar e analisar a morfologia de astrócitos corticais da prole;

- Realizar análise histológica no músculo gastrocnêmio dos filhotes;

- Dosar a atividade da enzima $Mg^{2+}$-ATPase em músculo esquelético da prole;

- Avaliar se a administração, durante a gestação, de determinadas substâncias com efeito antioxidante como a vitamina E, vitamina C e melatonina, poderia promover efeito protetor sobre os danos neurológicos e musculares causados aos filhotes expostos ao excesso de metionina durante a vida pré-natal.

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